Carvacrol after status epilepticus (SE) prevents recurrent SE, early seizures, cell death and cognitive decline

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Abstract

Objective: Carvacrol is a naturally occurring a monoterpenic phenol which has been suggested to have an action at transient receptor potential cation subfamily M7 channels (TRPM7), GABA(A) receptors and sodium channels, and has been shown to be anti-inflammatory. Carvacrol is neuroprotective in *in vivo* and *in vitro* models of cerebral ischemia, probably through its action at TRPM7 channels. We therefore aimed to determine the effect of carvacrol on status epilepticus (SE), chronic epilepsy, cell death and post-SE cognitive decline.

Methods: We performed long-term, continuous wireless EEG monitoring *in vivo* in rats who underwent perforant path stimulation (PPS) to induce SE and were then randomised to treatment with carvacrol or saline. We also evaluated TRPM7 receptor expression and quantified seizure-induced cell death. The alternating T-maze paradigm was used to assess memory function.

Results: Immunostaining showed that TRPM7 channels are widely expressed in neurons within the hippocampus. We found that carvacrol inhibited recurrent SE and early seizures in vivo, but had no detectable effect in the hippocampus on paired-pulse inhibition or the fiber volley, indicating that it was not acting through sodium channel inhibition or GABA receptors. Although the development and severity of chronic epilepsy was not altered by carvacrol, cognitive decline was significantly improved in animals treated with carvacrol. In keeping with preserved memory functions in animals treated with carvacrol, carvacrol had a protective effect against

SE-induced cell death in CA1 and hilus, the hippocampal regions most affected by cell loss in the PPS epilepsy model.

Significance: Carvacrol, a naturally occurring inhibitor of TRPM7 channels, is a novel, promising treatment to prevent early recurrence of SE, SE related neuronal damage and cognitive decline.

Key words: TRPM7, cell death, refractory status epilepticus, seizures, epilepsy, cognitive decline

INTRODUCTION

Carvacrol is a monoterpenic phenol, which is present in oregano and thyme oil. It has been widely used in the food industry as a flavoring and food preservative. Carvacrol has been shown to have bactericidal, fungicidal and insecticidal activity.[1–3] More recently it has been shown to have neuroprotective effects in both *in vitro* and *in vivo* animal models of cerebral ischemia,[4–7] probably through inhibition of TRPM7 channels.[6] TRPM7 channels are ubiquitously expressed transmembrane ion channels, which are constitutively active and suppressed by high intracellular concentrations of free magnesium and $Mg^{2+}ATP$,[8] and so act as magnesium sensors.[9] TRPM7 channels are expressed in the brain[10] where they have been shown to be important in mediating neuronal anoxic cell death.[11] Following initial reports, many studies have supported the importance of TRPM7 channels in brain ischemia.[4,12] More recently a pathological role of TRPM7 channels in traumatic brain injury has been reported.[5] Both brain ischemia and traumatic brain injury are profound tissue injuries resulting in excitotoxicity and overt necrotic cell death. We

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asked whether this neuroprotective effect extends to prolonged seizure activity (status epilepticus) and its consequences - chronic epilepsy and cognitive decline. Excessive neuronal activity is the hallmark and defining feature of seizures and SE,[13] and ion channels are crucial in generating such activity. TRPM7 channels therefore may represent an important target in epilepsy, but surprisingly very little is known about the role of TRPM7 channels in SE, seizures, epilepsy and epilepsy comorbidities. Whereas cell death has been a focus of most studies investigating TRPM7 function, studies evaluating the role of TRPM7 channels in the sequelae of neuronal cell death such as memory function are lacking. More recently, the study of TRPM7 channel function has been facilitated through the characterization of potent TRPM7 agonists and antagonists.[7] Transient pharmacological manipulation of TRPM7 currents is particularly interesting, since global deletion of TRPM7 is lethal[14] and therefore permanent genetic knock down is less attractive as a therapeutic intervention. We show here that carvacrol prevents recurrent SE, early seizures post-SE and neuronal cell death in the hippocampus. Importantly, carvacrol prevented post status epilepticus memory decline, suggesting that TRPM7 channels are a potential target for future drug development treating SE and its sequelae, and that a well-tolerated, natural oil, carvacrol, should be further investigated for use in this condition.

