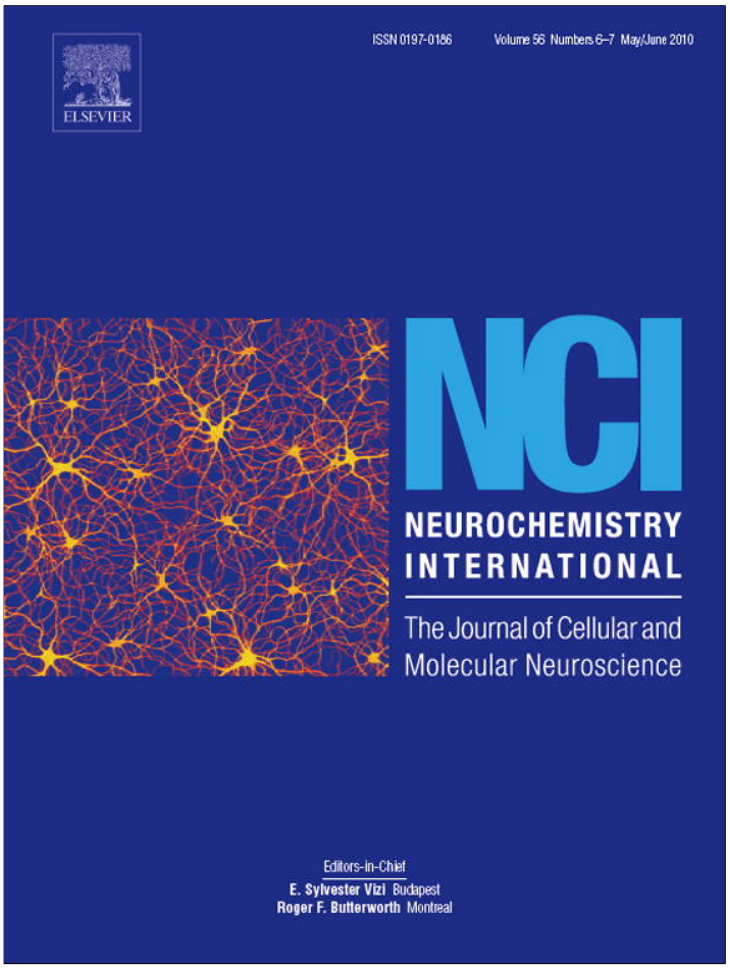

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Matrix metalloproteinase-9 activity increased by two different types of epileptic seizures that do not induce neuronal death: A possible role in homeostatic synaptic plasticity

Eszter Takács [a,*], Rita Nyilas [a], Zsuzsanna Szepesi [a], Péter Baracskay [a], Bente Karlsen [b], Tina Røsvold [b], Alvhild A. Bjørkum [b], András Czurkó [a,e], Zsolt Kovács [c], Adrienna K. Kékesi [a,d], Gábor Juhász [a]

[a] Laboratory of Proteomics, Institute of Biology, Eötvös Loránd University, Budapest, Hungary
[b] Bergen University College, Faculty of Health and Social Sciences, Norway
[c] Department of Zoology, The University of West Hungary, Savaria Campus, Szombathely, Hungary
[d] Department of Physiology and Neurobiology, Eötvös Loránd University, Budapest, Hungary
[e] Institute of Medical Chemistry, University of Szeged, Szeged, Dóm tér 8, H-6720, Hungary

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ABSTRACT

Matrix metalloproteases (MMPs) degrade or modify extracellular matrix or membrane-bound proteins in the brain. MMP-2 and MMP-9 are activated by treatments that result in a sustained neuronal depolarization and are thought to contribute to neuronal death and structural remodeling. At the synapse, MMP actions on extracellular proteins contribute to changes in synaptic efficacy during learning paradigms. They are also activated during epileptic seizures, and MMP-9 has been associated with the establishment of aberrant synaptic connections after neuronal death induced by kainate treatment. It remains unclear whether MMPs are activated by epileptic activities that do not induce cell death. Here we examine this point in two animal models of epilepsy that do not involve extensive cell damage. We detected an elevation of MMP-9 enzymatic activity in cortical regions of secondary generalization after focal seizures induced by 4-aminopyridine (4-AP) application in rats. Pro-MMP-9 levels were also higher in Wistar Glaxo Rijswijk (WAG/Rij) rats, a genetic model of generalized absence epilepsy, than they were in Sprague–Dawley rats, and this elevation was correlated with diurnally occurring spike-wave-discharges in WAG/Rij rats. The increased enzymatic activity of MMP-9 in these two different epilepsy models is associated with synchronized neuronal activity that does not induce widespread cell death. In these epilepsy models MMP-9 induction may therefore be associated with functions such as homeostatic synaptic plasticity rather than neuronal death.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of inducible brain proteases. MMP-2 and MMP-9, also known as gelatinase-A and -B, are Zn^{2+}-dependent members of this family with recognized roles in proteolysis of the extracellular matrix (ECM) (Birkedal-Hansen et al., 1993; Chakraborti et al., 2003; Dzwonek et al., 2004; Nagase and Woessner, 1999; Sternlicht and Werb, 2001; Van den Steen et al., 2002). They degrade ECM proteins, including adhesion and signaling molecules (Gall and Lynch, 2004; Kaczmarek et al., 2002; Michaluk et al., 2007; Wang et al., 2008; Yamada et al., 2001; Yong et al., 1998), as well as neurotransmitter receptors (Michaluk et al., 2009) and growth factors (Nagase and Woessner, 1999; Page-McCaw et al., 2007).

Expression of MMPs increases in pathological processes including tumorgenesis (Bodey et al., 2001; Egeblad and Werb, 2002; Rooprai and McCormick, 1997; Yu and Stamenkovic, 2000), inflammation (Rosenberg, 2002) and epilepsies (Jourquin et al., 2003; Konopacki et al., 2007; Szklarczyk et al., 2002; Wilczynski et al., 2008; Zhang et al., 1998). However MMPs may also be activated in physiological situations including learning paradigms (Bozdagi et al., 2007; Meighan et al., 2006; Wright et al., 2003; Wright et al., 2007), synaptic plasticity (Meighan et al., 2007; Nagy et al., 2006) and retinal light adaptation (Papp et al., 2007). The

Abbreviations: MMP, matrix metalloproteinases; 4-AP, 4-aminopyridine; WAG/Rij, Wistar Glaxo Rijswijk; SWDs, spike and wave discharges; ACSF, artificial cerebrospinal fluid; ECM, extracellular matrix; TIMPs, tissue inhibitors of MMPs; SPRD, Sprague–Dawley.

* Corresponding author at: Laboratory of Proteomics, Institute of Biology, Eötvös Loránd University, H-1117 Budapest, Pázmány P. stny. 1/C, Hungary.
Tel.: +36 1 372 2500x8110.
E-mail addresses: neuroproteome@gmail.com, gjuhasz@dec001.geobio.elte.hu (E. Takács).

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effects of MMP activation include alterations of neuronal excitability and synaptic efficacy (Bozdagi et al., 2007; Meighan et al., 2007; Nagy et al., 2006; Wilczynski et al., 2008) via changes in synaptic structure (Reeves et al., 2003; Shiosaka and Yoshida, 2000; Szklarczyk et al., 2002; Wang et al., 2008).

MMP-9 is induced during the status epilepticus after kainate (Jourquin et al., 2003; Konopacki et al., 2007; Szklarczyk et al., 2002; Zhang et al., 1998, 2000) or pilocarpine (Kim et al., 2009) treatment. In these models, MMP-9 appears to contribute to neuronal death (Jourquin et al., 2003; Kim et al., 2009), pruning of dendritic spines (Poolos, 2008; Wilczynski et al., 2008) and the establishment of aberrant synaptic contacts (Szklarczyk et al., 2002; Wilczynski et al., 2008). It remains unclear whether the enzymatic activity of MMP-2 and MMP-9 is enhanced by epileptiform activities that do not induce neuronal death.

