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ARTICLE

Triheptanoin protects against status epilepticus-induced hippocampal mitochondrial dysfunctions, oxidative stress and neuronal degeneration

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Abstract

Triheptanoin, the triglyceride of heptanoate, is anaplerotic (refills deficient tricarboxylic acid cycle intermediates) via the propionyl-CoA carboxylase pathway. It has been shown to be neuroprotective and anticonvulsant in several models of neurological disorders. Here, we investigated the effects of triheptanoin against changes of hippocampal mitochondrial functions, oxidative stress and cell death induced by pilocarpine-induced status epilepticus (SE) in mice. Ten days of triheptanoin pre-treatment did not protect against SE, but it preserved hippocampal mitochondrial functions including state 2, state 3 ADP, state 3 uncoupled respiration, respiration linked to ATP synthesis along with the activities of pyruvate dehydrogenase complex and oxoglutarate dehydrogenase complex 24 h post-SE. Triheptanoin prevented the SE-induced reductions of

hippocampal mitochondrial superoxide dismutase activity and plasma antioxidant status as well as lipid peroxidation. It also reduced neuronal degeneration in hippocampal CA1 and CA3 regions 3 days after SE. In addition, heptanoate significantly reduced hydrogen peroxide-induced cell death in cultured neurons. *In situ* hybridization localized the enzymes of the propionyl-CoA carboxylase pathway, specifically *Pccα*, *Pccβ* and *methylmalonyl-CoA mutase* to adult mouse hippocampal pyramidal neurons and dentate granule cells, indicating that anaplerosis may occur in neurons. In conclusion, triheptanoin appears to have anaplerotic and antioxidant effects which contribute to its neuroprotective properties.

Keywords: mitochondrial function, neuroprotection, oxidative stress, pilocarpine, triheptanoin.

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Triheptanoin, the triglyceride of heptanoate, has unique metabolic effects. After lipolysis in the gastro-intestinal tract, heptanoate can either enter the blood and brain directly or is metabolized in the liver to form C₅-ketone bodies (Gu *et al.* 2010) or glucose via gluconeogenesis (Marin-Valencia *et al.* 2013). Subsequently, all heptanoate derivatives are metabolized into acetyl-CoA and anaplerotic propionyl-CoA. The latter can replenish the levels of four-carbon tricarboxylic acid (TCA) cycle intermediates by forming succinyl-CoA, which is catalyzed by propionyl-CoA carboxylase (PCC) and methylmalonyl-CoA mutase (Kinman *et al.* 2006). A study using ¹³C-labeled heptanoate found that heptanoate is anaplerotic in the brain via this pathway (Marin-Valencia *et al.* 2013). Furthermore, triheptanoin has been shown to increase methylmalonyl-CoA levels and restore acetyl-CoA and propionyl-CoA levels in a chronic stage of ‘epilepsy’,

which develops in mice after pilocarpine-induced status epilepticus (SE; Willis *et al.* 2010).

Given the importance of energy production in cell survival, metabolic treatments including triheptanoin have been tested

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Abbreviations used: BSA, bovine serum albumin; CS, citrate synthase; MCM, methylmalonyl-CoA mutase; OGDHc, oxoglutarate dehydrogenase complex; PCC, propionyl-CoA carboxylase; PDHc, pyruvate dehydrogenase complex; SE, status epilepticus; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, tricarboxylic acid.

in several models of neurological disorders and found to be neuroprotective. In a mouse model of Canavan Disease, triheptanoin treatment reduced oxidative stress, prevented the loss of oligodendrocytes and improved motor function (Francis *et al.* 2014). Oral triheptanoin treatment also delayed motor neuron loss and the onset of symptoms in a mouse Amyotrophic Lateral Sclerosis model (Tefera *et al.* 2016). In addition, triheptanoin pre-treatment for 2 weeks prior to middle cerebral artery occlusion significantly reduced infarct area, improved neurological function and attenuated reductions in complex II and IV activities in mice, suggesting that preservation of mitochondrial function is neuroprotective (Schwarzkopf *et al.* 2015).

We have previously shown that 24 h after pilocarpine-induced SE, mitochondrial functions were impaired and lipid peroxidation was substantially increased in the hippocampal formations of mice (Carrasco-Pozo *et al.* 2015). It is well known that disturbances in mitochondrial functions can lead to excessive reactive oxygen species production which can further exacerbate perturbations in energy production. Here, we aimed to further elucidate the mechanisms underlying neuroprotective and anticonvulsant actions of triheptanoin by investigating the effects of triheptanoin pre-treatment on SE-induced pre-specified outcomes, namely changes of mitochondrial respiration and TCA cycle enzyme activities, oxidative stress and the cellular and plasma defence against oxidative stress. We also assessed the extent to which triheptanoin pre-treatment protects against neuronal degeneration in the hippocampal formations of mice 3 days after pilocarpine injection and whether heptanoate protects cultured neurons against oxidative stress. Last, we used *in situ* hybridization to localize the expression of enzymes central to the PCC pathway in naïve adult mouse brains.

Materials and methods

All chemicals and reagents were obtained from Sigma Aldrich (St Louis, MO, USA) unless stated otherwise. Please note that Research Resource Identifiers are not registered for the animals and catalogue numbers are provided where applicable.

Animals

All mice used were 7–8 week old male CD1 (35–40 g) outbred albino mice from the Animal Resource Centre (Murdoch, WA, Australia). They were housed individually in open top cages under a 12-h light-dark cycle with free access to food and water and all experiments involving animals were conducted during the light cycle. All experiments were approved by the animal ethics committee of the University of Queensland (SBMS/128/14) and followed the guidelines of the Queensland Animal Care and Protection Act 2001 to minimize the suffering of the animals. All mice were carefully monitored at least twice daily and any mouse with more than 20% weight loss after pilocarpine injection would be euthanized according to our approved ethics protocol. All work was conducted according to the Animal Research: Reporting of *In Vivo*

Experiments (ARRIVE) guidelines (Kilkenny *et al.* 2010). The choice of anesthetics used depends on the experiments. Isoflurane was used to induce light anesthesia prior to decapitation for mitochondria and enzyme related experiments because it is critical to extract and freeze the tissues as quickly as possible. An overdose of sodium pentobarbitone was used before animals were subjected to transcardial perfusion to ensure deep anesthesia throughout terminal perfusion, while the heart was still pumping.

Preregistration of study, randomization, blinding and power analysis

The study was not preregistered. All mice were randomly numbered by animal house staff and then randomly assigned to treatment groups using simple randomization. For each experiment forty mice were ordered from Animal Resource Centre and they arrived in groups of 20 per box. All mice were randomly numbered by animal house staff using computer-generated numbers before being pseudo-randomly assigned (with equal animal numbers in both groups) to treatment groups by another blinded experimenter. All experimenters remained blinded throughout the experiments until all analyses were completed. Using power analyses (Statmate, GraphPad Prism 2.0) and previously measured variabilities, the sample sizes in some assays only enabled us to detect 30–50% of differences to the mean with Student's *t*-tests at the 0.05 significance level with 80% power. However, by combining the data from two experiments, we improved the power to allow us to detect 20–30% of differences. Please note that the outcome of pilocarpine injections varies widely between experiments due to a steep dose-response curve. All mice that survived after pilocarpine injection were included in the experiments. In all experiments yielding parametric data that were normally distributed, values that were outside of two standard deviations of the mean were excluded.

Triheptanoin treatment

35E% oral triheptanoin (% total caloric intake; Ultragenyx Pharmaceuticals Inc., Novato, CA, USA) was mixed into mouse chow (SF11-79, Specialty Feeds, Glen Forrest, WA, Australia), while untreated mice were given regular chow (SF10-27) as previously described (Thomas *et al.* 2012). Mice in both groups received similar amounts of protein, vitamins and minerals relative to the caloric density of the chows (Willis *et al.* 2010) and were treated for 10 days prior to pilocarpine injection and thereafter until euthanized. Body weight changes of mice were monitored throughout the treatment phases. The experimental timelines are shown in Fig. 1.

