

Article

Targeted Reduction of Excessive Mitochondrial Superoxide by Mitoquinone Rescues Cognitive Impairment Without Affecting Spontaneous Recurrent Seizures in a Mouse Model of Temporal Lobe Epilepsy

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Abstract

Cognitive impairment is a major comorbidity in temporal lobe epilepsy (TLE), yet its underlying pathophysiology remains poorly understood and current therapies provide minimal benefit. While oxidative stress has traditionally been viewed as a precursor to cell death-mediated cognitive decline, cell death is absent in many patients and preclinical models with memory impairment. Here, we tested whether excessive mitochondrial reactive oxygen species (ROS) actively contribute to memory impairment through mechanisms distinct from cell death. Using Kv1.1 knockout (KO) mice, a TLE model with mitochondrial respiratory chain complex I (MRCI) impairment, we found elevated hippocampal mitochondrial superoxide, impaired recognition memory, deficits in synaptic plasticity, and abnormal sharp wave–ripple oscillations. Applying the MRCI inhibitor rotenone to wild-type hippocampal slices caused increased superoxide and mirrored electrophysiology deficits. Both acute and sub-chronic treatment with the mitochondria-targeted antioxidant mitoquinone (MitoQ) reduced superoxide levels, rescued synaptic plasticity, restored network activity, and normalized memory performance in KO mice—without altering seizure frequency, severity, or neuronal excitability. Our results identify mitochondrial superoxide as a reversible driver of hippocampal dysfunction in epilepsy and demonstrate that mitochondria-targeted antioxidant therapy can restore cognition despite persistent seizures. This study provides proof-of-concept for novel treatments improving cognitive comorbidities in TLE beyond seizure control.



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1. Introduction

In the United States there are approximately 3.4 million people with epilepsy (PWE) [1]. Cognitive dysfunction affects 60–70% of PWE across multiple domains such as visual and verbal memory, attention, executive function, visuospatial skills, and language [2,3]. Temporal lobe epilepsy (TLE), the most common drug-resistant epilepsy with ~30,000 new cases annually in the United States, is frequently accompanied by severe cognitive impairment [4,5]. These impairments are often more debilitating than seizures and substantially

reduce quality of life. Current cognitive therapy inadequately addresses these deficits [6], and no FDA-approved pharmacological treatments exist for cognitive impairments in PWE. Moreover, to date, there are no experimental therapies in randomized controlled trials as per the clinical trial databases (www.clinicaltrials.gov and www.cortellislabs.com).

The primary obstacle to developing effective therapies is the absence of an identified common mechanism underlying TLE-associated cognitive impairments [7]. For instance, cell death and subsequent neuronal remodeling are not present in all epilepsy patients or preclinical models exhibiting memory impairment; hippocampal cell loss and volume reduction do not always correlate with cognitive dysfunction [8–11]. These observations suggest that a more subtle pathophysiology may underlie cognitive impairment in TLE.

The hippocampus of human TLE exhibits mitochondrial dysfunction, oxidative stress, altered synaptic plasticity, inflammation, and neuronal loss [12–14]—neuropathology that also occurs in multiple animal models of TLE with comorbid cognitive impairment [13,15,16]. Traditionally, increased reactive oxygen species (ROS) has been viewed as a harbinger of cell death and implicated in memory decline [13]. However, the focus on oxidative stress-mediated neuronal death in epilepsy has overshadowed over 30 years of fundamental research, demonstrating mitochondrial and ROS involvement in synaptic transmission and plasticity, thereby obscuring their potential active role in cognitive dysfunction.

Studies have demonstrated that mitochondria regulate synaptic transmission through ATP production, ROS generation, and dynamic sequestration of cytosolic calcium [17]. Although mitochondrial contributions are synapse specific, ATP is generally necessary for vesicular release and recycling, low levels of ROS can act as signaling molecules promoting synaptic plasticity, and mitochondrial calcium buffering modulates neurotransmitter release probability. In control animals and tissue, experimentally increasing ROS acutely inhibits mitochondrial respiratory chain complex I (MRCI), subsequently reducing ATP production, and impairing mitochondrial calcium buffering, ATP-dependent synaptic vesicle recycling, AMPA and NMDA receptor trafficking, and short- and long-term synaptic plasticity [17–27]. These findings indicate that heightened ROS levels—even those insufficient to induce cell death but are ever present in living brain cells during epilepsy—may still detrimentally alter the physiology of surviving neurons.

Here, we test the hypothesis that mitochondria-generated ROS contributes to cognitive dysfunction in a preclinical TLE model. We used Kv1.1 knockout (KO) mice, a preclinical model of TLE with learning and memory deficits and impaired hippocampal mitochondrial bioenergetics [16,28,29]. Analogous to human TLE, the MRCI function is reduced in the KO hippocampus and mitochondrial ROS levels are elevated [16]. Superoxide, an important ROS, is generated when electrons are unsuccessfully transferred during electron transport and bind to molecular oxygen. To increase successful electron transport and to prevent excessive superoxide formation, we used mitoquinone (MitoQ) [30], a synthetic ubiquinone targeted to mitochondria.

2. Materials and Methods

2.1. Animals

C3HeB/FeJ mice heterozygous for Kv1.1 KO (Strain 003532) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) in 2009 and a colony was established and maintained in the animal research facility of Creighton University School of Medicine. Heterozygous mice were bred to produce homozygous wildtype (WT) and KO mice. Mice were kept on a 12-h light/dark cycle, room temperature was set at 22.2 °C and ranged between 21.1 and 23.3 °C, room humidity ranged between 30 and 70%, and mice had ad libitum access to food and water. Mice of each genotype were randomly assigned to

experimental groups. Male and female mice were used. At experiment termination, the mice were P40–47 (Figure 1). In this age range, the WT mice weighed ~23 g (range: 21–26 g) and the KO mice weighed ~19 g (range: 16–20 g). All experiments were approved by the Institutional Animal Care and Use Committee of Creighton University School of Medicine and in accordance with the Declaration of Helsinki.

2.2. Chemicals

MitoQ was purchased from Focus Biomolecules LLC (10-1363, Plymouth Meeting, PA, USA). The MitoQ stock solution (10 mM) was prepared in DMSO and diluted with artificial cerebrospinal fluid (aCSF) to a 500 nM perfusion solution for ex vivo studies. The 10 mM MitoQ stock solution was diluted with 20% DMSO in sterile 0.9% NaCl (Hospira Inc., Lake Forest, IL, USA) to a 1.473 mM solution for 5 mg/kg intraperitoneal in vivo injection [31–35]. Rotenone was purchased from Sigma-Aldrich (St. Louis, MO, USA). The rotenone stock solution (10 mM) was prepared in DMSO and diluted with aCSF to a 100 nM perfusion solution for ex vivo studies [28]. Unless specified, all other chemicals were purchased from Sigma-Aldrich.

2.3. Experimental Design

Mice were randomly assigned to one of four experimental groups (Figure 1). Experiment 1: P40–42 WT and KO mice were treated for 5 days followed by behavioral memory tasks. A preliminary theoretical power analysis for a two-way ANOVA suggested required sample sizes of 15 mice per group (G*Power v3.1.9.6) [36]. After initial experiments, the power analysis was refined for effect size and variation, and the required sample size was reduced to 6–7 mice per group; thus, the WT + vehicle and KO + vehicle groups had 15 mice each and the WT + MitoQ and KO + MitoQ groups had 6 and 7 mice, respectively. After the behavioral assays, the mice were euthanized and living hippocampal slices were immediately collected from a subset of mice for either superoxide detection or electrophysiology. Experiment 2: P33–35 KO mice were surgically implanted with subdural electrodes, allowed to recover for five days, and then treated with vehicle for two days followed by MitoQ for five days. Continuous video-EEG recordings were obtained during treatment. Based on our previous studies with these mice using similar techniques and treatment strategies [21,37], the power analysis for a paired *t*-test indicated required sample sizes of 5 mice per group. Experiment 3: P40–45 WT and KO mice were euthanized and living hippocampal slices were immediately collected for either superoxide detection or electrophysiology during acute treatments. Based on our previous studies using similar techniques [38,39], power analyses for large effect sizes indicated at least 3 slices per group for various endpoints. Experiment 4: P40–45 WT and KO mice were euthanized and hippocampal tissue was immediately collected for either RT-qPCR or mitochondria isolation with subsequent polarography. Based on our previous studies with these mice using similar techniques [16,40], power analyses indicated at least 4 mice per group for various endpoints.

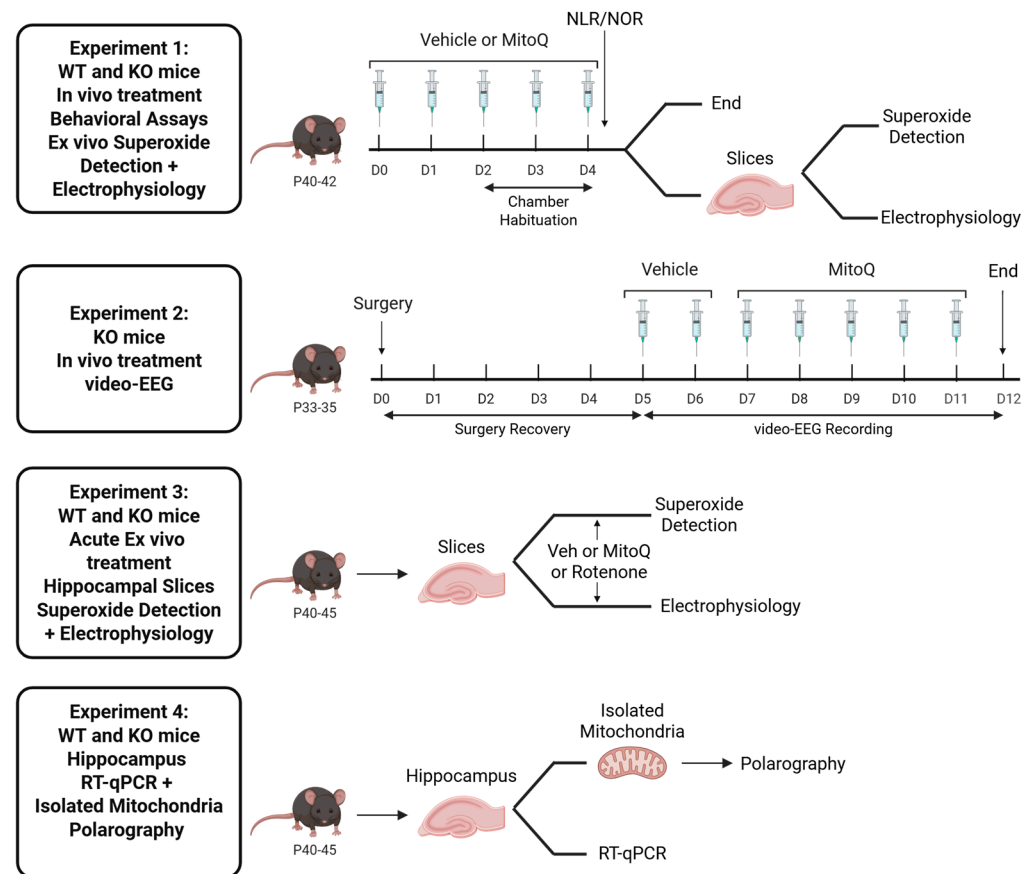


Figure 1. Illustration of experimental design and timeline. Created in BioRender. Simeone, T. (2026) <https://BioRender.com/j13o12c> [41].

