Published in final edited form as:

Neurobiol Dis. 2017 June ; 102: 1–10. doi:10.1016/j.nbd.2017.02.003.

A calpain inhibitor ameliorates seizure burden in an experimental model of temporal lobe epilepsy

Philip M. Lam, Jessica Carlsen, and Marco I. González*

Department of Pediatrics, Division of Neurology and Translational Epilepsy Research Program, University of Colorado School of Medicine, Aurora, CO, 80045 USA

Abstract

In this study, we used the pilocarpine model of epilepsy to evaluate the involvement of calpain dysregulation on epileptogenesis. Detection of spectrin breakdown products (SBDPs, a hallmark of calpain activation) after induction of pilocarpine-induced *status epilepticus* (SE) and before appearance of spontaneous seizure suggested the existence of sustained calpain activation during epileptogenesis. Acute treatment with the cell permeable inhibitor of calpain, MDL-28170, resulted in a partial but significant reduction on seizure burden. The reduction on seizure burden was associated with a limited reduction on the generation of SBDPs but was correlated with a reduction in astrogliosis, microglia activation and cell sprouting. Together, these observations provide evidence for the role of calpain in epileptogenesis. In addition, provide proof-of-principle for the use of calpain inhibitors as a novel strategy to prevent epileptic seizures and its associated pathologies.

Keywords

Epileptogenesis; calpain; proteolysis; epilepsy; spontaneous seizures

INTRODUCTION

Epilepsy is a chronic disease characterized by the occurrence of spontaneous recurrent seizures (SRS) arising from abnormal neuronal hyperexcitability and synchronization (Kumar and Buckmaster, 2006; El-Hassar et al., 2007; Fritschy, 2008; Williams et al., 2009; O'Dell et al., 2012). In humans, almost half of individuals experiencing *de novo status epilepticus* (SE) develop epilepsy after a seizure-free interval (Annegers et al., 1987; French et al., 1993; Tsai et al., 2009). A short seizure-free interval (latent period) also precedes the appearance of spontaneous seizures in chemoconvulsant (pilocarpine or kainate) models of temporal lobe epilepsy (TLE) (Sharma et al., 2007; Loscher and Brandt, 2010; O'Dell et al.,

*Corresponding Author: Dr. Marco I. González, Pharmacy Building (V20), Mail Stop 8605, 12850 E. Montview Blvd, Room 3117, Aurora, CO 80045, Telephone: (303) 724-4250, Fax: (303) 724-5466, marco.gonzalez@ucdenver.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2012). In addition to recurrent seizures, rodents enduring chemoconvulsant-induced epilepsy demonstrate brain lesions (neuronal loss, astrogliosis, mossy fiber sprouting and hippocampal sclerosis) highly isomorphic to the human condition (Sharma et al., 2007; Curia et al., 2008; Pitkanen and Lukasiuk, 2009).

The calcium-dependent proteases with papain-like activity (*calpains*) belong to a family of non-lysosomal cysteine proteases activated by calcium (Campbell and Davies, 2012; Ono and Sorimachi, 2012). Two major calpain isoforms are ubiquitously expressed in the brain: calpain-1 and calpain-2 (Liu et al., 2008; Saatman et al., 2010; Baudry and Bi, 2016). Following a brain injury, a sustained increase in intracellular calcium results in calpain activation (Liu et al., 2008; Saatman et al., 2010). Calpain activation can be readily detected following brain injury induced by chemoconvulsants (pilocarpine or kainate) typically used to triggered *SE* (Bi et al., 1996; Araujo et al., 2008; Wang et al., 2008). As such, there is evidence that sustained calpain activation contributes to both chronic and acute neurodegeneration in a wide range of pathologic conditions including *SE* (Vanderklish and Bahr, 2000; Bevers and Neumar, 2008; Vosler et al., 2008; Saatman et al., 2010). Pharmacological inhibition of calpain after chemoconvulsant-induced *SE* provides neuroprotection (Araujo et al., 2008; Wang et al., 2008), suggesting that calpains have an active contribution to the acute neurodegenerative process. Moreover, analysis of tissue obtained from patients with epilepsy showed increased calpain expression (Feng et al., 2011; Das et al., 2012). Despite this knowledge, the contribution of calpain overactivation to the epileptogenic process remains poorly studied.

Due to the existence of a close correlation between calpain activation and a broad range of proteins that can be cleaved by this protease, calpain inhibition is an attractive therapeutic target (Vosler et al., 2008; Saatman et al., 2010). As an additional feature, calpain inhibition is expected to have few side effects since the basal levels of calpain activation prevailing in the normal brain is relatively low (Saatman et al., 2010). Here, we report the effects of a calpain inhibitor on several aspects linked to epileptogenesis including seizure burden and cellular pathologies associated to seizure occurrence. Our findings suggest that pharmacological inhibition of calpain represents a novel therapeutic approach to reduce seizure burden.

METHODS

Pilocarpine-Induced Status Epilepticus

Male Sprague Dawley rats (Charles River, Wilmington, MA) were housed in a controlled environment with food and water *ad libitum*. Animal experiments were performed in accordance with Institutional Animal Care and Use Committee regulations and protocols approved by the University of Colorado Anschutz Medical Campus. *Status epilepticus* was induced at 8–9 weeks of age according to a previously reported protocol (Brooks-Kayal et al., 1998; Shumate et al., 1998). To reduce the peripheral effects of pilocarpine, an intraperitoneal (i.p.) injection of scopolamine methyl nitrate (1 mg/kg, Sigma, St. Louis, MO) was applied 30 min before administration of pilocarpine hydrochloride (385 mg/kg i.p., Sigma, St. Louis, MO). If rats did not exhibit convulsive seizures 1 h after pilocarpine injection, a second or third dose of pilocarpine (192.5 mg/kg) was administered in order to

achieve seizure equivalence between animals. To slow down seizure progression and decrease mortality, diazepam (6 mg/kg, i.p.; Hospira, Lake Forest, IL) was administered 1 h after *SE* onset and additional doses (3 mg/kg, i.p.) were administered every 2 h if seizures persisted. Control rats were handled similarly but received a subconvulsive dose of pilocarpine (38.5 mg/kg, i.p.) and 1/10 of the dose of diazepam (0.6 mg/kg, i.p.). As criteria for inclusion, all rats used had confirmed stage 5 behavioral seizures.