To answer this question, we therefore used two different animal models of epilepsy that do not involve cell damage. The first was the generalized cortical seizures induced by focal application of the K^+ channel blocker 4-aminopyridine (4-AP). Enzymatic activity was examined in distant regions as seizures generalized. We used halothane anesthesia since previous studies showed it reduces neuronal degeneration (Baracskay et al., 2008; Nishikawa and MacIver, 2000; Pena and Tapia, 1999; Slezia et al., 2004; Walker et al., 1999).

The second epilepsy model was the Wistar Glaxo Rijswijk (WAG/Rij) rat. After the age of four months, WAG/Rijs generate spike and wave discharges (SWDs) (Coenen and van Luijtelaar, 2003). No neuronal damage is detected in thalamic or cortical circuits underlying the absence-like seizures (Coenen and van Luijtelaar, 2003; Meeren et al., 2005, 2002). We compared enzymatic activity of MMP-2 and -9 in Sprague–Dawley and WAG/Rij rats at 6 weeks, before seizures emerge, and at 6 months when SWDs are established. Seizures in WAG/Rij rats follow a diurnal rhythm occurring most frequently at transitions between sleep and waking states (Coenen et al., 1991, 1992; Drinkenburg et al., 1991; van Luijtelaar and Coenen, 1988). We therefore compared MMP-9 activity in thalamic and cortical regions during wakefulness and the seizure-rich transition to sleep. Both MMP-9 and its precursor pro-MMP-9 were enhanced in regions of generalization of 4-AP-induced activity, and during periods of high seizure activity in adult WAG/Rij animals, in comparison to both young rats and Sprague–Dawley controls.

2. Experimental procedures

2.1. Animals and reagents

Experiments were carried out on male WAG/Rij (Wistar Albino Glaxo/Rijswijk, Netherlands) and Sprague–Dawley (Charles River Laboratories, Hungary) rats. Animals were housed in groups of 3–5 in standard conditions of a 12 h light/dark cycle (light from 06:00 h till 18:00 h). Animal care and treatment conformed to guidelines of the Council Directive 86/609/EEC and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII), and with local regulations for research animals. We took care to minimize pain, suffering and the number of animals used. EEG signals were recorded from 3 rats during 4-AP induced seizures, tissue from 35 animals was used for gel-based zymography. Three animals which showed no behavioural signs of generalized seizures were excluded from MMP measurements after 4-AP treatment. MMP activity was assayed in 4 WAG/Rij and 4 Sprague–Dawley rats aged 5–6 weeks, 4 WAG/Rij and 4 Sprague–Dawley rats aged 5–7 months. The absence of seizures was confirmed in EEG records from 2 WAG/Rij animals aged 6 weeks. Eight WAG/Rij rats were used to compare MMP activity at times of day associated with a high or low frequency of seizure occurrence and we examined this correlation in EEG records from 2 additional animals. Reagents were obtained from Sigma–Aldrich Co. (Hungary), unless otherwise indicated.

2.2. Tissue dissection

Animals were decapitated under halothane anesthesia (1–1.5% halothane in air), and brains dissected in cooled ACSF (artificial cerebrospinal fluid). Parietal and prefrontal cortices, thalamus and hippocampus in some experiments were dissected on an ice-cold plate. Tissue was stored at −80 °C before analysis. Animals were decapitated at 12:00 h, during the low-frequency seizure period for WAG/Rij animals, except 4 rats sacrificed at 18:00 h during the high-frequency seizure period.

2.3. Surgery procedures for 4-AP administration

4-AP experiments were carried out on male Sprague–Dawley rats (300–400 g) which were anesthetised by halothane (1–1.5% halothane in air) and placed in a stereotaxic frame (David Kopf, USA). A hole, of diameter 1.5–2 mm, was drilled in the skull above the parietal cortex (coordinates from bregma: anterior: −5.8, lateral: 3.2). The dura was removed and the hole covered with fibrin sponge (Spongostan) to prevent drying. After 30 min recovery, 4-AP (0.5 mg/kg) was placed on the parietal cortex surface. After 45 min, the cortical surface was washed repeatedly with ACSF at 36 °C, the wound was closed and animals removed to a quiet, dark and secure place for observation of the behavioural symptoms of epilepsy. In sham operated control animals, a hole was drilled, the dura was removed, but 4-AP was not applied, and the hole was washed in the same way after 45 min. Ipsilateral and contralateral parietal and frontal cortices and thalamic tissue samples were prepared on ice from 4-AP treated and control rats at three time points: $t = 0$ corresponding to the end of the 4-AP application, $t = 6$ h and $t = 24$ h ($n = 6$ for each group)

2.4. EEG recordings

Behavioural and electrical signs of seizures after focal 4-AP application were correlated in EEG records from three Sprague–Dawley rats. Screw electrodes were inserted above the frontal (cFC, iFC), somatosensory (cSS, iSS) and parieto-occipital cortices (cPO, iPO) through holes drilled in the skull with a wound clip placed under the skin as reference electrode. A hole was drilled to expose the parietal cortex for 4-AP application and a Teflon rod of 2 mm diameter was inserted into the dental acrylic cement so the hole remained accessible. Electrodes were attached to a 10-pin socket fixed to the skull with cement. 4-AP was applied after 1 week. A baseline EEG was recorded, and under halothane anesthesia the Teflon rod was removed so that 4-AP could be applied. The hole was washed thoroughly with ACSF after 45 min, covered with bone wax and anesthesia was stopped. EEG records (Grass EEG 8B, 0.3–70 Hz bandwidth) were made during 6 h after 4-AP application and for 1 h periods at 12 h and 24 h and 7 days. Data was stored using a CED 1401 system and the SPIKE2 v2.1 software from CED (Cambridge, UK). Power spectra were analyzed from records of duration 60 min using NeuroExplorer v.3.2 software (Nex Technologies, MA, USA).

In 3 WAG/Rij animals prolonged (11:00 h until 19:00 h, 8 h) EEG records were made to follow the circadian variation in the occurrence of spike-wave-discharges (SWDs). Since animals were maintained on a 12 h) light/dark cycle, this time included a period of sustained illumination followed by a transition to darkness. Procedures were similar to those for EEG records from 4-AP treated animals with screw electrodes inserted above the parietal, fronto-parietal and frontal cortices and identical EEG settings. Changes in SWD frequency were estimated with respect to the frequency between 11:00 h and 12:00 h. A one sample t-test was used to compare the number of SWDs with standard error of the mean (SEM), and significance levels of: $^*p < 0.05$, $^{**}p < 0.01$. Prolonged EEG records from young WAG/Rij rats ($n = 2$, 6 weeks), using similar implantation and recording procedures, confirmed that these animals did not generate seizures (data not shown).

2.5. Behavioural scoring

Animal behaviour after 4-AP application was scored according to the Racine scale (Racine, 1972) with modifications (Malhotra et al., 1997). Stage 0: behavioural arrest, hair rising, excitement and rapid breathing, Stage 1: salivation, unilateral movement of the lips, tongue, and vibrissae, Stage 2: head nodding, head and eye clonus, Stage 3: unilateral or bilateral forelimb clonus and "wet dog shakes", Stage 4: forelimb clonic seizures and clonic rearing, Stage 5: generalized clonic seizures, falling, uncontrollable jumping and later atonia.