2.4. Behavioral Assessments

Open Field: Mice were placed in the center of a 40 cm × 40 cm × 40 cm open field arena with visual cues and allowed to habituate to the arena for 5 min per day for two days prior to memory tests. The arena was cleaned with 70% isopropyl alcohol followed by Oxivir Tb disinfecting wipes (Diversey Holdings Ltd., Fort Mill, SC, USA). On the third day, animals were allowed to habituate to the arena for 5 min prior to the training session. Thigmotaxis, the tendency to prefer the periphery over the center of an open field, was assessed using the indices described by Simon et al. [42] (i.e., index of thigmotaxis, thigmotaxis time locomotor activity).

$$\text{Thigmotaxis Index} = \left(\frac{\text{distance travelled in the periphery (2.5 cm from the walls)}}{\text{total distance traveled in the arena}} \right) \times 100$$

$$\text{Thigmotaxis Time} = \left(\frac{\text{time spent in the periphery (2.5 cm from the walls)}}{\text{total time spent in the arena}} \right) \times 100$$

$$\text{Locomotion} = \left(\frac{\text{total distance traveled in the arena}}{\text{total time spent in the arena}} \right)$$

Novel location recognition and novel object recognition: On the third day, 20 min after the habituation phase, two identical objects were placed in the open field 6 cm from the back and side wall and the mice were placed facing the wall opposite to the objects. The mice were allowed 10 min to explore each object. The arena and the objects were cleaned with 70% isopropyl alcohol and Oxivir Tb disinfecting wipes between sessions. One hour after the training session, a novel location recognition (NLR) test was performed by moving one of the objects to the opposite corner without altering the position of the

other object and mice were allowed 10 min to explore (Figure 2a). One hour after the NLR test, a novel object recognition (NOR) test was performed by replacing the object moved during NLR with a new object and the mice were allowed 10 min to explore. The objects used were 50 mL Fisherbrand™ Easy Reader™ conical polypropylene centrifuge tubes (Cat no. 05–539-12; Fisher Scientific, Waltham, MA, USA) and Henke-Ject® 30 mL syringes (Cat no. 61LD31; Grainger, Omaha, NE, USA). Use and placement of the objects were counterbalanced across the experiments and groups. Testing was conducted in a dim red-lighting condition (10–20 lux), with an average temperature of ~21 °C and an average relative humidity of ~38%. All behavioral assays were conducted between zeitgeber time (ZT)2 and ZT6 during the light phase (lights on at ZT0; lights off at ZT12). Mouse identities were coded, and videos of the behavioral tests were analyzed using ANY-maze software v7.0 (Stoelting Co., Wood Dale, IL, USA). A mouse was scored as exploring an object when its head was oriented toward the object within 2.5 cm or when the nose was touching the object. The exploration time was recorded, and the discrimination index (DI) was calculated as follows [43]:

$$DI = \left(\left(\frac{D - C}{C + D} \right) - \left(\frac{B - A}{A + B} \right) \right) \times 100$$

where A = exploration time of familiar object during training, B = exploration time of second identical object during training, C = exploration time of familiar object and location during test, and D = exploration time of novel object or location during test. Range = −100 to +100; positive = discrimination for novel; zero = no discrimination; negative = discrimination for familiar (avoidance of novel). No sex differences were observed.

2.5. Acute Slice Preparation

Mice were anesthetized, decapitated, and their brains were removed using an ice-cold oxygenated (95% O₂/5% CO₂) cutting aCSF bath containing the following (in mM): 206 sucrose, 2.8 KCl, 0.5 CaCl₂, 4.8 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose (pH 7.4). Horizontal sections (200 μm for superoxide detection experiments and 400 μm for electrophysiology) of the ventral hippocampal–entorhinal cortex were sliced on a vibratome (Leica VT1200, Leica Microsystems Inc., Bannockburn, IL, USA) and transferred to a holding chamber for 1 h containing a warm (32 °C), oxygenated (95% O₂/5% CO₂) recording aCSF bath containing the following (in mM): 125 NaCl, 2.8 KCl, 2.4 CaCl₂, 2.5 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose (pH 7.4), as we have previously described [16,38,39].

2.6. Superoxide Detection with MitoSOX

Acute hippocampal slices (200 μm) were incubated in either aCSF, aCSF with MitoQ (500 nM), aCSF with rotenone (100 nM), or aCSF with MitoQ and rotenone for 10 min before bath application of 5 μM mitochondrial superoxide indicator (MitoSOX Red Invitrogen, M36008, Fisher Scientific, Waltham, MA, USA) for an additional 10 min [31]. Slices were immediately fixed with ice-cold 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C. Slices were washed, stained with 0.9 μM DAPI (MP Biomedicals, LLC, Santa Ana, CA, USA), and mounted onto slides with fluorescent mounting media (Fluoromount-G, SouthernBiotech, Birmingham, AL, USA). The stratum pyramidale layer of CA1 and CA3 and the dentate gyrus (DG) granule cell layer of the hippocampal sections were Z-stack imaged at 20× using a Nikon Ti-E confocal microscope, and parameters were held constant for sections of the same experiment. The MitoSOX/DAPI fluorescence intensity of the images was calculated using Fiji software [44]. Micrographs were imported into PowerPoint Microsoft 365 (Microsoft Corp., Redmond, WA, USA) for figure generation and uniformly sharpened using the picture format corrections tool.

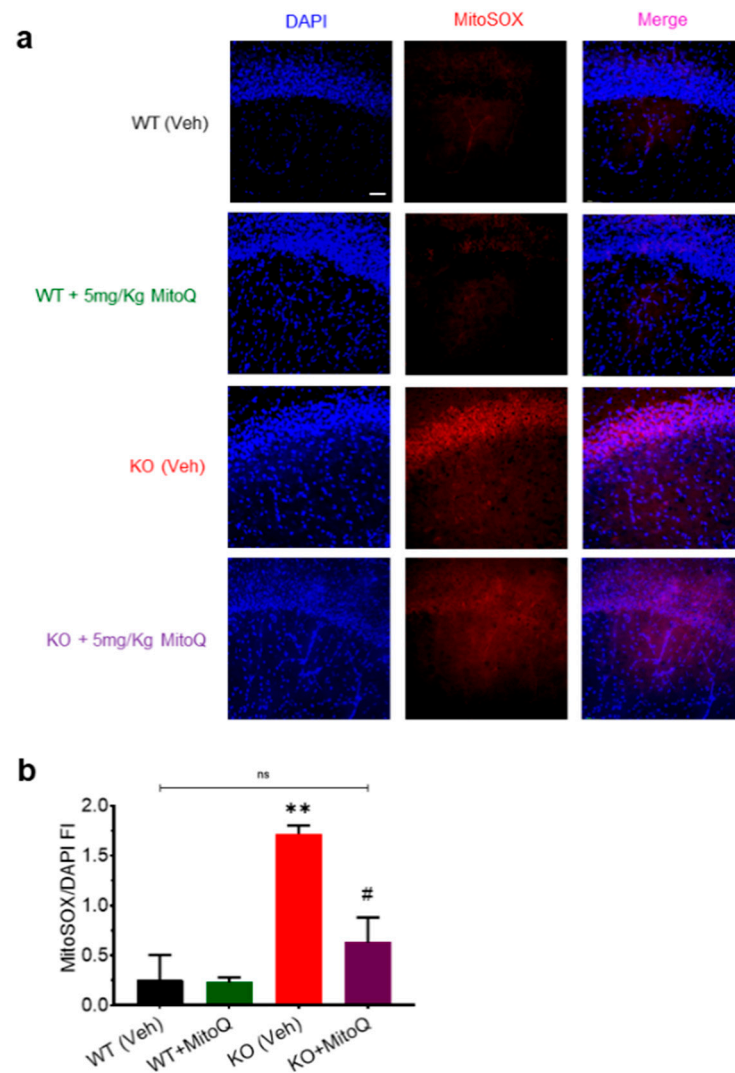


Figure 2. In vivo sub-chronic treatment with MitoQ reduces the high mitochondrial superoxide levels in epileptic KO mice CA1 pyramidal neurons. (a) A 5-day MitoQ (5 mg/Kg) treatment reduced excessive MitoSOX fluorescence signal (red) in the KO to WT levels. DAPI staining is blue. Merged images reveal overlapped staining as purple. Representative $20\times$ confocal images. The post-imaging brightness of the WT micrograph red channel was increased by 40% to demonstrate the presence of tissue. Scale bar 100 μm . (b) MitoQ treatment reduced the fluorescence intensity (FI) of the KO to WT levels. MitoSOX Red FI was quantified in relation to DAPI. Two-way ANOVA with a Tukey multiple comparisons post hoc test. WT: $n = 3$ mice, 3 slices; WT + MitoQ: $n = 3$ mice, 3 slices; KO: $n = 3$ mice, 3 slices; KO + MitoQ: $n = 3$ mice, 3 slices. ns: not significant; ** $p < 0.01$ compared to the WT; # $p < 0.05$ compared to the KO.

2.7. Multi-Electrode Array Recording

Acute hippocampal slices (400 μm) were positioned on a MED64 probe over a multi-microelectrode array and perfused with recording aCSF (1 mL min^{-1} flow rate). Spontaneous and evoked extracellular field recordings were acquired with Med64 Mobius software v3.02 (Alpha MED Scientific Inc., Osaka, Japan), as described in [21,39,45].

Evoked extracellular field recording: Input/output (I/O) curves were generated by stimulating the Schaffer collateral pathway and recording the field excitatory postsynaptic potentials (fEPSPs) in the cornu ammonis 1 (CA1) stratum radiatum and stratum pyramidale. A Boltzmann sigmoidal curve was used to fit the I/O data normalized to the maximum output to determine the stimulation intensity that resulted in 50% of the fEPSP slope (V50). Evoked potentials were generated by paired pulses (40 ms inter-stimuli in-

terval) and recorded. Slopes (10–90%) of the fEPSPs were used to quantify the responses. Paired-pulse ratios (PPR) were calculated by dividing the second response (slope 2) by the first response (slope 1). A steady control baseline was recorded for 15 min and slices in the treatment groups were perfused with either 500 nM MitoQ, 100 nM rotenone, or 500 nM MitoQ and 100 nM rotenone for 20–30 min until the effects plateaued. LTP was induced with a theta burst stimulation TBS protocol (8 bursts with 8 pulses each, 200 Hz burst frequency, 40 ms burst duration, 2 s inter-burst interval) [46] followed by a 60-min recording. Post-TBS, nonlinear regression was used to monoexponentially fit the averaged fEPSP data, and the span and plateau of the fit were used to calculate the amplitude of potentiation for STP and LTP, respectively. STP was calculated as the difference between the maximum and minimum values of the fit. LTP was calculated as the difference between the minimum value of the fit and the average of the 15 min normalized baseline.

