Int J Physiol Pathophysiol Pharmacol 2011;3(2):120-132
www.ijppp.org /ISSN:1944-8171/IJPPP1105002

Original Article

Suppression of TNF receptor-1 signaling in an *in vitro* model of epileptic tolerance

Simon J Thompson*,[1], Michelle D Ashley*,[1], Sabine Stöhr[1], Clara Schindler[1], Minghua Li[1], Kristin A McCarthy-Culpepper[1], Andrea N Pearson[1,3], Zhi-Gang Xiong[1,3], Roger P Simon[1,3], David C Henshall[2], Robert Meller[1,3]

*[1]RS Dow Neurobiology Laboratories, Legacy Research, 1225 NE 2nd Ave, Portland, Oregon, 97232, USA. [2]Department of Physiology & Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland. [3]Neuroscience Institute, Morehouse School of Medicine, 720 Westview Drive, Atlanta, GA, 30310-1495. *These authors contributed equally to this work.*

Received May 25, 2011; accepted June 10, 2011; Epub June 13, 2011; Published June 30, 2011

Abstract: Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine that can regulate cell survival, inflammation or, under certain circumstances, trigger cell death. Previous work in rat seizure models and analysis of temporal lobe samples from epilepsy patients has suggested seizures activate TNF receptor 1 (TNFR1). Here we explored the activation and functional significance of TNFR1 signaling in the mouse hippocampus using *in vitro* and *in vivo* models of seizure-induced neuronal injury. Focal-onset *status epilepticus* in mice upregulated TNFR1 levels and led to formation of TNFR1-TNFR-associated death domain (TRADD) and TRADD-Fas-associated death domain (FADD) binding. Seizure-like injury modeled *in vitro* by removal of chronic excitatory blockade in mouse hippocampal neurons also activated this TNFR1 signaling pathway. Prior exposure of hippocampal neurons to a non-harmful seizure episode, via NMDA receptor blockade, 24 h prior to injurious seizures significantly reduced cell death and modeled epileptic tolerance *in vitro*. TNFR1 complex formation with TRADD and TRADD-FADD binding were reduced in tolerant cells. Finally, TNFR1 signaling and cell death were reduced by PKF-242-484, a dual matrix metaloproteinase/TNFα converting enzyme inhibitor. The present study shows that TNFR1 signaling is activated in mouse seizure models and may contribute to neuropathology *in vitro* and *in vivo* while suppression of this pathway may underlie neuroprotection in epileptic tolerance.

Keywords: Seizure, epileptic tolerance, preconditioning, TACE, PKF242-484, cell death, neuroprotection, TNF

Introduction

Prolonged seizures in humans, and experimentally-induced *status epilepticus,* can result in profound damage to the hippocampus and other temporal lobe structures [1-2]. Seizure-induced neuronal death can be reduced if the brain is pre-exposed to brief, non-injurious seizures (seizure preconditioning) [3]. Termed epileptic tolerance, this has been demonstrated for a variety of preconditioning stimuli including electroshocks and low doses of kainic acid (KA) delivered in the days prior to *status epilepticus* [3]. The transcriptional profile of epileptic tolerance features down-regulation of genes associated with neurotransmission, calcium signaling and cell death [4]. Other molecular mediators of epileptic tolerance have been suggested, including nuclear factor κB (NFκB) [3, 5].

Seizure-induced neuronal death is caused by glutamate excitotoxicity and activation of signaling pathways associated with apoptosis [6]. Neuroinflammation is also recognized as an important component of the pathophysiology of seizure-damage and epileptogenesis [7]. A key mediator is tumor necrosis factor alpha (TNFα), a pleiotropic cytokine which functions through two cell surface-expressed receptors, p55 (TNFR1) and p75 (TNFR2) [8]. TNFα is synthesized as 26 kD protein that is cleaved by TNFα converting enzyme (TACE, also known as a disintegrin and metalloproteinase 17, ADAM-17), which releases the active 17 kD fragment of the

protein (soluble TNFα) [8]. The soluble form of TNFα has a higher affinity for TNFR1 whereas the membrane-bound pro-form of TNFα has a preference for TNFR2 [9]. Various CNS insults lead to production of TNFα, including seizures, and both neurons and glia can produce and react to TNFα [8, 10-11].

Several signaling pathways are activated by TNF α [8, 12]. The major responses are cell survival, including neuroprotection [13-16], and inflammation, mediated by binding of the receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2) to TNFR1, the main TNF receptor in brain, followed by downstream activation of nuclear factor κB and the c-Jun *N*-terminal kinase pathways [14, 17-21]. TNFα and TNFRs are also implicated directly in seizure generation [22-24].

TNFR1 can also trigger potentially harmful oxidation stress and reactive oxygen species (ROS) [25]. Moreover, TNFR1 contains a death domain and under certain conditions, for example prolonged activation or protein synthesis inhibition, TNFR1 can promote cell death via apoptosis signal-regulating kinase 1 (ASK1) and JNK [26], or via caspase-8 [12, 27]. For the latter, current models suggest a requirement of receptor internalization. Having recruited TNFR-associated death domain (TRADD), TRADD can, after dissociation from RIP1 and TRAF2, recruit the Fas associated death domain (FADD) and activate pro-caspase 8 [21, 28]. Indeed, the generation of an internalized deficient mutant TNFR1 inhibits cell death signaling [29-30].

TNFR1 signaling may contribute to the pathophysiology of seizure-induced neuronal death. Binding of TNFR1 to TRADD and TRADD to RIP is increased following *status epilepticus* in rats and seizure-induced neuronal death can be reduced by TNFα–neutralizing antibodies [31-32]. There is evidence supporting activation of caspase-8, as well as ASK1, by seizures [32-33], and active caspase trapping experiments show that caspase-8 activation is also an apical event following seizure-like insults *in vitro* [34]. Caspase-8 pseudosubstrate inhibitors are also reported to reduce seizure-induced neuronal death *in vitro* and *in vivo* [31, 34-35]. Last, TNFR1-TRADD and TRADD-FADD complexes are present in biopsy samples from resected hippocampi from patients with temporal lobe epilepsy [36]. In the present study, we further explored the role of this signaling pathway in mouse models of seizure-induced neuronal death using an *in vitro* model of epileptic tolerance in hippocampal cultures.

