Published in final edited form as:

Epilepsy Res. 2017 May ; 132: 50–58. doi:10.1016/j.eplepsyres.2017.02.021.

Increased metalloproteinase activity in the hippocampus following status epilepticus

Deepti Dubey^{a,*}, Paulette A. McRae^{b,*}, Elyse K. Rankin-Gee^a, Esther Baranov^b, Luke Wandrey^b, Stephanie Rogers^b, Brenda E. Porter^{a,b,c}

^aDepartment of Neurology, School of Medicine, Stanford University, 1201 Welch Road, P211 MSLS, Stanford CA 94305

^bThe Children's Hospital of Philadelphia, Department of Pediatrics and Division of Neurology 34th and Civic Center Boulevard, Philadelphia PA 19104

Abstract

Increased neuronal plasticity and neuronal cell loss has been implicated in the development of epilepsy following injury. Parvalbumin fast spiking inhibitory interneurons have a robust extracellular matrix coating their cell bodies and the proximal dendrites called the perineuronal net (PNN). The role of the PNN is not clear but it has been implicated in closing of the critical period, altering seizure thresholds and providing neuronal protection from oxidative stress. The PNN is susceptible to degradation following a prolonged seizure and there is an increase in proteolytic-fragments of the PNN enriched proteoglycan aggrecan (Dzwonek et al., 2004). Here we demonstrate an increase in matrix metalloproteinase (MMP) activity in the hippocampus following status epilepticus (SE). We further assessed MMP3 and 13, two of 24 identified MMPs, both MMP3 and 13 mRNA increase in the hippocampus after SE and MMP13 activity increases by functional assay as well as it co-localizes with PNN in rat brain. In contrast, two of the brain expressed ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs) also implicated in aggrecan degradation, did not consistently increase following SE though ADAMTS4 is highly expressed in glia and ADAMTS5 in neuronal cell bodies and their processes. The increase in MMP activity following SE suggests that in the future studies, MMP inhibitors are candidates for blocking PNN degradation and assessing the role of the PNN loss in epileptogenesis and cellular function.

Keywords

Perineuronal net; Epilepsy; Matrix metalloproteinase; Status epilepticus; ADAMTS

^cCorresponding Author: Brenda E. Porter MD, PhD, Associate Professor of Neurology, Stanford University Medical School, P211 MSLS, 1201 Welch Road, Stanford, CA, 94305, phone: 650-724-4179, fax: 650-498-6262, brenda2@stanford.edu.

*Co-first Authors

Conflict of Interest: None

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.

1. Introduction

Epilepsy prevention in high-risk patients has been a challenging problem. Drugs that effectively treat spontaneous seizures have been ineffective in preventing epilepsy. One reason may be that most anti-epileptic drugs dampen excitation or increase inhibition without focusing on the molecular and cellular changes that contribute to the development of epilepsy. Previously we published in a rodent model of epilepsy that perineuronal net (PNN) integrity is lost as animals develop epilepsy and remains diminished in animals with chronic epilepsy (McRae et al., 2012). The loss of the PNN may apply to other brain disorders such as stroke and schizophrenia (Karetko-Sysa et al., 2011, Mauney et al., 2013). The PNN is a multimeric proteoglycan complex that surrounds synapses on the cell body and proximal dendrites and is especially abundant around fast spiking-parvalbumin (FS-PV) interneurons.

The main components of the PNN are hyaluronic acid (HA), hyaluronan synthases (HASs), chondroitin sulfate proteoglycans - primarily lecticans, HA and proteoglycan link protein (HAPLNs), and tenascin-R (Galtrey et al., 2008, Carulli et al., 2010, Kwok et al., 2010, Giamanco and Matthews, 2012). Aggrecan is a PNN specific lectican, having several protein domains for cross linking to tenascin and HA and long stretches of chondroitin sulfate side chains. Hyaluronic acid is synthesized and remains attached to the plasma membrane by HASs (McRae and Porter, 2012). The PNN appears during the 2nd week of postnatal development and its components continue to increase throughout adulthood (McRae et al., 2010). The PNN has multiple functions, including neurotransmitter receptor synaptic stabilization, closing of the ocular dominance sensitive period, and neuroprotection (Cabungcal et al., 2013, Maroto et al., 2013). Proteoglycan degradation has been proposed as a therapeutic strategy to promote neuronal reorganization and recovery following trauma but also might contribute to the development of epilepsy (Kwok et al., 2008). Future studies will be needed to determine if disruption of the PNN contributes to epileptogenesis, memory problems and the changes in the electrophysiology of the FS-PV interneurons that contributes to seizure activity (Zhang and Buckmaster, 2009, Shiri et al., 2015, Toyoda et al., 2015).

Matrix metalloproteinases (MMPs), cleave extracellular proteins including aggrecan, are found throughout the CNS and are necessary for long-term potentiation and regulation of post-synaptic density morphology (Dzwonek et al., 2004). Recently, an increase in the MMP neo-epitopes of aggrecan following status epilepticus (SE) was demonstrated (Rankin-Gee et al., 2015). Only a few of the more than 24 MMPs have been studied in epilepsy, though not all are expressed in the brain. MMP9 and MMP13 are two MMPs found to increase following brain injury, although there are no prior studies exploring their role in degrading PNNs or aggrecan proteolysis in the brain (Nagel et al., 2005). In seizures, MMP9 mRNA and protein levels increase, accompanied by an increase in enzymatic activity (Konopacki et al., 2007, Mizoguchi et al., 2011, Hoehna et al., 2012). Furthermore, pentylenetetrazole (PTZ) kindling is inhibited in MMP9 knockout mice and kindling is increased in MMP9 overexpressing transgenic mice (Wilczynski et al., 2008). Following focal cerebral ischemia, there is up regulation and colocalization of MMP13 with aggrecan, suggesting PNN degradation may occur in response to a variety of brain injuries (Nagel et al., 2005). Another protease family known to cleave aggrecan but less studied in the CNS is ADAMTS (A

Disintegrin And Metalloproteinase with Thrombospondin motifs) proteases known as aggrecanases (Stanton et al., 2011, Gottschall and Howell, 2015). ADAMTS1, 4, 5 and 9, are expressed in the brain and are also candidates for aggrecan degradation following SE.