MATERIALS AND METHODS

Animals

All animal procedures were carried out subject to local ethical approval and followed the UK Home Office Animal (Scientific Procedures) Act, 1986. Male adult Sprague-Dawley rats (280-310g, Charles River, U.K.) were housed with a 12 h light-dark cycle and access to food and water *ad libitum*. The perforant path model of epilepsy was used to model SE and chronic epilepsy *in vivo* and has been described previously[15,16]. For the *in vitro* experiments, rats were culled with isoflurane overdose and then decapitated. To detect a difference of two standard deviations (a clinically significant change) with a power of 0.8-0.9 and an alpha error of 0.05 a sample size of 4-6 animals per group is needed.

Preparing acute brain slices

After decapitation, the rat brain was quickly removed and 350 μm horizontal brain slices comprising the hippocampus, dentate gyrus and entorhinal cortex were cut on the vibratome (Leica VT1200S, Leica mycrosystems, Nussloch, Germany). The slices were stored in aerated (95% O_2/5%CO_2) artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 0.9 mM NaH_2PO_4, 14 mM D-glucose, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2.6 mM $NaHCO_3$ and titrated to an osmolality of 296 mOsm/kg.

Evoked field EPSP experiments

Evoked extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from CA1 region of the hippocampus in *in vitro* combined hippocampal-enthorinal

cortex slices. The recording electrode, a borosilicate glass microelectrode of 1–2 MΩ resistance filled with ACSF was placed in stratum radiatum (for fEPSP slopes) or stratum pyramidale (for population spikes) of CA1 region. A bipolar stimulating electrode was placed in the Schaffer collaterals/commissural fibre region. Stimulation intensity was set to 0.3-0.8mA and single pulses [100µs width] with 20 s intervals were delivered. After 20 min of recording, drug or vehicle was added to the perfusion solution and recording continued for another 20 min. Recordings were filtered at 4 kHz, obtained using a MultiClamp 700B amplifier (Molecular Devices, USA) and digitized at 10 kHz. The LabView (National Instruments, Austin, TX) software was used for data acquisition and off-line analysis. Population spike data were acquired and analysed using WinWCP software (John Dempster, University of Strathclyde, Glasgow, UK).

Electrode implantation – In vivo experiments

All electrodes were purchased from Plastics One Inc, Bilaney Consultants UK Ltd. The animal was placed in a stereotaxic frame (David Kopf Instruments, USA) under isoflurane anaesthesia (2-3 % in 2 L/min O_2) and buprenorphine analgesia (0.2 mg/kg s.c.; Temgesic, Scherig-Plough, UK). Co-ordinates for the stimulation electrode were 8.1 mm caudal and 4.4 mm lateral from bregma at a depth of approximately 3.5 mm to stimulate the angular bundle, and 4 mm caudal and 2.5 mm lateral from the bregma for the recording electrode which was inserted (in all animals except those undergoing chronic EEG recordings) to lie within the dentate granule layer. The reference electrode was secured subcutaneously. A single pulse stimulus of 3.5 mA and 150 µs width was delivered from a Neurolog system (Digitimer Ltd, Welwyn Garden City, UK) and a stimulus isolator (Digitimer, UK). Recordings were

bandpass-filtered at 0.1-50 Hz and recorded using Spike 2 software (CED, Cambridge, UK). The optimal position for electrodes was determined by adjusting the electrode position to achieve a maximal population field excitatory postsynaptic potential (fEPSP; >2 mV population spike).

For those animals that were to undergo continuous EEG monitoring, a Wireless Subcutaneous Transmitter (A3019D, 0.3-160 Hz band-pass, 512 per second sample rate, Open Source Instruments) was implanted subcutaneously in the dorsal part of the rat body. The stimulation electrode was implanted as above. The reference and the recording electrodes were inserted to lie extradurally through drilled holes and held in place with skull screws. The recording electrode was placed 4 mm caudal and 2.5 mm lateral from the bregma to enable recordings from the cortex. The reference electrode was implanted in the contralateral side of the brain.

Subsequently anaesthesia was weaned, 5 ml 0.9% w/v saline was administered subcutaneously and rats were monitored until full recovery.