Materials and methods

Mouse seizure model

All animal procedures were performed in an AAALAC accredited facility in accordance with protocols approved by the Legacy Institutional Animal Care and Use Committee and the principles outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Studies were performed according to previously described methods with modifications [37-38]. Adult (20-22g) male C57Bl/6 mice (Charles River) underwent seizures induced by unilateral stereotaxic microinjection of kainic acid (KA) into the basolateral amygdala nucleus. Following anesthesia, the femoral vein was catheterized for administration of anticonvulsant and then animals were placed in a stereotaxic frame modified with a headpiece compatible for the mouse (Kopf Instruments, Tujunga, CA). Mice were maintained in the anesthetized condition with an adapted facemask using a mixture containing 68.5% N_2O, 30% O_2 and 1.5% isoflurane, and kept normothermic (37 ± 1°C) with a rectal thermometer connected to a feedback-controlled heating pad (Harvard Instruments, Holliston, MA) and heat lamp. Mice were affixed with three surface recording electrodes (Plastics One, Inc, Roanoke, VA) and a 26 -gauge steel guide cannula over the dura using dental cement (Plastic One Inc.). Anesthesia was discontinued, EEG recordings were commenced and then a 31-gauge internal cannula (Plastics One Inc.) was inserted into the lumen of the guide to inject KA (0.3 μg in 0.2 μl) into the amygdala. Non-seizure control animals underwent the same surgical procedure but received intraamygdala vehicle (phosphate buffered saline, pH 7.4) injection. The EEG was monitored until lorazepam (6 mg/kg, i.v.) administration 40 min after KA, and then recorded for up to 30 min thereafter. Mice were euthanized 0.5-72 h after anticonvulsant administration and brains were microdissected on ice or flash-frozen whole for histology.

Mouse in vitro seizure model using hippocampal neurons

Hippocampal neuron cultures were prepared from 1 day old C57 mouse pups (mixed sex).

Briefly, mouse pups were anaesthetized using isoflurane, decapitated and hippocampi were dissected and dissociated with papain (Worthington Biochemicals). For model set up, initial cultures of hippocampal cells were plated out in Neurobasal-A/B27 media (Invitrogen). Excitation in the culture was suppressed using either 10 mM kynurenic acid/ 5 mM $MgCl_2$ or 5 mM $MgCl_2$ as previously published [39-40]. Media was replenished every 2-3 days. Cells were used after 3 weeks in culture. Seizure-like activity was induced by incubating the cells in unsupplemented Neurobasal-A/ B27 media for 20 min. Seizures were terminated by replacing media with high Mg^{2+} media until assay/ harvesting.

Electrophysiological recordings

For electrophysiology experiments, cells were plated out on poly-D-lysine coated glass cover slips at a density of 400,000 cell/coverslip. Patch-clamp recordings were performed as described previously [41]. Patch electrodes were constructed from thin-walled borosilicate glass (1.5 mm diameter, WPI, Sarasota, FL) on a two-stage puller (PP83, Narishige, Tokyo, Japan). The patch electrodes had resistance between 3 to 5 MΩ when filled with the intracellular solution (140 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM Hepes, 11 mM EGTA, 4 mM MgATP, pH 7.3, using NaOH, 300 mOsm. Membrane potentials were recorded in current-clamp mode using Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Data were filtered at 2 kHz and digitized on-line using Digidata 1320A DAC units (Axon Instruments). The online acquisition was done using pClamp software (version 8.0, Axon Instruments).

Cells were maintained in an extracellular solution (140 mM NaCl, 5.4 mM KCl, 20 mM Hepes, 10 mM Glucose, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM kynurenic acid, pH 7.4 using NaOH; 320 - 335 mOsm). All electrophysiological experiments were done at room temperature (22-24 ^oC). Once cells were patched, a multi-barrel perfusion system (SF-77B, Warner Instrument Co.) was employed to achieve a rapid exchange of solutions containing kynurenic / Mg^{2+} to a kynurenic acid/ Mg^{2+} free solution.

Cell death assay

Evaluation of cell death was performed as described [42]. For cell death assays cells were grown in 24 well plates at a density of approximately 200,000 cell/well. Following withdrawal of excitation blocking media, lactate dehydrogenase (LDH) release from hippocampal cells was determined using a cytotoxicity detection kit (Roche, CA). Briefly, 100 µl of media was reacted with 100 µl of reagent, incubated at room temperature for 30 min and read on a spectrophotometer at 405 nm and 692 nm.

Western blotting

Immunoblotting was performed as previously described [43]. Tissue samples were lysed in a non-denaturing buffer containing protease inhibitors (100 µg/ml phenylmethylsulfonylfluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin,1 µg/ml pepstatin, 50 mM NaF, 2 mM Na_3VO_4 and phosphatase cocktail inhibitor (Sigma, St Louis, MO)). Protein concentration was determined by Bradford reagent spectrophotometrically at A^{595}. Protein samples (50 µg) were denatured in a gel-loading buffer at 100 ° C for 5 min and then loaded onto 12% SDS-polyacrylamide gels. Proteins were transferred to polyvinylodene difluoride (PVDF) membranes (Bio-Rad) and incubated with primary antibodies at 4 ^oC overnight. Membranes were incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology, Beverly, MA) followed by chemiluminesense detection (NEN Life Science Products, Boston, MA) either by exposure to film or direct capture using a Kodak image station. Film Images were digitized using a Dage 72 camera.

Immunoprecipation

Cells were lysed in a buffer (150mM NaCl, 50mM Tris-HCl, 1.0% NP-40, 1mM EDTA) containing protease inhibitors (aprotinin 1.0 µg/ ml, leupeptin 0.5 µg/ ml, pepstatin 1.0 µg/ ml, Phenylmethanesulfonyl fluoride 10 µg/ ml (Sigma)) and a phosphatase inhibitor cocktail (Sigma) 10 µl/ ml. Cells were centrifuged at 6000 rpm for 5 min at 4°C and protein levels determined using Bradford reagent (Sigma). Protein levels were normalized to approximately 500 µg/ sample. Samples were then incubated with protein A-agarose beads pre-conjugated with anti-TRADD or FADD antibodies (5 µg). Samples were incubated for one hour at room temperature and then precipitated by centrifugation at 1000 rpm for 1 min at 4 °C. Proteins

were washed with 1.0 ml PBS/ 0.1 % NP 40 (x3). Samples were denatured in a loading buffer containing SDS and β-mercaptoethanol prior to loading on a gel as per immunoblotting above.

Drugs and chemicals

The following antibodies were used for this study; Caspase-8 (BD Pharmingen, mf#559932), cleaved caspase 8 (Santa Cruz Biotechnology, mf #: sc-5263), TRADD (Santa Cruz, mf #: sc-7868) FADD (Upstate Biotech., mf #: 05-486) TNFR1 (Santa Cruz, mf #: sc-7895), α-tubulin (Santa Cruz, mf #: sc-8035). Primary antibodies were used at 1:1000-1:500 except cleaved caspase 8 (1:100). MK801 (dizocilpine, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate) and kynurenic acid were purchased from Tocris Cookson, L-685458 ((5S)-(t-Butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl)-L-leu-L-phe-amide) was purchased from Sigma. PKF242-484 (TNF484, [(2S,3R)-N4-((S)-2,2-Dimethyl-1-methylcarbamoyl-propyl)-N1-hydroxy-2-hydroxymethyl-3-(4-methoxy-phenyl)-succinamide]) was a kind gift from Novartis Institutes for BioMedical Research (Basel, Switzerland)). All other reagents were purchased from Sigma at molecular biology grade unless noted.