Pilocarpine model

After 10 days of treatment, the pilocarpine model was used to induce seizures in mice as previously described with all mice given the drug injection (Thomas *et al.* 2012; Carrasco-Pozo *et al.* 2015). Briefly, in a randomized order between 9 am and 1 pm, all mice were injected with methylscopolamine (2 mg/kg in 0.9% saline; i.p.) 15 min prior to pilocarpine injection (340 mg/kg in 0.9% saline; s.c.) to minimize peripheral side effects. After the single dose pilocarpine injection, mice were placed in clean clear cages lined with paper towels and seizure behaviors were scored from 0 to 5 for 90 min with 15 min intervals as previously described to obtain semi-quantified SE severity scores (Benson *et al.* 2015; Carrasco-Pozo *et al.* 2015). Ninety minutes after pilocarpine administration,

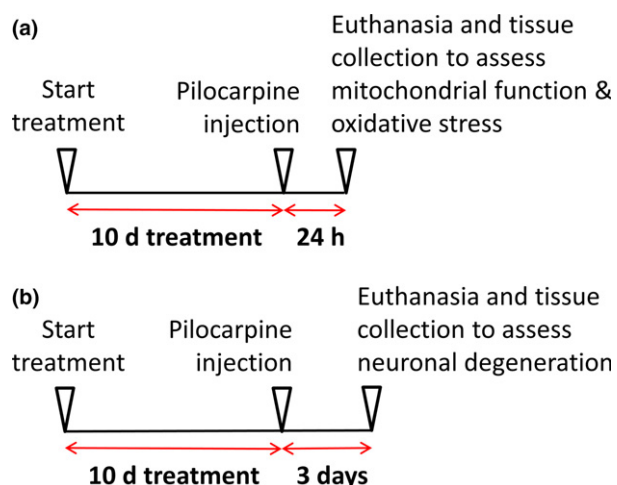


Fig. 1 Experimental timelines for triheptanoin treatment and pilocarpine injections. Mice were treated with triheptanoin (35E%) for 10 days prior to pilocarpine injection to induce status epilepticus. (a) Bilateral hippocampal formations, cortices and blood were collected 24 h post-pilocarpine injection for the assessment of mitochondrial functions, enzyme activity assays and oxidative stress. (b) Mice were euthanized 72 h after pilocarpine-induced status epilepticus and whole brains were collected for the histological assessment of neuronal degeneration.

seizures were terminated with pentobarbital (30 mg/kg in 0.9% saline; i.p.; Provet, Qld., Australia) followed by 1 mL of 4% dextrose in 0.18% saline (s.c.) and mice were returned to their home cages. This rehydration was continued twice daily for the next 3 days combined with handfeeding the respective diets.

Tissue and plasma collection

Mice were anaesthetized with isoflurane and euthanized by decapitation 24 h post-pilocarpine injection. Bilateral hippocampal formations were dissected, frozen on dry ice and stored at -80°C for lipid peroxidation and enzyme activity measurements. In a separate experiment, enriched mitochondrial fractions were prepared from freshly isolated bilateral hippocampal formations for the assessment of mitochondrial function using an extracellular flux analyzer. Blood was obtained from decapitated trunks in EDTA-coated tubes, centrifuged at 2000 g for 10 min at 4°C . The collected plasma was then stored at -80°C until analyzed.

Mitochondrial preparation

Mitochondria were enriched as previously described (Carrasco-Pozo *et al.* 2015) using buffer solutions that were chilled on ice. All centrifugations were done at 4°C . Briefly, using a Teflon dounce homogenizer, bilateral hippocampal formations or unilateral cortices from each mouse were homogenized individually in 25 volumes of MSHE buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES and 1 mM EGTA, pH 7.2) for 10 strokes on ice followed by centrifugation at 1000 g for 10 min. An aliquot was collected and stored at -80°C to assess lipid peroxidation. After adding 1 mL of MSHE with bovine serum albumin (BSA) buffer [0.5% (w/v) fatty acid-free BSA, pH 7.2] to the remaining supernatant, samples were centrifuged at 12 000 g for 10 min. The supernatant (cytosolic fraction) was collected. The pellet (mitochondrial fraction) was

washed with MSHE without BSA, centrifuged at 10 000 g for 10 min and reconstituted in the same buffer. Both fractions were stored at -80°C until analyzed. The mitochondrial fraction contains synaptic and non-synaptic mitochondria.

Mitochondrial coupling assay

Here, we enriched mitochondria as described above except that freshly isolated bilateral hippocampal formations and only MSHE buffer were used. Isolated mitochondria were kept on ice until needed and were used fresh for mitochondrial function analysis using the extracellular flux XF⁹⁶ Analyzer at 37°C (Seahorse Bioscience, MA, USA) as previously described (Carrasco-Pozo *et al.* 2015; Tan *et al.* 2017). Briefly, 3 μg of mitochondrial preparations in buffer [220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA] containing 10 mM succinate and 2 μM rotenone was added to each well in quadruplicates for each animal. Mitochondria were activated by the addition of 6 volumes of the same buffer at 37°C . Oxygen consumption rate was measured after sequential addition of 3 mM ADP, 2.5 $\mu\text{g}/\text{mL}$ oligomycin, 4 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone and 4 μM antimycin A to stimulate different states of mitochondrial respiration, namely state 3 ADP, state 4o, state 3u and non-mitochondrial respiration, respectively. Non-mitochondrial respiration was subtracted from every mitochondrial function parameter and respiration linked to ATP synthesis (state 3 ADP minus state 4o) was calculated as previously described (Carrasco-Pozo *et al.* 2015; Tan *et al.* 2017). All mitochondrial function parameters ($n = 7\text{--}15$ mice/group combined from two experiments) were normalized to protein content, which was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA; cat. no: 23225).

Ferric reducing ability of plasma

The antioxidant status of the plasma was assessed by measuring the ability of the plasma to reduce colorless ferric tripyridyltriazine adduct to the ferrous form which was measured spectrophotometrically at 593 nm after 30 min incubation at 37°C (Benzie and Strain 1996; Carrasco-Pozo *et al.* 2015). The antioxidant power was calculated based on a standard curve generated from FeSO_4 and expressed as $\mu\text{mol FeSO}_4/\text{L}$ plasma ($n = 8\text{--}17$ mice/group combined from two experiments).

Lipid peroxidation

Lipid peroxidation in the cortex and hippocampal formations was determined using a thiobarbituric acid reactive substances Assay Kit according to manufacturer's instructions, which quantifies the amounts malondialdehyde-thiobarbituric acid adduct colorimetrically at 535 nm (Cayman Chemical Company, Ann Arbor, MI, USA; cat. no: 10009055; $n = 3\text{--}9$ mice/group).

Catalase and superoxide dismutase activities

Isolated cytosol and mitochondrial preparations from the hippocampal formations described above were used to determine the activities of catalase in the cytosol ($n = 3\text{--}9$ mice/group) and superoxide dismutase (SOD) in both cellular fractions (cytosol: $n = 3\text{--}9$ mice/group and mitochondria: $n = 7\text{--}16$ mice/group combined from two experiments). The activities of these antioxidant enzymes were measured using the Catalase Assay Kit and Superoxide Dismutase

Assay Kit, respectively, according to the manufacturer's instructions (both from Cayman Chemical Company; cat. no: 707002 and 706002, respectively).

Enzyme activity assays

The activities of several enzymes including pyruvate dehydrogenase complex (PDHc; $n = 7$ – 15 mice/group combined from two experiments), oxoglutarate dehydrogenase complex (OGDHc; $n = 8$ – 17 mice/group combined from two experiments) and citrate synthase ($n = 3$ – 8 mice/group) were measured through continuous spectrophotometric assays. After adding 20 μg of mitochondrial protein to the reaction mixes (Table S1), reactions were started by addition of the respective initiating substrates just prior to measurement by a Spectramax 190 Microplate Reader (Molecular Devices, CA, USA). The linear portion of the reaction was used to determine the rate of change in absorbance and enzyme activity was then calculated, normalized to protein content and expressed as nmol/min/mg protein.

Mitochondrial extracts (20 μg) from the hippocampal formations of triheptanoin-treated and untreated naïve mice not subjected to the pilocarpine model were incubated with Fenton reaction mix containing 100 μM FeCl_3 , 50 μM ascorbic acid and 100 μM H_2O_2 at 37°C for 15 min. The activities of OGDHc were then measured as described above, except that reduction of NAD was quantified fluorometrically at 350 nm excitation and 460 nm emission using a multi-mode microplate reader POLARstar[®] Omega (BMG LABTECH, Ortenberg, Germany). Enzyme activities were normalized to protein content and expressed as units of relative fluorescence/min/mg protein ($n = 8$ – 9 mice/group).