Spontaneous network activity recording: Binary Med64 data of spontaneous activity recordings were imported to Spike2 (v7.19) software (Cambridge Electronic Design, Cambridge, UK), rescaled, and filtered to determine sharp waves and high-frequency oscillations (SPWs–HFOs). SPW features (amplitude, duration, and interval) were determined as described in [45]. Filtered spontaneous activity recordings were 100–600 Hz band pass filtered (−3 dB points = 80 Hz and 619 Hz; 1319 filter coefficients) to visualize the HFOs [21,39,45]. A time-frequency analysis of the HFOs was performed with a fast Fourier transform with a size of 2048 points and a hanging window. The time-frequency spectra data sweep of recording was gated with SPW events with a sweep length of 0.125 s and a pre-event time of 0.025 s. The spectral distribution obtained was normalized with power between 97 and 600 Hz to obtain a statistical distribution with unit area, $p(f)$, which was used to determine spectral indices, as described previously [47,48]. The following spectral indices were determined: (i) entropy (in bits)—a measure of frequency dispersion, $-\sum_f p(f) \log_2 p(f)$, maximum at $\log_2 N = 5.7$, with $N = 52$ bins; and (ii) fast ripple/ripple ratio—a measure of fast ripple band proportion = normalized power of fast ripple (200–600 Hz)/ripple (97–199 Hz).

2.8. Spontaneous Recurrent Seizure (SRS) Monitoring

Adult KO mice (P30) were housed individually in a transparent plexiglass cage and allowed to habituate for five days. Mice ~P35 were anesthetized with isoflurane (5% initiation and 3% maintenance) and two subdural, ipsilateral cortical electrodes were implanted at 1.2 mm anterior to the bregma and 1 mm lateral to the midline. A reference electrode was implanted 1.5 mm posterior to the bregma and 1 mm lateral to the midline, contralateral from the recording electrodes. EMG electrode wires were inserted into the nuchal muscles in all mice. Electrodes were soldered to the head mount (Pinnacle Technologies, Inc., Lawrence, KS, USA) and secured to the skull with superglue (Loctite 454). After five recovery days, the head mounts were attached to a tether and video–EEG–EMG recordings were started using a time-synched infrared video surveillance system (Pinnacle Technology, Inc., Lawrence, KS, USA), as previously described [16,37,49–51]. The mice were injected intraperitoneally (i.p.) with vehicle (sterile 20% DMSO in sterile saline) once daily between ZT2 and ZT6 for two days followed by five days of daily i.p. injections of MitoQ (5 mg/kg in 20% DMSO in sterile saline).

EEG recordings were imported into Spike2 v10 software (Cambridge Electronic Design, Cambridge, UK) and generalized seizures were identified using short-time fast Fourier transform time-frequency analysis which were confirmed with behavior from video recordings using Sirenia software (Pinnacle Technology, Inc., Lawrence, KS, USA). Generalized seizure severity (types 2–6) was manually scored, and seizure burden (seizure type $\times$ seizure duration) and seizure frequency were calculated as described by Deodhar

et al. [37]. Seizure behavior was scored using a modified Racine scale for generalized seizures: Type 1—myoclonic jerk; Type 2—head stereotypy; Type 3—bilateral clonus manifested as hunched, forelimb clonus with or without rearing; Type 4—hindlimb clonus with a head tilt, tail extension with 1 or 2 rearing and falling events; Type 5—bilateral clonus and continuous rearing and falling of 3 or more times; Type 6—tonic-clonic seizures involving running, energetic myoclonic jumping, falling, limb tonus and clonus.

2.9. Reverse-Transcriptase Quantitative PCR (RT-qPCR)

Micro-isolated hippocampi were flash-frozen in methyl butane. RNA was isolated from frozen hippocampi tissue using a kit (RNeasy Mini kit, 74104, QIAGEN N.V., Hilden, Germany). The isolated RNA was digested using DNase and the cDNA was prepared using 1 µg of RNA and an Agilent cDNA kit. The cDNA concentration was diluted to 1:1 cDNA dilution with nuclease-free water which was used for amplification with SYBR green (Agilent Technologies Inc., Santa Clara, CA, USA). Custom-designed PCR primers (reported 5' to 3') for superoxide dismutase [*Sod1*: (gene ID: 20655; forward: GCAATGTGACTGCTGGAAAG and reverse: CTCAGACCACACAGGGAATG), *Sod2*: (gene ID: 20656; forward: GTCTCACCATCTTCCTGTCATC and reverse: AGCGGGCAAGTGCTTATT), and ribosomal protein L30 [*Rpl30*: (gene ID: 19946; forward: AAGGCAAAGCGAAGTTG-GTT and reverse: ACCTGGGTCAATGATAGCCA)] were purchased from Integrated DNA Technologies (Newark, NJ, USA). Primers for *Sod3* (gene ID: 20657), catalase (*Cat*; gene ID: 12359), and glutathione peroxidase 3 (*Gpx3*; gene ID: 14778) were purchased from Bio-Rad (Hercules, CA, USA). Fold changes in mRNA transcripts were quantified relative to *Rpl30* and then normalized to WT using the $2^{-\Delta\Delta C_t}$ method. Samples were run in triple or quadruple technical replicates.

2.10. Mitochondrial Polarography

Hippocampal tissues were collected, quickly micro-dissected on ice, and homogenized to isolate mitochondria using differential centrifugation [16]. Protein concentrations of isolated mitochondria were determined with a Bradford assay (Bio-Rad). Mitochondria (100 µg) were resuspended in a KCl respiration buffer (in mM: 125 KCl, 20 HEPES, 2 MgCl₂, 2.5 KH₂PO₄, pH 7.2) and placed in a sealed, thermostatically controlled chamber at 37 °C. Mitochondria were energized via ATP-producing NADH as follows: coenzyme Q oxidoreductase (MRCI)-driven state III respiration with 5 mM pyruvate and 2.5 mM malate and concurrent activation of adenosine triphosphate synthase by 150 µM adenosine diphosphate. State III respiration with or without 100 nM rotenone or 400 µM ascorbic acid was determined using a standard Clark-type electrode (Hansatech Instruments, Ltd., Norfolk, UK), the rate of oxygen consumption was determined with the line of best-fit algorithm, and nmol of oxygen mg⁻¹ min⁻¹ was calculated as described previously [16].

2.11. Statistical Analysis

All data are presented as mean ± SEM. Kolmogorov–Smirnov and Bartlett's tests were used to test the normality and homoscedasticity of the residuals, respectively. No exclusions resulted. One-way or two-way ANOVA with a Tukey multiple comparisons post hoc test and unpaired and paired *t*-tests were performed where appropriate. All statistical analyses were conducted with Prism 10 software (GraphPad Software, Inc., La Jolla, CA, USA), and *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Excessive Mitochondrial Superoxide in Epileptic KO Mice Hippocampi Is Attenuated by MitoQ

To determine the levels of superoxide in the hippocampus, live ventral slices were incubated with MitoSOX Red, a fluorogenic dye targeted to mitochondria that fluoresces red when oxidized by superoxide and imaged. Excessive mitochondrial superoxide was evident in the CA1, CA3, and dentate gyrus (DG) hippocampal subregions of KO mice compared to WT (Figures 2a,b, S2 and S3), providing spatial resolution to our previous report of elevated superoxide levels in isolated hippocampal mitochondria [16]. In vivo administration of the mitochondria-targeted antioxidant MitoQ (i.p., 5 mg/kg/day for five days) significantly reduced the KO superoxide to WT levels. These findings indicate that, in this preclinical model of TLE, mitochondrial superoxide levels are abnormally high and can be attenuated with MitoQ treatment.

3.2. Sub-Chronic Treatment with MitoQ Rescues Impaired Memory and Synaptic Plasticity in KO Mice

Previous studies have demonstrated impairment of learning and memory in KO mice using the Barnes maze test [17]. Here, we found similar impairment in two hippocampal-dependent tests of spatial (NLR) and non-spatial (NOR) memory (Figure 3a). The KO mice failed to recognize the novel locations of previously introduced objects in the NLR test as represented by negative discrimination index scores (Figure 3b). The KO mice also failed to recognize newly introduced objects as novel in the NOR test (Figure 3c). The MitoQ treatment restored the KO performance to WT levels on both these memory tasks. The MitoQ treatment did not alter discrimination in the WT mice, suggesting beneficial effects only in pathologic environments.

Anxiety and motor deficits could affect the outcomes of NLR and NOR. Therefore, we evaluated mouse behavior in the open field chambers used for NLR and NOR during habituation and quantified indicators of anxiety (thigmotaxis index, thigmotaxis time, and locomotor activity). We found no significant differences between the groups for any parameter (Supplementary Figure S3).

Impairment of LTP at the CA3–CA1 synapse was previously demonstrated in KO hippocampal slices [28,29]. To determine whether mitochondrial superoxide contributes to reduced LTP, ex vivo hippocampal slices from the mice that received the in vivo treatment were placed on a multielectrode array. The CA3 Schaffer collaterals were activated with a theta burst stimulation (TBS) and the extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 *stratum radiatum* (Figure 3d). We found a similar impairment of LTP in the KO slices, and additional analyses also revealed reduced STP (Figure 3e–h). An examination of the slices from the mice treated with MitoQ indicated that the KO STP and LTP were restored and rescued, respectively. Interestingly, MitoQ reduced LTP in the WT, suggesting that typical levels of superoxide promote LTP at the WT synapses, which is in agreement with previous reports [19,52–54]. Collectively, these results indicate that elevated mitochondrial superoxide contributes to hippocampal-dependent memory deficits in KO mice, which are rescued with MitoQ.