Data analysis

Data are presented as mean ± standard error of the mean (S.E.M.) of *n* determinations. Data were analyzed using one way analysis of variance (ANOVA) with appropriate *post-hoc* tests (Graphpad Prism v 4.0). Significance was accepted at $p < 0.05$.

Results

Assembly of TNFR1 signaling scaffold following status epilepticus in mice

We previously reported TNFR1 signaling is activated by *status epilepticus* in rats evoked by intra-amygdala KA [31-32]. We recently characterized a mouse model using the same focal-onset approach and therefore began by exploring whether TNFR1 signaling is activated by *status epilepticus* in mice.

Seizures in adult C57BL/6 mice were evoked by unilateral intra-amygdala microinjection of KA and terminated by intravenous anticonvulsant to minimize morbidity and mortality. Analysis of tissue sections 24 h later revealed neuronal death within the ipsilateral hippocampal CA3 subfield and, to a lesser extent, CA1 and hilar regions (**Figure 1A**). The contralateral hippocampus of mice showed no significant neuronal death (**Figure 1A**).

Western blot analysis of whole cell lysates detected TNFα (~17 kD) and TNFR1 (~55kD) at very low levels in control mouse hippocampus (**Figure 1B**). Seizures had minimal effect on TNFα levels up to 72 h but triggered a time-dependent increase in expression of TNFR1 that peaked at 24-72 hours (**Figure 1B**). TRADD (~35 kD) and FADD (~28 kD) were also both present in control mouse hippocampus but at higher levels than either TNFα or TNFR1. Both showed small changes in expression over time, typically reduced levels ~4 – 8 h after seizures, with both recovering at later time points (24 – 72 h) to levels similar to control (**Figure 1B**).

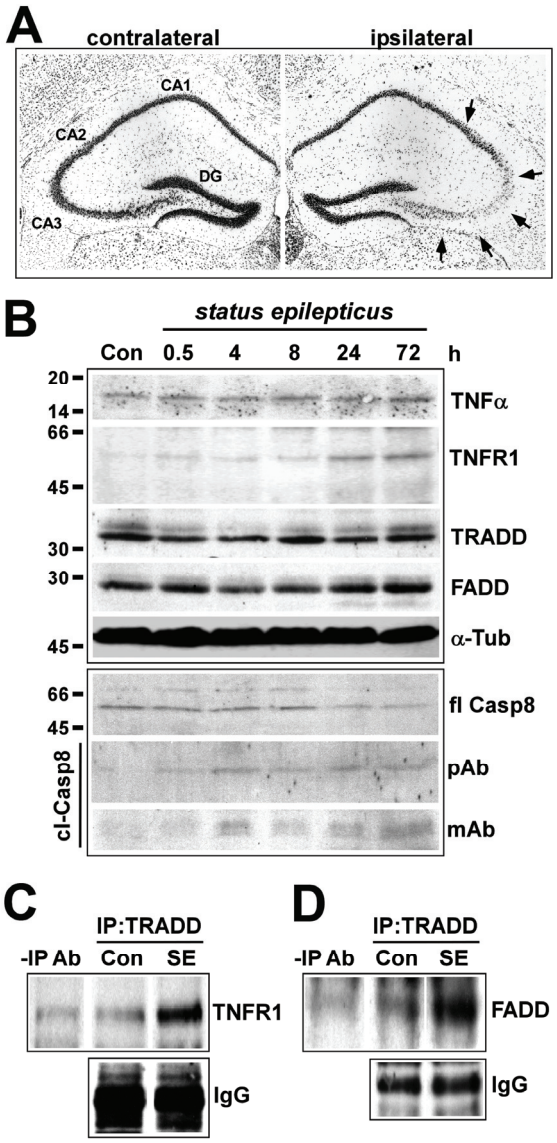
Caspase-8 (~55 kD) was expressed at very low levels in control mouse hippocampus. A rabbit polyclonal antibody detected a presumed cleaved fragment in all samples after seizures which was not present in controls (**Figure 1B**). Western blot analysis using a monoclonal antibody against the p20 subunit of caspase-8 also detected a band after seizures not present in controls (**Figure 1B**).

Finally, we undertook immunoprecipitation analyses to investigate whether seizures in mice induced assembly of TNFR1 signaling complexes. For these studies we used the anti-TRADD antibody to elute either TNFR1 or FADD. TRADD immunoprecipitates contained higher levels of TNFR1 (Figure 1C) and FADD (Figure 1D) in samples obtained 4 h after seizures compared to controls. Thus, *status epilepticus* in mice results in formation of a TNFR1 signaling scaffold.

Activation of TNFR1 signaling cascade in an in vitro model of seizure-induced neuronal death using primary mouse hippocampal neurons

To extend these *in vivo* data we sought to characterize an *in vitro* model with which to study the pathway using mouse hippocampal neurons. Based on work by Furshpan & Potter [40],

we previously modeled seizure-induced neuronal death in rat primary hippocampal cultures [34, 42]. In the model, neurons are cultured in an excitation-blocking medium for up to three weeks. On wash-out, this produces paroxysmal seizure-like activity and neuronal death that is partially caspase-8-dependent [34, 42]. Presently, we cultured mouse hippocampal neurons for 21 days *in vitro* (DIV) in the same excitation blocking media. Changing to media without excitation blockers resulted in induction of seizure-like activity (i.e. high frequency electrical activity 14.2 ± 3.4 Hz; **Figure 2A**). In addition, we observed cell depolarization by 29.25 ± 3.2 mV. Seizure-like activity of 20 min duration was sufficient to induce significant cell death, as determined by lactate dehydrogenase (LDH) release assay (**Figure 3E, 4D**).



To examine the TNFR1 pathway, we used immunoblotting and immunoprecipitation to determine the expression and formation of intracellular complexes. Western blotting detected expression of TNFR1, TRADD and FADD in primary mouse hippocampal neuron cultures (**Figure 2B**). There was a small increase in TNFR1 levels at 4 and 24 h after seizures (**Figure 2B**) while levels of TRADD and FADD remained similar to control (**Figure 2B**).

We next immunoprecipitated TRADD from lysates of mouse hippocampal cultures and immunoblotted them for the presence of TNFR1 and FADD. Binding of TNFR1 to TRADD increased in samples prepared 0.5 h after seizures compared to control, and remained elevated at 1, 4 and 24 h (**Figure 2C**). Similar results were obtained for binding of TRADD to FADD. There was very low level binding of TRADD to FADD in control cultures while binding was increased in all samples after seizures. Thus, there is a rapid formation of the TNFR1 signaling complex following injurious seizure-like activity in mouse hippocampal neurons.