Here we focus on MMP13 and MMP3, and ADAMTS4 and 5 since they are expressed in the brain and known to specifically cleave aggrecan in cartilage (Fosang et al., 1991, Flannery et al., 1992, Fosang et al., 1996, Dzwonek et al., 2004, Cross et al., 2006, Held-Feindt et al., 2006). The role of the PNN in epileptogenesis or its ability to alter FS-PV cell function is unknown. Identification of proteases involved in degradation of the PNN and extracellular matrix after a brain insult will allow for targeted therapies for prevention of epilepsy in high-risk patients.

2. Material and Methods

2.1 Animals

The Institutional Animal Care and Use Committees at the Children's Hospital of Philadelphia and Stanford University approved all the procedures. Male Sprague–Dawley (SD) rats approximately 250 gm in weight, from Charles River (Wilmington, MA, U.S.A.), were randomly assigned into either the control group or the status epilepticus (SE) group. Rats were single-housed in temperature and humidity controlled housing with a 12 hours light- 12 hours dark cycle and had *ad libitum* access to food and water.

2.2 Seizure Induction

Status epilepticus was induced in the experimental group using the pilocarpine chemoconvulsant rodent model of epilepsy following same protocol as described in Dubey and Porter, 2016 (Dubey and Porter, 2016). Briefly, male SD rats were injected with 1mg/kg methyl-scopolamine intraperitoneally (IP) (Sigma-Aldrich Corp., MO) followed by IP injection of 385mg/kg body weight of pilocarpine hydrochloride (Sigma-Aldrich Corp., MO). Control rats received sub-convulsive dose of 38.5mg/kg body weight of pilocarpine. Rats were monitored for behavioral seizures and SE induction onset was measured from the start of the first stage five seizure on Racine scale (Racine, 1972). The seizure group received an IP injection of diazepam (6 mg/kg) (Hospira Inc., IL) 1 h after the onset of Racine stage five seizure, while the control group received the same dose 1 h after the low-dose pilocarpine injection. The seizure group received an additional 1/2 dose of diazepam (3 mg/kg) 2 h after the initial diazepam injection if the animal had ongoing seizure activity.

2.3 MMP activity

Status epilepticus was induced in male SD rats as described above. Hippocampi were quickly dissected from SE-induced and control rats euthanized at different time points after SE induction and frozen in dry ice. Hippocampal lysates were prepared using reagents provided in the SensoLyte® Plus 520 MMP13 Assay Kit (Cat # AS-72019) and SensoLyte® 520 Generic MMP Assay Kit (Catalogue # 71158) (Anospec, Fremont, CA) and assay for the MMP13 activity and total MMP activity in SE and control samples was performed following manufacturer's instructions. The fluorescent intensity of the samples was measured at Excitation/Emission wavelength=490 nm/520 nm. All values were normalized

to the values from control animals and plotted. Statistical analysis was performed using an analysis of variance (ANOVA) and post hoc Bonferroni's multiple comparisons test comparing seizure and control animals using Prism 5.0 software.

2.4 Real Time-PCR (RT-PCR)

Real Time-PCR was performed as described in McRae *et al.*, 2012. Briefly, whole hippocampal tissue samples were homogenized with a sonicator on ice. RNA was extracted from the tissue using the *mirVana* Isolation Kit (Thermo Fisher Scientific, Waltham, MA). RNA concentrations were measured with a spectrophotometer (NanoDrop ND1000, ThermoFisher Scientific, Wilmington, DE). Five micrograms of purified RNA in 8 µl were reverse transcribed with the SuperScript II reverse transcription kit using random hexamer primers (Life Technologies, NY). The cDNA concentrations were quantified and diluted to 500 ng/2 µl sample that underwent RT-PCR in a 384-well plate. Each master mix was prepared using the Taqman Universal Master Mix (Life Technologies, NY) and a probe for MMP3 (RN00591740_m1), MMP13 (RN01448194_m1), ADAMTS4 (AIRR82F: GTCCCCCTGCAGTGCCCGATTCATCACTGACTTCCTGGACAATGGCTATGGACACT GCCTCTTAGACAAACCAGAGGCTCCCCTGCATCTGCCAGTGACT), or ADAMTS5 (RN01458488_m1) (Thermo Fisher Scientific, Waltham, MA). Each sample was assayed in triplicate to minimize error and matched to a standard curve of rat cortex cDNA. The RT-PCR assay was executed by an SDS 7900HT thermocycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA), comprised of a 2 min cycle at 50 °C, followed by a 10 min cycle at 95 °C, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Data was expressed as a percent change relative to control values in the same run. Statistical analysis was performed using an analysis of variance (ANOVA) and post hoc Bonferroni's multiple comparisons test comparing seizure and control animals using Prism 5.0 software.