Neuronal degeneration

SE was induced using pilocarpine after 10 days of treatment as described above (in the 'Pilocarpine model' section). Mice were then given 0.5 mL of 20% long-chain triglyceride or triheptanoin emulsion (s.c.; both from B. Braun Melsungen, Melsungen, Germany) after pentobarbital injection and twice daily thereafter. Mice were anesthetized using isoflurane and euthanized by decapitation 3 days post-pilocarpine injection. Whole brains were extracted and fixed in 4% paraformaldehyde for 24 h at 4°C before being dehydrated through an ascending series of ethanol and embedded in paraffin. Coronal sections (9 μm) between 1.8 and 2.5 mm bregma (midlevel hippocampi) were cut and stained with Fluoro-Jade[®] B (Merck Millipore, MA, USA) as previously described (Schmued and Hopkins 2000). Neuronal degeneration was assessed by an experienced experimenter blinded to the treatment groups by scoring the number of cells that fluoresced in the CA1 and CA3 regions (both $n = 8$ – 11 mice/group combined from two experiments) of the hippocampal formations (Carrasco-Pozo *et al.* 2015) using an Olympus BX81 upright fluorescent microscope with a 20 \times objective. A score of 0 represents no degeneration while scores of 1, 2 and 3 represent mild, moderate and severe degeneration, respectively, as previously described (Samala *et al.* 2008).

Cortical neuronal cultures

All culture media and reagents were obtained from Life Technologies (CA, USA) unless stated otherwise. Briefly, bilateral cerebral cortices were removed from E15 CD1 mouse embryos, triturated and seeded at a density of 7.5×10^5 cells per well in a 24-well plate with Neurobasal[®] media containing 25 mM glucose, 2 mM

glutamine, 10 $\mu\text{g}/\text{mL}$ gentamicin and serum-free B-27[®] Supplement. Neuronal cells were cultured for 12 days until confluent at 37°C with 5% CO_2 and culture media were changed every 5 days. Cells were treated with 50 μM of heptanoic acid (final concentration; pH 7.4; Sigma Aldrich) overnight prior to treatment with 100 μM of H_2O_2 (final concentration; Merck Millipore) for 24 h. Culture media were collected before and after H_2O_2 treatment from each well and cell death was assessed as the percentage of lactate dehydrogenase activity released into the media relative to the total amount of lactate dehydrogenase activity per well using a Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany; cat. no: 11644793001; $n = 3$ independent culture preparations).

In situ hybridization

Mice were anesthetized using an overdose of sodium pentobarbitone (120 mg/kg, i.p.; Troy Laboratories, NSW, Australia) and were subjected to transcardial perfusion with cold 0.9% saline containing 0.1% sodium nitrite followed by cold 4% paraformaldehyde solutions. Whole brains were extracted, processed and sectioned as described above (in the 'Neuronal degeneration' section).

Primers for genes of interest were designed based on the sequences available on GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>). T7 RNA polymerase promoter (5'-TAATACGACTCAC-TATAGGG-3') was added to the forward primers while T3 RNA polymerase promoter (5'-AATTAACCCTCACTAAAGGG-3') was added to the reverse primers (primer sequences and accession numbers in Table S2). Standard PCR was performed to synthesize templates for *in vitro* transcription using a cDNA sample from a naïve mouse brain under the following cycling conditions: initial hot start of 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min and 72°C for 7 min. PCR products were run on 1% agarose gel and only one band was visible for each primer pair. The bands corresponding to expected size amplicon were extracted using MinElute[®] Gel Extraction Kit (Qiagen, NRW, Germany; cat. no: 28604) followed by digoxigenin labeling (Roche; cat. no: 11277073910), both according to the manufacturers' instructions.

Hybridization was performed as previously described (Simmons *et al.* 2008) except that digoxigenin-labelled probes were diluted 1:600. The slides were incubated at 22°C until purple precipitations were formed (approximately 4 days) and the reaction was stopped using 1 \times phosphate-buffered saline. Then slides were counterstained with Nuclear Fast Red (1 mg/mL), dehydrated in ascending concentrations of ethanol followed by xylene and mounted in DPX mounting medium (Ajax Finechem, NSW, Australia). Images were obtained using ScanScope XT (Aperio, CA, USA).

Statistical analysis

All graphs show mean \pm SEM unless stated otherwise. All statistical comparisons of experiments with two variables and more than one cohort of mice were done using three-way ANOVAs with cohort as a random factor. Since cohort did not influence any outcomes, we then used two-way ANOVAs followed by Tukey's multiple comparisons *post hoc* tests at significance level of $p < 0.05$, except for SE severity which were evaluated by Mann-Whitney tests. The number of mice with no or only mild neurodegeneration scores in the pyramidal cell layers of CA1 and CA3 regions was compared using a Chi-square test. The significances for interaction, row and column factors from two-way ANOVA

are indicated in the figures. The number of stars in the figures and the p-values in the text indicate the significance of Tukey's *post hoc* tests unless stated otherwise. The sample sizes for each group are shown in the bar graphs. All analyses were performed using GraphPad Prism 7.02 (GraphPad Software, CA, USA) and IBM SPSS Statistics (Java Console, version 25, Armonk, NY, USA).

Results

Triheptanoin preserved mitochondrial functions in SE mice

Body weight was monitored during triheptanoin treatment and as previously published (Willis *et al.* 2010), no significant difference was observed between untreated and triheptanoin-treated mice after 10 days (e.g. 38.2 ± 3.1 g vs. 38.4 ± 3.2 g; mean \pm SD; $n = 20$ mice in one experimental group). The body weights of the two SE groups were also not significantly different at 24 h post-pilocarpine injection (vehicle: 35.8 ± 2.8 g, triheptanoin: 35.6 ± 2.7 g; mean \pm SD; $n = 9$ mice per group). There was no protection

against SE or SE-induced mortality. The total number of no SE, SE and SE-induced mortality in all experiments was 22, 24 and 38 mice in the vehicle group and 22, 29 and 31 mice, respectively, in the triheptanoin group. The seizure severity scores were not significantly different between the two groups in any experiment (median score; untreated: 11 and triheptanoin-treated mice: 13; $p = 0.19$, Mann–Whitney test; $n = 22$ and 28 mice, respectively). Various mitochondrial function parameters were assessed 24 h post-pilocarpine injection based on oxygen consumption rates using an extracellular flux analyzer and the raw data from one experiment are shown in Fig. 2a. Each mitochondrial parameter was quantified and data from two independent experiments are combined after normalizing to the no SE vehicle groups (Fig. 2b–d).

In accordance with our previous study, several mitochondrial function parameters were impaired in the hippocampal formations of untreated SE mice 24 h following pilocarpine injection (Carrasco-Pozo *et al.* 2015). Specifically, state 2