Figure 1. TNFR1 signaling *in vivo* following *status epilepticus* in mice. **A.** Representative photomicrographs of cresyl violet-stained contralateral and ipsilateral mouse hippocampi 24 h following *status epilepticus*. Note prominent loss of ipsilateral CA3 neurons (arrows). **B.** Panel of immunoblots showing expression of TNFα, TNFR1 and downstream components of the TNFR1 signaling pathway in mouse hippocampus 0.5-72 h following seizures, compared to control (Con, 4 h). Immunoblots are representative of results from at least two independent experiments, performed on sets of pooled hippocampi from 2 animals. α-Tubulin (α-Tub) is included as a guide to protein loading. fl, full-length; pAb, polyclonal antibody; mAb, monoclonal Ab; clCasp8, cleaved caspase-8. **C.** Representative immunoblot (n = 4 per group) showing increased TNFR1 binding to TRADD immunoprecipitates (IP) in mouse hippocampus 4 h after seizures compared to time-matched controls. **D.** Representative immunoblot (n = 4 per group) showing increased FADD binding to TRADD immunoprecipitates in mouse hippocampus 4 h after seizures compared to time-matched controls. -IP Ab; negative control in which IP antibody was omitted. IP immunoblots are representative of results from two independent experiments, performed on sets of pooled hippocampi from 4 animals.

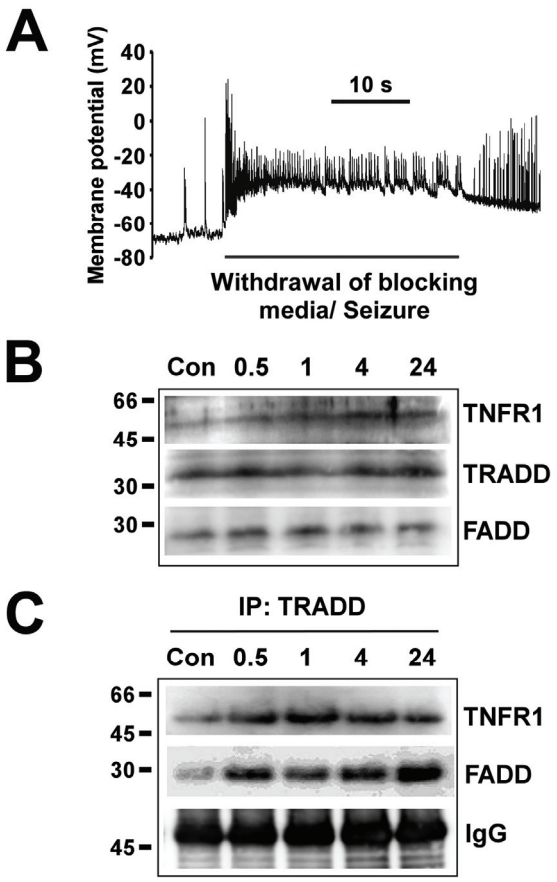


Figure 2. *In vitro* seizures modeled in mouse hippocampal neurons activate TNFR1 signaling. **A**. Representative electrophysiological recordings of mouse hippocampal neurons subjected to seizure induction by withdrawal of chronic excitation-blocking medium. **B**. Expression of TNFR1, TRADD and FADD in mouse hippocampus cells 0.5 - 24 h following seizures compared to Control (Con) cells. **C**. Immunoprecipitation experiments demonstrating increased TNFR1 binding to TRADD and TRADD binding to FADD, 0.5 - 24 h following seizures compared to Con. IP immunoblots are representative of results from 4 independent experiments.

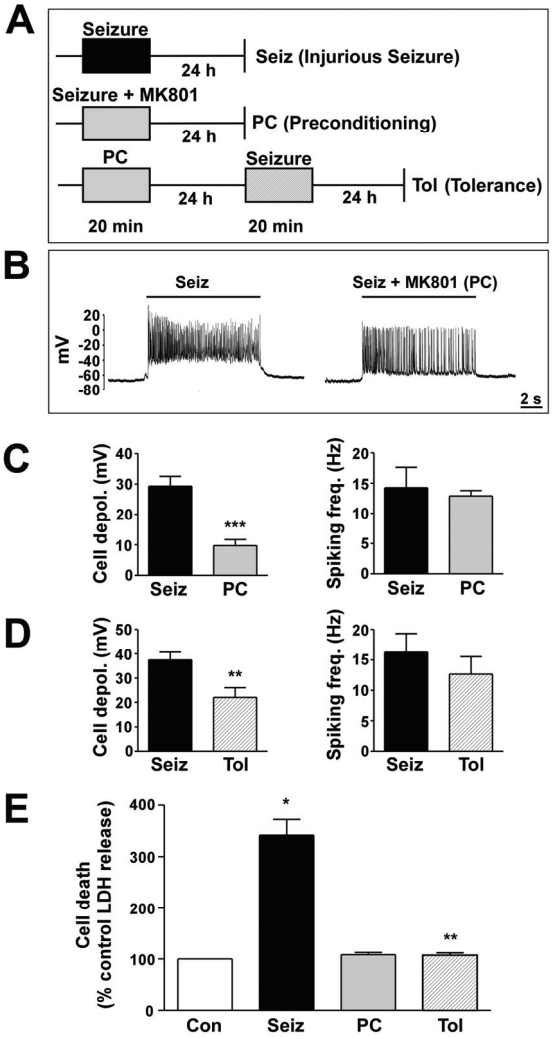
Characterization of in vitro mouse model of epileptic tolerance

We have previously modeled epileptic tolerance *in vivo* by exposure of mice to non-convulsive seizures induced by low-dose systemic KA one day before *status epilepticus*. This results in strong protection against seizure-induced neuronal death without affecting the severity of *status epilepticus* [44]. We next adapted our mouse model of seizure-induced neuronal death *in vitro* to model epileptic tolerance. Three experimental groups were prepared (**Figure 3A**). Injurious seizures were modeled as before by withdrawing excitation blocking media for 20 min. Non-harmful preconditioning seizures were modeled by including MK801 in the media during removal of the excitation blocking media, since it has previously been shown in rat primary neurons that the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK801 reduces seizure-induced cell depolarization and cell death in the present model without blocking seizure-like activity [34, 40, 42]. Finally, epileptic tolerance was modeled by exposing cultures to the PC stimulus followed 24 h later by the otherwise harmful period of 20 min seizures (**Figure 3A**).

When mouse primary hippocampal neurons were subject to 20 min seizure-like activity in the presence of MK801 (i.e. PC) this fully blocked seizure-induced cell death (**Figure 3E**). Incubating neurons with MK801 alone did not induce cell death (data not shown). Next we confirmed that neurons subject to this PC paradigm still underwent seizure-like activity. This preconditioning could also be observed when cells were subjected to seizure-like activity in the presence of the competitive NMDA receptor antagonist AP5 (data not shown). Using whole cell patch recordings we found that incubating cells with MK801 significantly reduced seizure-induced cell depolarization to 9.7 ± 2.0 mV (**Figure 3C**). However, consistent with our previous findings, the frequency of cell firing was not significantly reduced by MK801 (12.8 Hz ± 4.4) (**Figure 3C**).