2.5 Western blotting

Fresh or frozen, whole hippocampal tissue samples were homogenized on ice with a sonicator. Protein concentrations were analyzed using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using a spectrophotometer. Equal amount of protein was added to 4× NuPage LDS Sample Buffer (Life Technologies, NY) and 10× NuPage Sample Reducing Agent to bring samples to a concentration of 1 µg/µl. This mixture was placed on a dry bath at 72–80°C for 7–8 min. Samples were loaded into NuPage 4–12% Bis-Tris gels (Life Technologies, NY) and run at 200V in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and NuPage antioxidant (Life Technologies, NY). Protein size was assessed with Precision Plus Protein Kaleidoscope Standards (Bio-Rad). Proteins were transferred to nitrocellulose filters using the iBlot Gel Transfer Device and iBlot Transfer Stacks Mini (Life Technologies, NY). Membranes were blocked with 5% milk in low salt Tris Buffered Saline-Tween (TBS-T) at 4°C overnight before exposure to primary antibody MMP3 (ab18898, Abcam, 1:100), MMP13 (ab75606, Abcam, 1:100), ADAMTS4 (ab111905, Abcam, 1:500). Secondary antibodies were diluted in blocking solution and blot was incubated 1 h at room temperature. This was followed by 5 min washes: one in low salt TBS-T, three in high salt TBS-T, and a final wash in low salt TBS-T. Blots were visualized using SuperSignal West Pico Mouse IgG Detection Kit (Thermo Fisher Scientific, Waltham, MA). Membranes were stripped and reprobed with anti-tubulin (T5168, Sigma-Aldrich,

1:10,000) for loading control. Tubulin blots were exposed for approximately 1 s, the shortest we could accomplish. Quantitative analysis of the intensity of the bands of interest on the X-ray films was done using ImageJ software, and statistical analysis (AVOVA with Bonferroni multiple comparison) was performed using Prism 5.0.

2.6 Immunostaining

Under the influence of 3% isoflurane as anesthesia, rats underwent intra-cardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (Sigma-Aldrich Corp., MO). Rats were euthanized while deeply anesthetized, via exsanguination and vascular perfusion. The brains were harvested and post fixed overnight in 4% paraformaldehyde followed by dehydration with 30% sucrose (Sigma-Aldrich Corp., MO) for two days at 4°C. Forty-micron sections were cut on a cryostat, and free-floating sections were incubated at 4°C overnight in the primary antibodies ADAMTS4 (ab111905, Abcam, 1:50), ADAMTS5 (ab50749, Abcam, 1:50), MAP2A (mab378, EMD Millipore, 1:700) or GFAP (1:50; a gift from Dr. J. Grinspan) with 0.5% Triton X-100 in DMEM. The next day the sections were rinsed with phosphate buffer then incubated with Alexa fluorescent-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat secondary antibodies (Invitrogen, Carlsbad, CA, USA) and 0.5% Triton X-100 in DMEM. 4' 6-diamidino-2-phenylindole DAPI (1:1000; Molecular Probes, Carlsbad, CA, USA) with 0.5% Triton X-100 in DMEM was applied after the secondary antibody staining was complete. Sections were rinsed with phosphate buffer and mounted onto glass slides using Prolong Antifade mounting medium (Life Technologies, NY) or Vectashield mounting medium with DAPI (Vector Laboratories, CA). Stained sections were visualized using a Zeiss Axioplan microscope (Thornwood, NY, USA). Images were processed in ImageJ.

2.7 Wisteria Floribunda staining

Co-staining for MMP13 and PNN was done as described in (Rankin-Gee et al., 2015) with slight modifications. Rats were perfused with 4% paraformaldehyde via cardiac infusion. Brains were removed and kept in 4% paraformaldehyde overnight before being transferred to a 30% sucrose solution. Forty micrometer brain slices were collected using a microtome and were stored in cryopreservative. Slices were washed with PBS five times for 5 min each. Slices were blocked in PBS + 2% BSA for 1 h at room temperature. Sections were co-incubated in biotinylated Wisteria Floribunda Lectin (WFA) (20 µg/ml, B-1355, Vector Labs) and anti-MMP13 antibody (1:100, MAB13426, EMD Millipore Corp., CA) in PBS + 2% BSA overnight. Sections were incubated in DyLight 488 Streptavidin (1:500, SA-5488-1, Vector Labs) and 594 Alexa fluorescent-conjugated goat anti-mouse antibody for 3 h at room temperature after washes in PBS. Slices were then mounted with Vectashield mounting medium after staining with DAPI. Confocal images of stained sections were captured using Leica Microsystems, IL, USA with a 40X magnification oil lens. Images were processed in ImageJ.

3. Results

3.1. Matrix metalloproteinase activity increases in the hippocampus of animals following SE

In a recent study, Rankin-Gee *et al.*, 2015, demonstrated that a MMP cleavage product of aggrecan increases after SE. The MMP cleavage product was concentrated around parvalbumin-positive interneurons. To quantify MMP protease activity after SE we performed a fluorescent protease assay to measure total MMP protease activity in the hippocampal lysates of the SE-induced and control rats. As shown in Figure 1, there was an increase in the total MMP activity in the hippocampus of SE-induced animals at both 48 hours and 1-week post-SE relative to controls.

3.2. MMP13 enzymatic activity and expression level increases following SE

Since we found an increase in the total MMP activity in the hippocampus of SE induced animals, we next wanted to look at specific MMP candidates. To investigate if there is any difference in the expression level of MMP13 in SE-induced and control animals, we measured the mRNA and protein levels of MMP13 in the hippocampus of SE-induced and control animals at different time-points post-SE. As shown in Figure 2A, *MMP13* mRNA increased ~4-fold at 48 hours post-SE. At 1 week post-SE there were no significant differences between control and SE animals (Figure 2A). We next determined the protein expression level of MMP13 in the hippocampal lysates of control and SE-induced rats. We did not detect any difference in the MMP13 protein expression level at 48 hours post-SE (Figure 2B). However, we detected a ~2-fold increase in the protein level of MMP13 in the SE-induced animals at 1 week post-SE (Figure 2B).