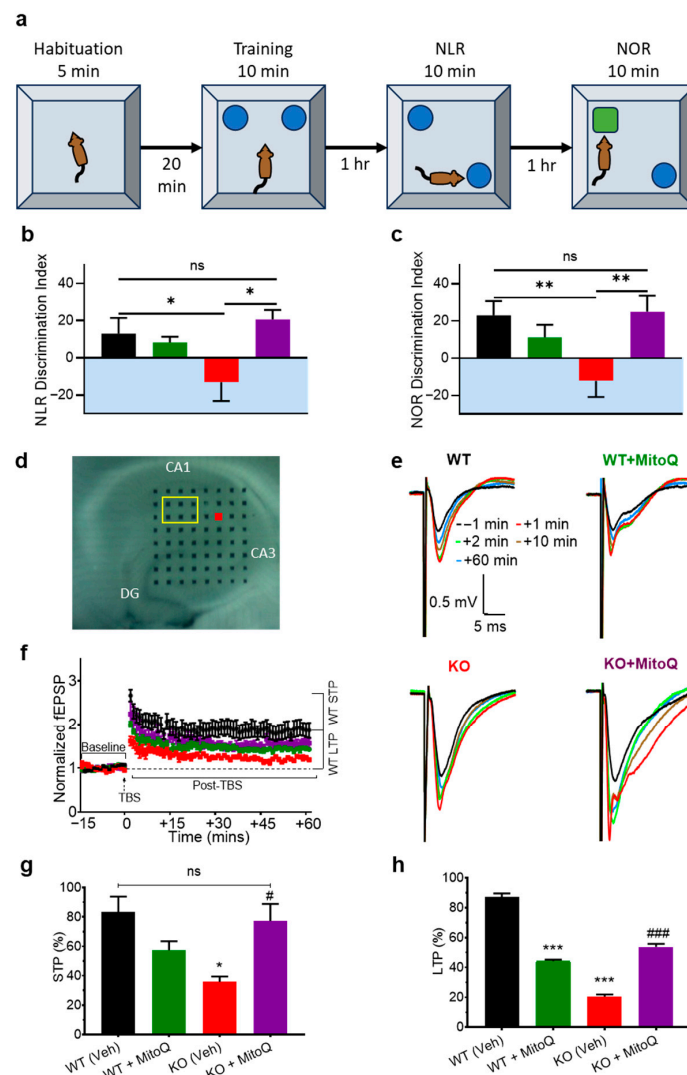


Figure 3. In vivo sub-chronic treatment of epileptic KO mice with MitoQ rescues impaired memory and synaptic plasticity. (a) Illustration of the sequence of procedures for NLR and NOR on the experimental day. The discrimination indices of (b) NLR and (c) NOR indicated KO mice displayed no discrimination in either test (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). MitoQ treatment restored discrimination to WT levels. Two-way ANOVA with a Tukey multiple comparisons post hoc test. WT: $n = 15$ mice; WT + MitoQ: $n = 6$ mice; KO: $n = 15$ mice; KO + MitoQ: $n = 7$ mice. ns: not significant, $* p < 0.05$, $** p < 0.01$. (d) A photomicrograph of a mouse hippocampal slice on a 64 multielectrode array. An electrode underlying the Schaffer collaterals is used for stimulation (filled red square) eliciting the fEPSPs recorded by electrodes in the CA1 stratum radiatum (open yellow rectangle). (e) Example fEPSPs from timepoints prior to and post-TBS stimulation for each genotype/treatment. (f) The KO CA3–CA1 synapse has reduced fEPSP plasticity after TBS (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). STP and LTP are presented as percentages. The reduced magnitude of the KO (g) STP and (h) LTP were rescued with MitoQ. WT + Veh: $n = 3$ mice, 3 slices, 7 electrodes; WT + MitoQ: $n = 3$ mice, 3 slices, 5 electrodes; KO + Veh: $n = 3$ mice, 4 slices, 5 electrodes; KO + MitoQ: $n = 3$ mice, 4 slices, 8 electrodes. ns: not significant, $* p < 0.05$ and $*** p < 0.001$ compared to the WT; # $p < 0.05$, ### $p < 0.001$ compared to the KO.

3.3. Sub-Chronic Treatment with MitoQ Has No Effect on the SRSs of Epileptic KO Mice

Seizures may detrimentally affect memory [55], raising the possibility that improvements in cognitive performance by reducing excessive superoxide in vivo are the result of preventing seizures rather than a direct effect on mechanisms of cognitive impairment. Hence, we monitored the SRSs in the KO mice using video-EEG and administered vehicle

for two days followed by MitoQ (5 mg/kg/day) for five days. KO mice experience multiple types of generalized convulsive seizures of varying durations, as we have described previously (Figure 4a) [37]. Therefore, we performed a number of seizure analyses to determine any effects that may be masked by a gross daily count. We analyzed the SRS frequency by hourly counts (Figure 4b), cumulative probability as a function of time post-injection, to account for any pharmacokinetic properties preventing the detection of transient anti-seizure effects (Figure 4c), seizure frequency normalized to vehicle in 4-h bins (Figure 4d), severity (i.e., seizure type), and burden (i.e., severity*duration) (Figure 4e). Regardless of the method of analysis, the MitoQ treatment had no significant effect on the SRSs. Together these data suggest excessive mitochondrial superoxide does not actively participate in seizure genesis, and that MitoQ-induced cognitive improvement is not an indirect effect of SRS reduction.

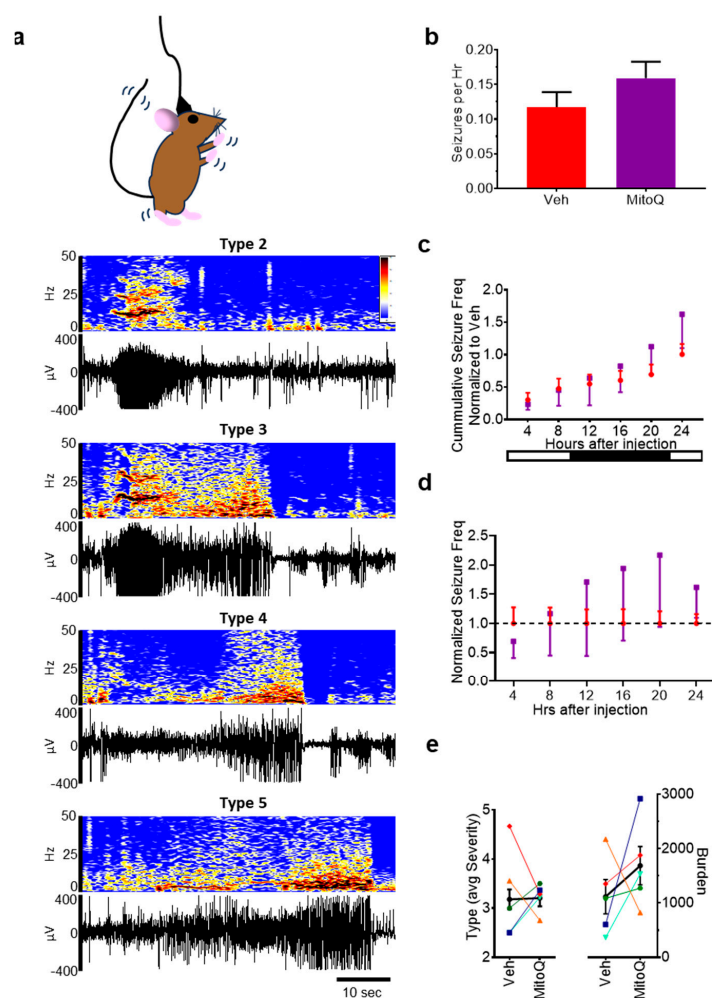


Figure 4. Sub-chronic treatment MitoQ has no effect on SRSs. (a) Illustration of a tethered mouse in a rearing Type 5 seizure. Time frequency analyses with corresponding trace of examples of seizure types recorded from one KO mouse. (b) Quantification of number of SRSs per hour of KO mice. (c) Cumulative SRS frequency over 24 hours post-injection of vehicle (red) or MitoQ (purple). Bottom bar represents light–dark periods (empty-filled, respectively) relative to the hours post-injection. (d) Normalized SRS frequency per 4-h bin post-injection of vehicle (red) or MitoQ (purple). (e) Average SRS types (left) and burdens (right) during vehicle and MitoQ. Individual mice represented by colored symbols and group means by filled black circles. Paired *t*-test or repeated measures by two-way ANOVA (*n* = 5 mice).

3.4. Acute Administration of MitoQ Ex Vivo Exerts Similar Effects as In Vivo Treatment at CA3–CA1 Synapses in WT and KO at Baseline

To determine whether the physiological effects of excessive superoxide require long-term exposure, we performed a series of experiments using ex vivo hippocampal slices from naïve mice and acutely administered MitoQ. Similar to sub-chronic in vivo treatment, acute application of MitoQ (500 nM for 10 min) significantly reduced the KO superoxide to WT levels throughout the hippocampal slice (Figures 5, S4 and S5).

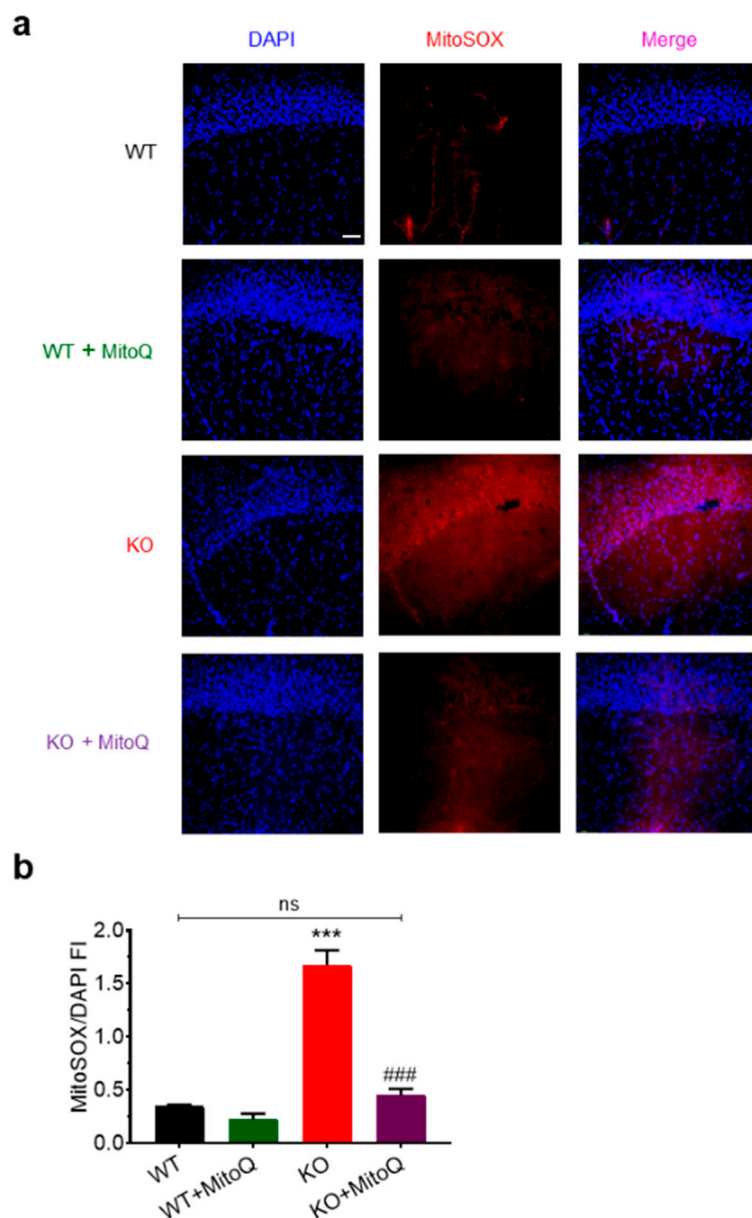


Figure 5. Acute application of MitoQ ex vivo reduces excessive superoxide in epileptic KO mice CA1. (a) Treatment of ventral hippocampal slices with MitoQ (500 nM) for 10 min reduced the significantly higher MitoSOX fluorescence signal (red) in the KO CA1 to WT levels. DAPI staining is blue. Merged images reveal overlapped staining as purple. Representative 20× confocal images. The post-imaging brightness of the WT and WT + MitoQ micrographs red channel was increased by 40% to demonstrate the presence of tissue. Scale bar 100 µm. (b) MitoQ treatment reduced the significantly higher fluorescence intensity (FI) of the KO to WT levels. MitoSOX Red FI was quantified in relation to DAPI. Two-way ANOVA with a Tukey multiple comparisons post hoc test. WT: $n = 3$ mice, 3 slices; WT + MitoQ: $n = 3$ mice, 3 slices; KO: $n = 3$ mice, 3 slices; KO + MitoQ: $n = 3$ mice, 3 slices. ns: not significant; *** $p < 0.001$ compared to the WT; ### $p < 0.001$ compared to the KO.