To model epileptic tolerance *in vitro*, we investigated the pairing of the PC stimulus (excitation blocking media withdrawal in the presence of MK801) with a subsequent injurious seizure 24 h later. The interval of 24 h between preconditioning and injurious seizure was designed to match our *in vivo* model [44] and the interval also effective for ischemic tolerance modeled *in vitro* [43]. Neurons preconditioned 24 h prior to injurious seizures showed significantly less cell death compared to non-preconditioned cultures (**Figure 3E**). Patch clamp recordings determined that neuronal depolarization during injurious seizures was reduced in tolerance neurons which had previously been preconditioned (**Figure 3D**). In contrast, the frequency of seizure

-like activity was not significantly different in between neurons exposed to injurious seizures and tolerance cultures (**Figure 3D**). Therefore, hippocampal neurons can be preconditioned by inducing seizure-like activity in the presence of MK801 resulting in tolerance to normally harmful high frequency electrical activity.



Suppression of TNFR1 signaling in in vitro epileptic tolerance

We next investigated whether TNFR1 signaling was altered in epileptic tolerance modeled in mouse hippocampal cultures. The expression of TNFR1 signaling components TNFR1, TRADD and FADD was not altered in tolerant neurons following seizures (data not shown). In contrast, seizure-induced binding of FADD to TRADD was prevented in neurons that were preconditioned 24 h prior to the harmful seizure-like activity (**Figure 4A**). Consistent with this observation, we noted a decrease in caspase-8 cleavage in tolerant cells compared to non-tolerant neurons following harmful seizure-like activity (**Figure 4B**). Taken together, these data suggest that preconditioning inhibited the ability of harmful seizure-like activity to activate TRADD-FADD binding and downstream signaling.

Pharmacological inhibition of TNFR1 signaling blocks seizure-induced neuronal death in mouse hippocampal cultures

Finally, we examined whether pharmacological inhibition of TNFR1 signaling could reduce seizure-induced cell death in mouse primary hippocampal neurons. TNFα converting enzyme (TACE), also known as ADAM-17, is responsible for TNFα cleavage and release of the soluble form. Here, we used two inhibitors of ADAMs: PKF242-484, to block TNFα processing and L685,485 a dual selective TACE inhibitor.

PKF242-484 reduced TNFR1 binding to TRADD following seizure-like activity *in vitro* (**Figure 4C**).

Figure 3. An *in vitro* model of epileptic tolerance. **A**. Schematic diagram of seizure tolerance paradigm. Standard harmful seizures are triggered by 20 min withdrawal of excitation-blocking medium (top, Seiz). Seizure preconditioning is modeled by inclusion of MK801 (10 μM) during a 20 min seizure (middle, PC). Epileptic tolerance is modeled by exposing hippocampal neurons to the PC stimulus 24 h before injurious seizures. **B**. Electrophysiologic traces of seizure-like spiking and cell depolarization during withdrawal of excitation-blocking medium (seizure) and PC, when MK801 was included. Not firing rate is minimally affected although cell depolarization is reduced by MK801. **C**. Quantification of cell depolarization and frequency determinations during normal seizures and seizures in the presence of MK801 (PC). Data are mean ± sem of 6 observations. ***$p < 0.001$. **D**. Quantification of cell depolarization and frequency determinations during normal seizures and during seizures in cultures subjected to PC 24 h earlier (Tol). **$p < 0.01$. **E**. Neuroprotection in seizure tolerant hippocampal cells as determined by LDH release. Cell death was measured by LDH assay. Seizures caused a large increase in LDH release which was blocked in PC neurons and in neurons subjected to PC followed 24 h later by harmful seizures (Tol). Data shown are mean ± sem ($n = 6$ per group) * $p < 0.05$, **$p < 0.01$ vs. seizure.

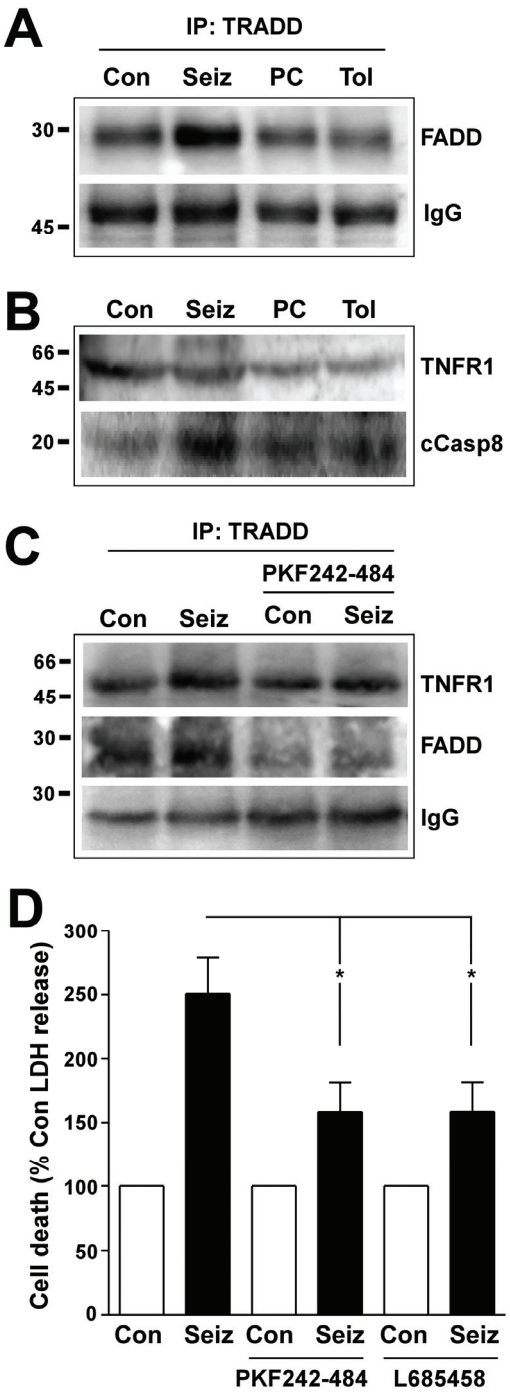


Figure 4. Blockade of TNFR signaling in *in vitro* epileptic tolerance and by pharmacological inhibition results in neuroprotection. **A**. Representative immunoblot showing the normal increase in TRADD binding to FADD after seizures is blocked in preconditioned cells and epileptic tolerance. IP immunoblots are representative of results from three independent experiments. **B**. Expression of TNFR1 and cleaved caspase 8 (cCasp-8) in mouse hippocampus cells in control seizure-treated, preconditioned (PC: seizure in presence of 10 μM MK801) and tolerant cells (Tol). Immunoblots are representative of results from at least three independent experiments. **C**. Representative immunoblots showing reduced FADD and TNFR1 binding to TRADD immunoprecipitates in PKF242-484-treated cells subjected to seizures compared to control cells. IP immunoblots are representative of results from three independent experiments. **D**. LDH release assay showing effect of inhibitors of MMP/TACE (PKF 242-484: 1μM) or γ-secretase (L685458: 1 μM). Cells were subjected to seizure in the presence of the compounds following a 10 min pretreatment. Cell death was determined 24 h later by LDH release assay. PKF242-484 and L658458 both significantly reduce LDH release compared to control seizures. Data shown are mean ± sem (n = 6), *p <0.05 vs. control seizure (Seiz).