2.6. Gelatin zymography

Proteins were prepared and in-gel MMP renaturation performed following procedures of Weeks et al. (1976) and Szklarczyk et al. (2002) with some modifications. Tissue was homogenized in ice-cold buffer with 10 mM $CaCl_2$ and 0.25% TritonX-100 (20 μL buffer/1 mg wet tissue). After centrifugation (20 m, 6000 × g, 4 °C) the pellet containing ECM-bound MMPs and a TritonX insoluble fraction, was separated from supernatants. It was re-solubilized in buffer containing 50 mM Tris–HCl and 0.1 mM $CaCl_2$ (pH 7.4) incubated for 15 min at 60 °C and centrifuged a second time (20 m, 10,000 × g, 4 °C). Insoluble supernatant fractions were precipitated with cold 60% ethanol (750 μl ethanol/500 μl sample), and centrifuged (5 m, 15,000 × g, 4 °C). Precipitates were solubilized in non-reducing SDS-sample buffer (125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromphenol blue; 5.3 μl/1 mg initial wet tissue) and incubated for 15 min at 37 °C. Protein concentrations were measured by Bicichoninic Acid Protein Assay Kit (Sigma–Aldrich Co.), using 25 μl samples removed immediately before precipitation. Samples were stored at −70 °C.

Equal amounts of proteins (60 μg from soluble and 20 μg from insoluble samples) from different brain areas were separated on 7.5% SDS-PAGE gel co-polymerized with 0.1% FITC-labeled gelatin. FITC labeling of the gelatin (Sigma–Aldrich Co.) followed the protocol of Hattori (Hattori et al., 2002). Gels were washed in 2.5% (v/v) TritonX-100 (2 × 15 min) in distilled water to remove SDS, and then incubated in activation buffer (50 mM Tris pH 7.5, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (v/v) TritonX-100, 0.2% (w/v) NaN_3) for 48 h) at 37 °C. Gels were digitally photographed in UV light (Geldoc1000 system, Bio-Rad Laboratories Inc., Hercules, CA, USA). Enzymatic activity causes dark bands with a bright fluorescent background on the gels (in contrast to Coomassie stained gels). Proteinase activity was quantified by densitometry of gelatinolytic bands (ImageQuant software, Amersham). Gelatinase levels from treated rats were compared to control samples loaded on the same gel.

Density differences were analyzed by a one-way ANOVA test with *post hoc* multiple comparison test (Tukey test). ANOVA was followed by a Student's *t*-test with significance levels of: $^{*}p < 0.05$, $^{**}p < 0.01$. Values represent mean densitometry data (optical density in control %) with the standard error of mean (SEM). Experimental groups were as follows: first paradigm: 4-AP treated at 0 h, 6 h, 24 h ($n = 6$ each), sham operated at 0 h, 6 h, 24 h (all $n = 5$), 2 absolute controls (non-operated animals); second paradigm: young WAG/Rij rats (6 weeks, $n = 4$), adult WAG/Rij rats (6 months, $n = 4$), young SPRD rats (6 weeks, $n = 4$) and adult SPRD rats (6 months, $n = 4$); third paradigm: adult WAG/Rij rats sacrificed at 12:00 h (low seizure frequency period, $n = 4$) and adult WAG/Rij rats at 18:00 h (high seizure frequency period, $n = 4$).

2.7. Western blot analysis

Tissue samples were homogenized in lysis buffer (10 mM $CaCl_2$, 0.25% TritonX-100, with the enzyme-inhibitors: 200 mM vanadate, 200 mM PMSF 100× and 10 mM leupeptin, 2.5 μg/ml aprotinin, 1 mg/ml pepstatin A 1000×; 20 μl buffer/1 mg wet tissue). After centrifugation (20 m, 6000 × *g*, 4 °C) supernatants were precipitated by ethanol. After a second centrifugation (5 min, 15,000 × *g*, 4 °C) precipitates were re-suspended in buffer (125 mM Tris–HCl, 4% SDS, 20% glycerol, 2% 2-merkaptoetanol, pH 6.8) and boiled at 95 °C for 5 m. Equal protein samples were separated in SDS-PAGE and then transferred into nitrocellulose membrane (Bio-Rad, Pure Nitrocellulose Membrane, 0.45 μm). Membranes were blocked for 1 h at room temperature in TBS-solution containing 5% non-fat milk and 0.05% TWEEN 20, then incubated for 2–4 h at 4 °C in blocking solution with the primary MMP-9 antibody (1:500, Torrey Pines Biolab, Houston, TX). Membranes were washed (4 × 10 m) in TBS-solution, containing 0.05% TWEEN 20, and incubated with HRP-conjugated donkey anti-rabbit IgG secondary antibody (DakoCytomation, Denmark; dilution 1:2000 in blocking buffer) at room temperature for 1 h. Protein bands were visualized using ECL chemiluminescence detection and medical X-ray films (ECL, PIERCE, Super Signal West Pico Chemiluminescent Substrate).

3. Results

3.1. Generalizing cortical epilepsy induced by 4-AP application: EEG and behaviour

Seizures were initiated focally by 4-AP application and then generalized into a secondary status epilepticus involving all cortical regions. At the focus the first paroxysmal events appeared at 15 min and they evolved into continuous spiking activity after 60 min. At this time, similar activity was detected at contralateral sites in parietal cortex, and seizures were generalized to all cortical regions at 3 h after 4-AP application (Fig. 1a). The generalized convulsive status epilepticus followed a similar progression to that described by Treiman et al. (1990) with the emergence at 6 h of a slow oscillation comparable to the periodic epileptiform discharge phase (Treiman et al., 1990).

At 6 h, spectral densities of EEG signals showed a peak in power close to 1 Hz (Fig. 1b), and large (300–350 μV) low-frequency (1–1.5 Hz) events predominated. On emergence from the anesthetic, all 4-AP treated animals exhibited severe epileptic seizures and that reached Stage 5 of the Racine scale.

Recovery from the status epilepticus occurred at 24–48 h after 4-AP treatment. Rat behaviour was normal, large, low-frequency events were absent from the EEG and the EEG power spectrum returned to its control form. At one week, no epileptiform activity was evident in EEG records and rat behaviour appeared to be normal. Tissue samples for measurements of MMP activity were prepared at a time point corresponding to the end of the 4-AP treatment ($t = 0$ h) and at 6 h and 24 h.

3.2. Thalamo-cortical epilepsy in WAG/Rij animals

Spike and wave discharges in WAG/Rij animals consisted of a spindle-like series of EEG waves at 8–10 Hz that were at least 2 times larger than control delta waves (Fig. 1c). Fig. 1d shows the variability in frequency of the occurrence of SWDs during prolonged EEG recordings made from 11:00 until 19:00 h that included a transition from light to darkness ($n = 4$ animals, 8 sessions per animal). In our animals, the lowest frequency of SWDs (6.32 ± 0.76/h) occurred between 11:00 h and 12:00 h and the highest frequency of SWDs occurred between 17:00 h and 18:00 h (17.46 ± 1.93/h, $p < 0.01$, $n = 3$). Tissue samples to compare MMP activity were prepared at time points corresponding to these periods of high and of low frequency of SWD occurrence.

3.3. The enzymatic activity of MMP-9 in the 4-AP model

Zymograms showed 2 bands of MMP-9 (gelatinase-B) enzymatic activity. A band of weight 92–100 kDa corresponds to the pro form (pro-MMP-9) and a band at 88 kDa represents active-MMP-9 (act-MMP-9), or perhaps a form intermediate between pro- and active-MMP-9 (Zhang et al., 1998). Since the intermediate form auto-catalytizes into the active form (Chakraborti et al., 2003; Page-McCaw et al., 2007) we will refer to the 88 kDa band as active-MMP-9. Our operating procedures might influence MMP activity, even distant from the site of 4-AP application. We tested this point in tissue from ipsi- and contralateral frontal cortex. A small increase in MMP-9 activity was detected at 6 h (Fig. 2d) in frontal cortex of sham operated rats as compared to rats that were not operated ($n = 2$). We therefore compared tissue from 4-AP treated animals to that from sham operated animals at each time point. We validated the 92–100 kDa MMP-9 band by Western blot analysis in the parietal cortex (Fig. 2e). MMP-2 (gelatinase-A) is evident as a band at ~65–72 kDa. We detected no changes in MMP-2 activity in any brain area.

Changes in enzymatic MMP-9 activity induced by 4-AP application were examined in samples of ipsilateral, I, and contralateral, C, parietal cortex (Fig. 2a), frontal cortex (Fig. 2b) and thalamus (Fig. 2c) at 6 h and 24 h. Enzymatic activity of MMP-9 in parietal cortex was also examined immediately after 4-AP application ceased ($t = 0$ h).