MDL-28170 is cell permeable peptide that inhibits both calpain-1 and calpain-2 (Markgraf et al., 1998; Thompson et al., 2010). To evaluate if MDL-28170 (50 mg/Kg, i.p., Bachem, Torrance, CA) prevented cellular alterations linked to epileptogenesis, two treatment paradigms were used: a *low*-dose treatment consisting of *two acute* injections applied at 1 and 5 h after *SE* onset with a final dose the following morning; and, a *high*-dose treatment that consisted of *four acute* doses at 1, 3, 5 and 9 h after *SE* onset with a final dose the following morning. A third group, *SE* plus vehicle (Veh), was composed of rats that received vehicle injections (DMSO) with the same frequency of the *low*- and *high*-dose. The vehicle group was predicted to undergo all pathophysiological events promoting SRS and was used as reference to evaluate possible disease-modifying effects of MDL-28170. To determine if MDL-28170 altered seizure burden, in addition to the acute doses, daily doses of the drug were administered at 1, 2 and 3 days post-*SE*. Animals were randomly assigned to each of these groups. The concentration and administration frequency were chosen based in studies describing MDL-28170 delivery to the brain (Li et al., 1998; Markgraf et al., 1998; Araujo et al., 2008). MDL-28170 has a plasma half-life of 1 to 2 hours and is capable of penetrating the blood-brain barrier and cell membranes. In naïve rats, a single intravenous bolus administration of MDL-28170 (30 mg/kg) resulted in protease inhibition within the brain in 30 min and declined over a period of 4 hours with a half-life of approximately 2 hours and no apparent toxicity (Markgraf et al., 1998).

Tissue Lysates

Following the rapid isolation of hippocampus from the rest of the brain, hippocampal slices (600 µm) were prepared using a McIlwan tissue chopper. Each individual slice was then microdissected to isolate the Cornu Ammonis 1 (CA1); the region of hippocampus where more prominent levels of calpain activation and neuronal death can be detected after *SE* (Araujo et al., 2008). For microdissection, the CA3 region was separated from the CA1 and DG; then, the CA1 region was separated from DG through the hippocampal sulcus (Silva et al., 2001). All CA1 pieces collected from the same rat were pooled together, frozen on dry ice and stored at -80°C. Whole tissue lysates were prepared by brief tissue sonication in RIPA buffer containing a mixture of protease and phosphatase inhibitors. To remove cell debris, lysates were cleared by centrifugation at 17,000 X g for 20 min. Protein concentration was determined using the Bio-Rad RC/DC reagent kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blot

Protein samples were separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.4) plus 0.05% Tween 20. Blots were then incubated overnight at 4°C with primary antibodies

diluted in 1% non-fat dry milk. A polyclonal rabbit antibody, AB38, which recognizes calpain-cleaved spectrin fragments of ~150 kDa was produced and characterized previously (Roberts-Lewis et al., 1994) and was a generous gift from Dr. David R. Lynch (University of Pennsylvania, PA). A rabbit monoclonal antibody that detects full-length and cleaved α -spectrin was obtained from Epitomics (Cat. No. 2507-1, Burlingame, CA). To estimate potential variability in protein content and loading, blots were re-probed with an anti-actin antibody (Sigma, St. Louis, MO). Following incubation with the primary antibody, blots were washed and then incubated at room temperature for 1 h with the appropriate secondary antibodies. Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase were from GE Health Care (Piscataway, NJ) or Jackson ImmunoResearch laboratories (West Grove, PA), respectively. Immunoreactive bands were visualized using Super Signal West Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and film. After scanning the films, immunoreactive bands of the appropriate size were quantified using ImageJ (NIH, Bethesda, MD, USA). Immunoreactivity for the bands of interest was normalized to actin immunoreactivity and compared to control values.

Histological Analysis

Rats were deeply anesthetized and transcardially perfused, first with ice-cold PBS and then with ice-cold 4% PFA in 0.1 M phosphate buffer pH 7.4. Brains were removed from the skull and post-fixed overnight in 4% PFA solution. Fixed brains were cryoprotected in 30% sucrose solution and embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA). Whole brains were serially sectioned to obtain 15 μ m coronal sections. For staining, three mounted sections were selected from a 1-in-15 series starting at approximately the same level of hippocampus (2.8 mm posterior to Bregma). For consistency and to minimize variability in the staining procedure, control and *SE* brains were processed and stained in parallel. Following staining, cell counts were conducted blinded to the administered treatment. The number of cells counted in three sections was averaged and the average number of cells is the reported value for each animal. Controls where the primary antibodies were omitted were run to confirm that the staining was dependent on the primary antibody. Images were obtained using a Nikon Eclipse TE2000-U fluorescence microscope.

To detect degenerating neurons, sections were stained with a simple, reliable, and sensitive technique using the anionic fluorochrome Fluoro-Jade B (FJB, Cat. No. 1FJB, Histo-Chem Inc, Jefferson, AR). Mounted sections were dried at room temperature and rehydrated with 100% ethanol for 10 min, 70% ethanol for 2 min and finally rinsed in distilled water for 2 min. Sections were immersed in 0.06% potassium permanganate for 10 min, rinsed with distilled water for 2 min and finally immersed in 0.0004% FJB staining solution for 10 min. Following staining, sections were rinsed with distilled water, dried and immersed in CitriSolv (Fisher, Pittsburgh, PA). After staining, tissue sections were mounted on slides using Permount (Fisher Scientific, Pittsburg, PA, USA).

To estimate astrogliosis, brain sections were stained with an antibody to detect GFAP (a marker for astrocytes). Tissue sections were blocked with PBS containing 10% normal goat serum, 0.1% BSA, 0.01% glycine and 0.3% Triton X-100. Sections were then incubated overnight with a mouse monoclonal anti-GFAP (Cat. No. G3893, Sigma, St. Louis, MO)

diluted 1:1000 in blocking buffer. The next day, slices were washed and incubated with a highly cross-adsorbed Alexa Fluor 568 goat anti-mouse secondary antibody. To estimate inflammation, brain slices were stained with an antibody to detect Iba-1 (a marker for microglia). Tissue sections were blocked with PBS containing 10% normal goat serum and 0.3% Triton X-100. Sections were then incubated overnight with a rabbit polyclonal anti-Iba1 antibody (Cat. No. 019-19741, Wako, Richmond, VA) diluted 1:500 in blocking buffer. Next day, slices were washed and incubated with a highly cross-adsorbed Alexa Fluor 568 goat anti-rabbit secondary antibody. After staining, tissue sections were mounted on slides using Vectorshield (Vector Laboratories, Burlingame, CA, USA).

To evaluate mossy fiber sprouting, brain slices were stained with an antibody to detect Zinc Transporter 3 (ZnT3), a reliable measure of mossy fiber sprouting (Chi et al., 2008; Hester and Danzer, 2013). Tissue sections were blocked with PBS containing 10% normal goat serum and 0.3% Triton X-100. Sections were then incubated overnight with a rabbit polyclonal anti-ZnT3 antibody (Cat. No. 197 002, Synaptic Systems GmbH, Gottingen, Germany) diluted 1:500 in blocking buffer. The next day, slices were washed and incubated with a highly cross-adsorbed Alexa Fluor 568 goat anti-rabbit secondary antibody. After staining, tissue sections were mounted on slides using Vectorshield.