Since we found increased levels of MMP13 protein in SE- induced animals, we performed an enzymatic fluorescent assay utilizing selective immunoprecipitation of MMP13 to look at its activity level. We did not detect any change in MMP13 activity in the hippocampus of SE-induced animals 48 hours after SE (Figure 2C). Interestingly, there was a significant increase in MMP13 activity 1 week after SE induction (Figure 2C).

3.3. MMP13 co-localizes with PNN in rat brain

The higher level of expression of MMP13 protein and its higher activity in SE-induced animals compared to control animals post-SE led us to hypothesize that it might be regulating the perineuronal net degradation in SE-induced animals. So, we performed immunostaining to determine the localization of MMP13 in the brain of SE-induced rats at 48 hours and 1 week post-SE. After co-immunostaining with the PNN detecting stain *wisteria floribunda* (WFA) and MMP13 antibody, we found that the MMP13 protein co-localized with the majority of the PNN positive cells both in the hippocampus and cortex, as shown in the overlay panels in Figure 3A and 3B.

3.4. MMP3 mRNA expression increased following SE

The expression level of *MMP3* mRNA and protein was measured in the hippocampus of SE-induced and control animals. The mRNA level of MMP3 was elevated ~5-fold at both 48 hours and 1 week post-SE as compared to control (Figure 4A). However, protein expression

of MMP3 in the hippocampal lysates of control and SE-induced rats did not significantly change at any of the time points measured, 48 hours or 1 week (Figure 4B).

3.5. ADAMTS4 and ADAMTS5 expression remain unaltered following SE

We measured mRNA levels of *ADAMTS4* and *5*, known aggrecan proteases, in the hippocampus of SE-induced or control rats at 48 hours and 1 week post-SE. As shown in Figure 5A and B, we did not detect a difference in the mRNA levels of either *ADAMTS4* or *ADAMTS5* after SE-induction at any of the time points tested.

To check the effect of SE-induction on the protein levels of ADAMTS4 and 5, we performed immunohistochemistry of the SE-induced and control rat brains. We found an inconsistent changes in the staining intensity of ADAMTS4 in the post-SE animals compared to controls (Figure 6A). We did not detect any alteration in the intensity of ADAMTS5 immunostaining between SE-induced and control animals at 1 week post-SE (Figure 6A). We also did immunoblot analysis to determine the expression level of ADAMTS4 and did not detect any difference in the expression of ADAMTS4 protein between control and SE-induced animals at both the tested time points (Figure 6B). We could not perform quantification of ADAMTS5 protein since the available antibodies did not produce a consistent band on the western blot.

3.6. ADAMTS4 localizes in astrocytes while ADAMTS5 localizes in the neuropils of the rat hippocampus

Both ADAMTS4 and 5 are expressed in the CNS (Cross et al., 2006, Held-Feindt et al., 2006), however, there are very few studies showing the localization of ADAMTS4 and 5 proteins in the hippocampus (Krstic et al., 2012). The ADAMTS4 antibody produced staining throughout the hippocampus that primarily colocalized with GFAP immunoreactivity as shown in the overlay of Figure 7A, however low level expression in other non-GFAP expressing cells was not ruled out. ADAMTS5 immunofluorescent staining colocalized mostly with the dendritic marker MAP2A-B (Figure 7B) suggesting that ADAMTS5 is localized in the neuropil, although there could be low-level of expression in other cell types.

4. Discussion

Here we show that there is an increase in global MMP activity and more specifically in MMP13 activity of the hippocampus following status epilepticus. The increase in MMP13 activity is not sufficient to explain the global increase in MMP activity as the time courses differ, total MMP activity increased at both 48 hours and 1 week after SE but MMP13 activity only increased at 1 week after SE. MMP3 is a candidate for increased MMP activity following SE as there was a ~ 5 fold increase in MMP3 mRNA but this same increase was not corroborated by an increase in MMP3 protein following SE. We did not identify an assay that could isolate MMP3 activity and therefore do not know the contribution of MMP3 to the increase in global MMP activity after SE.

As there are a large number of MMP's, at least 24, and many have been reported to be expressed in the brain, MMP2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 24 (Dzwonek et al.,

2004), it will be a daunting task to clarify which have altered activity following SE and their specific role in PNN degradation and epileptogenesis. The best-studied MMP in the CNS is MMP9, MMP9 knock-out and overexpression mice have shown a role for MMP9 in synapse stabilization and an increase in epileptogenesis that parallels the level of MMP9 (Mizoguchi et al., 2011, Hoehna et al., 2012). In addition to MMP9, MMP3 and MMP13 can cleave aggrecan to produce the neo-epitope reported by Buttner *et. al.*, 1998 and previously shown by us to be found in the hippocampus following SE (Buttner et al., 1998, Rankin-Gee et al., 2015). MMP3 and 13 have also been implicated in a variety of nervous system disorders especially stroke and neuroinflammation with limited information about their role in epilepsy (Kim et al., 2005, Nagel et al., 2005, Lu et al., 2009).

There is only handful of studies showing the role of MMP13 in the diseased or normal brain function (Nagel et al., 2005, Cuadrado et al., 2009) mostly focusing on cerebral ischemia. Nagel et al., 2005 have shown that focal cerebral ischemia in rats induces the expression of MMP13 and found close spacial association between MMP13 and aggrecan. Previous studies done in the lab (McRae et al., 2012, Rankin-Gee et al., 2015) showed that MMP cleavage products of aggrecan, which is a constituent of PNN, were present in the pilocarpine induced SE brains. In the current study we have shown that MMP13 expression and activity increase in rat brain post-SE, and that MMP13 is localized in close vicinity to the PNN positive cells. This data suggests that MMP13 is a strong candidate for the proteolytic degradation of PNN observed in the brain after induction of SE.