Changes in active and precursor forms of MMP-9 occurred most rapidly in the parietal cortex. At the $t = 0$ time point, 45 min after 4-AP application, the active form of MMP-9 increased by $161 \pm 5\%$ ($p < 0.05$, $n = 6$) in ipsilateral tissue and by $136 \pm 10\%$ contralaterally. At 6 h, pro-MMP-9 increased in ipsilateral parietal cortex by $211 \pm 8\%$ ($p < 0.05$, $n = 6$), while there was a significant decrease in active-MMP-9 by $18 \pm 2\%$ ($p < 0.01$, $n = 6$). At 6 h, there were no significant changes in active or pro-MMP-9 in tissue from any other site. In ipsilateral frontal cortex, the level of the pro-form was $149 \pm 21\%$ and that of the active form was $94 \pm 3\%$. Enzymatic activity of MMP-9 was unchanged in ipsilateral thalamus (pro-form: $100 \pm 7\%$, active form: $80 \pm 4\%$), or in the contralateral parietal cortex (pro: $142 \pm 23\%$; act: $79 \pm 5\%$), frontal cortex (pro: $91 \pm 8\%$, act: $105 \pm 10\%$) or thalamus (pro: $106 \pm 12\%$, act: $114 \pm 7\%$).

Enzymatic levels of both precursor and active-MMP-9 increased further at 24 h. In ipsilateral parietal cortex, near the site of 4-AP application, pro-MMP-9 increased to $2240 \pm 488\%$ ($p < 0.05$, $n = 6$) of control values. Significant, but smaller, increases occurred at sites recruited during seizure generalization. Pro-MMP-9 increased by $158 \pm 27\%$ in contralateral parietal cortex and by similar values in frontal cortex (I: $263 \pm 30\%$, $p < 0.05$, $n = 5$; C: $181 \pm 16\%$, $p < 0.05$, $n = 5$) and thalamus (I: $155 \pm 6\%$, $p < 0.05$, $n = 5$; C: $128 \pm 14\%$). Enzymatic levels of active-MMP-9 increased comparably in parietal cortex (I: $292 \pm 61\%$; C: $309 \pm 29\%$, $p < 0.05$, $n = 6$), frontal cortex (I: $332 \pm 21\%$, $p < 0.05$, $n = 5$; C: $226 \pm 45\%$) and thalamus

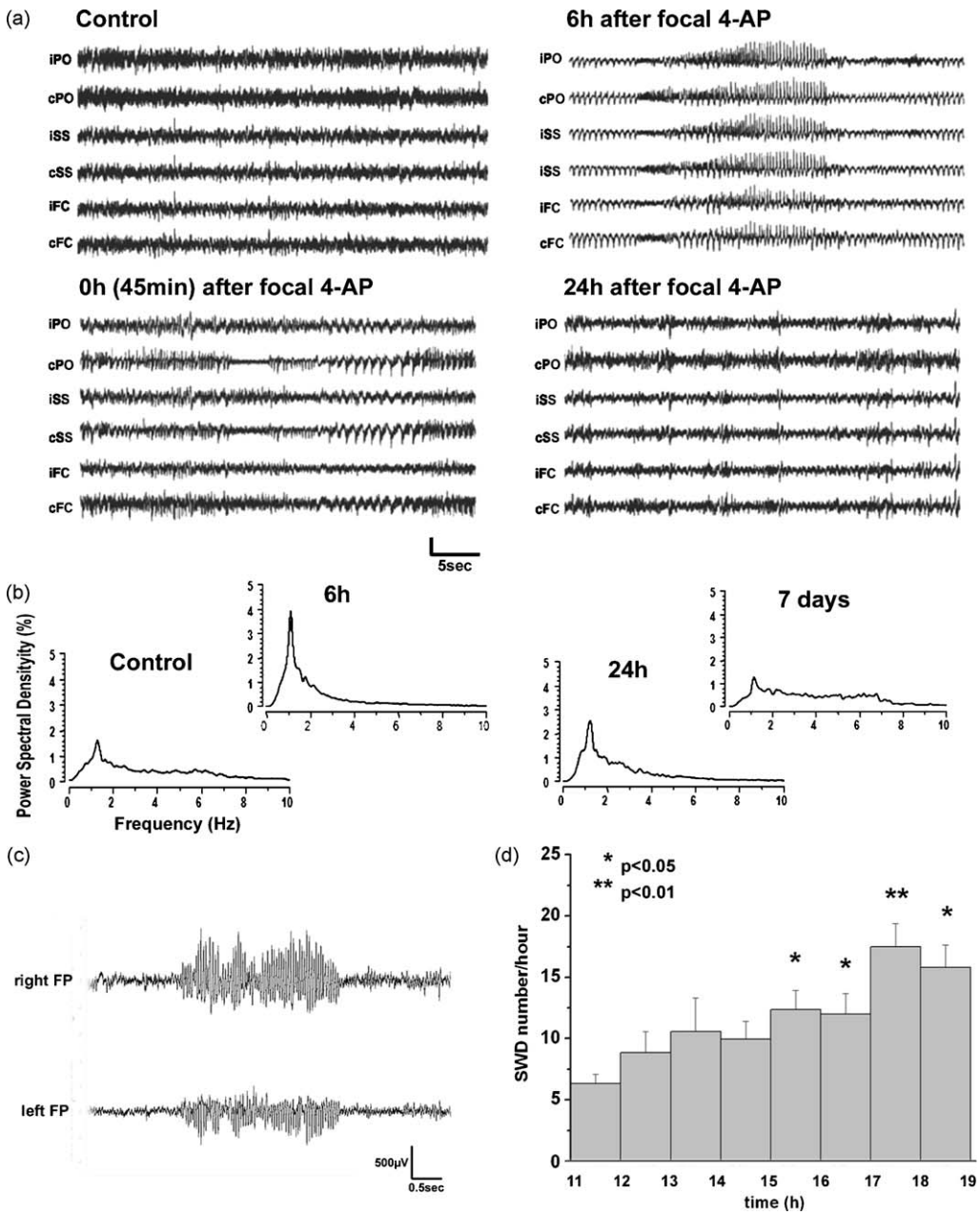

Fig. 1. Electrical signs of seizure activity in 4-AP treated and WAG/Rij rats. EEG records (a) and power spectrum analysis (b) after focal, cortical 4-AP treatment. Control traces and records made at the end of 4-AP application ($t = 0$) and at 6 h and 24 h later, showing the emergence and recovery from generalized epileptic seizures. Ictal and interictal periods alternate at 0 h, and seizures are generalized to all cortical regions at 6 h. At 24 h, large amplitude, low-frequency spikes associated with the generalized status epilepticus had disappeared from the EEG. Amplitude calibrations: 50 μV in control and 250 μV for records at 0, 6 and 24 h. Locations of EEG electrodes: cFC, contralateral frontal cortex; iFC, ipsilateral frontal cortex; cSS, contralateral somatosensory cortex; iSS, ipsilateral somatosensory cortex; cPO, contralateral parieto-occipital cortex; iPO, ipsilateral parieto-occipital cortex. (b) Power spectra (PSD) from control EEG records and at 6 h, 24 h and 10 days were derived from EEG records of duration 60 min. At 6 h a novel peak was detected at 1 Hz. This peak was much reduced at 24 h as the PSD shape returned to control. (c) In the gentically epileptic WAG/Rij rats we detected the tipical form of spike and wave discharges. (d) The number of SWDs per hour in the WAG/Rij animals used in this study was at a minimum at 11:00–12:00 h and increased at 17:00–18:00 h as the rats make the transition from sleep to waking.

(I: $287 \pm 11\%$, $p < 0.05$, $n = 5$; C: $148 \pm 16\%$). These data thus demonstrate significant, delayed increases in both active and precursor forms of the proteinase MMP-9, but not MMP-2, at sites of generalized seizure activity induced by focal 4-AP application.