In a separate cohort of slices, we evaluated basal CA3–CA1 synaptic transmission by stimulating the CA3 Schaffer collaterals and recording fiber volleys, a measure of recruited axons, and dendritic field excitatory postsynaptic potentials (fEPSPs) from the CA1 stratum radiatum (Figure 6a,b *top*). Input/output curves generated by plotting the slope of the dendritic fEPSP versus the stimulation intensity indicated that the KO synapse was more excitable, requiring 23% lower stimulation intensity to evoke a half-maximal (V_{50}) response (WT, $45.7 \pm 0.8 \mu\text{A}$ vs. KO, $35.1 \pm 0.5 \mu\text{A}$; $p < 0.001$) (Figure 6c). The KO fiber volleys and dendritic fEPSPs were 100% and 277% larger, respectively, than WT levels (Figures 6b and S6a–c), similar to our previous findings at the medial perforant path–CA3 and mossy fiber–CA3 synapses [39]. Nonlinear fits of normalized fEPSPs as a function of normalized fiber volleys (FV–E coupling) revealed a leftward shift of the coupling between presynaptic input to postsynaptic output, suggesting that the increased recruitment KO fibers was not entirely responsible for the increased dendritic fEPSPs (Figure 6d). We further examined the excitability of the CA1 neurons by recording the somatic fEPSPs and population spikes, a measure of elicited postsynaptic action potentials, from the CA1 stratum pyramidale (Figure 6a,b *bottom*). The KO somatic fEPSPs and population spikes were each 100% larger than WT levels (Supplementary Figure S6d,e). Quantifying the coupling of the somatic fEPSPs with population spikes (E–S coupling) revealed a leftward shift of the coupling between somatic depolarization and action potential firing, indicating that the KO CA1 neurons are more excitable than the WT (i.e., firing occurred with less excitatory input) (Figure 6d). The effects of MitoQ were apparent within 5 min after perfusion and plateaued in ~20 min. MitoQ increased the KO dendritic and somatic fEPSPs to a greater degree than the WT but did not affect fiber volleys or population spikes. Furthermore, MitoQ did not significantly affect the fEPSP V_{50} s, FV–E, or E–S couplings of either genotype (see Figures 6 and S6). Together these data indicate that excessive mitochondrial ROS reduces synaptic efficacy but does not affect excitability.

3.5. Acute Administration of MitoQ Ex Vivo Rescues KO Short- and Long-Term Potentiation (STP and LTP)

Finally, we assessed the effects of acute pretreatment of MitoQ on the STP and LTP of the CA1 fEPSPs (STP and LTP) following TBS. Similar to our *in vivo* treatment data (Figure 3h), acute perfusion of MitoQ reduced LTP in the WT slices (Figure 6h). This finding supports previous reports that normal levels of superoxide actively participate in the maintenance of LTP [19,52–54]. MitoQ restored the KO STP and increased the KO LTP to levels comparable to the WT (Figure 6f–h), suggesting that excessive superoxide interferes with mechanisms of STP and LTP maintenance.

3.6. Acute Administration of MitoQ Ex Vivo Does Not Impact Presynaptic Facilitation

Presynaptic mechanisms that increase neurotransmitter release probabilities can contribute to STP and LTP [56]. We examined the paired-pulse ratios (PPRs) after TBS to determine whether neurotransmitter release probabilities could account for reduced STP and LTP at the KO synapses. As expected, the PPRs decreased immediately following TBS in all groups, reflecting decreased facilitation and increased neurotransmitter release probabilities relative to baseline levels. The PPRs gradually returned to baseline levels, mirroring the transition of STP into LTP (Figure 6g). There were no differences between the groups, suggesting impaired KO STP and LTP and the restorative effects of MitoQ may not involve presynaptic changes in neurotransmitter release probability. Thus, impairment of STP and LTP may involve postsynaptic consequences of excessive mitochondrial superoxide. Together these data indicate that, post-TBS, superoxide has opposing effects at the WT and KO synapses, promoting LTP in the WT and interfering with STP and LTP in the KO.

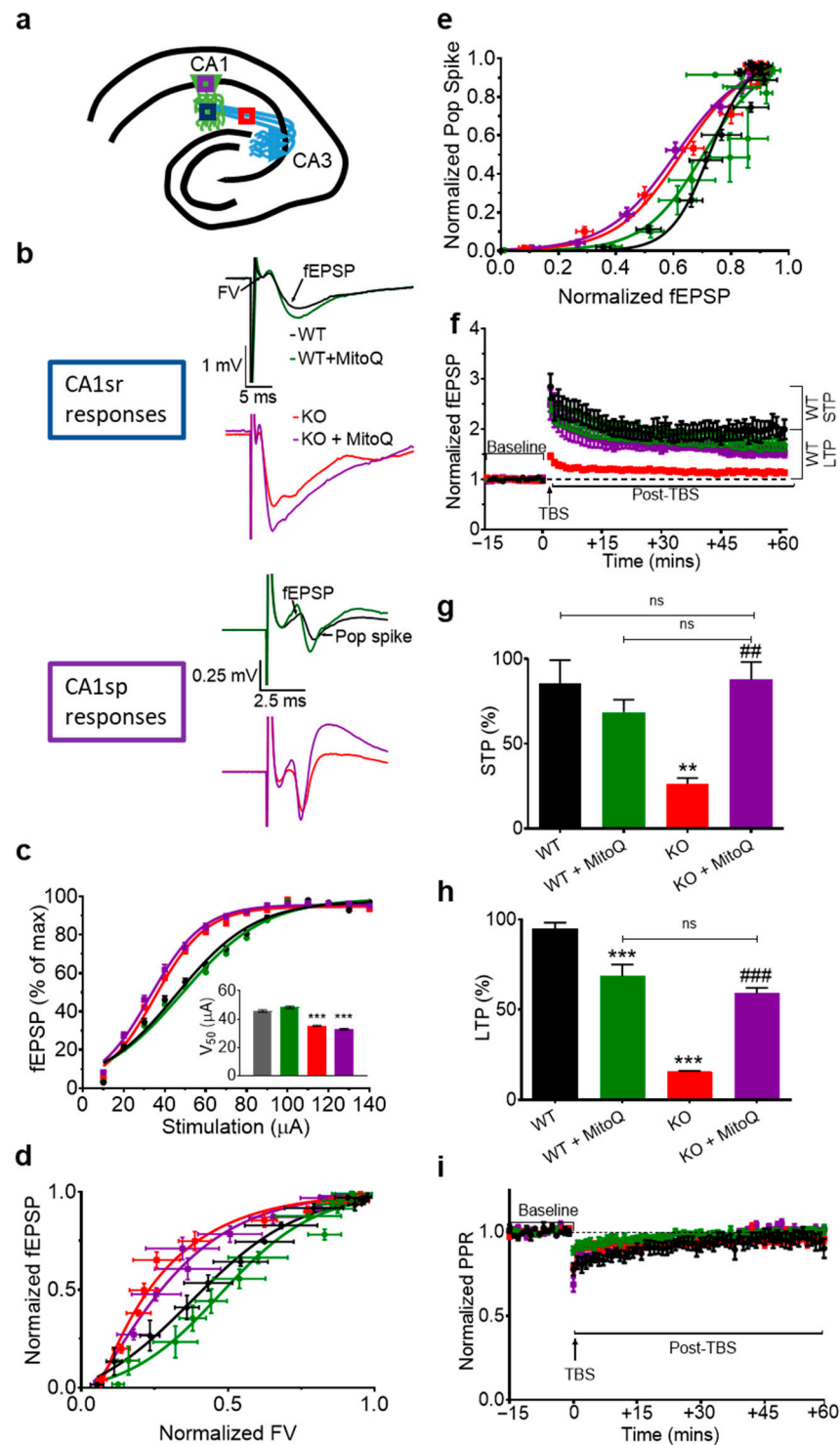


Figure 6. Acute MitoQ treatment ex vivo rescues STP and LTP but fails to limit hyperexcitability. (a) Schematic of a ventral hippocampal slice with a stimulation electrode (red) in the CA3 Schaffer collaterals, recording electrode (purple) in the CA1 stratum pyramidale (sp), and recording electrode (blue) in the CA1 stratum radiatum (sr). (b) Representative traces of the dendritic fEPSP and FV traces (top two) and the somatic fEPSP and population spike trace (bottom two) from the WT and KO slices before and after treatment with 500 nM MitoQ. (c) Synaptic I/O relationships indicate reduced V_{50} (inset) at the CA3–CA1 synapse of the KO compared to the WT. (WT, black; WT + MitoQ, green;

KO, red; KO + MitoQ, purple). MitoQ had no effect on V_{50} . WT: $n = 5$ mice, 7 slices, 28 electrodes; KO = 5 mice, 5 slices, 24 electrodes. (d) FV-fEPSP coupling differed between the KO and WT but was unaffected by MitoQ. (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). WT: $n = 3$ mice, 4 slices, 5 electrodes; KO = 4 mice, 4 slices, 6 electrodes. (e) fEPSP-to-spike (ES) coupling differed between the KO and WT but was unaffected by MitoQ. (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). WT: $n = 2$ mice, 2 slices, 3 electrodes; KO = 5 mice, 5 slices, 17 electrodes. Two-way ANOVA with a Tukey multiple comparisons post hoc test. (f) The KO CA3–CA1 synapse has reduced fEPSP plasticity after TBS (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). The reduced magnitude of KO (g) STP and (h) LTP were rescued with MitoQ. (i) The post-TBS PPR was not different between the groups. (WT, black; WT + MitoQ, green; KO, red; KO + MitoQ, purple). WT: $n = 4$ mice, 4 slices, 12 electrodes; WT + MitoQ: $n = 5$ mice, 5 slices, 12 electrodes; KO: $n = 4$ mice, 5 slices, 9 electrodes; KO + MitoQ: $n = 6$ mice, 6 slices, 13 electrodes. Two-way ANOVA with a Tukey multiple comparisons post hoc test. ns: not significant; ** $p < 0.01$ and *** $p < 0.001$ compared to the WT; ## $p < 0.01$ and ### $p < 0.001$ compared to the KO.