Until recently, there have been a limited number of compounds that have a high affinity and specificity for inhibition of specific MMP's making it difficult to identify a specific role of a single MMP species (Benjamin and Khalil, 2012). Prior studies have tried to address the role of MMP activity in epilepsy and epileptogenesis, using broad-spectrum MMP inhibitor doxycycline, an antibiotic with MMP and other off-target effects. Doxycycline delayed seizure genesis in a kindling model and suppressed pilocarpine-induced seizures though it is unclear if its anti-epileptic effects were via MMP inhibition (Nogueira et al., 2011, Pollock et al., 2014). Further studies are needed using more specific MMP inhibitors before there is a clear understanding of the importance of MMP activity in epileptogenesis.

The ADAMTS1, 4, 5 and 9, are proteases known as aggrecanases (Stanton et al., 2011). Song *et. al.*, 2007 showed that ADAMTS4 and 5 mediate the aggrecan degradation in human articular cartilage explants (Song et al., 2007). Both ADAMTS4 and 5 are expressed in brain (Gottschall and Howell, 2015). A prior study using kainate-induced SE found an increase in *ADAMTS4* mRNA in the hippocampus at time points less than 24 hours after SE but not at one week (Yuan et al., 2002). A different study in the mouse hippocampus reported an increase in ADAMTS4 staining in at least one animal following a kainite- induced seizure (Krstic et al., 2012). Here we used both mRNA measurements and immunohistochemistry to evaluate for changes in ADAMTS4 and 5 levels in the hippocampus following SE. We could not detect an increase in the mRNA or immunofluorescence of either ADAMTS4 or 5. There was however variability in ADAMTS4 staining across animals but no consistent change in immunofluorescent staining following SE at the time points we studied. A recent report suggested that *ADAMTS8* and *14* mRNA's are enriched in FS-PV interneurons making

them potential regulators of the PNN, supporting this possibility ADAMTS-8 *in vitro* can cleave aggrecan (Collins-Racie et al., 2004, Rossier et al., 2015).

Immunostaining of ADAMTS4 and 5 differed in their cellular distribution, ADAMTS4 co-stained prominently with GFAP positive astrocytes and ADAMTS5 co-stained with MAP2A-B in neuronal process, however low-level expression of these protein in cell types other than discussed here can not be ruled out. Our ADAMTS4 and 5 staining was relatively similar to that found in the aged mouse hippocampus though they did not report cellular co-staining (Krstic et al., 2012). The staining patterns for ADAMTS4 was similar to that seen previously in the spinal cord astrocytes, but ADAMTS5 also co-localized with astrocyte markers (Cross et al., 2006, Demircan et al., 2013). Emerging is a highly localized and cell type specific expression of ADMATS family members in the central nervous system suggesting they may have regional, cellular and disease specific roles in matrix remodeling.

There is converging evidence that remodeling of the ECM including the PNN is increased following an episode of SE at least partially as a result of increased MMP activity including now MMP13. There remains limited data though on the role of MMP activity in the development and preservation of epilepsy. Both pharmacologic and molecular tools will need to be developed to test the role of blocking MMP activity in epilepsy prevention.

Acknowledgment

Author contributions: PAM, EKR, LW and EB performed experiments. BEP, DD, PAM and EKR analyzed and interpreted the data. DD, PAM and BEP prepared the manuscript. Funding: This work was supported by NIH R01NS056222 grant to BEP and NIH diversity supplement to NIH R01NS056222 and CURE postdoctoral Taking Flight Award to PAM.

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
FS-PV	Fast spiking-parvalbumin neurons
GFAP	Glial fibrillary acidic protein
IP	Intraperitoneal
MAP2	Microtubule- associated protein 2
MMP	Matrix metalloproteinase
PNN	Perineuronal net
RT-PCR	Real time- polymerase chain reaction
SE	Status epilepticus

6. References

Benjamin MM, Khalil RA (2012) Matrix metalloproteinase inhibitors as investigative tools in the pathogenesis and management of vascular disease. *EXS* 103:209–279. [PubMed: 22642194]