3.4. Enzymatic activity of MMP-9 and MMP-2 in young and adult WAG/Rij rats

Genetically selected WAG/Rij rats generate absence-like epileptiform activity after reaching the adult stage. We therefore compared enzymatic levels of the gelatinases MMP-2 and MMP-9 in young (6 weeks) and adult (6 months) animals of this strain (Fig. 3a) and also with Sprague–Dawley rats which show no spontaneous absence-like behaviour (Fig. 3c). Levels of both gelatinases were assayed in the thalamus and parietal cortex which contribute to spike-and wave discharges and also in hippocampus and frontal cortex.

The enzymatic activity of MMP-2 was significantly lower in adults than in young animals in parietal cortex ($58 \pm 7\%$, $p < 0.01$, $n = 4$), frontal cortex ($44 \pm 8\%$, $p < 0.01$, $n = 4$) and thalamus ($50 \pm 6\%$,

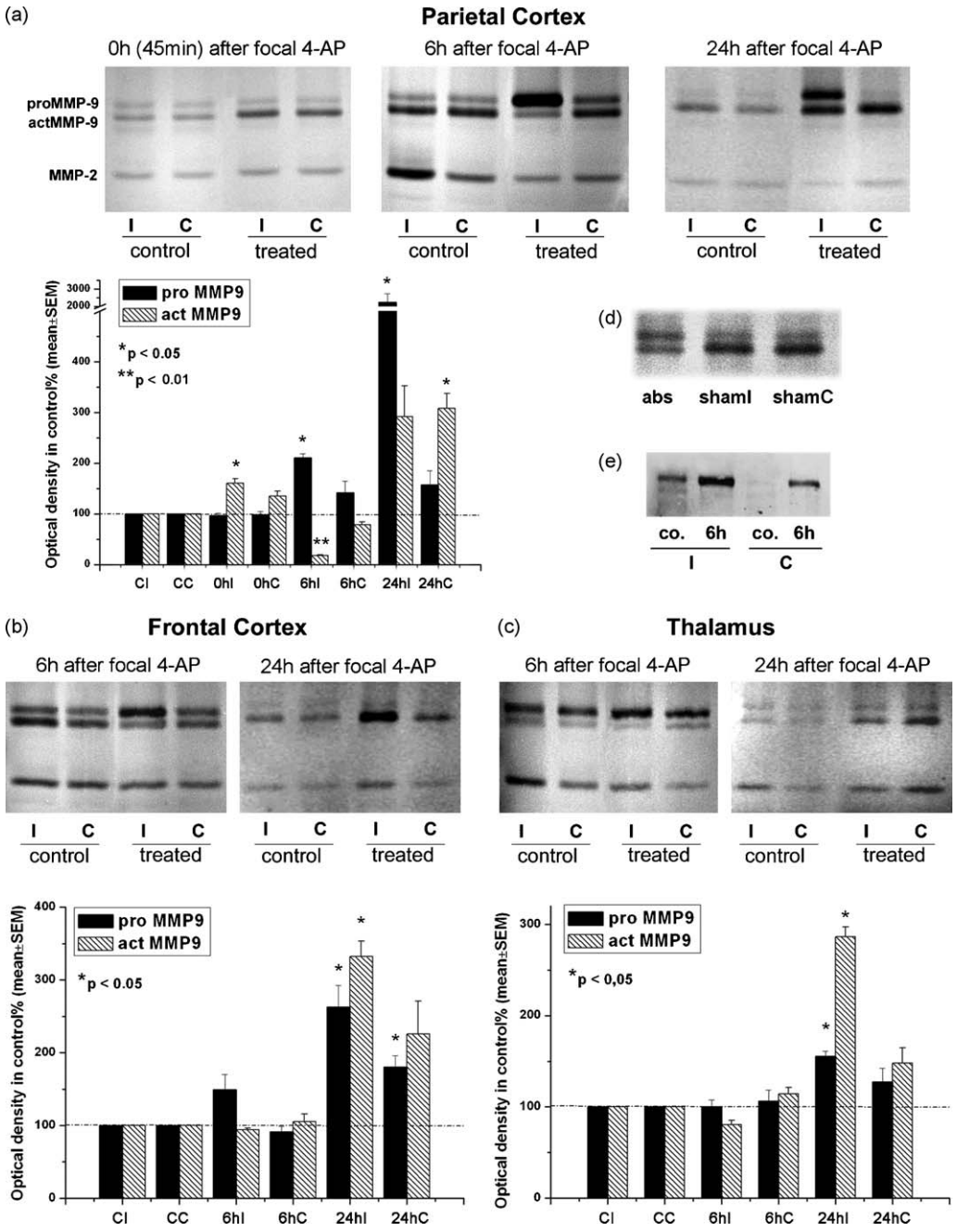

Fig. 2. Enzymatic activity of MMP-9 in 4-AP treated animals. (a–c) Gelatine zymography and densitometry of pro- and active-MMP-9 at ipsilateral, I, and contralateral, C, sites of parietal cortex (a), frontal cortex (b) and thalamus (c). Enzymatic activity was measured at the termination of focal 4-AP application (t = 0 h) and at 6 h and 24 h. Gelatinase levels are expressed as percentage of sham operated controls. Pro-MMP-9 activity increased by more than 20 times in ipsilateral parietal cortex at 24 h (a). (d) The effect of the operating procedures on MMP activity in frontal cortex, distant from the site of 4-AP application. A small increase in MMP-9 enzymatic activity was detected at 6 h in sham operated rats, both sides (shamI and shamC), as compared to absolute control rats that were not operated (abs). (e) Western blot analysis from the parietal cortex confirmed the MMP-9 origin of the 92–100 kDa gelatinolytic band in controls (co.) and 4-AP treated (6 h) animals, both sides.

$p < 0.01$, $n = 4$), and slightly higher in hippocampus (117 ± 7%). The enzymatic activity of active-MMP-9 in adult rats was higher than in young animals in the thalamus (129 ± 4%, $p < 0.05$, $n = 4$) and frontal cortex (133 ± 13%) and was lower in the adult parietal cortex (51 ± 14%, $p < 0.05$, $n = 4$) and hippocampus (43 ± 5%, $p < 0.05$, $n = 4$). Enzymatic levels of pro-MMP-9 were significantly higher in adult than young animals in all brain regions. The largest difference was detected in the hippocampus (901 ± 236%, $p < 0.05$, $n = 4$), but pro-MMP-9 levels were also enhanced in thalamus (421 ± 20%, $p < 0.01$), parietal cortex (394 ± 90%, $p < 0.05$, $n = 4$) and frontal cortex (280 ± 60%, $p < 0.05$, $n = 4$).