Similarly, FADD binding to TRADD following seizure-like activity was also reduced by PKF242-484 (**Figure 4C**). We next investigated the effect of PKF242-484 on cell death in the model. PKF242-484 was incubated with neurons during injurious 20 min seizure-like activity and the subsequent (24 h) recovery period. PKF242-484 had no significant effect on basal LDH release from cells, but significantly reduced LDH release from cells following seizures (**Figure 4D**). To confirm that blocking TACE inhibits seizure-induced cell death, we investigated a second compound, L685,485 which has TACE blocking properties. L685,485 also reduced cell death following seizure-like activity (**Figure 4D**) without affecting basal release of LDH from neuronal cultures. Hence, our data suggested that blocking TNF receptor signaling reduces cell damage from seizure-like activity *in vitro*.

Discussion

The present study provides mouse model data supporting the formation of intracellular TNFR1 signaling complexes after injurious seizures *in vitro* and *in vivo*. We show blocking TNFR signaling with TNFα converting enzyme (TACE) inhibitors blocks seizure-induced cell death *in vitro* and we introduce an *in vitro* model of epileptic tolerance using mouse hippocamapl cultures and show this is associated with abrogation of the TNFR1 signaling pathway and cell death. These studies suggest TNFR1 signaling may contribute to the pathophysiology of seizure-induced neuronal death.

TNFα and TNFR1 signaling are important regulators of cell survival, inflammation, synaptic strength and cell death in the brain [10-11, 45]. TNFα levels are increased by brief and prolonged seizures [46-47]. Animals subjected to long-term TNFα administration also show a greater degree of spontaneous excitatory activity in brain and an increased susceptibility to pentylenetetrazol-induced seizures [48]. TNFR1 signaling may mediate these effects and contribute to the pathophysiology of seizure-induced neuronal death. TNFR1 signaling also appears to be active in chronic epilepsy. Indeed, several components including TNFR1, TRADD and ASK1 are over-expressed and complexes of TRADD-FADD are present in human brain samples from patients with intractable temporal lobe seizures [36]. The present study extends reports in rat models [31-32] and shows TNFR1 signaling occurs during seizure-induced neuronal death *in vivo* in mice. The mouse data here follow a similar spatio-temporal profile as was observed in rats subject to *status epilepticus* by intra-amygdala KA [32]. Thus, seizures caused TNFR1 binding to TRADD along with TRADD binding to FADD. This complex may be capable of recruiting caspase-8 [28] and we detected caspase-8 cleavage fragments after seizures *in vivo*. The weak expression of caspase-8 in mouse brain and the minimal cleavage we detected suggests, however, it may not be sufficient to mediate caspase-dependent cell death *in vivo* [8]. Indeed, there is evidence supporting the importance of the Bcl-2 family-regulated mitochondrial pathway in this model [49-50]. Nevertheless, caspase-8 may promote this pathway by cleaving Bid, which is detected over a time frame compatible with the observed activation of the TNFR1 pathway here [51].

The present study also characterized an *in vitro* mouse model of seizure-induced neuronal death using primary hippocampal neurons. The model was adapted from work in rat hippocampal neurons and based on the observation that when cells are chronically grown in an excitation-blocking media that on removal this triggers paroxysmal seizure-like activity and neuronal death [40, 42]. The mouse model bears several similarities to the rat model. These include the degree of neuronal death triggered, and the protection afforded by NMDA receptor blockade [42]. The role of intrinsic versus extrinsic pathway caspases was not a focus in the present study but the mouse model is consistent with the rat [34] in that increased cleavage of caspase-8 is detected As with the *in vivo* findings, the *in vitro* model was associated with an increase in TRADD-FADD binding supportive of activated TNFR1 signaling. Since we observe TNFR1 receptor scaffold formation in cultured hippocampal neurons, the acute signaling response to TNFα is likely mediated by neuron-to-neuron signaling. Glia are present in these cultures, however, and their influence may be important [11]. Indeed, gliosis may be the main source of TNFα in experimental and human epilepsy [11]. TNFα may also contribute to epileptogenesis given that this cytokine enhances basal synaptic transmission and affects ion channel function in neurons, which may result in hyper-excitability [11]. Thus, the *in vitro* studies here support *in vivo* findings that seizures activate TNFR1 signaling.

The functional significance of TNFα signaling was also demonstrated presently. PKF242-484 (also known as TNF484) is an experimental TACE inhibitor with some activity against metalloproteinases. PKF242-484 analogues are currently being investigated to improve bioavailability of the compound, although PKF242-484 has shown anti-inflammatory properties in a number of disease models [52]. For example, PKF242-484 reduces tissue damage in intestine and airways due to the inhibition of TNFα-induced inflammation [53-54]. Our *in vitro* studies showed that PKF242-484 blocked activation of TNFR1 signaling and seizure-induced cell death. These findings suggest that TNFα signaling in this model, as inferred by *in vivo* studies, may contribute to neuronal death and could be targeted for neuroprotection. TNFR1 signaling is not ordinarily capable of activating cell death unless cell protective gene expression is blocked, for example because of protein synthesis inhibition. Indeed, inhibitors of transcription or translation must normally be co-applied with TNFα to trigger cell death. A simple explanation for why TNFα-TNFR1 signaling promotes neuronal death in the present model is that protein synthesis is inhibited by seizures. Indeed, inhibition of protein synthesis by seizure activity *in vivo* and seizure-like insults *in vitro* was recognized many years ago [55-58].

Epileptic tolerance, whereby prior exposure of the brain to brief seizures reduces damage caused by a subsequent and otherwise harmful seizure, is a powerful paradigm for exploring

endogenous pathways of neuroprotection [4]. Most insights have been obtained using *in vivo* models. The present study introduces a suitable *in vitro* model of epileptic tolerance which should accommodate certain experimental manipulations not readily available to the researcher *in vivo.* We preconditioned neurons with a benign seizure by blocking NMDA receptors during removal of excitation-blocking media. A noteworthy feature of the model is the similar temporal interval between preconditioning and challenge in which the tolerance was acquired compared to both our *in vivo* model [44], and our previously reported *in vitro* model of ischemic tolerance [43]. Some differences are also apparent. The neuroprotection in our model was complete, in contrast to the ~50-60% protection modeled *in vivo*. The explanation for this difference may lie with the effect of preconditioning on the subsequent neuronal depolarization in response to the harmful seizure. Whereas *in vivo* the severity of *status epilepticus* is unchanged in preconditioned mice [44], the present neuron culture model is associated with a reduction in neuronal depolarization in preconditioned cells although spiking frequency is not significantly changed. Nevertheless, we cannot exclude that the completeness of the neuroprotection in the tolerance model *in vitro* is achieved, at least in part, by a suppression of the subsequent seizure episodes.

The molecular mechanisms underlying neuroprotection in epileptic tolerance remain incompletely understood. Exploratory transcriptional profiling of the present *in vitro* model reported a large number of differentially expressed genes which included cell signaling, metabolism, and transcription [59]. The present study points to a potential for role of the suppression of TNFR1 signaling. Indeed, we found that TRADD-FADD binding was blocked in seizure tolerance *in vitro* and there was reduced cleavage of caspase-8. What remains to be determined is whether preventing the various survival-promoting effects and inflammatory consequences of TNFR1 signaling in this model could have otherwise detrimental effects in the longer term.

In conclusion, the present study suggests TNFR1 signaling is a pathophysiologic feature of seizure-induced neuronal death in *in vitro* and *in vivo* mouse models. The introduction of an *in vitro* model of epileptic tolerance enables additional molecular manipulations and supports suppression of TNFR1 signaling as a potential mechanism underlying the neuroprotection. These studies contribute to our understanding of the relationship between seizures and TNFR1 signaling and identify potential therapeutic targets for neuroprotection and, possibly, anti-epileptogenesis.

Acknowledgements

The authors would like to thank Akitaka Yamamoto and Veronica Jessick for technical support. This work was supported by NIH/ NINDS (NS039016-RPS and NS047622-RPS) and by Science Foundation Ireland grant 08/IN1/ B1875 (to DCH). The authors declare no conflicts of interest. PKF242-484 was a gift from Dr. Neumann (Novartis).

Address correspondence to: Dr. Robert Meller, Neuroscience Institute, Morehouse School of Medicine, 720 Westview Drive, Atlanta, GA, 30310-1495, E-mail: rmeller@msm.edu

References

[1] Meldrum BS. Implications for neuroprotective treatments. Prog Brain Res 2002; 135: 487-495.

[2] Fujikawa DG. Prolonged seizures and cellular injury: understanding the connection. Epilepsy Behav 2005; 7 Suppl 3: S3-11.

[3] Jimenez-Mateos EM and Henshall DC. Seizure preconditioning and epileptic tolerance: models and mechanisms. Int J Physiol Pathophysiol Pharmacol 2009; 1: 180-191.

[4] Jimenez-Mateos EM, Hatazaki S, Johnson MB, Bellver-Estelles C, Mouri G, Bonner C, Prehn JH, Meller R, Simon RP and Henshall DC. Hippocampal transcriptome after status epilepticus in mice rendered seizure damage-tolerant by epileptic preconditioning features suppressed calcium and neuronal excitability pathways. Neurobiol Dis 2008; 32: 442-453.

[5] Blondeau N, Widmann C, Lazdunski M and Heurteaux C. Activation of the nuclear factor-kappaB is a key event in brain tolerance. J Neurosci 2001; 21: 4668-4677.

[6] Engel T and Henshall DC. Apoptosis, Bcl-2 family proteins and caspases: the ABCs of seizure-damage and epileptogenesis? Int J Physiol Pathophysiol Pharmacol 2009; 1: 97-115.

[7] Vezzani A, French J, Bartfai T and Baram TZ. The role of inflammation in epilepsy. Nat Rev Neurol 2011; 7: 31-40.

[8] Wajant H, Pfizenmaier K and Scheurich P. Tumor necrosis factor signaling. Cell Death

Differ 2003; 10: 45-65.
[9] McCoy MK and Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflammation 2008; 5: 45.
[10] Clark IA, Alleva LM and Vissel B. The roles of TNF in brain dysfunction and disease. Pharmacol Ther 2010; 128: 519-548.
[11] Stellwagen D. The contribution of TNFalpha to synaptic plasticity and nervous system function. Adv Exp Med Biol 2011; 691: 541-557.
[12] Chen G and Goeddel DV. TNF-R1 signaling: a beautiful pathway. Science 2002; 296: 1634-1635.
[13] Cheng B, Christakos S and Mattson MP. Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron 1994; 12: 139-153.
[14] Liu ZG, Hsu H, Goeddel DV and Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 1996; 87: 565-576.
[15] Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW and Mattson MP. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nat Med 1996; 2: 788-794.
[16] Gary DS, Bruce-Keller AJ, Kindy MS and Mattson MP. Ischemic and excitotoxic brain injury is enhanced in mice lacking the p55 tumor necrosis factor receptor. J Cereb Blood Flow Metab 1998; 18: 1283-1287.
[17] Hsu H, Xiong J and Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF- kappa B activation. Cell 1995; 81: 495-504.
[18] Westwick JK, Weitzel C, Minden A, Karin M and Brenner DA. Tumor necrosis factor alpha stimulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 1994; 269: 26396-26401.
[19] Natoli G, Costanzo A, Ianni A, Templeton DJ, Woodgett JR, Balsano C and Levrero M. Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science 1997; 275: 200-203.
[20] Varfolomeev EE and Ashkenazi A. Tumor necrosis factor: an apoptosis JuNKie? Cell 2004; 116: 491-497.
[21] Wilson NS, Dixit V and Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009; 10: 348-355.
[22] Balosso S, Ravizza T, Perego C, Peschon J, Campbell IL, De Simoni MG and Vezzani A. Tumor necrosis factor-alpha inhibits seizures in mice via p75 receptors. Ann Neurol 2005; 57: 804-812.
[23] Stellwagen D and Malenka RC. Synaptic scaling mediated by glial TNF-alpha. Nature 2006; 440: 1054-1059.
[24] Balosso S, Ravizza T, Pierucci M, Calcagno E, Invernizzi R, Di Giovanni G, Esposito E and Vezzani A. Molecular and functional interactions between tumor necrosis factor-alpha receptors and the glutamatergic system in the mouse hippocampus: implications for seizure susceptibility. Neuroscience 2009; 161: 293-300.
[25] Shen HM and Pervaiz S. TNF receptor superfamily-induced cell death: redox-dependent execution. Faseb J 2006; 20: 1589-1598.
[26] Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997; 275: 90-94.
[27] Wang L, Du F and Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell 2008; 133: 693-703.
[28] Micheau O and Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003; 114: 181-190.
[29] Schutze S, Machleidt T, Adam D, Schwandner R, Wiegmann K, Kruse ML, Heinrich M, Wickel M and Kronke M. Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling. J Biol Chem 1999; 274: 10203-10212.
[30] Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, Adam D, Mentlein R, Kabelitz D and Schutze S. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 2004; 21: 415-428.
[31] Henshall DC, Araki T, Schindler CK, Shinoda S, Lan JQ and Simon RP. Expression of death-associated protein kinase and recruitment to the tumor necrosis factor signaling pathway following brief seizures. J Neurochem 2003; 86: 1260-1270.
[32] Shinoda S, Skradski SL, Araki T, Schindler CK, Meller R, Lan JQ, Taki W, Simon RP and Henshall DC. Formation of a tumour necrosis factor receptor 1 molecular scaffolding complex and activation of apoptosis signal-regulating kinase 1 during seizure-induced neuronal death. Eur J Neurosci 2003; 17: 2065-2076.
[33] Henshall DC, Bonislawski DP, Skradski SL, Lan JQ, Meller R and Simon RP. Cleavage of Bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. Neurobiol Dis 2001; 8: 568-580.
[34] Meller R, Clayton C, Torrey DJ, Schindler CK, Lan JQ, Cameron JA, Chu XP, Xiong ZG, Simon RP and Henshall DC. Activation of the caspase