Electrode Implantation and Electroencephalogram (EEG) Acquisition

Rats were implanted with intracranial EEG electrodes approximately one week before *SE* induction. Two screws were used as subdural electrodes and placed bilaterally at ~ 2.5 mm lateral from midline and 4 mm caudal to Bregma over the temporolimbic cortices. In addition, a polyamide coated stainless-steel wire (Plastics-One, Roanoke, VA) was used as a depth electrode and placed ~ 3.3 mm caudal to Bregma, 1.69 mm lateral from the midline and 2.6 mm below the skull in the right hippocampus. Reference and ground electrodes were placed on the back of the skull slightly behind lambda. Dental acrylic was used to secure a plastic connector (Plastics-One, Roanoke, VA) attached to the electrodes according to standard methods (Zhang et al., 2004; Grabenstatter et al., 2014). Animals were allowed to recover from surgery for one week before further experimentation. For EEG recording, animals were placed in a recording chamber and connected to flexible cables with a commutator (i.e., electric swivel) to allow free movement. Recordings were obtained 24 h/day using an automatic Pinnacle digital video-EEG system (Pinnacle Technology Inc., Lawrence, KS). A trained technician blinded to treatment examined electrographic recordings off-line in order to identify electrographic seizures and potential artifacts. Seizure activity differed from background noise by the presence of EEG signals with progression of spike frequency, large-amplitude and high-frequency activity lasting at least 10 sec. Behavioral characterization of seizures was done accordingly with the Racine scale (Racine, 1972) which is based on the behaviors observed during a seizure episode and classify seizures in five categories: mouth and facial movements, stage 1; head nodding, stage 2; forelimb clonus, stage 3; rearing, stage 4; and, rearing and falling, stage 5. Seizures with an electrographic component associated with subtle or no behavioral manifestations were scored as class 2 or below and labeled as “non-convulsive” while seizures with overt behavioral manifestations were scored as class 3 or above and labeled as “convulsive” (Krook-Magnuson et al., 2013; Grabenstatter et al., 2014).

Power Analysis

Lab Chart software (AD Instruments, Colorado Springs, CO) was used to calculate the power of EEG signals between 1 and 50 Hz. Power was calculated for individual EEG epochs of 1 min in duration. A value for baseline recordings collected before SE was obtained as follows: total power for each one-minute-epoch within a 2 hour period of EEG recording was calculated and then averaged in order to obtain a single power value that represented the averaged baseline. The average baseline value was then used to normalize the one-minute-epoch values obtained from the analysis of EEG recordings obtained before, during and after SE. The EEG power values from the Lab Chart software in mV^2 were log-transformed to dB units using the formula: $10 \times \log_{10} \text{mV}^2$ (Cahn et al., 2010). A similar approach for power analysis has been proposed as standardized method for quantitative analysis of electrographic SE (Lehmkuhle et al., 2009). To calculate Integral Power, raw power values obtained with Lab Chart were used. In this case, the values for each one-minute-epoch were added and reported as $\log \text{mV}^2/\text{Hz}$. Integral Power was calculated for periods of time encompassing either the first one-hour of SE (0 to 1 hour, before diazepam administration), or the first six hours of SE (0 to 6 hours, including a period of time after the diazepam administration).

Statistical analysis

Data is presented as the mean $\pm$ SEM. For statistical evaluation, either parametric or non-parametric tests were used depending on data distribution. Student's *t*-test was used when to assess differences between two groups. Analysis of variance (ANOVA) followed by *post hoc* testing was used to assess differences when more than two groups were compared. Values of $p < 0.05$ were considered significant. GraphPad InStat software (GraphPad Software, Inc., San Diego, CA, USA) was used to perform statistical analysis.

RESULTS

In this study, we evaluated the role that calpain overactivation might have on epileptogenesis. Western blot analysis was used to characterize the time-course of calpain activation during the “silent” period following pilocarpine-induced SE. Calpain activation was estimated by detecting formation of α -spectrin breakdown products (SBDPs), a hallmark of calpain activity. Two different antibodies were used for this analysis: the first one, antibody AB38, detects SBDPs generated by specific calpain cleavage; the second one, anti- α -spectrin antibody, detects both the full-length protein and SBDPs generated after cleavage (Figure 1). Western blot analysis of tissue lysates obtained at different time points along the epileptogenic period showed a time-dependent increase in SBDPs that was similarly detected with by both antibodies. The AB38 antibody detected a significant increase in SBDPs within 24 h of SE induction that is maintained 4 days post-SE and appears to decline at 8 days post-SE (Figure 1). Although in these experiments both antibodies detected a significant increase in the formation of SBDPs, the results obtained with AB38 antibody were more consistent than those revealed by the α -spectrin antibody.

To evaluate the effects of calpain inhibition on the generation of SBDP a *low*- and *high*-dose treatment with MDL-28170 (50 mg/Kg, i.p.) was used. The dosage and concentration

applied was chosen accordingly with previous studies describing MDL-28170 delivery to the brain (Li et al., 1998; Markgraf et al., 1998; Araujo et al., 2008). When compared to control animals, vehicle treated rats showed a dramatic increase in SBDPs generation (Figure 1). Samples from animals treated with both the *low*- and *high*-dose of MDL-28170 showed a partial but significant reduction in SBDPs as detected by the AB38 antibody (Figure 1E). The results obtained with the α -spectrin antibody were more variable and despite a downward trend were not statistically significant (Figure 1F).

The limited reduction in SBDPs formation detected with the AB38 antibody suggested that, as previously described, MDL-28170 treatment could be used to at least partially block calpain overactivation and prevent neuronal death (Araujo et al., 2008; Wang et al., 2008). To corroborate the effect of MDL-28170 on neuronal degeneration, FJB staining was used to assess neuronal death following *SE* induction. Tissue sections of vehicle-treated rats showed a variable but consistent increase in the number of FJB⁺ cells detected in the CA1 region of hippocampus while no obvious staining was detected in tissue from control animals (Figure 2). The number of FJB⁺ positive cells in the *low*-dose group was variable and more similar to vehicle-treated tissue but the number of FJB⁺ cells in the *high*-dose group was significantly decreased, suggesting that calpain inhibition following *SE* reduced the number of degenerating cells. The observation that the *high*-dose treatment with MDL-28170 prevents neurodegeneration after chemoconvulsant-induced *SE* is consistent with prior evidence suggesting that frequent administration of MDL-28170 over a short period of time is necessary to prevent neuronal death (Araujo et al., 2008).