3.5. The enzymatic activity of MMP-9 and MMP-2 in Sprague–Dawley rats

As in WAG/Rij animals, the enzymatic activity of MMP-2 was reduced in the adult (Fig. 3b). Levels in animals aged 6 months expressed in terms of levels measured in young animals (6 weeks) were significantly lower in the thalamus (29 ± 7%, $p < 0.01$, $n = 4$), frontal cortex (49 ± 11%, $p < 0.01$, $n = 4$), parietal cortex (56 ± 8%, $p < 0.01$, $n = 4$) and hippocampus (65 ± 8%, $p < 0.05$, $n = 4$). In contrast, pro-MMP-9 levels tended to be higher, although not significantly so, in adults than in young SPRD rats. Changes measured

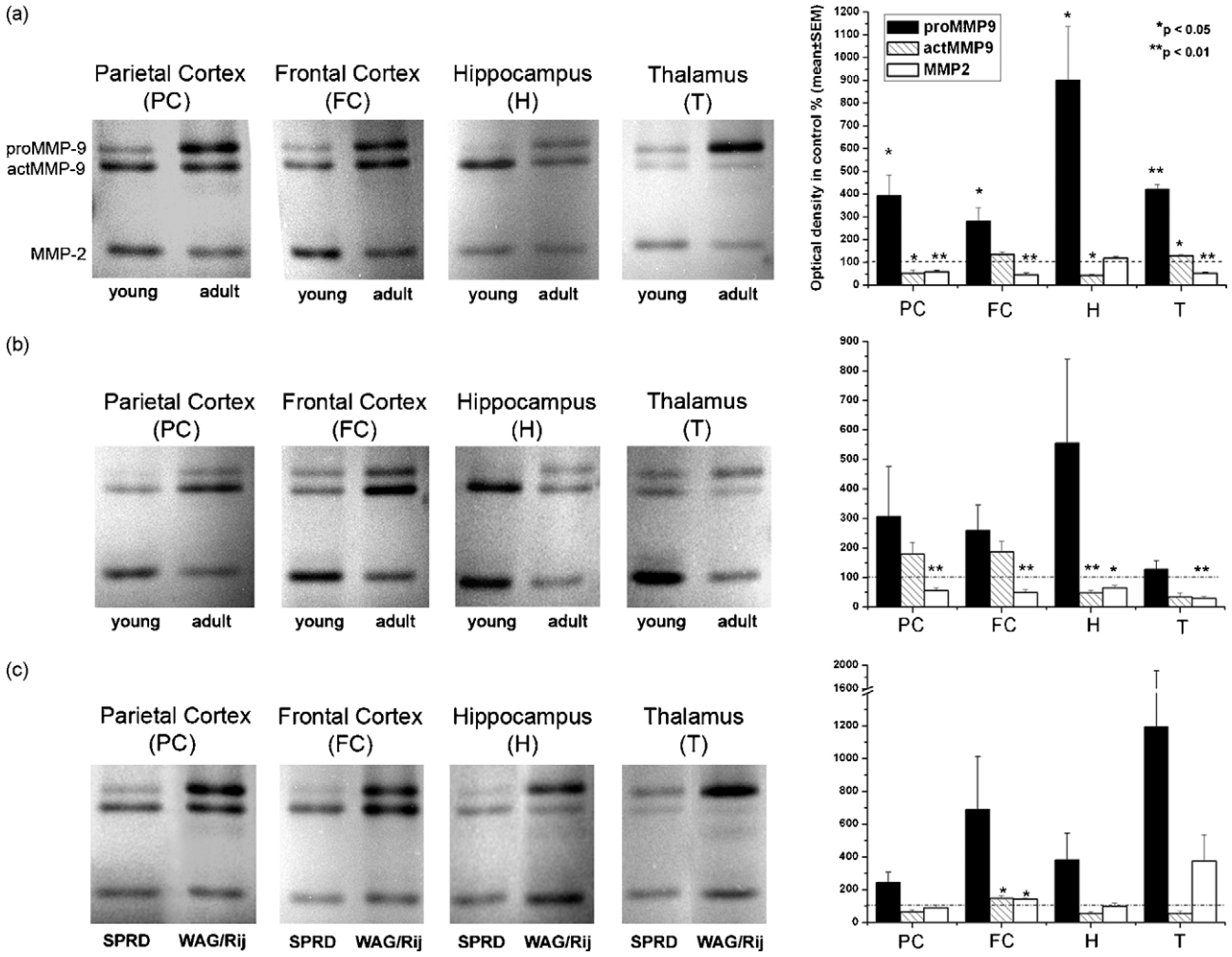

Fig. 3. Enzymatic levels of MMP-2 and MMP-9 in WAG/Rij and SPRD rats. (a and b) Gelatine zymography and densitometry for the two gelatinases in different brain areas of genetically epileptic WAG/Rij rats (a) at 6 weeks and 6 months and non-epileptic adult SPRD rats (b) at 6 weeks and 6 months. (c) Enzymatic levels of gelatinases in adult WAG/Rij rats were also compared to adult SPRD rats. Gelatinase levels are expressed as percentage of enzyme levels in young rats (a and b) or non-epileptic adult SPRD rats (c). Pro-MMP-9 activity increased by 3–9 times between the two ages in different brain areas of WAG/Rij animals, but only by 1–6 times in SPRD rats. The largest increase in pro-MMP-9 activity occurred in the hippocampus which was the site showing the most significant decrease in active-MMP-9. Enzymatic activity of MMP-2 decreased homogeneously in all brain areas of adult rats, except for the hippocampus of WAG/Rij animals.

for the different brain regions were: thalamus (128 ± 29%), frontal cortex (259 ± 87%), parietal cortex (307 ± 168%) and hippocampus (555 ± 285%). Alterations in enzymatic activity of the active form of MMP-9 did not show a consistent pattern. Measured changes were: thalamus (34 ± 13%) frontal cortex (187 ± 36%), parietal cortex (180 ± 39%) and hippocampus (47 ± 9%, $p < 0.01$, $n = 4$).

3.6. Comparison of enzymatic MMP levels in adult SPRD and WAG/Rij rats

We detected some differences in the enzymatic activity of MMP-2 in adult WAG/Rij rats and control SPRD animals (Fig. 3c). MMP-2 levels were higher in the thalamus (374 ± 161%) and frontal cortex (144 ± 6%, $p < 0.05$, $n = 4$) but essentially similar in the parietal cortex (91 ± 13%) and hippocampus (99 ± 18%).

In contrast, levels of pro-MMP-9 activity were consistently higher in adult WAG/Rij rats than in control SPRD rats (Fig. 3c). Differences were, in the thalamus (1195 ± 713%), in frontal cortex (689 ± 324%), in parietal cortex (244 ± 62%) and in the hippocampus (383 ± 161%). Enzymatic levels of active-MMP-9 however were mostly lower in WAG/Rij rats than in SPRD animals: thalamus (56 ± 14%), parietal cortex (64 ± 12%) and hippocampus (56 ± 10) although frontal cortex levels were higher (146 ± 14%, $p < 0.05$, $n = 4$).

Overall, these data reveal an age-dependent reduction in MMP-2 activity in both WAG/Rij and SPRD rats. In contrast pro-MMP-9 increased significantly between young WAG/Rij animals which did not and older animals which did generate absence-like seizures. Pro-MMP-9 was also elevated in adult WAG/Rij rats as compared to age-matched SPRD animals, but levels of active-MMP-9 were not generally higher in WAG/Rij animals.

3.7. The enzymatic activity of MMP-9 correlates with seizure genesis in the WAG/Rij rat

We next searched for dynamic changes in enzymatic MMP-9 activity correlated with the circadian variation in SWD frequency (Coenen et al., 1991; van Luijtelaar and Coenen, 1988). As shown in Fig. 1d, SWD frequency for WAG/Rij animals used in this study was at a minimum at 11:00–12:00 h and increased at 17:00–18:00 h as the rats make the transition from sleep to waking. We therefore compared the enzymatic activity of MMP-9 in tissue prepared at 12:00 h (used as control) and tissue obtained at 18:00 h (Fig. 4).