- Buttner FH, Hughes CE, Margerie D, Lichte A, Tschesche H, Caterson B, Bartnik E (1998) Membrane type 1 matrix metalloproteinase (MT1-MMP) cleaves the recombinant aggrecan substrate rAgg1mut at the 'aggrecanase' and the MMP sites. Characterization of MT1-MMP catabolic activities on the interglobular domain of aggrecan. *Biochem J* 333 (Pt 1):159–165. [PubMed: 9639575]
- Cabungcal JH, Steullet P, Morishita H, Kraftsik R, Cuenod M, Hensch TK, Do KQ (2013) Perineuronal nets protect fast-spiking interneurons against oxidative stress. *Proc Natl Acad Sci U S A* 110:9130–9135. [PubMed: 23671099]
- Carulli D, Pizzorusso T, Kwok JC, Putignano E, Poli A, Forostyak S, Andrews MR, Deepa SS, Glant TT, Fawcett JW (2010) Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. *Brain* 133:2331–2347. [PubMed: 20566484]
- Collins-Racie LA, Flannery CR, Zeng W, Corcoran C, Annis-Freeman B, Agostino MJ, Arai M, DiBlasio-Smith E, Dorner AJ, Georgiadis KE, Jin M, Tan XY, Morris EA, LaVallie ER (2004) ADAMTS-8 exhibits aggrecanase activity and is expressed in human articular cartilage. *Matrix Biol* 23:219–230. [PubMed: 15296936]
- Cross AK, Haddock G, Surr J, Plumb J, Bunning RA, Buttle DJ, Woodroffe MN (2006) Differential expression of ADAMTS-1, -4, -5 and TIMP-3 in rat spinal cord at different stages of acute experimental autoimmune encephalomyelitis. *J Autoimmun* 26:16–23. [PubMed: 16303287]
- Cuadrado E, Rosell A, Borrell-Pages M, Garcia-Bonilla L, Hernandez-Guillamon M, Ortega-Aznar A, Montaner J (2009) Matrix metalloproteinase-13 is activated and is found in the nucleus of neural cells after cerebral ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 29:398–410.
- Demircan K, Yonezawa T, Takigawa T, Topcu V, Erdogan S, Ucar F, Armutcu F, Yigitoglu MR, Ninomiya Y, Hirohata S (2013) ADAMTS1, ADAMTS5, ADAMTS9 and aggrecanase-generated proteoglycan fragments are induced following spinal cord injury in mouse. *Neurosci Lett* 544:25–30. [PubMed: 23562508]
- Dubey D, Porter BE (2016) CRTC1 nuclear localization in the hippocampus of the pilocarpine-induced status epilepticus model of temporal lobe epilepsy. *Neuroscience* 320:57–68. [PubMed: 26844388]
- Dzwonek J, Rylski M, Kaczmarek L (2004) Matrix metalloproteinases and their endogenous inhibitors in neuronal physiology of the adult brain. *FEBS Lett* 567:129–135. [PubMed: 15165905]
- Flannery CR, Lark MW, Sandy JD (1992) Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human articular cartilage. *J Biol Chem* 267:1008–1014. [PubMed: 1730630]
- Fosang AJ, Last K, Knauper V, Murphy G, Neame PJ (1996) Degradation of cartilage aggrecan by collagenase-3 (MMP-13). *FEBS Lett* 380:17–20. [PubMed: 8603731]
- Fosang AJ, Neame PJ, Hardingham TE, Murphy G, Hamilton JA (1991) Cleavage of cartilage proteoglycan between G1 and G2 domains by stromelysins. *J Biol Chem* 266:15579–15582. [PubMed: 1874716]
- Galtrey CM, Kwok JC, Carulli D, Rhodes KE, Fawcett JW (2008) Distribution and synthesis of extracellular matrix proteoglycans, hyaluronan, link proteins and tenascin-R in the rat spinal cord. *Eur J Neurosci* 27:1373–1390. [PubMed: 18364019]
- Giamanco KA, Matthews RT (2012) Deconstructing the perineuronal net: cellular contributions and molecular composition of the neuronal extracellular matrix. *Neuroscience* 218:367–384. [PubMed: 22659016]
- Gottschall PE, Howell MD (2015) ADAMTS expression and function in central nervous system injury and disorders. *Matrix biology : journal of the International Society for Matrix Biology* 44–46:70–76.
- Held-Feindt J, Paredes EB, Blomer U, Seidenbecher C, Stark AM, Mehdorn HM, Mentlein R (2006) Matrix-degrading proteases ADAMTS4 and ADAMTS5 (disintegrins and metalloproteinases with thrombospondin motifs 4 and 5) are expressed in human glioblastomas. *Int J Cancer* 118:55–61. [PubMed: 16003758]
- Hoehna Y, Uckermann O, Luksch H, Stefovskaya V, Marzahn J, Theil M, Gorkiewicz T, Gawlak M, Wilczynski GM, Kaczmarek L, Ikonomidou C (2012) Matrix metalloproteinase 9 regulates cell death following pilocarpine-induced seizures in the developing brain. *Neurobiol Dis* 48:339–347. [PubMed: 22782080]

- Karetko-Sysa M, Skangiel-Kramska J, Nowicka D (2011) Disturbance of perineuronal nets in the perilesional area after photothrombosis is not associated with neuronal death. *Exp Neurol* 231:113–126. [PubMed: 21683696]
- Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, Joh TH (2005) Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. *J Neurosci* 25:3701–3711. [PubMed: 15814801]
- Konopacki FA, Ryłski M, Wilczek E, Amborska R, Detka D, Kaczmarek L, Wilczynski GM (2007) Synaptic localization of seizure-induced matrix metalloproteinase-9 mRNA. *Neuroscience* 150:31–39. [PubMed: 17928157]
- Krstic D, Rodriguez M, Knuesel I (2012) Regulated proteolytic processing of Reelin through interplay of tissue plasminogen activator (tPA), ADAMTS-4, ADAMTS-5, and their modulators. *PLoS One* 7:e47793. [PubMed: 23082219]
- Kwok JC, Afshari F, Garcia-Alias G, Fawcett JW (2008) Proteoglycans in the central nervous system: plasticity, regeneration and their stimulation with chondroitinase ABC. *Restor Neurol Neurosci* 26:131–145. [PubMed: 18820407]
- Kwok JC, Carulli D, Fawcett JW (2010) In vitro modeling of perineuronal nets: hyaluronan synthase and link protein are necessary for their formation and integrity. *J Neurochem* 114:1447–1459. [PubMed: 20584105]
- Lu DY, Yu WH, Yeh WL, Tang CH, Leung YM, Wong KL, Chen YF, Lai CH, Fu WM (2009) Hypoxia-induced matrix metalloproteinase-13 expression in astrocytes enhances permeability of brain endothelial cells. *J Cell Physiol* 220:163–173. [PubMed: 19241444]
- Maroto M, Fernandez-Morales JC, Padin JF, Gonzalez JC, Hernandez-Guijo JM, Montell E, Verges J, de Diego AM, Garcia AG (2013) Chondroitin sulfate, a major component of the perineuronal net, elicits inward currents, cell depolarization, and calcium transients by acting on AMPA and kainate receptors of hippocampal neurons. *J Neurochem*.
- Mauney SA, Athanas KM, Pantazopoulos H, Shaskan N, Passeri E, Berretta S, Woo TU (2013) Developmental pattern of perineuronal nets in the human prefrontal cortex and their deficit in schizophrenia. *Biol Psychiatry* 74:427–435. [PubMed: 23790226]
- McRae PA, Baranov E, Rogers SL, Porter BE (2012) Persistent decrease in multiple components of the perineuronal net following status epilepticus. *Eur J Neurosci*.
- McRae PA, Baranov E, Sarode S, Brooks-Kayal AR, Porter BE (2010) Aggrecan expression, a component of the inhibitory interneuron perineuronal net, is altered following an early-life seizure. *Neurobiol Dis* 39:439–448. [PubMed: 20493259]
- McRae PA, Porter BE (2012) The perineuronal net component of the extracellular matrix in plasticity and epilepsy. *Neurochem Int* 61:963–972. [PubMed: 22954428]
- Mizoguchi H, Nakade J, Tachibana M, Ibi D, Someya E, Koike H, Kamei H, Nabeshima T, Itohara S, Takuma K, Sawada M, Sato J, Yamada K (2011) Matrix metalloproteinase-9 contributes to kindled seizure development in pentylentetrazole-treated mice by converting pro-BDNF to mature BDNF in the hippocampus. *J Neurosci* 31:12963–12971. [PubMed: 21900575]
- Nagel S, Sandy JD, Meyding-Lamade U, Schwark C, Bartsch JW, Wagner S (2005) Focal cerebral ischemia induces changes in both MMP-13 and aggrecan around individual neurons. *Brain Res* 1056:43–50. [PubMed: 16112096]
- Nogueira CR, Damasceno FM, de Aquino-Neto MR, de Andrade GM, Fontenele JB, de Medeiros TA, Viana GS (2011) Doxycycline protects against pilocarpine-induced convulsions in rats, through its antioxidant effect and modulation of brain amino acids. *Pharmacol Biochem Behav* 98:525–532. [PubMed: 21382396]
- Pollock E, Everest M, Brown A, Poulter MO (2014) Metalloproteinase inhibition prevents inhibitory synapse reorganization and seizure genesis. *Neurobiol Dis* 70:21–31. [PubMed: 24946277]
- Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 32:281–294. [PubMed: 4110397]
- Rankin-Gee EK, McRae PA, Baranov E, Rogers S, Wandrey L, Porter BE (2015) Perineuronal net degradation in epilepsy. *Epilepsia* 56:1124–1133. [PubMed: 26032766]
- Rossier J, Bernard A, Cabungcal JH, Perrenoud Q, Savoye A, Gallopin T, Hawrylycz M, Cuenod M, Do K, Urban A, Lein ES (2015) Cortical fast-spiking parvalbumin interneurons enwrapped in the

perineuronal net express the metallopeptidases Adamts8, Adamts15 and Neprilysin. *Mol Psychiatry* 20:154–161. [PubMed: 25510509]

Shiri Z, Manseau F, Levesque M, Williams S, Avoli M (2015) Interneuron activity leads to initiation of low-voltage fast-onset seizures. *Ann Neurol* 77:541–546. [PubMed: 25546300]

Song RH, Tortorella MD, Malfait AM, Alston JT, Yang Z, Arner EC, Griggs DW (2007) Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. *Arthritis Rheum* 56:575–585. [PubMed: 17265492]

Stanton H, Melrose J, Little CB, Fosang AJ (2011) Proteoglycan degradation by the ADAMTS family of proteinases. *Biochim Biophys Acta* 1812:1616–1629. [PubMed: 21914474]

Toyoda I, Fujita S, Thamattoor AK, Buckmaster PS (2015) Unit Activity of Hippocampal Interneurons before Spontaneous Seizures in an Animal Model of Temporal Lobe Epilepsy. *J Neurosci* 35:6600–6618. [PubMed: 25904809]

Wilczynski GM, Konopacki FA, Wilczek E, Lasiecka Z, Gorlewicz A, Michaluk P, Wawrzyniak M, Malinowska M, Okulski P, Kolodziej LR, Konopka W, Duniec K, Mioduszevska B, Nikolaev E, Walczak A, Owczarek D, Gorecki DC, Zuschratter W, Ottersen OP, Kaczmarek L (2008) Important role of matrix metalloproteinase 9 in epileptogenesis. *J Cell Biol* 180:1021–1035. [PubMed: 18332222]

Yuan W, Matthews RT, Sandy JD, Gottschall PE (2002) Association between protease-specific proteolytic cleavage of brevican and synaptic loss in the dentate gyrus of kainate-treated rats. *Neuroscience* 114:1091–1101. [PubMed: 12379262]

Zhang W, Buckmaster PS (2009) Dysfunction of the dentate basket cell circuit in a rat model of temporal lobe epilepsy. *J Neurosci* 29:7846–7856. [PubMed: 19535596]

Highlights

- Total MMP activity increases after status epilepticus
- MMP13 activity increases after status epilepticus
- MMP3 and MMP13 mRNA levels increase after status epilepticus
- MMP13 colocalizes with perineuronal net positive cells in rat brain
- ADAMTS4 and ADAMTS5 expression remain unaltered following status epilepticus

Figure 1: MMP activity increases in the Status Epilepticus - induced animals (SE) compared to control animals (CT):

Hippocampal lysates from SE or CT rats were prepared and total MMP activity assay was performed. End-point fluorescent intensity was measured and the fluorescent intensity readings were normalized to controls and plotted as the fold change in activity. SE animals (N=5) showed a significant increase in the total MMP activity at both 48 hours and 1 week post-SE induction compared to CT animals (N=5). Graphs were plotted as mean $\pm$ SEM. ANOVA with Bonferroni multiple comparison was performed. p-values for Bonferroni's multiple comparison **p-value= 0.0065 (48 hours) and **p-value= 0.0059 (1 week)