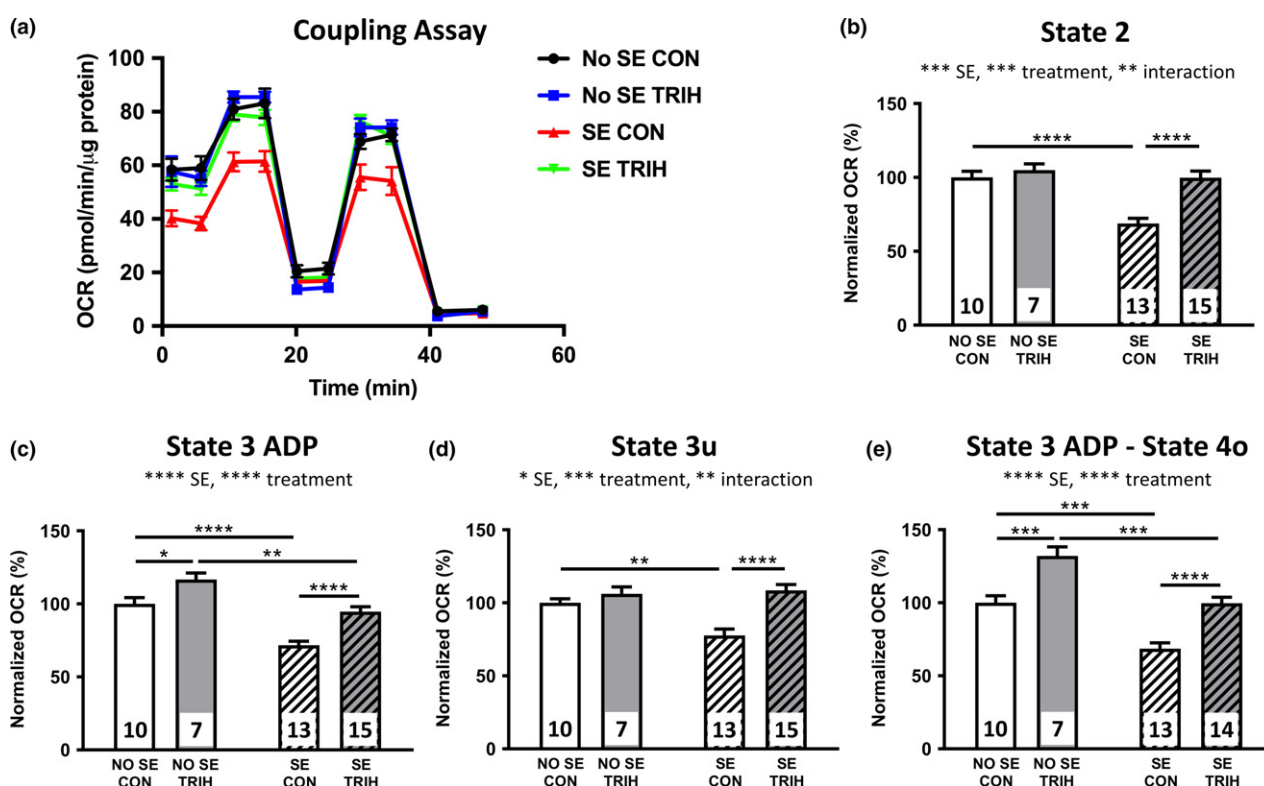


Fig. 2 Triheptanoin preserved mitochondrial functions in the hippocampal formations of pilocarpine-induced status epilepticus (SE) mice. Mice were given 35E% triheptanoin treatment 10 days prior to SE induction with pilocarpine and mitochondrial functions were assessed using an extracellular flux analyzer 24 h after pilocarpine injection. (a) In the presence of 10 mM succinate as substrate, oxygen consumption rates (OCR) at different states of mitochondrial respiration are shown. Various mitochondrial function parameters were impaired in untreated (CON) SE mice 24 h post-pilocarpine injection,

including (b) state 2, (c) state 3 (after the addition of ADP; state 3 ADP) and (d) state 3 uncoupled respiration (state 3u) as well as (e) respiration linked to ATP synthesis (state 3 ADP – state 4o). Triheptanoin (TRIH) treatment improved all impairments in mitochondrial functions in the hippocampal formations of SE mice. (b–e) All two-way ANOVAs followed by Tukey's *post hoc* tests, stars indicate Tukey's *post hoc* test significances, $n = 7$ –15 mice/group; combined data from two independent experiments after normalizing to no SE vehicle groups. Sample sizes for each group are as shown within each bar.

respiration decreased by 31% ($p < 0.0001$; Fig. 2b), state 3 respiration (after the addition of ADP) by 28% ($p < 0.0001$; Fig. 2c), state 3u by 22% ($p = 0.003$; Fig. 2d) and respiration linked to ATP synthesis by 31% ($p = 0.0001$; Fig. 2e) relative to untreated mice that did not develop SE. Triheptanoin-treated SE mice showed improvements in all mitochondrial function parameters compared to untreated SE mice. Reductions in state 2 ($p < 0.0001$; Fig. 2b) and state 3u respiration ($p < 0.0001$; Fig. 2d) were abolished while state 3 ADP ($p < 0.0001$; Fig. 2c) and respiration linked to ATP synthesis were increased in the SE group following triheptanoin treatment ($p < 0.0001$; all comparisons by two-way ANOVAS; $n = 7-15$ mice/group; Fig. 2e). It is also noteworthy that triheptanoin treatment increased state 3 ADP and respiration linked to ATP synthesis by 17% ($p = 0.04$) and 32% ($p = 0.0008$), respectively, in no SE mice.

Triheptanoin reduced oxidative stress in SE mice

Consistent with our previous findings, the reducing ability of the plasma decreased (14%; $p = 0.005$; $n = 8-17$ mice/group; Fig. 3a) while the levels of malondialdehyde increased in the cortex (30%; $p = 0.02$; $n = 3-9$ mice/group; Fig. 3b) and, to a

greater extent, in the hippocampal formations (101%; $p < 0.0001$; $n = 3-8$ mice/group; Fig. 3c) of SE mice 24 h post-pilocarpine injection. Triheptanoin treatment prevented the decrease in the reducing ability of the plasma ($p = 0.0007$; $n = 8-17$ mice/group; Fig. 3a) and abolished the increases in malondialdehyde levels found in both the cortex ($p = 0.0006$; $n = 3-9$ mice/group; Fig. 3b) and hippocampal formations ($p < 0.0001$; $n = 3-8$ mice/group; Fig. 3c). The activities of catalase (Fig. 3d) and cytosolic SOD (both $n = 3-9$ mice/group; Fig. 3e) were not altered by SE and triheptanoin treatment although triheptanoin attenuated the reduction in the activities of mitochondrial SOD ($p = 0.0004$; Fig. 3f) observed in untreated SE mice ($p = 0.0007$; $n = 7-16$ mice/group; all two-way ANOVAS).

Triheptanoin preserved TCA cycle enzyme activities in SE mice

Several enzymes central to the TCA cycle are known to be susceptible to oxidative stress. As anticipated, the activities of PDHc and OGDHc were reduced in untreated SE mice by 56% ($p = 0.0002$; Fig. 4a) and 39% ($p = 0.001$; Fig. 4b), respectively, but the activities of citrate synthase were unaltered relative to no SE mice ($p = 0.99$; Fig. 4c). Triheptanoin

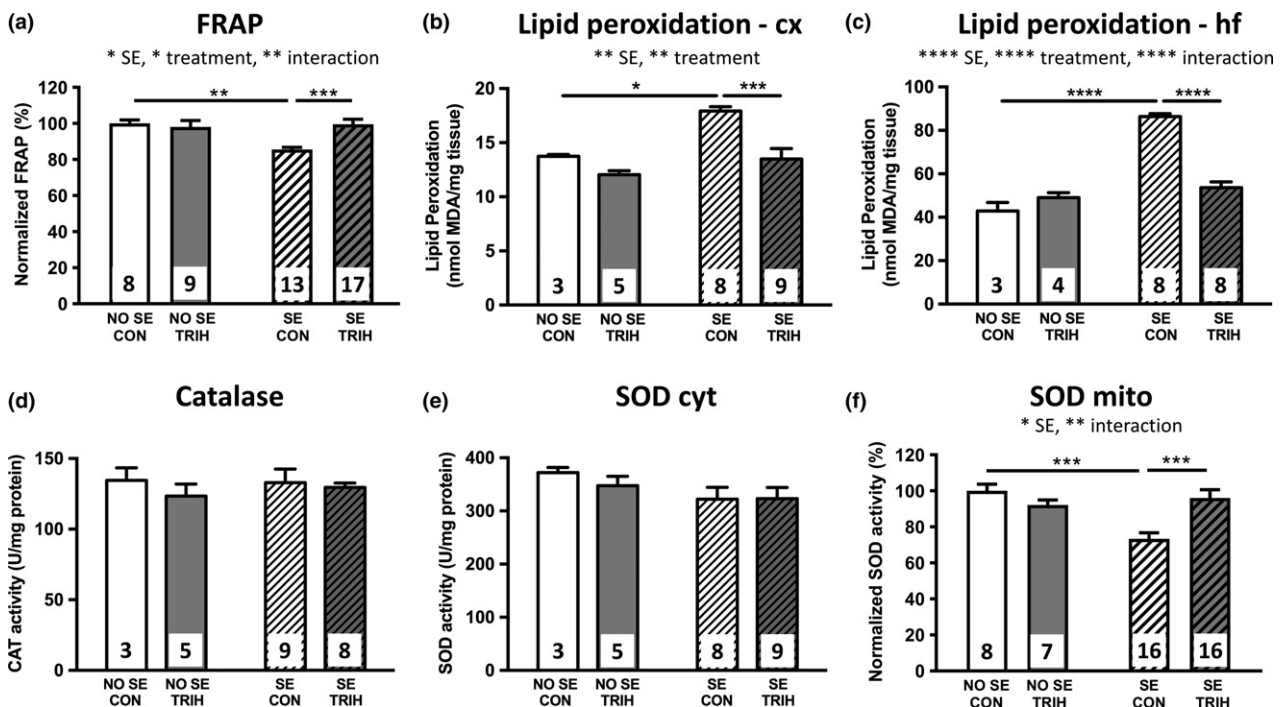


Fig. 3 Triheptanoin reduced oxidative stress in mice 24 h after pilocarpine-induced status epilepticus (SE). (a) The antioxidant capacity of the plasma decreased in untreated (CON) SE mice in a ferric reducing ability of plasma (FRAP) assay. Lipid peroxidation, measured as the amount of malondialdehyde (MDA) present, was increased in both the (b) cortex (cx) and (c) hippocampal formations (hf) of untreated SE mice. Although the activities of (d) catalase and (e) superoxide dismutase (SOD) in the cytosol (cyt) remained unaltered by