3.7. Acute Administration of MitoQ Ex Vivo Has a More Profound Impact on KO Hippocampal Network Oscillations

Previously, we found that the KO hippocampi exhibit a higher incidence of spontaneous SPWs–HFOs that are generated in the CA3 and propagate to the CA1 [52,53]. SPWs associated with HFOs in the ripple band (100–200 Hz) are network correlates of memory consolidation [57–60]. Here, we examined the SPW-ripples in the CA1 of the KO and WT hippocampi (Figure 7a). Similar to our previous results, the KOs displayed faster and smaller SPW-ripples, with a mean interevent interval 47% shorter than the WTs and 85% smaller amplitude (Figure 7b,c).

Acute treatment with MitoQ modestly increased the interevent interval of the WT SPW-ripples by 11%, thereby reducing their incidence, and the amplitude by 8%, suggesting basal superoxide levels minimally but significantly contribute to normal SPW-ripple oscillations. MitoQ exerted an even greater impact on the KO SPW-ripples, slowing the incidence to near-WT levels (i.e., increasing the interevent interval by 58%), and increasing the amplitude by 113%. These findings suggest that excessive mitochondrial superoxide has an active role interfering with the architecture of memory-associated network oscillations in the KO hippocampi. Specifically, superoxide is primarily responsible for more frequent KO SPW-ripple complexes and contributes to the reduced amplitude.

3.8. Acute Administration of MitoQ Ex Vivo Does Not Impact KO Pathologic High-Frequency Oscillations

The KO hippocampi also exhibit pathologic fast ripples, high-frequency oscillations > 200 Hz that are associated with hyperexcitable networks and are biomarkers for seizure-onset zones in epilepsy [38,39,61]. Ripples and fast ripples can be visualized with time-frequency analyses, demonstrating that these oscillations in the KO CA1 have a disorganized power spectrum (Figure 7d). Using analytical methods to measure entropy (i.e., spectral dispersion) and the fast ripple/ripple ratio within the signal [47,48], we found that the KO entropy and fast ripple/ripple ratio were 36% and 277% larger than the WT, respectively (Figure 7e,f). MitoQ did not affect either parameter, suggesting that excessive mitochondrial superoxide does not participate in ongoing pathologic high frequency network activity. The lack of effect on ex vivo measures of synaptic, cellular, and network hyperexcitability mirrors and supports our findings of a lack of effect on in vivo SRSs.

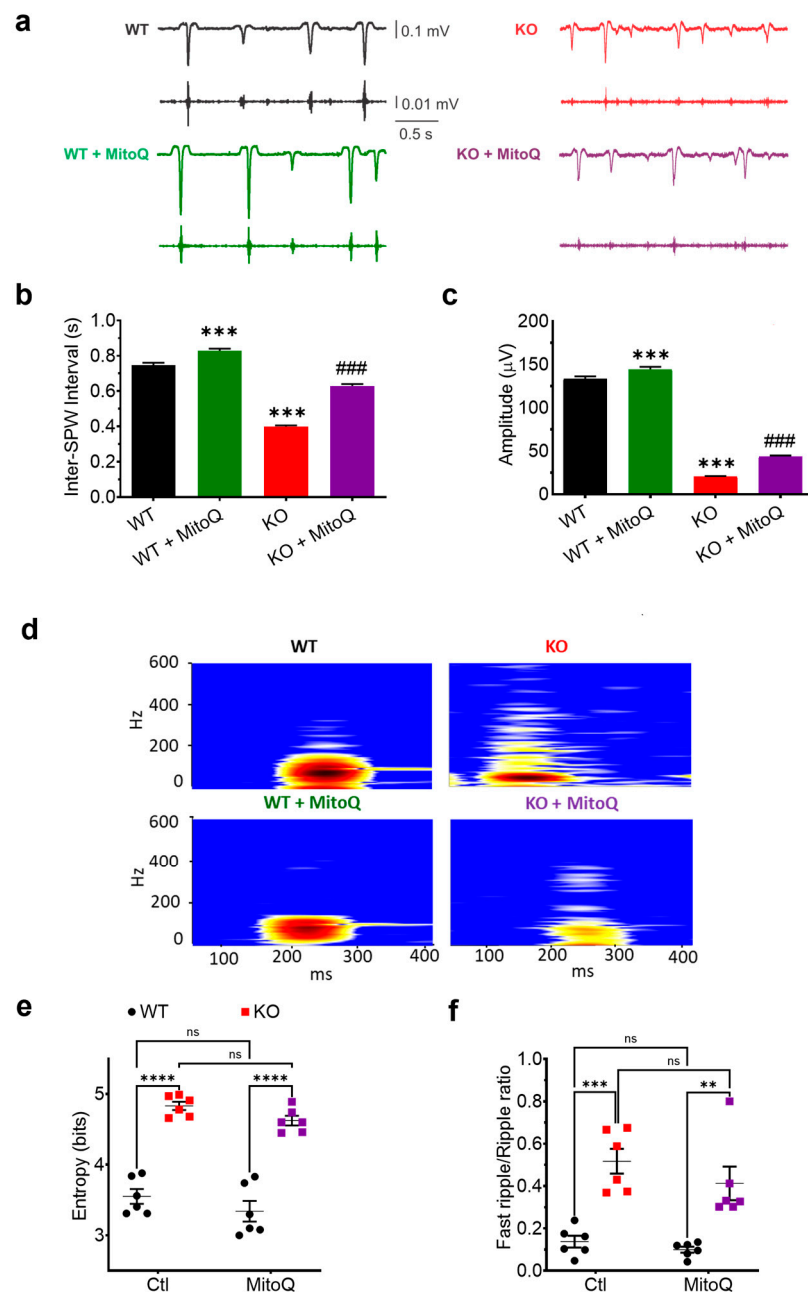


Figure 7. Excessive superoxide alters the SPW architecture but is not involved in pathologic fast ripples in the CA1 *stratum radiatum* of epileptic KO mice. (a) Representative spontaneous SPW recordings (top traces) from the KO and WT slices before and after treatment with MitoQ (500 nM). Band pass filtering revealed concurrent HFOs (bottom traces). (b) Inter-SPW intervals were shortened significantly in the KO compared to the WT, indicating an increased SPW incidence. MitoQ treatment increased the inter-SPW interval significantly in both genotypes. (c) SPW amplitudes were significantly reduced in the KO compared to the WT. MitoQ treatment significantly increased the SPW amplitude in both genotypes. (d) Representative time-frequency spectra from the WT and KO slices before and after treatment with MitoQ (500 nM). (e) Spectral entropy. The KO high-frequency oscillation (HFO) spectra was significantly dispersed compared to the WT, which was not restored with MitoQ. (WT+MitoQ, purple). (f) The KO had a significantly larger fast ripple/ripple ratio compared to the WT control, which was not reduced with MitoQ. (WT+MitoQ, purple). WT: $n = 5$ mice, 6 slices; KO: $n = 6$ mice, 6 slices. Two-way ANOVA with a Tukey multiple comparisons post hoc test. ns: not significant; ** $p < 0.001$, *** $p < 0.001$ and **** $p < 0.001$ compared to the WT; ### $p < 0.001$ compared to the KO.

3.9. KO Hippocampi Have Less Superoxide Dismutase

As a reductant, superoxide typically undergoes dismutation by superoxide dismutase (SOD) to form hydrogen peroxide (which is considered less reactive than superoxide). In experiments to determine the source of elevated levels of superoxide in the KO hippocampus, we determined the mRNA expression of key antioxidant genes. We found reduced expression of cytosolic/mitochondrial copper/zinc superoxide dismutase and mitochondrial manganese superoxide dismutase (*Sod1* and *Sod2*, respectively), but normal levels of extracellular copper/zinc superoxide dismutase (*Sod3*), mitochondrial glutathione peroxidase (*Gp3x*), and peroxisomal/mitochondrial catalase (*Cat*), suggesting a potential mechanism contributing to increased superoxide (Figure 8a).

3.10. KO Hippocampi Exhibit ROS-Mediated Inhibition of Mitochondrial Respiratory Complex I (MRCI)

ROS can inhibit MRCI activity [62]. Two ROS species are superoxide, and their dismutation products are hydrogen peroxide. We have previously demonstrated that the isolated KO hippocampal mitochondria have reduced MRCI-driven respiration, which is mimicked in the WT mitochondria by exposure to hydrogen peroxide [16]. We therefore tested the hypothesis that ROS inhibition contributes to KO MRCI dysfunction. Here, we isolated the mitochondria and found that the KO MRCI-driven respiration was 37% lower than the WT, supporting our previous findings (Figure 8b) [16]. Following application of the general antioxidant ascorbic acid (AA, 400 μ M), O₂ consumption by the WT and KO mitochondria increased by 46% and 80%, respectively; thus, reducing the ROS acutely restored KO respiration to WT levels (Figure 8b). These data suggest that MRCI experiences a baseline level of ROS-dependent inhibition which was significantly more pronounced in the KO mitochondria.

3.11. Inhibition of WT MRCI Recapitulates the KO Ex Vivo Phenotype

To determine whether MRCI inhibition contributes to the observed effects in the KO mice, the MRCI inhibitor rotenone was applied to the WT slices and the effects on hippocampal synaptic plasticity were determined. MRCI inhibition promoted the excessive generation of mitochondrial superoxide, supporting previous reports [63] (Supplementary Figure S7a,b). In the presence of rotenone, fEPSPs, STP, and LTP were significantly reduced by 16%, 49%, and 48% (Figure 8c–f), respectively. Rotenone increased the incidence of network SPWs–HFOs by reducing the mean interevent interval by 61% and decreased the SPW amplitude by 23% compared to baseline levels (Figure 8g,h), resembling the KO phenotype. Rotenone also promoted spectral dispersion, as entropy increased by 21% and the fast ripple/ripple ratio increased by 181%, suggesting enhanced network excitability (Figure 8i,j). Co-application of MitoQ with rotenone prevented the overproduction of superoxide and rescued fEPSP, STP, LTP, and SPW incidence and amplitude, but did not affect HFO entropy or the fast ripple/ripple ratio. These data support the acute detrimental effects by mitochondrial superoxide on synaptic plasticity and indicate that, while intact MRCI function limits ROS production, basal levels participate in the normal hippocampal synaptic plasticity, network excitability, and rhythms associated with learning and memory.