Analysis of the enzymatic activity of this tissue revealed an increase in active form of MMP-9 during periods of high SWD occurence in areas associated with SWD generation: in the thalamus (199 ± 19%, $p < 0.05$, $n = 4$) and frontal cortex

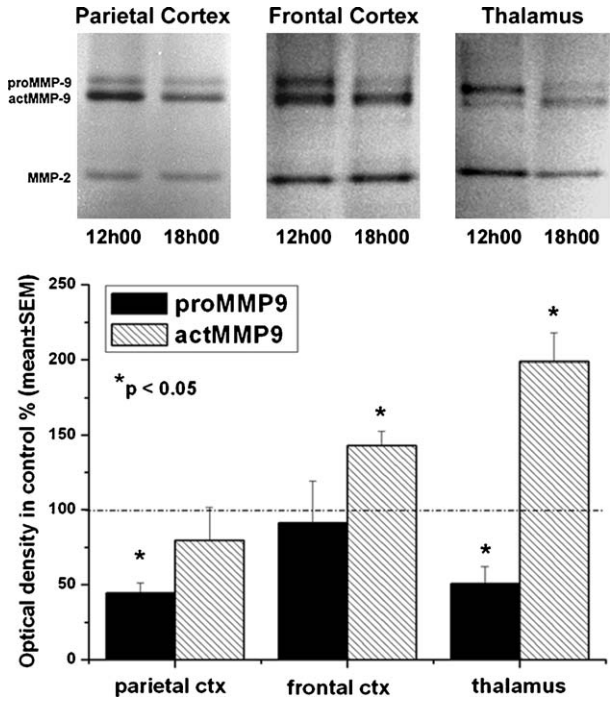

Fig. 4. Correlation of MMP-9 activity and seizure genesis in WAG/Rij rats. Gelatine zymography and densitometry comparing MMP-9 enzymatic activity in parietal cortex, frontal cortex and thalamus from tissue obtained at a period of low SWD frequency (12:00 h, used as control) and at a period of high SWD frequency (18:00 h). Active-MMP-9 increased significantly in frontal cortex and thalamus. Pro-MMP-9 decreased in parietal cortex and thalamus.

($143 \pm 10\%$, $p < 0.05$, $n = 4$), but no change in the parietal cortex (80 ± 22). In contrast, pro-MMP-9 activity was decreased in the thalamus ($51 \pm 11\%$, $p < 0.05$, $n = 4$) and parietal cortex ($44 \pm 7\%$, $p < 0.05$, $n = 4$) but unchanged in frontal cortex ($91 \pm 28\%$). These results therefore suggest that changes in enzymatic actions of the active form of MMP-9 may vary with seizure frequency in areas involved in seizure generation.

4. Discussion

Data presented here suggest in two different animal models of epilepsy that synchronized cortical seizures alone suffice to enhance both the precursor form and the active form of MMP-9. We detected no comparable increase in enzymatic activity of MMP-2. The first epilepsy model consisted of generalizing cortical seizures induced by focal application of the convulsant 4-AP. The second was the genetic absence seizure animal WAG/Rij. In these animals MMP-9 activity was considerably enhanced during the age-range at which thalamo-cortical absence-like seizures were first generated. Furthermore, MMP-9 activity showed a diurnal increase in areas involved in seizure generation that was correlated with a time period at the transition from sleep to wakefulness when seizure activity is elevated.

4.1. Neuronal death and MMP enzymatic activity

MMP-9 mRNA is significantly upregulated after kainate injection induced *status epilepticus* that lead to neuronal death in the hippocampal CA1 region and the hilus (Konopacki et al., 2007; Szklarczyk et al., 2002). In contrast, bicuculline, the $GABA_A$ antagonist, induces epileptiform discharges accompanied by little or no neuronal loss (Ben-Ari et al., 1981; Zhang et al., 1998) while MMP-9 levels are increased at 12 h (Zhang et al., 1998).

No cell death is associated with the emergence of thalamo-cortical seizures which occur in WAG/Rij rats aged about 4 months (Coenen and van Luijtelaar, 2003; Meeren et al., 2005, 2002) and which we show are associated with an increase in MMP-9 activity. Furthermore we showed that enzymatic activity of both precursor and active forms of MMP-9 was enhanced in the contralateral parietal cortex, frontal cortex and thalamus, regions of secondary generalization of cortical seizures induced by focal 4-AP application. Our previous data revealed no detectable neuronal loss in these regions (Baracskay et al., 2008; Gallyas et al., 2008).

Epileptiform activity induced by 4-AP is not associated with extensive neuronal damage (Pena and Tapia, 1999; Pena and Tapia, 2000; Slezia et al., 2004). We chose to apply 4-AP by placing a crystal on the cortical surface. In this way we avoided tissue damage due to a chronic cannula as well as possible side-effects of a vehicle solution. The absence of these factors may have improved our study, since tissue damage and the ensuing inflammation activate MMPs (Rosenberg, 2002; Rosenberg and Mun-Bryce, 2004; Candelario-Jalil et al., 2009)

We chose to apply 4-AP under halothane, since this anesthetic limits neuronal death as well as acute seizure spread (Baracskay et al., 2008; Walker et al., 1999). We detected only sporadic "dark" neurons at distant sites up to 6 h after 4-AP application using the Gallyas silver stain (Gallyas et al., 1993) which marks cells with a cytoskeletal collapse associated with neuronal compaction (Baracskay et al., 2008). Detailed light- and electron-microscopic analysis of tissue obtained at 1–3 days suggests that "dark" neurons in the intermediate area of the lesion due to 4-AP recover and do not die (Attilio et al., 1983; Gallyas et al., 2008). The hypothesis that neurons stained by the Gallyas procedure can recover, is supported by work showing that 99% of compacted granule cells of the hippocampal dentate region regain their normal volume within a few hours of injury (Csordás et al., 2003).

Our data suggests that MMP-9 enzymatic activity is enhanced during this period of neuronal recovery (Baracskay et al., 2008; Gallyas et al., 2008). If so, MMP-9 activation at sites distant from 4-AP application may be associated with the abnormal neuronal discharges corresponding to the epileptiform activity, rather than processes associated with cell death. Even so, MMP-9 enzymatic activity was maximal near the application site in parietal cortex, where some neuronal necrosis does occur (Gallyas et al., 2008) so we cannot completely exclude a contribution from cell death.

Changes in MMP-2 were considerably less than those detected in MMP-9 as in previous work on epilepsy (Jourquin et al., 2003; Szklarczyk et al., 2002; Wilczynski et al., 2008; Zhang et al., 1998) and in studies on the effects of light stimulation in rat retina (Papp et al., 2007).

4.2. Developmental changes in gelatinase activity

Since both MMP-2 and MMP-9 contribute to developmental processes (Vu and Werb, 2000), their enzymatic activity could be expected to be reduced in adult animals. While the enzymatic activity of MMP-2 was largely reduced in the adult, we detected an increase in the pro-MMP-9 pool in the adult for both SPRD and WAG/Rij rats. Possibly the adult brain uses less of the inducible pro-MMP-9 pool than the young brain.

Baseline levels of pro-MMP-9 increased considerably in hippocampus, both cortical regions examined and the thalamus between 6 weeks and 6 months, in both SPRD and WAG/Rij rats (Fig. 3a and b). This increase was largest in the hippocampus and we assume that it results from developmental mechanisms. However comparison of adult levels of MMP-9 in WAG/Rij and Sprague–Dawley animals (Fig. 3c) revealed much larger differences in the thalamus and frontal cortex which are involved in SWD generation (Meeren et al., 2002, 2005) than in the hippocampus.

4.3. Pro-MMP-9 enzymatic activity is enhanced in seizure-generating adult WAG/Rij rats

Different spatial patterns of changes in pro- and active forms of MMP-9 were detected in response to the thalamo-cortical seizures (van Luijtelaar and Coenen, 1988) of WAG/Rij rats and the generalized cortical seizures induced by focal 4-AP application (Barna et al., 1999; Medina-Ceja et al., 2000; Szente and Pongracz, 1981). We found consistently higher levels of pro-MMP-9 in adult WAG/Rij animals, especially in the thalamus, than in adult SPRD rats. An enhanced pool of pro-MMP-9 in WAG/Rij rats could provide a rapidly available pool of MMP-9 in one of the generator areas for thalamo-cortical seizures. Increased MMP-9 activation from its pro-form during periods of higher seizure frequency might result directly from neuronal depolarization, although we note that significant increases in MMP-9 after 4-AP induced seizure activity occurred with a rather longer latency.

We have provided data on changes in both the pro- and the active form of MMP-9. Previous work suggests that the constitutive pool of pro-MMP-9 may be rapidly transformed into the active form of the proteinase (Szklarczyk et al., 2002). Further the pool of pro-MMP-9 seems likely to be dynamically replenished by gene transcription induced by a seizure (Konopacki et al., 2007; Szklarczyk et al., 2002). We note that the pro-MMP-9 level remained increased at 24 h after seizures induced by 4-AP in SPRD rats and was consistently elevated in WAG/Rij rats at ages after the onset of genetic absence seizures. Conceivably an increased availability of MMP-9 may be functionally important after a seizure.