To evaluate MDL-28170's effects on seizure burden, continuous EEG and video recordings were collected. Initial analysis of the EEG recordings revealed that all three groups of animals (vehicle, *low*- and *high*-dose) presented seizure activity, suggesting that MDL-28170 did not abolish appearance of SRS following pilocarpine-induced *SE*. However, differences in seizure frequency were detected between vehicle and MDL-28170-treated rats. In vehicle-treated animals, the number of seizures detected on a *per day* bases steadily increased during the first two weeks post-*SE* (epileptogenic period) to then, as previously described, enter into a cycle of low seizure frequency during weeks three and four (Goffin et al., 2007; Grabenstatter et al., 2014). Vehicle-treated animals showed a prominent peak in the average number of seizures *per day* that was detected during the second week of recording. In addition, the number of animals that presented higher seizure frequency (20 seizures per day) was more prominent in the vehicle-treated group (Figure 3A). In contrast, the average number of seizures *per day* detected in the *low*-dose group did not show a prominent peak and remained flat during all four weeks of recording. When compared to the vehicle group, the number of seizures detected during the second week of recording was significantly reduced in the *low*-dose group (Figure 3). Unexpectedly, and despite a trend towards reduction, the number of seizures detected in the *high*-dose group was not significantly different from the vehicle-treated group. A further analysis that included all seizures detected during four weeks of recording showed that the total number of seizures detected in the *low*-dose group was significantly reduced when compared to vehicle group, suggesting a net reduction in the number of seizures detected in *low*-dose group. It is noteworthy to mention that, as previously described (Goffin et al., 2007; Grabenstatter et al., 2014); there is a large variability in the number of seizures on any given day. Thus it is

possible that, despite a trend, the high levels of inter- and intra-animal variability obscured possible differences.

Analysis of the seizures detected on EEG recordings in conjunction with analysis of the corresponding video recordings according with the Racine scale (Racine, 1972) helped to score and classify individual seizure events in two broad groups: convulsive (Racine's scale 3–5 or generalized seizures) and non-convulsive (Racine's scale 1–2 or partial seizures). Classification of seizures into convulsive and non-convulsive demonstrated that the majority of behaviors associated with EEG abnormalities corresponded to convulsive seizures (Racine's scale 3–5) and that the number of convulsive seizures was the one which changed the most (Figure 3). Further analysis showed a preponderant effect of MDL-20817 in preventing the occurrence of convulsive seizures and a less evident effect on non-convulsive seizures. Analysis of the overall number of convulsive seizures detected during the four weeks of monitoring showed a significant decrease in the *low*-dose group but, surprisingly, no significant change was detected in the *high*-dose group (Figure 3C). In addition, the percentage of behavioral and non-behavioral seizures detected in vehicle-treated and *low*-dose rats was similar (Figure 3D). Another parameter analyzed was seizure duration and, overall, seizure duration was similar in all groups analyzed and did not change with treatment (Figure 3E). When convulsive seizures were analyzed independently, seizure duration was unchanged. In summary, a *low*-dose treatment with MDL-28170 was mostly effective in reducing seizure occurrence during the early phase of the disease when seizure frequency is higher.

The first dose of MDL-28170 was given one hour after *SE* onset, at the same time the first dose of diazepam was given to interrupt *SE* and reduce mortality. This was done in order to reduce the possibility that MDL-28170 treatment might modify the initial *SE*-triggered insult. To further evaluate if MDL-28170 treatment altered *SE*, a power analysis was conducted. Total power calculated during the first 12 h of *SE* shows that all groups analyzed show a similar pattern of power increase and evolution in hippocampus and cortex (Figure 4). Furthermore, treatment with MDL-28170 did not significantly reduce the integral power detected during the first hour of *SE* (before diazepam administration, 0 to 1 h) or during the first six hours of *SE* (before and after diazepam administration, 0 to 6 h). Since no major differences in power were noted, it appears that *SE* severity was equivalent between the different groups analyzed.

Epileptogenesis is commonly associated with neuroinflammation, a pathology that can be detected in brain tissue as reactive gliosis. To determine a possible correlation between reduced SRS and reduced inflammation as a consequence of MDL-28170 treatment, the immunoreactivity for GFAP (astrogliosis) and Iba-1 (microgliosis) was analyzed in tissue obtained 24 h after *SE*. In the vehicle-treated group, immunostaining with antibodies for both GFAP and Iba-1 showed a dramatic increase in the number of immunopositive cells (Figure 5). A semi-quantitative analysis of the CA1 region of hippocampus showed that the *low*-dose treatment significantly reduced the number of GFAP and Iba-1 positive cells. However, despite a downward trend, the reduction in the number of GFAP and Iba-1 positive cells in the *high*-dose group was not statistically significant.

A long-term effect of pilocarpine-induced *SE* is the appearance of aberrant mossy fiber sprouting in granule cells of dentate gyrus. Previous studies have shown that detection of ZnT3 immunoreactivity in the axon terminals of granule cells from the dentate gyrus provides a reliable measure of mossy fiber sprouting (Chi et al., 2008; Hester and Danzer, 2013). To test if treatment with the calpain inhibitor blocked the formation of mossy fiber sprouting, tissue from a subset of chronically epileptic animals was collected and stained to detect ZnT3 immunoreactivity. In chronically epileptic animals treated with vehicle after *SE* induction, intense ZnT3 staining was detected (Figure 6). Acute treatment with MDL-28170 following *SE* significantly reduced ZnT3 staining, suggesting a reduction in sprouting formation in the *low*-dose group. No significant reduction in sprouting was detected in the *high*-dose group, which correlates with a lack of effect on the reduction on seizure burden.

DISCUSSION

The molecular mechanisms involved in the transformation of a normal brain into an epileptic one are not fully understood. In this study, we used the pilocarpine model of epilepsy to evaluate the involvement of calpain dysregulation on epileptogenesis. The generation of SBDPs (a hallmark of calpain activation) was detected following pilocarpine-induced *SE*, before and during appearance of spontaneous seizures, suggesting the existence of sustained calpain activation during epileptogenesis. Administration of MDL-28170, a cell permeable pharmacological inhibitor of calpain, resulted in a partial but significant decrease in SBDPs detection. Treatment with MDL-28170 also resulted in a significant reduction on seizure burden. This reduction on seizure occurrence was correlated with a decrease in tissue inflammation and cell sprouting providing a possible link between seizure occurrence and changes at the cellular level.

In the brain, calpain overactivation is generally observed after excitotoxic conditions like physical trauma, hypoxic/ischemic insult or chemical challenge (Liu et al., 2008). Calpain-dependent cleavage of functional and structural proteins required for proper brain function a main component of the cellular damage that commonly follows excitotoxic injury (Liu et al., 2008; Curcio et al., 2016). Previous reports have demonstrated that administration of MDL-28170 blocks the appearance of SBDPs and neuronal death (Araujo et al., 2008; Vosler et al., 2008; Wang et al., 2008). Here, the role of calpain overactivation on epileptogenesis was evaluated. Treatment with MDL-28170 after induction of *SE* resulted in a reduction on seizure burden. However, the beneficial effect of this inhibition was not straightforward and appears to be dose dependent. From the two groups treated with the calpain inhibitor, the *low*-dose group showed a significant reduction on seizure burden associated with a reduction in the detection of markers for inflammation and sprouting. Puzzlingly, the *high*-dose group, a group of animals treated with a higher amount of MDL-28170 did not show a significant reduction on seizure frequency or a significant reduction on the manifestation of the markers for inflammation and sprouting.