Figure 2: MMP13 enzymatic activity and expression levels increases following Status Epilepticus (SE):

Hippocampi from control (CT) and SE rats were harvested at different time points post-SE. (A) RNA was extracted from the hippocampi harvested from the SE and CT rats at different time points post-SE. Quantitative PCR was performed to check the expression level *MMP13* and fold change was plotted. A ~4- fold increase in *MMP13* mRNA expression at 48 hours was seen in SE animals (N=7–9) compared to CT animals (N=5–10). ANOVA with Bonferroni multiple comparison was performed (* $p < 0.05$, Bonferroni's multiple comparison). (B) Western blot analysis was performed and the MMP13 protein levels were determined by the band intensity on the blot. The intensities were plotted after normalization with tubulin. Representative western blots were shown below the graphs. MMP13 protein level increased ~2 fold in the SE animals (N=5–8) compared to CT animals (N=6–8) 1-week post-SE. ANOVA with Bonferroni multiple comparison was performed (** $p < 0.0005$, Bonferroni's multiple comparison). (C) MMP13 protein was immunoprecipitated and an activity assay was performed. End-point fluorescent intensity was measured and the fluorescent intensity readings were normalized to controls and plotted as the fold change in activity. MMP13 activity was unchanged at 48 hours and showed a ~2-fold increase in the SE animals (N=5) 1-week post-SE induction compared to controls (CT) (N=5). ANOVA with Bonferroni multiple comparison ** p -value=0.006 (1 week) All graphs were plotted as mean $\pm$ SEM.

Figure 3: MMP13 co-localizes with PNN expressing cells in rat brain:

Anaesthetized rats were perfused and the brains were harvested and co-stained for MMP13 protein (red) using anti-MMP13 antibody and PNN (green) using wisteria floribunda stain. MMP13 showed colocalization with the PNN in dentate gyrus (subgranular zone) region and cortex (layer IV/V) of SE-induced rats at both (A) 48 hours post-SE and (B) 1 week post-SE. The area in the inset is blown out on the right hand side of each figure to show the colocalization of the MMP13 in PNN expressing cells. Scale bar 20 μm.

Figure 4: *MMP3* mRNA expression increased following Status Epilepticus (SE): Hippocampi were collected from SE and control (CT) rats at different time points after SE induction. **(A)** *MMP3* mRNA showed a increased expression in SE animals (N =8–9) relative to CT animals (N=9–11) across all the time points tested. A ~5 fold increase was seen at 48 hours and 1 week post-SE. ANOVA with Bonferroni multiple comparison was performed (** $p < 0.005$, and * $p < 0.05$ Bonferroni's multiple comparison). **(B)** Equal amounts of proteins were loaded to perform western blot analysis and the protein levels were determined by the band intensity on the blot. The intensities were plotted after normalization with tubulin as mean $\pm$ SEM. Representative western blots were shown below the graphs. *MMP3* protein did not show any significant difference in the protein levels between SE (N =5–8) and CT animals (N=4–6) across different time points.

Figure 5: *ADAMTS4* and *ADAMTS5* mRNA expression does not change on Status Epilepticus (SE)-induction:

RNA was extracted from the hippocampi harvested from the SE and control (CT) rats at different time points. Quantitative PCR was performed to check the expression level of *ADAMTS4* and *ADAMTS5*. Graphs were plotted for fold change normalized to control values as mean $\pm$ SEM. **(A)** *ADAMTS4* mRNA does not show any difference in expression level between SE and CT animals at 48 hours and 1 week post-SE. **(B)** Similar to *ADAMTS4*, *ADAMTS5* did not show a difference in mRNA expression level between SE and CT animals across the three time points tested. ANOVA with Bonferroni multiple comparison was performed for *ADAMTS4* and *5* mRNA. SE (N=7–9) and CT (N=10–7)

Figure 6: Immunohistochemical staining of ADAMTS5 protein is unchanged at 1 week post-SE induction:

Immunostaining using ADAMTS4 or ADAMTS5 antibody was done and the representative fluorescent images are shown. (A) ADAMTS4 staining in the dentate gyrus (hilar region) of the status epilepticus- induced (SE) and control rats is shown at different magnifications (10X and 40X) at one week post-SE. Brighter staining was detected in the brains of some SE animals; however, this pattern was not consistent in all animals tested. ADAMTS5 staining did not show any difference in the staining pattern between SE and control rats at the time

point tested in the dentate gyrus (hilar and CA4 regions). Scale bar 100 μm (marked in black). **(B)** Equal amounts of proteins were loaded to perform western blot analysis and the protein levels were determined by the band intensity on the blot. The intensities were plotted after normalization with tubulin as mean $\pm$ SEM. Representative western blots were shown below the graphs. ADAMTS4 protein did not show any significant difference in the protein levels between SE (N =4) and CT animals (N=3–4) across different time points.

Figure 7: ADAMTS4 localizes in the glia while ADAMTS5 localizes in the neuropil dendrites in rat hippocampus:

Anaesthetized rats were perfused and the brain were harvested and immunostained for (A) ADAMTS4 (red) localized in the glial cells in CA1 (Stratum oriens) and dentate gyrus (subgranular zone) regions and shows colocalization with the GFAP (green), a marker for glia. (B) ADAMTS5 (red) which localized in the neuropil regions of CA1 and dentate gyrus (hilar region) and colocalized with the MAP2 (green), a marker for dendrites as shown in the overlay. Scale bar 20 μm .