The activation of MMP-9 varied between cortical regions in a way potentially consistent with a differential participation in absence seizure activity. While SWDs are recorded from widespread cortical and thalamic regions (Meeren et al., 2002; Midzianovskaia et al., 2001; van Luijtelaar and Coenen, 1988), they appear to emerge from facial somatosensory cortex and in general, frontal regions show more, sometimes larger SWDs, than do parietal regions (Polack et al., 2007; Meeren et al., 2002). A correlation between elevated pro-MMP-9 levels in WAG/Rij rats and regions of SWD generation could explain our data showing different levels of enzymatic activation in frontal and parietal cortex.

This correlation might be verified by testing changes in MMP-9 levels in animals treated with the anti-absence drug ethosuximide (ETX) which suppresses SWDs in WAG/Rij rats (Blumenfeld et al., 2008). However long-term ETX administration interferes with sleep pattern organization (Declerck and Wauquier, 1991; Gören and Onat, 2007) which have been shown in turn to decrease MMP-9 mRNA levels in the hippocampus (Taishi et al., 2001).

4.4. Dynamics of MMP-9 activation and synthesis

In the 4-AP model MMP-9 was activated rapidly, at 45 m, after application in the parietal cortex corresponding to the seizure focus. It seems probable that this ipsi- and contralateral elevation resulted from activating proteolysis of existing pro-MMP-9 pools rather than protein synthesis. Synthesis may also have contributed to the delayed increase, at 6 h and 24 h, in MMP-9 levels detected in distant areas. We note that the delayed involvement of epileptiform activity as well as potentially distinct mechanisms of MMP-9 regulation may contribute to the delayed upregulation of the zymogen in the thalamus and frontal cortex.

We noted interesting reciprocal changes in the pro- and active forms of MMP-9 in parietal cortex at 6 h after 4-AP application followed by considerable increases in both forms at 24 h. The reduction followed by rebound in the active form may depend on multiple factors that control the conversion of pro- to active-MMP-9 or the level of active-MMP-9. For instance, increased proteolysis or the formation of macro-molecular complexes between MMP-9 and its substrates or inhibitory factors such as TIMP-1 (Tissue Inhibitor of Matrix Metalloproteases) would reduce enzymatic activity detected on zymograms (Chakraborti et al., 2003; Dzwonek et al., 2004; Jaworski, 2000; Rivera et al., 1997).

While elevations of intracellular Ca^{2+} during epileptiform activity seem inevitable and Ca^{2+} entry alone induces MMP-9 release (Huang et al., 2006) our results showed also an enhanced synthesis and prolonged accumulation of the precursor form of MMP-9. These longer term elevations of the pro-form would tend to produce increased levels of functional MMP-9. Furthermore the delayed induction of MMP-9 in pre-frontal cortex and thalamus seems unlikely to depend on immediate increases in neuronal Ca^{2+} levels.

We detected both an activation and an increased synthesis of MMP-9 during and after seizures. It seems likely that these processes are independently controlled and our data do not discriminate between the relative roles of epileptiform spiking or of seizure-induced plasticity. Possibly the most important activators of MMP-9 are Ser-Proteases including tPA/plasmin and thrombin, which are quickly upregulated during KA induced seizures (Qian et al., 1993; Endo et al., 1999) Synthesis of MMP-9 could proceed via activation of neuronal intermediate early genes (Zagulska-Szymczak et al., 2001) or indirectly via proinflammatory cytokines such as interleukin-1 β (IL-1β) and tumor necrosis factor-α (TNF-α) which are liberated from glial cells (Chakraborti et al., 2003; Młodzikowska-Albrecht et al., 2007; Vezzani et al., 2008; Vezzani, 2008) and which activate transcription in neurons. In particular cytokines may activate MMP-9 transcription via the MAPK/Erk pathway (Chakraborti et al., 2003; Nagase and Woessner, 1999; Wu et al., 2009)

4.5. Possible homeostatic role of MMP-9 during seizures that do not induce cell death

Our data from these two epilepsy models suggests that MMP-9 elevation results from elevated neuronal activity since neuronal death does not occur in the WAG/Rij model of absence seizures and since we detected no neuronal death in zones of propagation of seizures induced by 4-AP. Sustained depolarization induces both pathological and protective changes (Greene et al., 2007; Holopainen, 2008; Ryu et al., 2007). The extracellular proteolysis mediated by MMPs may contribute to both types of process. Consistently elevated pro-MMP-9 levels should enhance proteolysis of extracellular matrix components and other extracellular proteins. Proteolytic control of peri-neuronal structures could provide a rapid external pathway to achieve homeostatic changes in synaptic efficacy. Similarly MMP-9 induction following neuronal depolarization may provide a feedback control of cellular excitability. MMP-9 is also activated during LTP (Nagy et al., 2006; Okulski et al., 2007; Wang et al., 2008) and retinal light adaptation (Papp et al., 2007).

Recent ideas on homeostatic synaptic plasticity suggest that the nervous system initiates plastic changes that tend to counteract any alteration in neuronal excitability (Turrigiano and Nelson, 2004; Turrigiano, 2008; Zahn et al., 2008). Seizures correspond to major increase in neuronal activity. How might MMPs contribute to restore lower levels of activity? It seems unlikely that MMPs affect synaptic connectivity during the 24 h period corresponding to our measurements. Furthermore, neither epilepsy model is associated with those plastic processes like aberrant synaptogenesis or axonal sprouting (Chi et al., 2008) or structural effects on dendritic spine density (Ouyang et al., 2007). Even more severe seizures induced in non-anesthetized mice by 4-AP lead only to moderate loss of spines and dendritic swelling (Rensing et al., 2005).

MMP-9 may participate in such a protective homeostatic plasticity via its extracellular substrates which include the integrins (Gall and Lynch, 2004; Nagase and Woessner, 1999; Nagy et al., 2006), cadherins (Steinhusen et al., 2001) and β-dystroglycan (Kaczmarek et al., 2002; Michaluk et al., 2007; Yamada et al., 2001). Such pathways exert structural effects at the synapse by controlling spine shape (Wilczynski et al., 2008), via β-dystroglycan (Michaluk et al., 2007) or ICAM-5 (Tian et al., 2007). These changes may be depend on an activity-dependent dendritic transport of MMP-9 mRNA that is stimulated after kainate administration (Konopacki et al., 2007). A reversible spine loss after seizures would reduce neuronal excitability and aid recovery from injury (Muller et al., 1993). The finding that MMP-9 deficiency reduces seizure-evoked pruning of dendritic spines in adult MMP-9 KO mice can also reinforce this idea (Wilczynski et al., 2008).

Spine pruning has not been demonstrated in absence epilepsies, however elevated pro-MMP-9 levels may help to explain the lower efficacy of kainate in producing secondary generalized seizures in these rats (Gurbanova et al., 2008) as well as their resistance to kindling (Akman et al., 2008; Eşkazan et al., 2002; Onat et al., 2007). The reduction in ictogenic potential of 4-AP observed in kainate-treated chronic epileptic rats may also suggests that homeostatic plasticity has been initiated after seizures (Zahn et al., 2008).

MMP-9 also influences synaptic signaling by acting directly on both NMDA (Michaluk et al., 2009) and AMPA subtypes of glutamate receptor (Gawlak et al., 2009). These changes at ionotropic glutamate receptors would act homeostatically to reduce the efficacy of glutamatergic synapses after multiple 4-AP induced seizures (Világi et al., 2009; Borbély et al., 2009).

Further studies are needed to specify the functions of elevated MMP-9 enzymatic activity in these two models, but they should be valuable in studies on homeostatic functions of MMP-9 activity in the epileptic brain.

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