It is well known that in animal models of epilepsy, induction of *SE* with chemoconvulsants causes a rapid and intense inflammatory cascade (Vezzani et al., 2015). Interleukin-1 (IL-1) is one of the several inflammatory molecules involved in the damaging responses observed after *SE* and both of its isoforms, IL-1 α and IL-1 β have been associated with the

inflammatory response detected in epilepsy. In hippocampus and forebrain of adult mice and rats, elevated levels of IL-1 β transcripts can be detected immediately after *SE* onset (30–60 min). This augmented expression precedes the glial inflammatory response typically observed during epileptogenesis (Vezzani et al., 2015). In addition, increased IL-1 α immunoreactivity has been detected in tissue obtained from the temporal lobe of epileptic patients (Sheng et al., 1994; Kan et al., 2012). Furthermore, single nucleotide polymorphisms leading to increased IL-1 α expression have been associated to epilepsy (Dominici et al., 2002; Salzmann et al., 2008). Interestingly, both IL-1 α and IL-1 β are expressed as precursor proteins that become fully activated after proteolytic processing by calpains and caspases, respectively (England et al., 2014). These observations raise the possibility that augmented processing of IL-1 α by calpain might offer a link between calpain activation and appearance of IL-1-related inflammation.

Hippocampal damage is thought to be critical for the development of TLE and neuroprotective drugs have been considered as a potential strategy to block epileptogenesis (Loscher and Brandt, 2010). Based on the concept that neuroprotection might be beneficial after *SE*, prevention of neuronal loss was adopted as a viable strategy to prevent epilepsy (Walker, 2007). However, under some particular instances neuroprotection do not prevents spontaneous seizures, strongly suggesting that neurodegeneration might not be a prerequisite for epileptogenesis (Brandt et al., 2003). Prior studies have shown that MDL-28170 reduces neuronal death following *SE* (Araujo et al., 2008; Wang et al., 2008) and the results presented here confirm calpain involvement in neurodegeneration. However, the most marked reduction on seizure burden was detected under conditions where neuronal death was not prevented. This raise the possibility that, as previously suggested, extensive neuronal loss could also prevent seizures by preventing formation of a (dys)functional network necessary to sustain epileptic activity (Walker, 2007). Thus, it is possible that while the *low*-dose treatment did not significantly prevent cell loss, the *high*-dose treatment prevented loss of cells that abnormally integrated into the network and contributed to seizure generation and cancelled some of the beneficial effects of the treatment. Indeed, there is evidence that endogenous neuroprotective mechanisms may be proepileptogenic because they promote axonal reorganization while extensive loss of neurons might reduce seizure susceptibility (Walker, 2007).

Aberrant mossy fiber sprouting is a common neuropathological feature of chronically epileptic tissue (Sutula et al., 1989; Buckmaster et al., 2002). However, although some studies have found a correlation between the extent of mossy fiber sprouting and seizure frequency, others have not (Buckmaster and Lew, 2011). Rapamycin, a drug that inhibits the signaling pathway involving activation of the mammalian target of rapamycin, suppresses mossy fiber sprouting and seizure frequency in some rat models of TLE but not all (Zeng et al., 2009). Also, in some instances, rapamycin blocked mossy fiber sprouting without affecting seizure frequency (Buckmaster and Lew, 2011; Heng et al., 2013). Thus, experiments using rapamycin suggest that mossy sprouting might not be absolutely necessary for epileptogenesis to occur. In the current study, a significant reduction in mossy fiber sprouting was detected in the *low*-dose group and correlated with a reduction on seizure frequency. Despite a downward trend, the *high*-dose group did not show a clear reduction in sprouting or seizure frequency. Since it is unclear if mossy fiber sprouting is

pro- or anti-epileptogenic and spontaneous seizures during the chronic period can occur in the absence of mossy fiber sprouting (Buckmaster and Lew, 2011; Buckmaster, 2014), it is possible that acute calpain inhibition interferes with the manifestation of mossy fiber sprouting during the chronic stage by reducing the recurrence of chronic spontaneous seizures.

The lack of a more prominent effect on seizure reduction in animals treated with higher amounts of MDL-28170 was unexpected since the initial prediction was that more efficient calpain inhibition would be of more benefit. The reason for this dichotomy is unknown, but some plausible explanations include: (i) higher doses of inhibitor might result in more wide spread calpain inhibition that in turn will promote detrimental effects. One can speculate that a *low*-dose of calpain inhibitor only targets “undesirable” effects of calpain overactivation but higher dosage also blocks “desirable” effects that require calpain activity. Along these lines, there is evidence suggesting that calpain activity is required for membrane repair and wound healing (Mellgren et al., 2007; Nassar et al., 2012). Thus, complete blockade of calpain activity may inhibit membrane and cell repair mechanisms necessary to maintain cellular homeostasis delaying recovery and result in adverse effects. (ii) The most abundant calpain subtypes found in the mammalian brain are calpain-1 and calpain-2 (Vanderklisch and Bahr, 2000; Bevers and Neumar, 2008; Liu et al., 2008; Vosler et al., 2008; Saatman et al., 2010). The calpain activity detected in the injured brain as SBDPs more likely results from the activation of both calpain-1 and calpain-2 and the specific contribution of each calpain subtype is mostly undistinguishable. Recent evidence suggests that calpain-1 and calpain-2 play opposite roles in neuroprotection and neurodegeneration (Seinfeld et al., 2016; Wang et al., 2016) with calpain-1 activation being necessary for neuroprotection while calpain-2 activation resulting in neurodegeneration (Baudry and Bi, 2016). Thus, it is possible that despite the fact that both calpain subtypes have similar affinities for the inhibitor used in this study, calpain-1 is preferentially inhibited by *low*-dose treatment and the *high*-dose treatment inhibits both calpain-1 and calpain-2. Unfortunately, the results obtained in the current study cannot determine the contribution of calpain-1 or calpain-2 but a natural progression of the current studies is to further investigate if there is an independent role for calpain-1 or calpain-2 during the epileptogenic process.

CONCLUSION

This study found that treatment with a calpain inhibitor partially reduced seizure burden and that the reduction on seizure burden was correlated with a decrease in the detection of markers of associated with tissue inflammation and cell sprouting. These observations provide some evidence to suggest that calpain inhibitors could be used as agents to block the appearance of chronic epileptic seizures. We propose that pharmacological inhibition of calpain might represent a novel therapeutic approach to reduce seizure burden in post-traumatic epilepsy.

Acknowledgments

The authors gratefully acknowledge Hien Dohan, Amelia Zommer and Janeen Williams for their contribution during the initial stages of these studies. We also would like to thank the *Rodent In Vivo Neurophysiology Core* at the University of Colorado Anschutz Medical Campus for providing the facilities to acquire and review the EEG/

Video data. The AB38 antibody was a generous gift from Dr. David R. Lynch (University of Pennsylvania, PA). Grants K01-NS069583 and R01-NS089698 from the National Institutes of Health to MIG supported this work.