SE, the activities of (F) SOD in the mitochondrial preparations (mito) were significantly reduced in the hippocampal formations of untreated SE mice. Triheptanoin abrogated all these changes observed in the untreated group. (a-f) All two-way ANOVAS followed by Tukey's *post hoc* tests, stars indicate Tukey's *post hoc* test significances. (a and f) Combined data from two experiments after normalizing to no SE vehicle group; $n = 7-17$ mice/group. (b-e) $n = 3-9$ mice/group. Sample sizes for each group are as shown within each bar.

treatment preserved the activities of both PDHc ($p = 0.0002$; Fig. 4a) and OGDHc ($p = 0.0006$; Fig. 4b; all two-way ANOVAS; $n = 7$ – 17 mice/group) in SE mice. Mitochondrial extracts from untreated mice or mice given triheptanoin treatment for 10 days were subjected to the Fenton reaction, which produces hydroxyl radicals (Halliwell and Gutteridge 1992), followed by the measurement of OGDHc activities. Although the activities of OGDHc were reduced in both untreated- and triheptanoin-treated mice in the presence of hydroxyl radicals (both $p < 0.0001$; Fig. 4d), the activities of OGDHc were 23% higher in the triheptanoin group ($p = 0.046$; two-way ANOVA; $n = 8$ – 9 mice/group; Fig. 4d), indicating that brain tissue derived from triheptanoin-treated mice is better protected against oxidative stress.

Neuroprotection *in vivo* and *in vitro*

Neuronal degeneration was assessed 3 days after pilocarpine injection using Fluoro-Jade[®] B staining. Examples of hippocampal formations with no degeneration (score of 0; Fig. 5a) and severe degeneration (score of 3; Fig. 5B) are

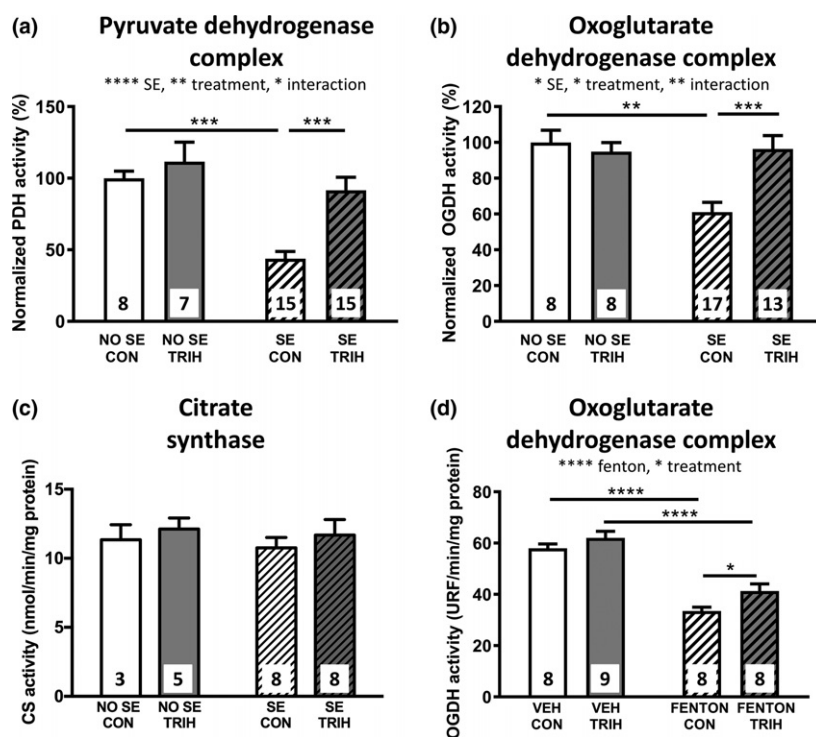


Fig. 4 Triheptanoin preserves enzyme activities in the hippocampal formations of mice after pilocarpine-induced status epilepticus (SE). Triheptanoin abolished the reductions in the activities of (a) pyruvate dehydrogenase complex and (b) oxoglutarate dehydrogenase complex following seizure induction (both combined data from two experiments after normalizing to no SE vehicle group; $n = 7$ – 17 mice/group). (c) The activities of citrate synthase were not altered by both SE and triheptanoin treatment ($n = 3$ – 9 mice/group). (d) Although incubation of

hippocampal extracts from untreated (CON) mice or triheptanoin-treated (TRIH) mice not subjected to SE induction with Fenton reaction mix, which produces hydroxyl radicals, reduced the activities of oxoglutarate dehydrogenase complex in both groups, the enzyme activities were higher in triheptanoin group ($n = 8$ – 9 mice/group). (a–d) All two-way ANOVAS followed by Tukey's *post hoc* tests, stars indicate Tukey's *post hoc* test significances. Sample sizes for each group are as shown within each bar. URF, units of relative fluorescence.

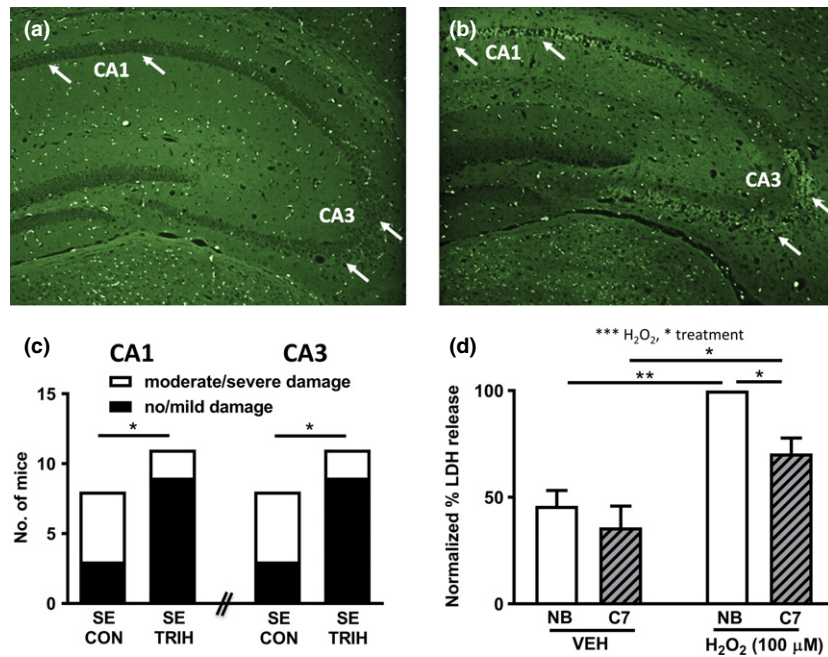


Fig. 5 Neuroprotective effects of triheptanoin *in vivo* and *in vitro*. Fluoro-Jade[®] B was used to assess neuronal degeneration in the hippocampal formations of mice with status epilepticus (SE) 3 days after pilocarpine injection. Examples of CA1 and CA3 regions in the hippocampal formations with (a) no damage (score of 0) and (b) severe degeneration (score of 3) are shown. Triheptanoin (TRIH) treatment significantly increased the number of mice with no or mild neuronal degeneration in both the (c) CA1 and CA3 regions compared to

untreated (CON) mice. (d) Heptanoic acid pre-treatment (C7; 50 μM final) protected neuronal cultures against hydrogen peroxide- (H₂O₂; 100 μM) induced cytotoxicity, normalized to the maximal % lactate dehydrogenase (LDH) release in the vehicle group. (c) Chi-square tests, $n = 8-11$ mice/group (combined data from two experiments). (d) Two-way ANOVA followed by Tukey's *post hoc* tests, stars indicate Tukey's *post hoc* test significances, $n = 3$ independent culture preparations.

Localization of anaplerotic enzymes in mouse hippocampal formations

A previous study showed that two of the anaplerotic enzymes in the PCC pathway, PCC α and methylmalonyl-CoA mutase are expressed in neurons but not astrocytes in adult rat brains (Ballhausen *et al.* 2009). Using *in situ* hybridization, we found that both *Pcc α* (Fig. 6a) and *Mcm* (Fig. 6c) are expressed in the hippocampal formations of mouse brain, specifically the hippocampal CA1 and CA3 pyramidal cell layers as well as the granule cell layer of the dentate gyrus. We also found that the β -subunit of *Pcc* (*Pcc β*) is expressed in the major hippocampal neuronal layers of adult mouse (Fig. 6e). The expression of these enzymes appears to have a similar distribution to *Pdh* (Fig. 6g), which is localized in the same neuronal layers. This is in contrast to the glutamate transporter 1 (*Glt1*; Fig. 6i), which is largely confined to astrocytes.

Discussion

Triheptanoin has been shown to be neuroprotective and anticonvulsant in several acute and chronic mouse seizure models (Willis *et al.* 2010; Thomas *et al.* 2012; Kim *et al.*

2013) and has been tested in several clinical trials as an add-on treatment for various neurological conditions, including epilepsy, glucose transporter 1 deficiency (Pascual *et al.* 2014; Mochel *et al.* 2016) and Huntington's disease (Mochel *et al.* 2010; Adanyeguh *et al.* 2015). It has been proposed that triheptanoin exerts its protective effects due to its unique metabolism, specifically via the refilling of four-carbon intermediates in the TCA cycle through the PCC pathway (anaplerosis; Kinman *et al.* 2006; Marin-Valencia *et al.* 2013). Here, we describe so far unknown neuroprotective effects of triheptanoin against SE-induced hippocampal mitochondrial impairments, lipid peroxidation and neuronal degeneration as well as *ex vivo* oxidative stress-induced neuronal degeneration and OGDHc activity. To which extent these effects of triheptanoin are antioxidant or are mediated via the preservation of energy metabolism still needs to be investigated.

We showed that various parameters of mitochondrial function, including state 2, state 3 ADP and state 3u respiration, respiration linked to ATP synthesis as well as the activities of PDHc and OGDHc were impaired in hippocampal formations 24 h post-SE induction, similar to our previous findings (Carrasco-Pozo *et al.* 2015). These

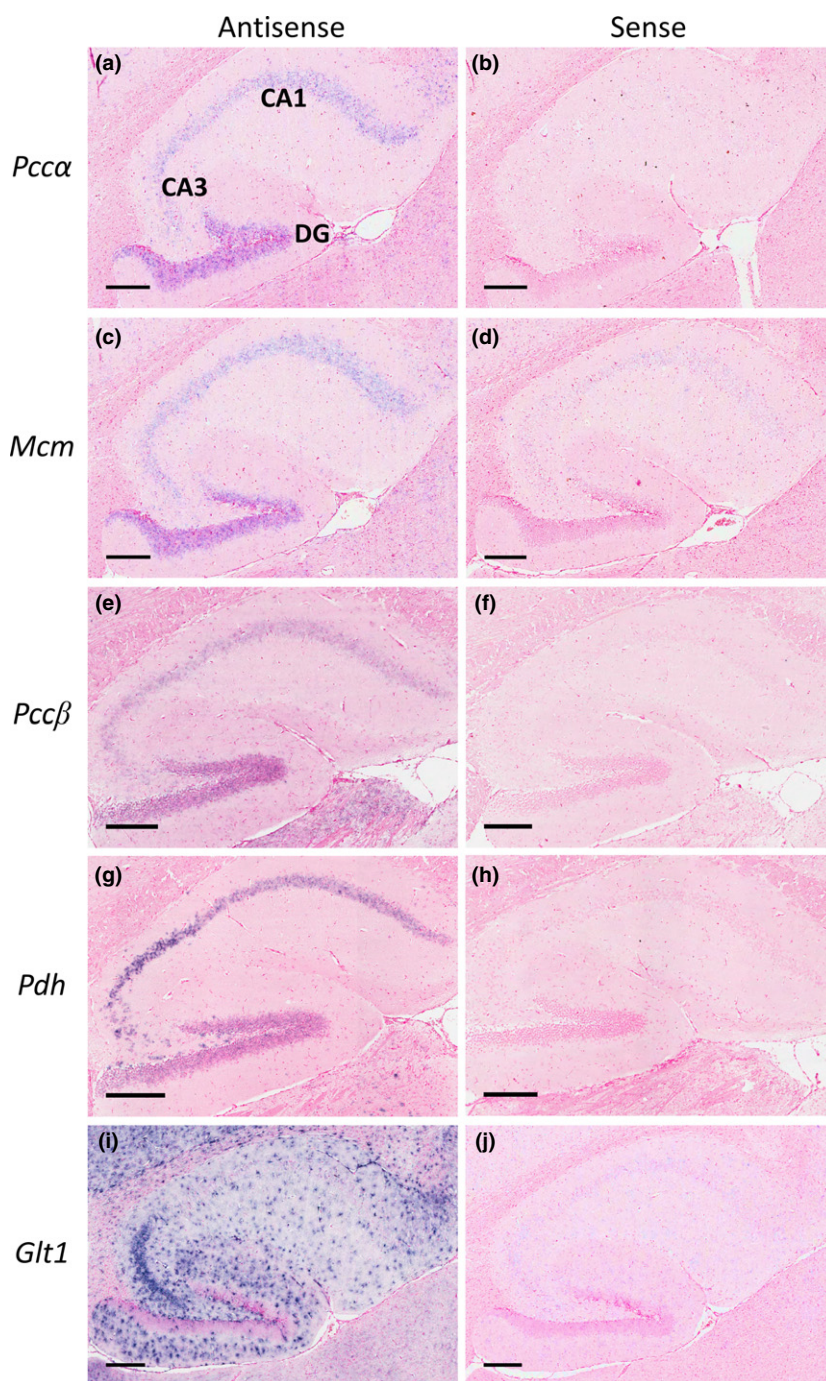


Fig. 6 Localization of anaplerotic enzymes in mouse hippocampal formations. The expression of anaplerotic enzymes in the propionyl-CoA carboxylase (PCC) pathway was examined using *in situ* hybridization. (a) Staining representative for the expression of the α -subunit of PCC (*Pcca*), (c) methylmalonyl-CoA mutase (*Mcm*) and (e) β -subunit of PCC (*Pcc β*) can be seen in the pyramidal cell layer of CA1 and CA3 regions as well as the granule cell layer of dentate gyrus (DG). The localization of these enzymes is similar to that of (g) pyruvate dehydrogenase (*Pdh*), which is mainly expressed in the neurons, but not that of (i) glutamate transporter 1 (*Glt1*), which is localized to astrocytes. Scale bar represents 250 μ m.

impairments are unlikely to be caused by neuronal cell loss because there is no significant neuronal cell death at 24 h in this model (Borges *et al.* 2003), since maximum cell death occurs at 48–72 h. Thus, the impairments observed are likely to be a reflection of the degeneration process. Mitochondria are the main ATP generators in cells and the impairments described here are likely to have a large impact on ATP production and consequently, neuronal signaling. The role of brain energy metabolism in epilepsy is evident from the

increase in cerebral glucose uptake upon seizure initiation and reduction between seizure events in the epileptic foci (Kuhl *et al.* 1980; Chugani and Chugani 1999) as well as epileptic phenotypes that are often exhibited by patients with mutations in metabolic enzymes (Bonnefont *et al.* 1992; Kerrigan *et al.* 2000; Horváth *et al.* 2006) and glucose transporter 1 deficiency (Mullen *et al.* 2011; Scheffer 2012). The dysfunctions in energy metabolism found here are therefore likely to contribute to the development of

spontaneous seizures, which occurs within days in this mouse model.

Metabolic treatments are beneficial

Triheptanoin pre-treatment before seizure induction did not alter the severity of SE. It is interesting that pilocarpine-induced seizure activity was not affected. This might be due to the fact that pilocarpine is a strong convulsant and anticonvulsant compounds do not typically show effectiveness in all seizure models. However, triheptanoin preserved all mitochondrial parameters assessed in SE mice and increased state 3 ADP and respiration linked to ATP synthesis in no SE mice. This furthers our previous findings that triheptanoin restored the levels of acetyl-CoA, propionyl-CoA and β -hydroxypentanoate and improved TCA cycling in chronic SE mouse brains (Willis *et al.* 2010; Hadera *et al.* 2014). Similar to triheptanoin, other metabolic treatments have been shown to protect against seizures and/or associated damage. This includes ketogenic diets, which supply ketones as additional fuel to glucose, or supplementation of TCA cycle intermediates, including pyruvate (Kim *et al.* 2007; Carvalho *et al.* 2011; Simeone *et al.* 2014; Popova *et al.* 2017), α -ketoglutarate (Yamamoto 1990; Yamamoto and Mohanan 2003), oxaloacetate (Carvalho *et al.* 2011) and succinate (Yue *et al.* 2002). It is still unknown to which extent triheptanoin and other metabolic treatments may be anti-epileptogenic. Oral pyruvate administration was found to abolish epileptic phenotypes in three different rodent epilepsy models (Popova *et al.* 2017) and improve mitochondrial functions and reduce seizure severity in Kv1.1 knockout mice (Simeone *et al.* 2014). In addition, the findings that succinate was shown to dose-dependently reduce seizures in pentylenetetrazol- and amygdala-kindled rats (Yue *et al.* 2002) and triheptanoin pre-treatment was found to delay corneal kindling in mice (Willis *et al.* 2010), are also promising although it is not clear if this is due to anticonvulsant and/or anti-epileptogenic effects. Taken together, these findings support the notion that preservation of brain energy metabolism is beneficial in epilepsy, including SE. It is now important to utilize this knowledge to develop new anti-seizure and anti-epileptic metabolic treatments, as these drugs are unlikely to have any central or sedative side effects.

Mechanism of antioxidant effects of triheptanoin

Apart from ATP production, mitochondria are also involved in the generation of reactive oxygen species which can impair mitochondrial function. We consistently showed that 1 day after SE, the antioxidant status of the plasma decreased while the levels of malondialdehyde increased substantially in both the cortex and hippocampal formations of SE mice. We also found that the activities of mitochondrial SOD were reduced in SE mice, indicating that mitochondria and TCA cycle enzymes, which are known to be highly susceptible to

oxidative stress are even more vulnerable after SE. These findings also support the notion that oxidative stress contributes to the observed mitochondrial dysfunctions, including those of the electron transport chain, respiration, ATP synthesis and the impaired activities of PDHc and OGDHc (Welter *et al.* 1996; Nulton-Persson and Szweida 2001), further affirming the role of mitochondria and oxidative stress in the pathophysiology of epilepsy (Rowley and Patel 2013).

All changes related to oxidative stress that were observed in SE mice were abrogated by triheptanoin treatment which might be ascribed to antioxidant activities. While it is unlikely that heptanoate is a direct scavenger of reactive oxygen or nitrogen species based on its structure, it is possible that triheptanoin upregulates the expression of antioxidant genes as was found previously with tridecanoin treatment (Tan *et al.* 2017). Here we did not find enhanced antioxidant status of the plasma or activities of catalase and SOD in triheptanoin-treated no SE mice and we did not measure the expression of other antioxidant genes. However, triheptanoin treatment preserved the activity of mitochondrial SOD during SE. Also, in extracts obtained from triheptanoin-treated mouse brains, PDHc and OGDHc were protected against loss of activity by hydroxyl radicals produced from the Fenton reaction. Another study found that supplementation of triheptanoin to a ketogenic diet (39E%) increased the mRNA levels of *Sirt1*, *Pparg*, *Sod1* and *Sod2* in a mouse model of Alzheimer's Disease (Aso *et al.* 2013). Taken together, these results suggest that triheptanoin has some antioxidant effects, which mechanisms remain to be explored.

Since triheptanoin attenuated impairments in mitochondrial function and induction of oxidative stress, we investigated its effects on neuronal degeneration in the hippocampal formations. Although triheptanoin did not alter seizure severity, it reduced neuronal degeneration in both the CA1 and CA3 regions 3 days post-seizure induction. In addition, heptanoate consistently protected neuronal cell cultures against H₂O₂-induced cell death, further affirming the neuroprotective effects of triheptanoin, which have been found previously in other models (Francis *et al.* 2014; Schwarzkopf *et al.* 2015; Tefera *et al.* 2016).

Anaplerosis via the PCC pathway may occur in neurons

Using *in situ* hybridization, this study found that *Pcc α* , *Pcc β* and *Mcm*, which are involved in the anaplerotic PCC pathway, are expressed in the hippocampal formations of adult mouse brains. Although we did not include any neuronal or astrocytic markers, these mRNAs were localized to the pyramidal cell layers of the CA1 and CA3 regions as well as in the granule cell layer of the dentate gyrus, indicating that the enzymes are mainly expressed by neurons. The pattern of expression is consistent with a previous study, which demonstrated that PCC α and methylmalonyl-CoA

mutase are expressed in NeuN-positive neurons but not in glial cells expressing glial fibrillary acidic protein or myelin basic protein, using *in situ* hybridization combined with immunohistochemistry as well as double immunofluorescence (Ballhausen *et al.* 2009). Taken together, these results further substantiate the view that anaplerosis via the PCC pathway can occur in neurons. However, another study that investigated the metabolism of heptanoate, by infusing [5,6,7]-¹³C₃-heptanoate in conscious wild type and glucose transporter I deficient mice, found that ¹³C was mostly incorporated into glutamine, but not glutamate (Marin-Valencia *et al.* 2013). Although this finding strongly suggests that [5,6,7]-¹³C₃-heptanoate was metabolized in glial cells due to the higher concentration of ¹³C₃-glutamine and glutamine after the infusion, the study cannot refute the possibility that heptanoate can first be metabolized in the neurons into glutamate before being taken up by the glial cells to be further metabolized into and stored as glutamine. In addition, unlike long-chain fatty acids, the β-oxidation of heptanoate does not require carnitine palmitoyltransferase, which has higher activity in astrocytes (Blázquez *et al.* 1998), further suggesting that heptanoate can be utilized by neurons.

Limitations of the study

Our study was performed exclusively in male mice, which disregards at least half of the population. Several of the experiments would have benefitted from higher sample sizes. However, we repeated the experiments giving rise to all major findings.

Conclusion

Here, we demonstrated that triheptanoin pre-treatment protected the hippocampus against SE-induced mitochondrial dysfunctions and loss of mitochondrial enzyme activities, oxidative stress and neuronal degeneration. Similarly, heptanoate improved cell survival during oxidative stress *in vitro* and PDHc and OGDHc activities in extracts obtained from triheptanoin-treated mouse brains were protected against the effects of oxidative attack by the Fenton reaction. Our *in situ* hybridization localized several anaplerotic enzymes in the PCC pathway to the main hippocampal neuronal cell layers. Taken together, our results further indicate that anaplerosis via the PCC pathway can occur in neurons and that triheptanoin has antioxidant effects which contribute to its protective effects toward mitochondrial functions, oxidative stress and cell death.

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Authors' contributions

KT, KB and CC planned and designed the experiments. KT, DS and CC performed the experiments and analyzed the data. KT drafted the manuscript and KT, DS, KB and CC revised the manuscript.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Reaction mixes for enzyme activity assays.

Table S2. Primer sequences without T7 or T3 RNA polymerase promoter for *in situ* hybridization probe synthesis.

References

- Adanyeguh I. M., Rinaldi D., Henry P. G., Caillet S., Valabregue R., Durr A. and Mochel F. (2015) Triheptanoin improves brain energy metabolism in patients with Huntington disease. *Neurology* **84**, 490–495.
- Aso E., Semakova J., Joda L., Semak V., Halbaut L., Calpena A., Escolano C., Perales J. C. and Ferrer I. (2013) Triheptanoin supplementation to ketogenic diet curbs cognitive impairment in APP/PS1 mice used as a model of familial Alzheimer's disease. *Curr. Alzheimer Res.* **10**, 290–297.
- Ballhausen D., Mittaz L., Boulat O., Bonafe L. and Braissant O. (2009) Evidence for catabolic pathway of propionate metabolism in CNS: expression pattern of methylmalonyl-CoA mutase and propionyl-CoA carboxylase alpha-subunit in developing and adult rat brain. *Neuroscience* **164**, 578–587.
- Benson M. J., Thomas N. K., Talwar S., Hodson M. P., Lynch J. W., Woodruff T. M. and Borges K. (2015) A novel anticonvulsant mechanism via inhibition of complement receptor C5ar1 in murine epilepsy models. *Neurobiol. Dis.* **76**, 87–97.
- Benzie I. F. and Strain J. J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **239**, 70–76.
- Blázquez C., Sánchez C., Velasco G. and Guzmán M. (1998) Role of carnitine palmitoyltransferase I in the control of ketogenesis in primary cultures of rat astrocytes. *J. Neurochem.* **71**, 1597–1606.
- Bonnefont J. P., Chretien D., Rustin P. *et al.* (1992) Alpha-ketoglutarate dehydrogenase deficiency presenting as congenital lactic acidosis. *J. Pediatr.* **121**, 255–258.
- Borges K., Gearing M., McDermott D. L., Smith A. B., Almonte A. G., Wainer B. H. and Dingledine R. (2003) Neuronal and glial pathological changes during epileptogenesis in the mouse pilocarpine model. *Exp. Neurol.* **182**, 21–34.
- Carrasco-Pozo C., Tan K. N. and Borges K. (2015) Sulforaphane is anticonvulsant and improves mitochondrial function. *J. Neurochem.* **135**, 932–942.