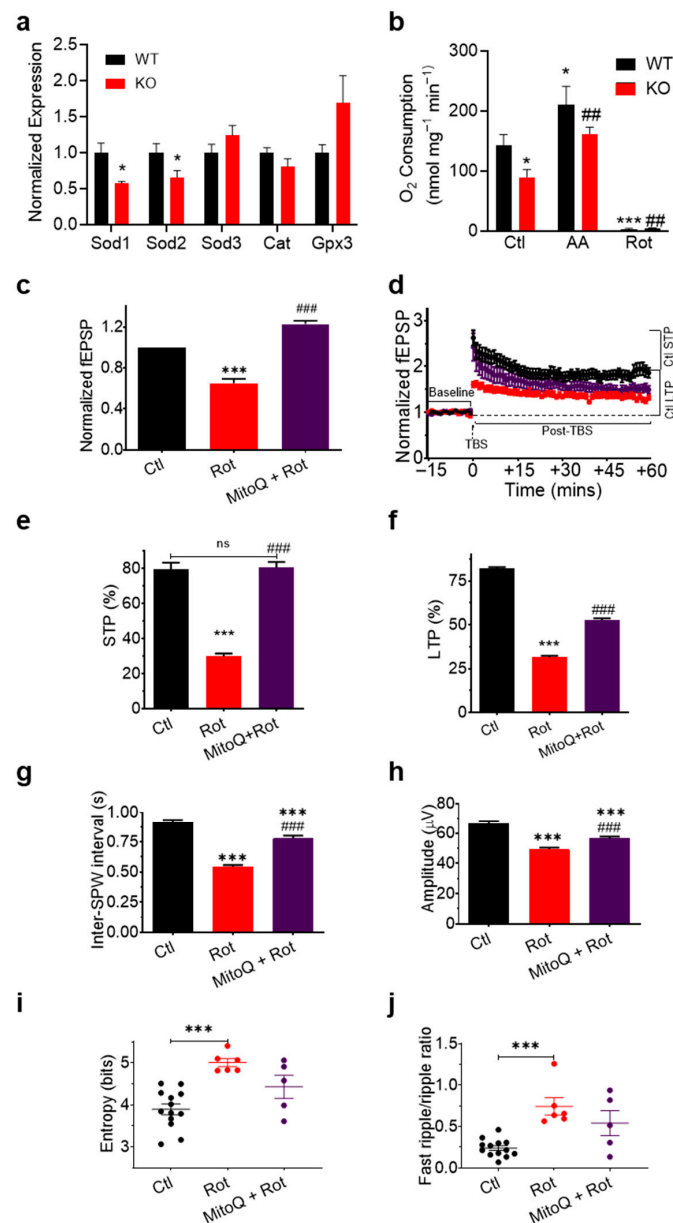


Figure 8. MRCI inhibition impairs plasticity and alters network oscillation but it is the resulting superoxide that is responsible. (a) The KO hippocampi have decreased mRNA transcripts for *Sod1* and *Sod2*. Gene expression was quantified relative to *Rpl30* and normalized to the WT. Unpaired *t*-test with Welch's correction ($n = 5\text{--}12$ mice per group). (b) MRCI O₂ consumption of isolated hippocampal mitochondria is reduced in the KO, increased by ascorbic acid (AA, 400 μM) and decreased by rotenone (Rot, 100 nM). Two-way ANOVA with a Sidak multiple comparisons test ($n = 4\text{--}8$ mice per group). (c) Rotenone (100nM) reduces the fEPSP which is prevented by MitoQ. One-way ANOVA with a Tukey multiple comparisons post hoc test. Control (Ctl): $n = 4$ mice, 4 slices, 29 electrodes; Rotenone (Rot): $n = 4$ mice, 4 slices, 29 electrodes; Rot + MitoQ: $n = 4$ mice, 4 slices, 15 electrodes. (d) Rotenone reduced fEPSP plasticity after TBS (control, black; rotenone, red; rotenone + MitoQ, purple). The reduced magnitude of rotenone (e) STP and (f) LTP were rescued with MitoQ. One-way ANOVA with a Tukey multiple comparisons post hoc test. Ctl: $n = 4$ mice, 4 slices, 17 electrodes; Rot: $n = 4$ mice, 4 slices, 14 electrodes; Rot + MitoQ: $n = 4$ mice, 4 slices, 9 electrodes. MitoQ also rescued rotenone reduction of (g) SPW interval and (h) amplitude. Rotenone increased (i) spectral entropy and (j) the fast ripple/ripple ratio. MitoQ did not affect these parameters. One-way ANOVA with a Tukey multiple comparisons post hoc test. Ctl: $n = 9$ mice, 13 slices; Rot = 5 mice, 6 slices; Rot + MitoQ = 4 mice, 5 slices. * $p < 0.05$ and *** $p < 0.001$ compared to the WT; ## $p < 0.01$ and ### $p < 0.001$ compared to the KO.

4. Discussion

This study demonstrates that cognitive impairment in a preclinical model of TLE is reversible through a mitochondria-targeted antioxidant treatment. Epileptic KO mice exhibited elevated hippocampal mitochondrial ROS, impaired recognition memory, disrupted short- and long-term synaptic plasticity, and abnormal hippocampal SPW-ripple oscillations. Both acute and sub-chronic treatments with the mitochondria-targeted antioxidant MitoQ restored memory performance, synaptic plasticity, and network activity to wild-type levels while reducing the mitochondrial superoxide burden. These effects occurred in the absence of altering seizures and neuronal or network excitability, indicating that cognitive impairments are mechanistically separable from hyperexcitability and the seizure phenotype.

4.1. Mitochondrial Function and Superoxide Generation

Among the complex functions of mitochondria, movement of electrons and the generation of ATP and superoxide are particularly important. During normal electron transport chain function, electrons flow from the substrates NADH and FADH₂ at MRCI and MRCII, respectively, to ubiquinone, then to MRCIII, and ultimately to MRCIV, where they reduce oxygen to water. This process shuttles protons to the inner membrane space and establishes a proton gradient which drives ATP production. However, electrons can escape and bind molecular oxygen to form superoxide. The majority of superoxide is generated at MRCI [64]. Superoxide undergoes dismutation to hydrogen peroxide via SOD1 (located in the mitochondria and cytosol) and SOD2 (restricted to the mitochondria) but can also cross membranes to participate in cell signaling. Of note, MRCI is not only the primary superoxide generator, but it is also susceptible to ROS interference [16,63]. Here, we found ROS inhibition to underlie the KO MRCI dysfunction.

MRCI dysfunction, associated with several neurological disorders, increases electron formation and superoxide production while dissipating the proton gradient [65]. This impairs two processes critical for synaptic transmission: mitochondrial calcium buffering and ATP production. Consequently, altered local levels of superoxide, calcium, and ATP have the potential to directly impact both presynaptic and postsynaptic signaling.

4.2. MitoQ Mechanism and Effects on Superoxide

MitoQ is a ubiquinone designed to cross the blood–brain barrier and to accumulate in the mitochondria via its lipophilic cationic triphenylphosphonium tail [30]. Within the mitochondria, MitoQ facilitates electron transport to MRCIII, increasing successful electron transfer and reducing superoxide-forming leaked electrons. In this study, MitoQ rescued deficient basal synaptic functions, plasticity, and oscillations important for learning and memory, implicating mitochondrial superoxide in the mechanism of impairment.

In the epileptic hippocampus of the KO mice, both *Sod1* and *Sod2* expression were reduced, corresponding to elevated superoxide levels. This is consistent with prior observations that excitotoxic conditions, like those occurring during seizures, suppress the neuronal PKD1/IKK/NF- κ B/SOD2 pathway to reduce *Sod2* expression [66]. Sub-chronic MitoQ treatment effectively reduced superoxide production by improving electron transport efficiency. Remarkably, superoxide levels decreased within 10 min of acute MitoQ application, suggesting superoxide generation had been occurring at a higher rate than dismutation by the existing (yet diminished) SOD enzymes. MitoQ presumably re-established the homeostatic generation–dismutation balance, normalizing the KO superoxide levels to WT levels.

4.3. Superoxide and Memory Function

Experimental elevation of superoxide (and its subsequent reactive species) through *Sod2* knockout in specific cell types impairs synaptic plasticity, LTP, and hippocampal-dependent short-term memory [67,68]. Studies in preclinical models of other neurological disorders, including Alzheimer's disease, Angelman syndrome, and traumatic brain injury, have demonstrated that synaptic dysfunction and memory deficits can be restored by MitoQ administration or *Sod2* overexpression [31–35]. These findings strongly support the role of elevated mitochondrial superoxide in synaptic dysfunction and memory deficits.

The current study extended these findings to preclinical TLE mice, revealing similar synaptic dysfunction and memory deficits. Sub-chronic MitoQ treatment restored the performance of the KO mice on two hippocampal-dependent memory tasks: spatial (NLR) and non-spatial (NOR). MitoQ treatment did not alter discrimination in the WT mice, suggesting its beneficial effects may be limited to pathological conditions.

4.4. Superoxide Effects on Basal Synaptic Function and Synaptic Plasticity

Mitochondria are essential for both pre- and postsynaptic functions. They are dynamic organelles that travel along neuronal processes [69]. Presynaptic neurotransmitter release depends on numerous factors, but one significant factor is that the neurotransmitter vesicle number correlates with mitochondria abundance in hippocampal synaptosomes [70,71]. Postsynaptically, the magnitude of an elicited response is determined in part by the presence of mitochondria at the synapse [69].

In this study, superoxide actively inhibited the postsynaptic CA1 dendritic fEPSPs following single stimulation at both the WT and KO CA3–CA1 synapses, which was relieved with MitoQ. Superoxide is known (i) to promote phosphorylation of several protein kinases, (ii) to inhibit activated phosphatases, and (iii) to diffuse into cytosol and extracellular space to interact with sodium channels, AMPA receptors, and GABA_A receptors, thereby influencing neuronal excitability [19,72–75]. Further studies are needed to discern the pertinent mechanism through which superoxide regulates synaptic transmission in our TLE model. Interestingly, excessive superoxide did not contribute to the heightened CA1 fEPSP response in the KO hippocampi. Previously, we found similar heightened fEPSP responses at the medial perforant path—dentate granule cell synapses and the dentate mossy fiber—CA3 synapses, suggesting this phenomenon may be due to loss of the Kv1.1 [39].

Specific stimulation patterns induce neurons to store information in two distinct phases of plasticity, STP and LTP. Mitochondria and MRCI activity modulate both phases [27,70]. This study revealed that superoxide has opposite effects depending on the concentration and plasticity phase. In the WT mice, normal superoxide levels did not affect STP, whereas LTP was promoted, as evidenced by the MitoQ reduction of LTP in the WT slices. This aligns with prior research demonstrating that removing homeostatic ROS in control animals impairs both LTP and contextual memory [20,22,53,76–78]. By contrast, excessive superoxide in the KO mice actively interfered with both STP and LTP—impairments were acutely rescued with MitoQ.