Abbreviations used

TLE	Temporal Lobe Epilepsy
SE	status epilepticus
CA1	Cornus Ammonis 1
DG	Dentate Gyrus
FJB	Fluoro-Jade B
GFAP	glial fibrillary acidic protein
Iba-1	ionized calcium-binding adaptor molecule 1
EEG	electroencephalogram
SBDPs	α -spectrin breakdown products
ZNT3	Zinc Transporter 3
IL-1	Interleukin 1
PBS	phosphate buffered saline

References

- Annegers JF, Hauser WA, Shirts SB, Kurland LT. Factors prognostic of unprovoked seizures after febrile convulsions. *N Engl J Med*. 1987; 316:493–498. [PubMed: 3807992]
- Araujo IM, Gil JM, Carreira BP, Mohapel P, Petersen A, Pinheiro PS, Soulet D, Bahr BA, Brundin P, Carvalho CM. Calpain activation is involved in early caspase-independent neurodegeneration in the hippocampus following status epilepticus. *J Neurochem*. 2008; 105:666–676. [PubMed: 18088374]
- Baudry M, Bi X. Calpain-1 and Calpain-2: The Yin and Yang of Synaptic Plasticity and Neurodegeneration. *Trends Neurosci*. 2016; 39:235–245. [PubMed: 26874794]
- Beyers MB, Neumar RW. Mechanistic role of calpains in postischemic neurodegeneration. *J Cereb Blood Flow Metab*. 2008; 28:655–673. [PubMed: 18073773]
- Bi X, Chang V, Siman R, Tocco G, Baudry M. Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. *Brain Res*. 1996; 726:98–108. [PubMed: 8836550]
- Brandt C, Potschka H, Loscher W, Ebert U. N-methyl-D-aspartate receptor blockade after status epilepticus protects against limbic brain damage but not against epilepsy in the kainate model of temporal lobe epilepsy. *Neuroscience*. 2003; 118:727–740. [PubMed: 12710980]
- Buckmaster PS. Does mossy fiber sprouting give rise to the epileptic state? *Adv Exp Med Biol*. 2014; 813:161–168. [PubMed: 25012375]
- Buckmaster PS, Lew FH. Rapamycin suppresses mossy fiber sprouting but not seizure frequency in a mouse model of temporal lobe epilepsy. *J Neurosci*. 2011; 31:2337–2347. [PubMed: 21307269]
- Buckmaster PS, Zhang GF, Yamawaki R. Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit. *J Neurosci*. 2002; 22:6650–6658. [PubMed: 12151544]
- Cahn BR, Delorme A, Polich J. Occipital gamma activation during Vipassana meditation. *Cogn Process*. 2010; 11:39–56. [PubMed: 20013298]

- Campbell RL, Davies PL. Structure-function relationships in calpains. *Biochem J.* 2012; 447:335–351. [PubMed: 23035980]
- Chi ZH, Wang X, Cai JQ, Stoltenberg M, Danscher G, Wang ZY. Zinc transporter 3 immunohistochemical tracing of sprouting mossy fibres. *Neurochem Int.* 2008; 52:1305–1309. [PubMed: 18406010]
- Curcio M, Salazar IL, Mele M, Canzoniero LM, Duarte CB. Calpains and neuronal damage in the ischemic brain: The swiss knife in synaptic injury. *Prog Neurobiol.* 2016; 143:1–35. [PubMed: 27283248]
- Curia G, Longo D, Biagini G, Jones RS, Avoli M. The pilocarpine model of temporal lobe epilepsy. *J Neurosci Methods.* 2008; 172:143–157. [PubMed: 18550176]
- Das A, Wallace GC, Holmes C, McDowell ML, Smith JA, Marshall JD, Bonilha L, Edwards JC, Glazier SS, Ray SK, Banik NL. Hippocampal tissue of patients with refractory temporal lobe epilepsy is associated with astrocyte activation, inflammation, and altered expression of channels and receptors. *Neuroscience.* 2012; 220:237–246. [PubMed: 22698689]
- Dominici R, Cattaneo M, Malferrari G, Archi D, Mariani C, Grimaldi LM, Biunno I. Cloning and functional analysis of the allelic polymorphism in the transcription regulatory region of interleukin-1 alpha. *Immunogenetics.* 2002; 54:82–86. [PubMed: 12037600]
- El-Hassar L, Esclapez M, Bernard C. Hyperexcitability of the CA1 hippocampal region during epileptogenesis. *Epilepsia.* 2007; 48(Suppl 5):131–139. [PubMed: 17910593]
- England H, Summersgill HR, Edye ME, Rothwell NJ, Brough D. Release of interleukin-1alpha or interleukin-1beta depends on mechanism of cell death. *J Biol Chem.* 2014; 289:15942–15950. [PubMed: 24790078]
- Feng ZH, Hao J, Ye L, Dayao C, Yan N, Yan Y, Chu L, Shi FD. Overexpression of mu-calpain in the anterior temporal neocortex of patients with intractable epilepsy correlates with clinicopathological characteristics. *Seizure.* 2011; 20:395–401. [PubMed: 21315622]
- French JA, Williamson PD, Thadani VM, Darcey TM, Mattson RH, Spencer SS, Spencer DD. Characteristics of medial temporal lobe epilepsy: I. Results of history and physical examination. *Ann Neurol.* 1993; 34:774–780. [PubMed: 8250525]
- Fritschy JM. Epilepsy, E/I Balance and GABA(A) Receptor Plasticity. *Front Mol Neurosci.* 2008; 1:5. [PubMed: 18946538]
- Goffin K, Nissinen J, Van Laere K, Pitkanen A. Cyclicity of spontaneous recurrent seizures in pilocarpine model of temporal lobe epilepsy in rat. *Exp Neurol.* 2007; 205:501–505. [PubMed: 17442304]
- Grabenstatter HL, Del Angel YC, Carlsen J, Wempe MF, White AM, Cogswell M, Russek SJ, Brooks-Kayal AR. The effect of STAT3 inhibition on status epilepticus and subsequent spontaneous seizures in the pilocarpine model of acquired epilepsy. *Neurobiol Dis.* 2014; 62:73–85. [PubMed: 24051278]
- Heng K, Haney MM, Buckmaster PS. High-dose rapamycin blocks mossy fiber sprouting but not seizures in a mouse model of temporal lobe epilepsy. *Epilepsia.* 2013; 54:1535–1541. [PubMed: 23848506]
- Hester MS, Danzer SC. Accumulation of abnormal adult-generated hippocampal granule cells predicts seizure frequency and severity. *J Neurosci.* 2013; 33:8926–8936. [PubMed: 23699504]
- Kan AA, de Jager W, de Wit M, Heijnen C, van Zuiden M, Ferrier C, van Rijen P, Gosselaar P, Hessel E, van Nieuwenhuizen O, de Graan PN. Protein expression profiling of inflammatory mediators in human temporal lobe epilepsy reveals co-activation of multiple chemokines and cytokines. *J Neuroinflammation.* 2012; 9:207. [PubMed: 22935090]
- Krook-Magnuson E, Armstrong C, Oijala M, Soltesz I. On-demand optogenetic control of spontaneous seizures in temporal lobe epilepsy. *Nat Commun.* 2013; 4:1376. [PubMed: 23340416]
- Kumar SS, Buckmaster PS. Hyperexcitability, interneurons, and loss of GABAergic synapses in entorhinal cortex in a model of temporal lobe epilepsy. *J Neurosci.* 2006; 26:4613–4623. [PubMed: 16641241]
- Lehmkuhle MJ, Thomson KE, Scheerlinck P, Pouliot W, Greger B, Dudek FE. A simple quantitative method for analyzing electrographic status epilepticus in rats. *J Neurophysiol.* 2009; 101:1660–1670. [PubMed: 19129295]