Induction of SE and carvacrol injection

7-10 days postoperatively the perforant path was stimulated through the implanted bipolar electrodes. Animals were randomised into 3 groups: "controls" which underwent surgery but not stimulation, "epileptic saline" which were stimulated and given repeated injections of saline and "epileptic carvacrol" which were stimulated and given repeated doses of carvacrol (75 mg/kg ip; Sigma Aldrich, UK).

The stimulation current was set to elicit a population spike that was 50-75 % of the maximum. Monopolar 20 Hz stimulation was delivered from a Neurolog system for 2 hours after an initial 20 min baseline recording.

During stimulation and subsequent self-sustaining status epilepticus (SSSE), behaviour, seizure type and severity were monitored, recorded for every 15 min and graded according to the Racine scale[17]. After 2 hours stimulation, the recording was continued and monitored for 20 min to ensure that the animal had developed SSSE. Afterwards diazepam (10 mg/kg i.p.) was injected and EEG recording continued for 20-30 min to ensure that SSSE was aborted. Following this, the carvacrol group received 75 mg/kg i.p. of carvacrol. The dose was chosen based on previous experiments in vivo [4,18]. The epileptic saline group received the same amount of saline (i.p.). Carvacrol is a lipophilic substance and is quickly metabolized and excreted. In rats only minimal amounts of carvacrol and its metabolites were found in urine 24h after oral ingestion. [19] Thus, another two doses of carvacrol (epileptic carvacrol group; 75 mg/kg i.p.) or saline (epileptic saline group) were administered every 8 hours, so that each animal received 3 injections during the first 24 hours after SE induction, when neuronal damage is greatest.

EEG monitoring of seizure activity

Animals that had implanted subcutaneous transmitters were then transferred to a telemetry unit (Open Source Instruments, Inc). Continuous EEG recordings were performed for up to 8 weeks. Neuroarchiver tool in long wire data acquisition (LWDAQ) software (Open Source Instruments, Inc.) was used to record and analyse seizures

(see www.opensourceinstruments.com/Electronics/A3018/Seizure_Detection.html).

Seizure analysis

Seizure-analysis was carried out in a semi-automated fashion. The Event Classifier program in Neuroarchiver was used to detect seizures. It automatically detects events based on a comparison of pre-selected events in an event library. The Classifier operates on the baseline power and the interval metrics which have been shown to be superior to coastline analysis. The algorithm was validated by visual EEG seizure analysis and adjusted to detect all seizure events. Each event highlighted by the Event Classifier was visually confirmed by AK, experienced in EEG seizure analysis, to exclude false positive results. All data was analysed off-line blinded to treatment group. Seizures were quantified on a weekly basis for seven weeks starting one week after induction of status epilepticus. Seizures were analysed by AK blinded to the animal group.

Immunostaining, imaging and cell counts

Three to four weeks after electrical induction of SSSE, a group of controls, epileptic saline and epileptic carvacrol groups were perfused with phosphate-buffer saline (PBS) and subsequently 4% paraformaldehyde (PFA). Animals were culled and brains were kept in 4% PFA overnight. For immunohistochemistry 45 µm coronal slices were cut using Leica vibratome (Leica microsystems, Nussloch, Germany). To ensure that similar regions within the hippocampus were compared between groups, slices were selected at the same distance (anterior-posterior) in all three groups. Slices were incubated with 40,6-diamidino-2-phenylindole (DAPI; 5 µg/ml, Sigma Aldrich, UK), NeuN (1:400, Merck Millipore) and TRPM7 antibody (1:300, Abcam) as

appropriate and secondary antibodies Alexa Fluor 546 (1:500, Molecular probes, Invitrogen) and Alexa Fluor 488 (1:500, Molecular probes, Invitrogen). All images were acquired at a resolution of 1024x1024 on a confocal microscope (Zeiss 710 VIS LSM, Oberkochen, Germany). The 20x objective was used to image CA1 and CA3 region and DG. DAPI emission spectra were imaged at 460 nm using a 405-nm excitation laser. Alexa Fluor 488 was excited with the 488 nm argon laser and emission was collected at 500-550 nm. Alexa Fluor 546 was excited with the 543 laser measuring light emitted >560 nm. Cell counts were performed by AK blinded to the treatment group. Cells counts were expressed as cell counts per length (CA1 and CA3 area) or as cell densities (see Fig. 4; hilar cell counts). In CA1 and CA3 regions, the number of cells per length were counted: for this purpose three areas of 300 µm length each were selected, each ~50 µm apart, representing the whole length of CA1/CA3. Cells that were within 45 µm of the main CA1/CA3 cell cluster were also included. Counting was performed blinded to the animal groups.

Rewarded alternating T-maze test

To assess memory function, the rewarded alternating T-maze test was used. Perforant path stimulated rats were tested 3-4 month following SE induction. The T-maze (Med Associates, Inc., USA; UK distributor: Sandown Scientific, UK; Standard T-Maze Package for Rat) experiments were conducted blinded to the treatment group and following a modified protocol described by Deacon and Rawlins.[20] The rewarded T-maze test consisted of the following parts: habituation, training and main experiments. Animal were weighed 2 subsequent days before starting a restricted diet to obtain a baseline weight. Then animals were weighed on a daily basis to

ensure that they maintained 90-95% of their free-feeding body weight. The main training was conducted as follows: Access to the correct choice arm was restricted. Animals were placed in the start arm. The sample arm was baited with one pellet and the choice arm with 2 pellets. Once animal reached the end of sample arm and consumed the pellet, it was placed back to the start arm not facing the T junction. Then the door to the choice arm was opened and the animal was allowed to choose an arm (Fig. 4A). During the main test the animal was given 60 second retention interval (the time period between exposure to sample and choice phase) before making a choice of arm. The correct arm choices were counted blinded to animal group.

Statistical analyses

Statistical analyses (Paired t-test, one way ANOVA, two-way ANOVA, Post hoc Tukey's test, Fisher's exact test, Loglinear mixed model followed by pairwise comparison with sequential Bonferroni correction) were performed using SPSS 17.0 (Chicago, IL, USA). Shapiro-Wilk and Anderson Darling tests were carried out to check for normality. The significance level was set at $p < 0.05$ and all data are given as mean ± standard error of the mean (SEM).

RESULTS

Carvacrol has no effect on fiber volley or paired pulse inhibition

Carvacrol acts on TRPM7 channels, but has also been reported to decrease ion conductance in peripheral sodium channels. [21] Moreover, it has been shown that

carvacrol can facilitate the action of low GABA concentrations on $GABA_A$ receptors.[22,23] To determine if these effects are relevant to hippocampal circuitry, we tested the effect of a high carvacrol concentration (1 mM) on fEPSPs in stratum pyramidale (SP) and radiatum (SR) of the CA1 region after stimulation of the Schaffer collaterals. The normalised mean slope of fEPSPs recorded in SR increased significantly during carvacrol treatment compared to baseline ($p<0.05$ paired t-test). There was, however, no significant change in fiber volley slope after carvacrol was added (Suppl Fig. 1) indicating that carvacrol does not affect sodium channels in acute brain slices. There was also no change in paired-pulse inhibition, as measured by population spike amplitude (one way ANOVA), which indicates that carvacrol does not significantly affect GABAergic inhibition (Suppl. Fig. 2).[24]

Expression pattern of TRPM7 in the hippocampal formation

We next determined qualitative expression levels of TRPM7 in the hippocampal formation. Although studies have demonstrated that mRNA levels of TRPM7 are high throughout the entire brain,[10] there has been no detailed study of neuronal TRPM7 expression in the hippocampus. We therefore co-stained for TRPM7 and NeuN within the hippocampus. This showed that there was robust expression of TRPM7 channels throughout the hippocampal formation (CA1, CA3 and hilus). This co-localized with the NeuN staining indicating that TRPM7 channels are expressed in neurons. TRPM7 channel expression was particularly high in CA1 and was least expressed in CA3, with hilus, and DG showing intermediate expression levels (Fig.1).

Carvacrol treatment prevents reoccurrence of early SE

Self-sustaining status epilepticus was induced in animals (n=16) which were then randomised to receive either three doses of saline or carvacrol (total dose 225mg/kg) over 24 hours. One animal dropped out (epileptic saline group) as the headpiece came off soon after stimulation. We first examined any possible anti-seizure effect of carvacrol in the first 24 hours). In particular, we examined recurrent SE, defined as continuous seizure activity) lasting more than 30 min or repetitive seizures with an inter-seizure interval of less than two minutes (for example see Fig. 3C). We found that 12 hours after induction of SE, and after diazepam injection as per protocol, six out of seven animals (~86 %) in the saline-treated group developed recurrent SE. This is in striking contrast to animals that were injected with carvacrol of which two out of eight (25%) animals developed recurrent SE (p < 0.05, Fisher's exact test; Fig. 2).

Carvacrol alters early seizure frequency but not chronic epilepsy

We next analysed whether there was a difference in the development of epilepsy between the two groups. In the PPS model of epilepsy that we used, animals develop seizures and chronic epilepsy immediately after SE. We quantified the seizure frequency on a weekly basis one week after SE; at that time any acute effects of carvacrol will have passed.[19] A generalized linear model (with a Poisson probability distribution, week as a within-subject effect and treatment as the between-subject factor) was used to compare seizure frequency changes over time between the two

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groups. This showed that overall there was no statistical difference between the groups (Wald-Chi Square 1.77, DF (1), p>0.05). However, the interaction of treatment with week was significant (Wald-Chi Square 37.8, DF (6), p<0.001) indicating that there is a difference between the groups depending on the week. Post hoc Mann-Whitney test showed that there was a significant difference in the seizure frequency in only the second week between the epileptic saline and epileptic carvacrol groups (Fig. 3B; p<0.05). Two animals (one saline treated and one treated with carvacrol) had to be excluded after the evaluation of status epilepticus, as there was a problem with the recording equipment after two days. The remaining saline-treated animals (n=6) had a median of 5 seizures whereas the animals treated with carvacrol (n=7) had a median of 0 seizures at week 2. This effect was not sustained in the following weeks. Analysis of seizure duration showed that there was no significant difference between epileptic saline and epileptic carvacrol groups (Fig. 3C).

Carvacrol prevents hippocampal cell death

Although, there was no effect on the development of chronic epilepsy, we asked whether carvacrol had an effect on neuronal death. The greatest cell death in the PPS model is observed in the first 1-2 days following initial SE and continues for one week with minimal cell death after this.[25] In separate groups of animals, we quantified neuronal cell death in CA1, CA3 and hilus in controls, epileptic saline and epileptic carvacrol animals (all n=6) 3-4 weeks after SE. We observed significant differences between the three groups in both hilar (Fig. 4C and D; [F (2,15)=8.28; p<0.01]) and

CA1 regions (Fig.4 A and B; [$F(2,15) = 47$; $p<0.001$]; both one way ANOVA). There was a ~ 20% reduction of cell counts in CA1 regions (Post hoc Tukey's test; $p<0.001$) and a ~50% reduction in the hilus (Post hoc Tukey's test; $p<0.01$) in epileptic saline-treated animals compared to controls. Neuronal cell counts were not significantly different between controls and epileptic carvacrol-treated animals; however, the cell count was significantly higher in the epileptic carvacrol group compared to the epileptic saline group (Post hoc Tukey's test; $p<0.001$), suggesting that inhibition of TRPM7 channels with carvacrol completely prevented neuronal cell death in both CA1 and hilar regions. No significant difference in the degree of cell loss was observed between the three groups in the CA3 area (Fig. 4E; one way ANOVA [$F(2,15) = 3.18$, $p>0.05$,]).

Cognitive decline is prevented by carvacrol

Finally, we asked whether preventing recurrent SE, early seizures and cell death with the TRPM7 blocker carvacrol has an impact on memory. We tested epileptic saline, epileptic carvacrol and control animals after ~3 months using an alternating reward T-maze protocol (Fig.5A). There was a significant difference in the percentage of correct arm choice between the three groups [One-way ANOVA, $F(2,14) = 9.7$; $p<0.01$]. The mean percentage of correct arm choices in the T-maze was 95.3±3.9% in controls versus 63.4±14.5 % in epileptic saline-treated animals (post hoc Tukey's test; $p<0.01$) suggesting that spatial memory is impaired in epileptic animals. There was no significant difference between control and epileptic animals treated with the TRPM7 blocker carvacrol (post hoc Tukey's test; $p>0.05$; Fig.5B), however the mean percentage of correct arm choices in the epileptic carvacrol group (86.5±15.3 %) was

significantly higher compared to the epileptic saline group (post hoc Tukey's test; p<0.05), suggesting that carvacrol was able to entirely prevent seizure-induced memory dysfunction.

DISCUSSION

We have shown that carvacrol, a TRPM7 channel blocker, effectively inhibited recurrent SE and early seizures after SE in the perforant path model of epilepsy, improving disease severity in the early stages. Carvacrol did not prevent subsequent development of chronic epilepsy, but efficiently prevented cognitive decline post-SE. TRPM7 channels are regulators of magnesium homeostasis. Hypomagnesaemia has been documented in patients suffering from epilepsy[26] and acute symptomatic seizures are precipitated by metabolic conditions that induce hypomagnesaemia such as alcohol consumption.[27] In addition, an *in vitro* epilepsy model – the low magnesium model of epilepsy – exerts its effect through depletion of extracellular magnesium. These findings indicate that magnesium homeostasis is important in epilepsy.

In the current study, we used carvacrol as an inhibitor of TRPM7 channel function. Carvacrol, a food additive, has antimicrobial properties likely via its toxicity against bacterial membranes.[28] In addition, carvacrol also has anti-inflammatory effects at high concentrations via PPARgamma-dependent suppression of COX-2 promoter activity.[29] With particular importance to our study, carvacrol has been shown to be a potent antagonist at TRPM7 channels in vertebrates in addition to being an antagonist at TRPL channels in drosophila[6]. Carvacrol also activates "thermo" TRP channels: TRPV3 and TRPA1, channels which are expressed at primary sensory

nerve terminals such as the tongue and nasal epithelium where they provide information about thermal changes.[30] The action on TRPM7 channels is likely to be their main effect in preventing recurrence of SE, seizures after SE and cognitive decline in our study. There is, however, a possibility that carvacrol's anti-inflammatory actions might contribute to these effects as well.

Also inhibition of peripheral sodium channels by carvacrol has been reported.[21,31] In our experiments carvacrol did not inhibit, but indeed potentiated fEPSPs in hippocampal-entorhinal cortex slices and also had no effect on the fiber volley, suggesting that a significant inhibitory effect on sodium channels did not occur. This could possibly be explained by different variants of sodium channels in peripheral nerves and CNS. Two studies have also suggested that carvacrol has an effect on GABA(A) receptors exposed to very low GABA concentrations (1-2 µM) [22,23]; these concentrations are orders of magnitude lower than GABA concentrations at the synapse. We, therefore, asked whether such an effect is relevant to hippocampal circuitry; carvacrol has no effect on paired-pulse inhibition of population spike amplitude, indicating that carvacrol is having no significant effect on GABAergic inhibition.[24]

A recent study indicates that carvacrol has an anti-seizure effect, as it completely prevented seizures in the 6 Hz, 32 mA partial seizure model with an ED50 of 35.8 mg/kg i.p.[32] In addition, carvacrol was tested against acute seizures in two further seizure models.[33] In this study, carvacrol increased the latency for the development of convulsions in the pentelenetetrazol model and prevented tonic convulsions in the maximum electroshock model. These acute anti-seizure effects were only observed with much higher concentrations of carvacrol (200 mg/kg i.p.) than those used in our

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study. Carvacrol´s effects on chronic epilepsy were not investigated in the study. Interestingly, despite high doses of carvacrol administered i.p. in their study, Quintans-Júnior and colleagues were unable to show a GABA(A) receptor related effect, since flumazenil, a potent GABA(A) receptor antagonist, did not prevent anti-seizure effects in their study. Taken together with our results, this suggests that carvacrol does not modulate GABAergic inhibition *in vivo* and rather supports the notion that carvacrol acts through blocking TRPM7 channels.

To our knowledge there have been no studies looking at modulation of key regulators of magnesium homeostasis such as the TRPM7 channel in chronic epilepsy. However, there are several studies which have looked at the role of TRPM7 channels in brain hypoxia and traumatic brain injury.[4,5,12] Those studies show that inhibiting TRPM7 channels is neuroprotective in these conditions. The mechanism by which TRPM7 channels contribute to neuronal death in these conditions has been shown to relate to an increase of Ca^{2+}-permeable nonselective cation conductances.[5,11] We have previously shown that epileptiform activity is characterized by repetitive Ca^{2+} oscillations and that prolonged seizure activity leads to Ca^{2+} overload and subsequent cell death.[34] It is likely that TRPM7 channels contribute to this process, and the current findings highlight a neuroprotective effect of the TRPM7 channel blocker carvacrol on hippocampal cell death in an *in vivo* epilepsy model.

Interestingly, we show that blocking TRPM7 channels reduced not only cell death in epilepsy but also early recurrence of status epilepticus. Recurrence of status epilepticus occurs in up to 50% of patients given benzodiazepines.[35] Early seizures after status epilepticus were also significantly suppressed by carvacrol in our study, whereas chronic seizure frequency remained unaltered. Carvacrol is cleared within

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24 hours,[19] indicating that this effect is not directly due to any acute anti-seizure effects of carvacrol. Indeed, these findings imply that acute hyperexcitability/seizures and chronic epilepsy after SE depend upon different mechanisms.[36,37]

We found that memory decline after SE as measured by the alternating T-maze test was prevented by inhibiting TRPM7 channels with carvacrol. To our knowledge this is the first report which shows that blocking TRPM7 channels can prevent the long-term sequelae of neuronal injury. This effect may be mediated through a direct effect on TRPM7 channels or through its suppression of recurrent status epilepticus. Previous studies have shown that suppression of status epilepticus e.g. via lamotrigine or valproate is effective in preventing neuronal cell death and cognitive dysfunction. [38,39] Moreover, the separation of epileptogenesis from neuronal death and cognitive deficits is supported by other animal studies.[40] The TRPM7 channel antagonist carvacrol is a food additive and has been used previously in animal models of stroke and traumatic brain injury.[4,5,18] We chose carvacrol in this study as it is likely that this is easily translatable into clinical trials in humans. It should be noted that TRPM7 channels are involved in many important physiological processes such as cytoskeletal organization, cell adhesion and cell migration,[41] processes which also occur in the adult brain. However, carvacrol is a reversible, short lasting antagonist at the TRPM7 channel,[6] and so acute administration is unlikely to impact upon these longer-term processes.

Our findings here indicate that administration of carvacrol, a naturally occurring oil, which blocks TRPM7 channels, can improve outcomes after status epilepticus, and is a potential disease-modifying treatment.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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KEY POINT BOX

- Carvacrol, a natural occurring oil, given after experimental status epilepticus prevents recurrence of the status epilepticus.

- Carvacrol administration prevents neuronal death in the hippocampus following status epilepticus

- Carvacrol administration has no impact on epileptogenesis, but prevents memory deficits that occur following status epilepticus.

- Since carvacrol is an TRPM7 antagonist, TRPM7 may play a role in the neuronal death and cognitive decline following status epilepticus.

LEGENDS OF FIGURES:

Figure 1: *TRPM7 channels are expressed on neurons in the hippocampal formation*

Representative confocal images showing immunhistochemistry staining of neurons (NeuN), TRPM7 channels (TRPM7) and co-localization of the two stainings (merge) in hilar (A) CA3 (B) and CA1 (C) regions.

Figure 2: *Recurrence of status epilepticus after performant path stimulation*

Pie charts summarizing recurrence of status epilepticus in epileptic animals treated with saline (A) and epileptic animals treated with carvacrol (B). Representative EEG trace in an animal with recurrent status epilepticus after performant path stimulation (C).

Figure 3: *Chronic seizures after performant path stimulation (PPS)*

Representative EEG trace of a seizure after PPS (A). Box plots summarizing median, 25 and 75 interquartile range (box) and minimum and maximum (whiskers) seizure counts per week (B). Bar charts with mean ± SEM seizure duration (C) in epileptic animals treated with saline and epileptic animals treated with carvacrol.

Figure 4: *The impact on carvacrol on cell death in the hippocampal formation*

Representative confocal images of immunohistochemistry of CA1 (A) and hilar regions (C) in control animals, epileptic saline-treated animals and epileptic animals treated with carvacrol. Bar charts summarising cell counts (mean ± SEM) in CA1 (B), hilar (D) and CA3 region in the three groups.

Figure 5: *Carvacrol treatment rescues memory dysfunction in the perforant path model of epilepsy*

Schematic drawing illustrating the experimental setup in the alternating reward T-maze paradigm (A). Bar chart summarizing correct arm choices (mean±SEM) in the alternating reward T-maze test in control animals, epileptic saline-treated animals and epileptic animals treated with carvacrol.