8 pathway mediates seizure-induced cell death in cultured hippocampal neurons. Epilepsy Res 2006; 70: 3-14.
[35] Li T, Lu C, Xia Z, Xiao B and Luo Y. Inhibition of caspase-8 attenuates neuronal death induced by limbic seizures in a cytochrome c-dependent and Smac/DIABLO-independent way. Brain Res 2006; 1098: 204-211.
[36] Yamamoto A, Schindler CK, Murphy BM, Bellver-Estelles C, So NK, Taki W, Meller R, Simon RP and Henshall DC. Evidence of tumor necrosis factor receptor 1 signaling in human temporal lobe epilepsy. Exp Neurol 2006; 202: 410-420.
[37] Araki T, Simon RP, Taki W, Lan JQ and Henshall DC. Characterization of neuronal death induced by focally evoked limbic seizures in the C57BL/6 mouse. J Neurosci Res 2002; 69: 614-621.
[38] Shinoda S, Araki T, Lan JQ, Schindler CK, Simon RP, Taki W and Henshall DC. Development of a model of seizure-induced hippocampal injury with features of programmed cell death in the BALB/c mouse. J Neurosci Res 2004; 76: 121-128.
[39] Furshpan EJ. A cell-culture approach to the study of seizure activity. Q J Exp Physiol 1989; 74: 975-985.
[40] Furshpan EJ and Potter DD. Seizure-like activity and cellular damage in rat hippocampal neurons in cell culture. Neuron 1989; 3: 199-207.
[41] Xiong Z, Lu W and MacDonald JF. Extracellular calcium sensed by a novel cation channel in hippocampal neurons. Proc Natl Acad Sci U S A 1997; 94: 7012-7017.
[42] Meller R, Schindler CK, Chu XP, Xiong ZG, Cameron JA, Simon RP and Henshall DC. Seizure-like activity leads to the release of BAD from 14-3-3 protein and cell death in hippocampal neurons in vitro. Cell Death Differ 2003; 10: 539-547.
[43] Meller R, Minami M, Cameron JA, Impey S, Chen D, Lan JQ, Henshall DC and Simon RP. CREB-mediated Bcl-2 protein expression after ischemic preconditioning. J Cereb Blood Flow Metab 2005; 25: 234-246.
[44] Hatazaki S, Bellver-Estelles C, Jimenez-Mateos EM, Meller R, Bonner C, Murphy N, Matsushima S, Taki W, Prehn JH, Simon RP and Henshall DC. Microarray profile of seizure damage-refractory hippocampal CA3 in a mouse model of epileptic preconditioning. Neuroscience 2007; 150: 467-477.
[45] Vezzani A, Balosso S and Ravizza T. The role of cytokines in the pathophysiology of epilepsy. Brain Behav Immun 2008; 22: 797-803.
[46] de Bock F, Dornand J and Rondouin G. Release of TNF alpha in the rat hippocampus following epileptic seizures and excitotoxic neuronal damage. Neuroreport 1996; 7: 1125-1129.
[47] De Simoni MG, Perego C, Ravizza T, Moneta D, Conti M, Marchesi F, De Luigi A, Garattini S and Vezzani A. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 2000; 12: 2623-2633.
[48] Lee RH, Mills EA, Schwartz N, Bell MR, Deeg KE, Ruthazer ES, Marsh-Armstrong N and Aizenman CD. Neurodevelopmental effects of chronic exposure to elevated levels of pro-inflammatory cytokines in a developing visual system. Neural Dev 2010; 5: 2.
[49] Engel T, Murphy BM, Hatazaki S, Jimenez-Mateos EM, Concannon CG, Woods I, Prehn JH and Henshall DC. Reduced hippocampal damage and epileptic seizures after status epilepticus in mice lacking proapoptotic Puma. FASEB J 2010; 24: 853-861.
[50] Murphy BM, Engel T, Paucard A, Hatazaki S, Mouri G, Tanaka K, Tuffy LP, Jimenez-Mateos EM, Woods I, Dunleavy M, Bonner HP, Meller R, Simon RP, Strasser A, Prehn JH and Henshall DC. Contrasting patterns of Bim induction and neuroprotection in Bim-deficient mice between hippocampus and neocortex after status epilepticus. Cell Death Differ 2010; 17: 459-468.
[51] Engel T, Caballero-Caballero A, Schindler CK, Plesnila N, Strasser A, Prehn JH and Henshall DC. BH3-only protein Bid is dispensable for seizure-induced neuronal death and the associated nuclear accumulation of apoptosis-inducing factor. J Neurochem 2010; 115: 92-101.
[52] Janser P, Neumann U, Miltz W, Feifel R and Buhl T. A cassette-dosing approach for improvement of oral bioavailability of dual TACE/MMP inhibitors. Bioorg Med Chem Lett 2006; 16: 2632-2636.
[53] Souza DG, Ferreira FL, Fagundes CT, Amaral FA, Vieira AT, Lisboa RA, Andrade MV, Trifilieff A and Teixeira MM. Effects of PKF242-484 and PKF241-466, novel dual inhibitors of TNF-alpha converting enzyme and matrix metalloproteinases, in a model of intestinal reperfusion injury in mice. Eur J Pharmacol 2007; 571: 72-80.
[54] Trifilieff A, Walker C, Keller T, Kottirsch G and Neumann U. Pharmacological profile of PKF242-484 and PKF241-466, novel dual inhibitors of TNF-alpha converting enzyme and matrix metalloproteinases, in models of airway inflammation. Br J Pharmacol 2002; 135: 1655-1664.
[55] Cotman CW, Banker G, Zornetzer SF and McGaugh JL. Electroshock effects on brain protein synthesis: relation to brain seizures and retrograde amnesia. Science 1971; 173: 454-456.
[56] Wasterlain CG. Inhibition of cerebral protein synthesis by epileptic seizures without motor

manifestations. Neurology 1974; 24: 175-180.
[57] Dwyer BE and Wasterlain CG. Selective focal inhibition of brain protein synthesis during generalized bicuculline seizures in newborn marmoset monkeys. Brain Res 1984; 308: 109-121.
[58] Cheung MK. The specificity of glutamate inhibition of protein synthesis in synaptosomal fractions from rat cerebral cortex. Neurochem Int 1989; 15: 293-300.
[59] Simon R, Henshall D, Stoehr S and Meller R. Endogenous mechanisms of neuroprotection. Epilepsia 2007; 48 Suppl 8: 72-73.