- Li PA, Howlett W, He QP, Miyashita H, Siddiqui M, Shuaib A. Postischemic treatment with calpain inhibitor MDL 28170 ameliorates brain damage in a gerbil model of global ischemia. *Neurosci Lett*. 1998; 247:17–20. [PubMed: 9637399]
- Liu J, Liu MC, Wang KK. Calpain in the CNS: from synaptic function to neurotoxicity. *Sci Signal*. 2008; 1:re1. [PubMed: 18398107]
- Loscher W, Brandt C. Prevention or modification of epileptogenesis after brain insults: experimental approaches and translational research. *Pharmacol Rev*. 2010; 62:668–700. [PubMed: 21079040]
- Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD. Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke*. 1998; 29:152–158. [PubMed: 9445345]
- Mellgren RL, Zhang W, Miyake K, McNeil PL. Calpain is required for the rapid, calcium-dependent repair of wounded plasma membrane. *J Biol Chem*. 2007; 282:2567–2575. [PubMed: 17121849]
- Nassar D, Letavernier E, Baud L, Aractingi S, Khosrotehrani K. Calpain activity is essential in skin wound healing and contributes to scar formation. *PLoS One*. 2012; 7:e37084. [PubMed: 22615899]
- O'Dell CM, Das A, Wallace Gt, Ray SK, Banik NL. Understanding the basic mechanisms underlying seizures in mesial temporal lobe epilepsy and possible therapeutic targets: a review. *J Neurosci Res*. 2012; 90:913–924. [PubMed: 22315182]
- Ono Y, Sorimachi H. Calpains: an elaborate proteolytic system. *Biochim Biophys Acta*. 2012; 1824:224–236. [PubMed: 21864727]
- Pitkanen A, Lukasiuk K. Molecular and cellular basis of epileptogenesis in symptomatic epilepsy. *Epilepsy Behav*. 2009; 14(Suppl 1):16–25. [PubMed: 18835369]
- Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol*. 1972; 32:281–294. [PubMed: 4110397]
- Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci*. 1994; 14:3934–3944. [PubMed: 8207497]
- Saatman KE, Creed J, Raghupathi R. Calpain as a therapeutic target in traumatic brain injury. *Neurotherapeutics*. 2010; 7:31–42. [PubMed: 20129495]
- Salzmann A, Perroud N, Crespel A, Lamercy C, Malafosse A. Candidate genes for temporal lobe epilepsy: a replication study. *Neurol Sci*. 2008; 29:397–403. [PubMed: 19066720]
- Seinfeld J, Baudry N, Xu X, Bi X, Baudry M. Differential Activation of Calpain-1 and Calpain-2 following Kainate-Induced Seizure Activity in Rats and Mice. *eNeuro*. 2016:3.
- Sharma AK, Reams RY, Jordan WH, Miller MA, Thacker HL, Snyder PW. Mesial temporal lobe epilepsy: pathogenesis, induced rodent models and lesions. *Toxicol Pathol*. 2007; 35:984–999. [PubMed: 18098044]
- Sheng JG, Boop FA, Mrak RE, Griffin WS. Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. *J Neurochem*. 1994; 63:1872–1879. [PubMed: 7931344]
- Silva AP, Carvalho AP, Carvalho CM, Malva JO. Modulation of intracellular calcium changes and glutamate release by neuropeptide Y1 and Y2 receptors in the rat hippocampus: differential effects in CA1, CA3 and dentate gyrus. *J Neurochem*. 2001; 79:286–296. [PubMed: 11677256]
- Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L. Mossy fiber synaptic reorganization in the epileptic human temporal lobe. *Ann Neurol*. 1989; 26:321–330. [PubMed: 2508534]
- Thompson SN, Carrico KM, Mustafa AG, Bains M, Hall ED. A pharmacological analysis of the neuroprotective efficacy of the brain- and cell-permeable calpain inhibitor MDL-28170 in the mouse controlled cortical impact traumatic brain injury model. *J Neurotrauma*. 2010; 27:2233–2243. [PubMed: 20874056]
- Tsai MH, Chuang YC, Chang HW, Chang WN, Lai SL, Huang CR, Tsai NW, Wang HC, Lin YJ, Lu CH. Factors predictive of outcome in patients with de novo status epilepticus. *QJM*. 2009; 102:57–62. [PubMed: 19015144]
- Vanderklish PW, Bahr BA. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. *Int J Exp Pathol*. 2000; 81:323–339. [PubMed: 11168679]

- Author Manuscript
- Author Manuscript
- Author Manuscript
- Author Manuscript
- Vezzani A, Dingledine R, Rossetti AO. Immunity and inflammation in status epilepticus and its sequelae: possibilities for therapeutic application. *Expert Rev Neurother*. 2015; 15:1081–1092. [PubMed: 26312647]
- Vosler PS, Brennan CS, Chen J. Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Mol Neurobiol*. 2008; 38:78–100. [PubMed: 18686046]
- Walker M. Neuroprotection in epilepsy. *Epilepsia*. 2007; 48(Suppl 8):66–68.
- Wang S, Shan P, Song Z, Dai T, Wang R, Chi Z. Mu-calpain mediates hippocampal neuron death in rats after lithium-pilocarpine-induced status epilepticus. *Brain Res Bull*. 2008; 76:90–96. [PubMed: 18395616]
- Wang Y, Lopez D, Davey PG, Cameron DJ, Nguyen K, Tran J, Marquez E, Liu Y, Bi X, Baudry M. Calpain-1 and calpain-2 play opposite roles in retinal ganglion cell degeneration induced by retinal ischemia/reperfusion injury. *Neurobiol Dis*. 2016; 93:121–128. [PubMed: 27185592]
- Williams PA, White AM, Clark S, Ferraro DJ, Swiercz W, Staley KJ, Dudek FE. Development of spontaneous recurrent seizures after kainate-induced status epilepticus. *J Neurosci*. 2009; 29:2103–2112. [PubMed: 19228963]
- Zeng LH, Rensing NR, Wong M. The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. *J Neurosci*. 2009; 29:6964–6972. [PubMed: 19474323]
- Zhang G, Raol Y, Hsu F, Coulter D, Brooks-Kayal A. Effects of Status Epilepticus on Hippocampal GABAA Receptors are Age-Dependent. *Neuroscience*. 2004; 125:299–303. [PubMed: 15062973]

HIGHLIGHTS

- *Status epilepticus* promotes calpain overactivation.
- Acute treatment with a pharmacological inhibitor of calpain ameliorates seizure burden.
- Pathologies associated with epileptogenesis were reduced by a calpain inhibitor.