STP is a calcium-dependent, unstable phase that declines over 30 min. It is primarily driven by elevated intracellular calcium and does not require protein synthesis. Calcium release from the mitochondria is a critical regulator of STP in a synapse-specific manner [24–26,79,80]. Presynaptically, residual calcium enhances neurotransmitter release probability and mobilizes synaptic vesicles from reserve pools, while the mitochondria buffer and release calcium to modulate local concentrations near the release sites [79,81,82]. Postsynaptically, calcium triggers immediate phosphorylation of existing AMPA receptors via calcium/calmodulin-dependent kinase II, enhancing receptor conductance [83,84]. LTP represents the sustained phase of synaptic strengthening that can persist for hours to days.

It typically requires new protein synthesis and may involve redox-sensitive transcription factors, structural changes in dendritic spine morphology, and alterations in postsynaptic density composition [85]. The transition from STP to LTP involves calcium-dependent signaling pathways linking NMDA receptor activation to AMPA receptor insertion [79,85]. Hippocampal superoxide is involved in LTP induction via NMDA receptor activation, which triggers calcium influx and mitochondrial ROS generation [77]. ROS activates the factors crucial for LTP, including ryanodine receptor 3, which amplifies calcium signaling, protein kinase C, and mitogen-activated protein kinases likely through redox-sensitive sites on these signaling molecules [19,52,54]. These kinases facilitate AMPA receptor trafficking by promoting receptor insertion, lateral diffusion, and stabilization at synapses.

Given the absence of changes in paired-pulse facilitation (a measure of presynaptic release probability), impaired STP and LTP in KO mice likely reflect postsynaptic remodeling deficits [81]. The acute reversibility of deficits with MitoQ indicates that excessive superoxide is the mechanistic effector for mitochondrial dysfunction-mediated STP and LTP attenuation in KO mice. Here, we found that inhibition of the KO MRC1 was due to ROS, and inhibiting the WT MRC1 with rotenone reduced STP and LTP mimicking the KO. Thus, dysfunctional mitochondria and superoxide upset a critical source of metabolic support resulting in reduced calcium sequestration and ATP production for ion pumps and signaling cascades. Our findings demonstrate a hormetic dose-effect of superoxide on synaptic plasticity—where physiological levels are beneficial for LTP but excessive levels are detrimental to both STP and LTP. Determining the molecular switches that are responsible for these concentration-dependent effects require further investigation, but may be related to excessive ROS interfering with calcium-mediated phosphorylation and recruitment of AMPA receptors to synapses [86].

As stated above, the published studies and our findings indicate that reducing superoxide to below normal levels in control animals is detrimental to LTP [20,22,53,76–78]. Thus, superoxide reductions should impair memory in normal animals. This prediction has support [76,78]; however, MitoQ did not significantly reduce the WT performance in our NOR and NLR assays. This disparity needs further investigation, as results may have important implications for the use of antioxidants (i.e., beneficial in pathologic conditions, but detrimental to normal neurophysiology). Future studies will expand these findings to other learning and memory modalities and to other mitochondrial-targeted antioxidants and general antioxidants. An alternative possibility is that there may be a threshold for LTP reduction that is required to be reached before obvious cognitive impairment is observed, and MitoQ reduction of the WT LTP does not reach this threshold. In agreement with this conjecture, MitoQ fully rescued the cognitive performance of the KO mice, even though MitoQ only restored the KO LTP to the level of the WT LTP in the presence of MitoQ (i.e., both were lower than the WT LTP, but not as low as the KO LTP). Dissociation of LTP and memory has been previously demonstrated [87]. Further studies are needed to explore this possibility.

4.5. Superoxide and Hippocampal Oscillations

While reactive oxygen species reportedly play important roles in oscillatory rhythms (such as synchronizing cardiomyocytes activity [88]), mitochondrial contributions to spontaneous hippocampal oscillations are poorly understood. To our knowledge, this is the first study implicating superoxide in these oscillations. In human epilepsy, SPW-ripples occur at higher rates and pathological fast ripples emerge [61,62]. Both phenomena are considered biomarkers of a hyperexcitable network [57–62], but their presence may also disrupt processes important for cognitive function and memory consolidation, such as normal network dynamics and synaptic plasticity [89,90]. The findings of the current study suggest

that cognitive impairment associated with fast ripples likely operates through two distinct, separable mechanisms. Elevated superoxide—whether from epileptic pathology in the KO hippocampus or acute WT mitochondrial inhibition with rotenone—consistently degraded the quality of SPW-ripples by increasing their incidence and reducing their amplitude, creating a weakened substrate for memory consolidation. MitoQ rescued these superoxide-dependent abnormalities, normalizing SPW-ripple incidence and partially restoring amplitude. However, fast ripples persisted despite the reduction of superoxide, indicating other factors contribute to their emergence independent of ongoing oxidative stress.

Fast ripples arise when spike timing reliability fails [47,48]. In the case of acute mitochondrial inhibition with rotenone, this could occur during reduced ATP generation [91], whereas neuronal loss or the lack of Kv1.1 reduce spike timing reliability in the KO hippocampus [21,39,47]. Further studies are needed to determine the relevant mechanisms and to confirm that the superoxide effects of SPWs–HFOs in vivo regulate performance on behavioral learning and memory tasks. Nevertheless, this finding challenges the assumption that fast ripples inevitably preclude normal cognition [57–72,89,90]—instead, it appears that, when oxidative stress is neutralized, the brain can compensate for or filter out the “noise” introduced by pathological HFOs.

4.6. MitoQ Does Not Affect Seizures

A recent comprehensive meta-analysis of clinical studies concluded that antioxidants are not significantly efficacious against seizures [92]. Similarly, we found that, although MitoQ rescued aspects of learning, memory, and plasticity, the treatment did not impact seizures, seizure-associated pathological fast ripples, or CA1 excitability, suggesting that superoxide may not contribute to these metrics of epilepsy-related hyperexcitability. Rather, seizures may persist due to structural/molecular changes as stated above for fast ripples, whereas cognitive impairment may reflect ongoing metabolic dysfunction superimposed on those structural/molecular changes. Thus, MitoQ can rescue cognitive processes without reversing structural epileptic remodeling. These findings improve our understanding of the clinical observations that some patients have well-controlled seizures but persistent cognitive problems, and some patients have frequent seizures but relatively preserved cognition.

4.7. Limitations

Our study has several limitations. First, only one model of TLE was used. In order to generalize our findings, superoxide detection and mitochondrial-targeted antioxidant treatment should be assessed in multiple models, including models of acquired epilepsy. Second, we performed only two recognition memory assays. As stated above, experiments involving an array of learning and memory modalities are needed to determine which aspects of cognition excessive superoxide impacts. Third, the dosing schedule for MitoQ was limited. Perhaps chronic dosing or dosing early in disease progression would reveal that MitoQ prevents seizures. Fourth, extracellular recordings provide low resolution details concerning the mechanisms of synaptic physiology. Patch-clamp recordings are needed for an in depth understanding of the presynaptic and postsynaptic effects of superoxide and MitoQ. Fifth, only MitoQ was used. Performing similar experiments with mitochondrial-targeted and general antioxidants will clarify which ROS are detrimental to cognition. Despite these limitations, our demonstration that oxidative stress drives cognitive impairment independently of seizures provides both mechanistic insight and a novel therapeutic target for future clinical investigation.

5. Conclusions

Collectively, MitoQ rescue suggests that local superoxide levels impact specific elements of synaptic plasticity and oscillations sufficient to reverse impairment of the temporal information processing involved in associative learning and memory consolidation in this mouse model of TLE. However, it is important to note that if reducing superoxide (and subsequent reactive species) removes interference with MRCI function, and thereby freeing it to function efficiently and to re-establish the proton gradient and subsequent calcium sequestration capacities and ATP production, then observed improvements may also result from the indirect restoration of local calcium buffering or ATP availability. Parsing these mechanisms is a topic for future studies.

Our results demonstrate that mitochondrial superoxide is a key driver of ongoing hippocampal neuronal and cognitive dysfunction in epilepsy. Its effects are acutely reversible and thus distinct from the role of ROS-induced cell death in cognitive decline [13]. MitoQ has been investigated in animal models and clinical trials for diseases with underlying mitochondrial dysfunction, such as cancer, pulmonary disease, liver disease, cardiovascular disease, chronic kidney disease, infertility, and ototoxicity, among other neurological disorders [93]. By demonstrating that mitochondria-targeted antioxidant treatment rescues memory and plasticity despite ongoing seizures and pathologic fast ripples, this work provides proof-of-concept that cognitive comorbidities may be therapeutically dissociated from the epileptic phenotype. This dissociation suggests that the optimal treatment of epilepsy may require parallel approaches, including traditional antiepileptic strategies for seizure control and metabolic/mitochondrial support for cognitive preservation. The complete cognitive rescue despite persistent seizures and fast ripples fundamentally reframes epileptic cognitive impairment as a treatable metabolic disorder rather than an inevitable consequence of having seizures. These findings suggest new treatment avenues aimed not only at seizure reduction but at improving the quality of life in patients with TLE.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020259/s1>, Figure S1: In vivo sub-chronic treatment with MitoQ reduces the high mitochondrial superoxide levels in epileptic KO mice CA3 pyramidal neurons. Figure S2: In vivo sub-chronic treatment with MitoQ reduces the high mitochondrial superoxide levels in epileptic KO mice dentate gyrus (DG) granule neurons. Figure S3: Memory impairment in epileptic KO mice and improvement with in vivo sub-chronic dose of MitoQ is independent of anxiety. Figure S4: Acute application of MitoQ reduces the high mitochondrial superoxide levels in epileptic KO mice CA3 pyramidal neurons. Figure S5: Acute application of MitoQ reduces the high mitochondrial superoxide levels in epileptic KO mice dentate gyrus (DG) granule neurons. Figure S6: Epileptic KO mice have larger dendritic and somatic fEPSPs, fiber volleys, and population spikes. Figure S7: MRCI inhibition generates mitochondrial superoxide in WT CA1 pyramidal neurons which are prevented with acute co-application of MitoQ and rotenone.

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Abbreviations

The following abbreviations are used in this manuscript:

CA1	cornu ammonis 1
DG	dentate gyrus
EEG	electroencephalography
EMG	electromyography
fEPSP	field excitatory postsynaptic potential
HFO	high-frequency oscillation
KO	Kv1.1 knock-out
LTP	long-term potentiation
MitoQ	mitoquinone
MRCI	mitochondrial respiratory chain complex I
NLR	novel location recognition
NOR	novel object recognition
PPR	paired-pulse ratio
PWE	people with epilepsy
ROS	reactive oxygen species
SPW	sharp wave
STP	short-term potentiation
TLE	temporal lobe epilepsy
WT	wild-type
ZT	zeitgeber time

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