- Carvalho A. S., Torres L. B., Persike D. S., Fernandes M. J., Amado D., Naffah-Mazzacoratti Mda G., Cavalheiro E. A. and da Silva A. V. (2011) Neuroprotective effect of pyruvate and oxaloacetate during pilocarpine induced status epilepticus in rats. *Neurochem. Int.* **58**, 385–390.
- Chugani H. T. and Chugani D. C. (1999) Basic mechanisms of childhood epilepsies: studies with positron emission tomography. *Adv. Neurol.* **79**, 883.
- Francis J. S., Markov V. and Leone P. (2014) Dietary triheptanoin rescues oligodendrocyte loss, dysmyelination and motor function in the *nur7* mouse model of Canavan disease. *J. Inherit. Metab. Dis.* **37**, 369–381.
- Gu L., Zhang G. F., Kombu R. S., Allen F., Kutz G., Brewer W. U., Roe C. R. and Brunengraber H. (2010) Parenteral and enteral metabolism of anaplerotic triheptanoin in normal rats. II. Effects on lipolysis, glucose production, and liver acyl-CoA profile. *Am. J. Physiol. Endocrinol. Metab.* **298**, E362–E371.
- Hadera M. G., Smeland O. B., McDonald T. S., Tan K. N., Sonnewald U. and Borges K. (2014) Triheptanoin partially restores levels of tricarboxylic acid cycle intermediates in the mouse pilocarpine model of epilepsy. *J. Neurochem.* **129**, 107–119.
- Halliwell B. and Gutteridge J. M. (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett.* **307**, 108–112.
- Horváth R., Abicht A., Holinski-Feder E., Laner A., Gempel K., Prokisch H., Lochmuller H., Klopstock T. and Jaksch M. (2006) Leigh syndrome caused by mutations in the flavoprotein (Fp) subunit of succinate dehydrogenase (SDHA). *J. Neurol. Neurosurg. Psychiatry* **77**, 74–76.
- Kerrigan J. F., Aleck K. A., Tarby T. J., Bird C. R. and Heidenreich R. A. (2000) Fumaric aciduria: clinical and imaging features. *Ann. Neurol.* **47**, 583–588.
- Kilkenny C., Browne W. J., Cuthill I. C., Emerson M. and Altman D. G. (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412.
- Kim T. Y., Yi J. S., Chung S. J., Kim D. K., Byun H. R., Lee J. Y. and Koh J. Y. (2007) Pyruvate protects against kainate-induced epileptic brain damage in rats. *Exp. Neurol.* **208**, 159–167.
- Kim T. H., Borges K., Petrou S. and Reid C. A. (2013) Triheptanoin reduces seizure susceptibility in a syndrome-specific mouse model of generalized epilepsy. *Epilepsy Res.* **103**, 101–105.
- Kinman R. P., Kasumov T., Jobbins K. A. *et al.* (2006) Parenteral and enteral metabolism of anaplerotic triheptanoin in normal rats. *Am. J. Physiol. Endocrinol. Metab.* **291**, E860–E866.
- Kuhl D. E., Engel J., Phelps M. E. and Selin C. (1980) Epileptic patterns of local cerebral metabolism and perfusion in humans determined by emission computed tomography of 18FDG and 13NH3. *Ann. Neurol.* **8**, 348–360.
- Marin-Valencia I., Good L. B., Ma Q., Malloy C. R. and Pascual J. M. (2013) Heptanoate as a neural fuel: energetic and neurotransmitter precursors in normal and glucose transporter I-deficient (G1D) brain. *J. Cereb. Blood Flow Metab.* **33**, 175–182.
- Mochel F., Duteil S., Marelli C. *et al.* (2010) Dietary anaplerotic therapy improves peripheral tissue energy metabolism in patients with Huntington's disease. *Eur. J. Hum. Genet.* **18**, 1057–1060.
- Mochel F., Hainque E., Gras D. *et al.* (2016) Triheptanoin dramatically reduces paroxysmal motor disorder in patients with GLUT1 deficiency. *J. Neurol. Neurosurg. Psychiatry* **87**, 550–553.
- Mullen S. A., Marini C., Suls A. *et al.* (2011) Glucose transporter 1 deficiency as a treatable cause of myoclonic astatic epilepsy. *Arch. Neurol.* **68**, 1152–1155.
- Nulton-Persson A. C. and Szveda L. I. (2001) Modulation of mitochondrial function by hydrogen peroxide. *J. Biol. Chem.* **276**, 23357–23361.
- Pascual J. M., Liu P., Mao D. *et al.* (2014) Triheptanoin for glucose transporter type I deficiency (G1D): modulation of human icogenesis, cerebral metabolic rate, and cognitive indices by a food supplement. *JAMA Neurol.* **71**, 1255–1265.
- Popova I., Malkov A., Ivanov A. I. *et al.* (2017) Metabolic correction by pyruvate halts acquired epilepsy in multiple rodent models. *Neurobiol. Dis.* **106**, 244–254.
- Rowley S. and Patel M. (2013) Mitochondrial involvement and oxidative stress in temporal lobe epilepsy. *Free Radic. Biol. Med.* **62**, 121–131.
- Samala R., Willis S. and Borges K. (2008) Anticonvulsant profile of a balanced ketogenic diet in acute mouse seizure models. *Epilepsy Res.* **81**, 119–127.
- Scheffer I. E. (2012) GLUT1 deficiency: a glut of epilepsy phenotypes. *Neurology* **78**, 524–525.
- Schmued L. C. and Hopkins K. J. (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.* **874**, 123–130.
- Schwarzkopf T. M., Koch K. and Klein J. (2015) Reduced severity of ischemic stroke and improvement of mitochondrial function after dietary treatment with the anaplerotic substance triheptanoin. *Neuroscience* **300**, 201–209.
- Simeone K. A., Matthews S. A., Samson K. K. and Simeone T. A. (2014) Targeting deficiencies in mitochondrial respiratory complex I and functional uncoupling exerts anti-seizure effects in a genetic model of temporal lobe epilepsy and in a model of acute temporal lobe seizures. *Exp. Neurol.* **251**, 84–90.
- Simmons D. G., Rawn S., Davies A., Hughes M. and Cross J. C. (2008) Spatial and temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not associated with their position in the locus. *BMC Genom.* **9**, 352.
- Tan K. N., Carrasco-Pozo C., McDonald T. S., Puchowicz M. and Borges K. (2017) Tridecanoin is anticonvulsant, antioxidant, and improves mitochondrial function. *J. Cereb. Blood Flow Metab.* **37**, 2035–2048.
- Tefera T. W., Wong Y., Barkl-Luke M. E., Ngo S. T., Thomas N. K., McDonald T. S. and Borges K. (2016) Triheptanoin protects motor neurons and delays the onset of motor symptoms in a mouse model of amyotrophic lateral sclerosis. *PLoS ONE* **11**, e0161816.
- Thomas N. K., Willis S., Sweetman L. and Borges K. (2012) Triheptanoin in acute mouse seizure models. *Epilepsy Res.* **99**, 312–317.
- Welter R., Yu L. and Yu C. A. (1996) The effects of nitric oxide on electron transport complexes. *Arch. Biochem. Biophys.* **331**, 9–14.
- Willis S., Stoll J., Sweetman L. and Borges K. (2010) Anticonvulsant effects of a triheptanoin diet in two mouse chronic seizure models. *Neurobiol. Dis.* **40**, 565–572.
- Yamamoto H. (1990) Protection against cyanide-induced convulsions with alpha-ketoglutarate. *Toxicology* **61**, 221–228.
- Yamamoto H. A. and Mohanan P. V. (2003) Effect of alpha-ketoglutarate and oxaloacetate on brain mitochondrial DNA damage and seizures induced by kainic acid in mice. *Toxicol. Lett.* **143**, 115–122.
- Yue W., Liu Y. X., Zang D. L., Zhou M., Zhang F. and Wang L. (2002) Inhibitory effects of succinic acid on chemical kindling and amygdala electrical kindling in rats. *Acta Pharmacol. Sin.* **23**, 847–850.