Figure 1. Calpain activation during epileptogenesis

Tissue from the CA1 region of hippocampus was collected at different time points during epileptogenesis (1, 4 and 8 days post SE). Western blot analysis was performed using two antibodies to detect SBDPs (AB38 and anti- α -spectrin) and one antibody to detect actin immunoreactivity. **(A)** Representative western blots for the detection of SBDPs and actin during the epileptogenic period. **(B)** Quantitation of SBDPs detected with the AB38 antibody (n=6). **(C)** Quantitation of SBDPs detected with the α -spectrin antibody (n=6). SBDP immunoreactivity was normalized to actin and compared to the signal detected at 4 days post SE. Data is presented as the mean $\pm$ SEM. **(D)** Representative western blots for the detection of SBDPs and actin following MDL-28170 treatment. **(E)** Quantitation of SBDPs detected with the AB38 antibody following MDL-28170 treatment (n=8). **(F)** Quantitation of SBDPs detected with the α -spectrin antibody after MDL-28170 treatment (n=8). Data is presented as the mean $\pm$ SEM. SBDP immunoreactivity was normalized to actin and compared to the signal detected in the vehicle group (Veh). SBDPs immunoreactivity in the different groups was compared by ANOVA, * p <0.05 or *** p <0.001 represent a significant difference.

Figure 2. Effect of MDL-28170 treatment on neuronal degeneration

Fixed brains were serially sectioned to obtain 15 μ m coronal sections. Three mounted sections were selected from a 1-in-15 series starting at approximately the same level of hippocampus. (A) To detect degenerating neurons, sections were stained with a simple and sensitive technique using the anionic fluorochrome Fluoro-Jade B (FJB). (B) The number of FJB positive (FJB⁺) cells was counted on three sections and averaged to represent the number of FJB⁺ cells in a particular animal. Cell counts were conducted blinded to the administered treatment. Data is presented as the mean $\pm$ SEM of controls (n=6), SE plus vehicle (n=13), low-dose (n=8) and high-dose (n= 8) MDL-28170. The number of FJB⁺ cells was compared by ANOVA, * p <0.05 represents a significant difference when compared to vehicle-treated animals. Scale bar represents 50 μ m.

Figure 3. Effect of MDL-28170 treatment on seizure burden

Rats were implanted with electrodes to obtain continuous EEG recordings using an automatic video-EEG system. A trained technician blinded to treatment examined EEG recordings off-line in order to identify electrographic seizures. Behavioral characterization of seizures was based on the Racine scale as follows: seizures scored as class 2 or below were considered non-convulsive and seizures scored as class 3 or above were categorized as convulsive. **(A)** Graphical representation of the number of seizures *per day* detected in vehicle ($n=14$), *low*-dose ($n=11$) and *high*-dose ($n=12$) groups. **(B)** In the vehicle-treated group, the main increase in the average seizure number (seizures *per day*) was detected during the second week and this increase was mostly due to an increase in the number of convulsive seizures. Data is presented as the mean $\pm$ SEM of groups: SE plus vehicle ($n=14$), *low*-dose ($n=11$) and *high*-dose ($n=12$). The number of seizures was compared by ANOVA, $*p<0.05$ represents a significant difference when compared to vehicle-treated animals. **(C)** The total number of seizures detected during the four weeks of recording in the *low*-dose group was significantly different from the number of seizures detected in the vehicle-treated group. Seizure number in the different groups was compared by ANOVA, $*p<0.05$ represents a significant difference when compared to vehicle-treated animals. **(D)** The relative proportion (percentage) of convulsive and non-convulsive seizures was not

affected by the *low*-dose treatment. **(E)** The duration of convulsive seizures detected in all three groups was similar; a trend towards a reduction in seizure duration for non-convulsive seizures was present in the *low*-dose group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Effect of MDL-28170 treatment on status epilepticus

Rats were implanted with electrodes to obtain EEG recordings during SE induction. (A) Representative EEG traces obtained from hippocampus and cortex. Diazepam was administered at 1 and 3 h after SE onset (arrows). Electrographic recordings from hippocampus (B) or cortex (D) were analyzed off-line to obtain normalized total power. Traces represent normalized power for: SE plus vehicle (red, n=8), low-dose (green, n=4) or high-dose (blue, n=4). Integral power was calculated by adding raw power values corresponding to one-min-epochs during the first hour of SE (0 and 1 h) or during the first 6 h of SE (0 to 6 h). Integral power for hippocampus (C) or cortex (E) was calculated for vehicle (n=4), low-dose (n=4), and high-dose (n=4) groups. Values were compared to the corresponding controls using a *t*-test and did not show a significant decrease on the brain regions analyzed.

Figure 5. Effect of MDL-28170 treatment on inflammation

Fixed brains were sectioned to obtain 15 μm coronal sections. Three sections were selected from a 1-in-15 series starting at approximately the same level of hippocampus. Sections were stained with antibodies to detect GFAP or Iba-1. Representative images of tissue sections stained with either GFAP (**A**) or Iba-1 (**C**) antibodies. The number of GFAP (**B**) or Iba-1 (**D**) positive cells was counted on the three sections and averaged to represent the number of cells in a particular animal. Cell counts were conducted blinded to the administered treatment. Data is presented as the mean ± SEM of controls (n=6), SE plus vehicle (n=13), low-dose (n=8) and high-dose (n=8). The number of positively stained cells was compared by ANOVA, * $p < 0.05$ represents a significant difference when compared to vehicle-treated animals. Scale bar represents 50 μm.

Figure 6. Effect of MDL-28170 treatment on mossy fiber sprouting

Fixed brains from chronically epileptic animals were sectioned to obtain coronal sections. Three sections starting at approximately the same level of hippocampus were selected from a 1-in-15 series. Sections were stained with antibodies to detect ZnT3. **(A)** Representative images of ZnT3 staining on the DG showing a prominent staining for ZnT3 around the granular cell layer (GCL). **(B)** The area of ZnT3 immunoreactivity was quantified on three sections and averaged to represent the area of covered by “sprouting” in a particular animal. Quantitations were conducted blinded to the administered treatment. Data is presented as the mean ± SEM of controls (n=5), SE plus vehicle (n=6), *low*-dose (n=5) and *high*-dose (n=7).

The area of positive staining was compared by ANOVA, * $p < 0.05$ represents a significant difference when compared to vehicle-treated animals. Scale bar represents 100 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript