Acta Neurol. Belg., 2009, **109**, 298-304

Mossy fiber sprouting, hippocampal damage and spontaneous recurrent seizures in pentylenetetrazole kindling rat model

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Abstract

Aim: The aim of this study was to determine the correlations among hippocampal damage, spontaneous recurrent seizures (SRS), and mossy fiber sprouting (MFS) using pentylenetetrazole (PTZ) kindling model.

Methods: Chronic epileptic model was established by administration of PTZ. Behaviour and EEG seizure activity were recorded. Rats' hippocampus were analyzed with haematoxylin and eosin (H&E) stain for histological lesions and evaluated for MFS with Timm stain.

Results: Prominent MFS was observed in area CA3 rather than the inner molecular layer in PTZ treated rats and the degree of MFS progressed with the development of behavioral kindled seizures. MFS preceded the occurrence of spontaneous seizures. No obvious neuronal necrosis and loss were observed in different regions of the hippocampus during kindling progression.

Conclusion: MFS is not the outcome of SRS. Severe hippocampal damage is not required in the development of MFS and SRS.

Key words: Temporal lobe epilepsy; mossy fiber sprouting; pentylenetetrazole; spontaneous recurrent seizures; hippocampus.

1. Introduction

Mossy fiber sprouting (MFS) is a pathological phenomenon commonly observed in both animal models of temporal lobe epilepsy (TLE) (Bausch, 2006) and brain sections of epileptic patients (Andrade-Valença *et al.*, 2008). The sprouted mossy fibers could either abnormally innervate the dentate inner molecular layer (IML) (Buckmaster *et al.*, 2002), or form new synapses with apical dendrites of CA3 pyramidal cells (Muramatsu *et al.*, 2008). In spite of the consensus that kindled seizures can induce MFS, the relationship between this neuropathology and spontaneous recurrent seizures (SRS) remains unclear. Some studies showed that the degree of aberrant MFS correlated with SRS in kainate models of TLE (Nadler, 2003). Some demonstrated that MFS was not necessarily related to the onset and progression of SRS (Nissinen *et al.*, 2001; Gorter *et al.*, 2001). Some even claimed that MFS was neither the cause nor the outcome of SRS (Longo and Mello, 1999). Another pathological marker of TLE is hippocampal sclerosis which is characterized by extensive neuronal loss in the dentate hilus, the CA1 and CA3 subfields (Morimoto *et al.*, 2004). The correlations among the extent of hippocampal damage, the frequency of SRS, and the degree of MFS in TLE are also under debate due to variable findings in different animal models and human studies. A study by Homles *et al.* (1999) showed prominent sprouting in area CA3 in the absence of significant decrease in CA3, CA1 and hilar neuron numbers. While another study showed that rats with widespread hippocampal injury exhibited more robust MFS than rats with moderate hippocampal injury (Rao *et al.*, 2006). An earlier study demonstrated an association between seizure progression and the extent of hilar neuron loss (Gorter *et al.*, 2001), although studies in another kindling model and TLE patients failed to find a clear association between the occurrence of SRS and the severity of hilar cell death (Thom *et al.*, 2005). The obscurity of their correlations is partly due to lack of knowledge of their alterations during epileptogenesis. Here we addressed the question by observing the degree of MFS, the occurrence of SRS and the extent of hippocampal damage at different time points of kindling. Kindling has been widely adopted

as a model of synaptic rearrangement and neuronal plasticity. Pilocarpine or kainic acid induced status epilepticus (SE) is responsible for high mortality in these two models. On the other hand, PTZ, a widely used convulsant, could induce sprouting of the hippocampal mossy fibers during kindling progression (Homles *et al.*, 1999; Tian FF *et al.*, 2008) with lower experimental animal mortality. Therefore we used PTZ as the convulsant agent in the present study.

2. Material and methods

2.1. Animals grouping and kindling

120 adult male Sprague-Dawley (SD) rats (Central South University, China) weighing 180~220 g were equally divided into control and PTZ group, each containing five subgroups of 12 rats. Rats of the PTZ group received a dose of 30 mg/kg PTZ intraperitoneally, once per day till rats were kindled or sacrificed, while the control rats were injected with an equal dose of saline. Rats were considered kindled when seizure attack (score ≥ 3) occurred after each PTZ injection for five consecutive days. The PTZ treated rats met the kindling criterion after an average of 26.1 ± 1.6 days PTZ injection. At time points 3 days, 1 week, 2 weeks, 4 weeks and 6 weeks, respectively, after the first injection, rats of the five subgroups were perfused for the following histology: a. Timm staining and scoring; b. haematoxylin and eosin (H&E) staining and scoring.

2.2. Behaviour and EEG monitoring

Rats were observed for at least 2 h/day by an investigator for the occurrence of PTZ-induced seizures before kindled. Kindled rats were monitored for the occurrence of SRS 24 h/day, using a video camera that was positioned above the cages, till they were sacrificed at aforementioned time points. The convulsive behaviour was classified in the following stages, modified to that proposed by Racine (Pavlova *et al.*, 2006): 0 – no behavioral changes; 1 – facial movements, ear and whisker twitching; 2 – myoclonic convulsions without rearing; 3 – myoclonic convulsions with rearing; 4 – clonic convulsion with loss of posture; 5 – generalized clonicotonic seizures. Each rat received an implantation of screws for a 20 min EEG recording right before perfusion.Using 10% chloral hydrate anesthesia, we surgically implanted three stainless steel screws into rats' skulls. Two screws on either side of the midline anterior to the coronal sutures served as active electrodes, and a screw anterior to the lambdoidal suture to the right of midline was used as a ground electrode. A Video/EEG monitoring system (Nuocheng Instruments, China) was used to record EEG from each animal.

2.3. Timm staining

At 3 days, 1 week, 2 weeks, 4 weeks and 6 weeks, respectively, after the first injection, rats were deeply anaesthetized with 10% chloral hydrate and perfused intracardially with 150 ml of saline, followed by 200 ml of 0.1 mol/L phosphate buffer (pH7.2~7.6) containing 0.4% sodium sulfide and 250 ml of 4% paraformaldehyde at 4°C. The brains were removed and put in 4% paraformaldehyde to fix for 24 h, then transferred into 0.1 mol/L phosphate buffer containing 30% sucrose, embedded by optimum cutting temperature compound after sinking, at last cut at a vibratome into 25 μm coronal sections. The microscope slides with brain slices were put in Timm developer for 60~80 min at 26°C in the dark. Then they were taken out for a 5 min wash in water to terminate the reaction. After that, the slides were dehydrated, cleaned and mounted with gum routinely. The distribution of Timm granules in the IML and stratum oriens of CA3 was rated on a scale of 0 to 5 according to published criteria (Cavazos *et al.*, 1991; Holmes *et al.*, 1999).

2.4. H&E staining and evaluation

Rats were anaesthetized with 10% chloral hydrate by intraperitoneal injection, then perfused intracardially with saline and 4% paraformaldehyde successively. The brains were removed and post-fixed. Thereafter, tissue was sliced, routinely processed, and embedded in paraffin wax. 5 μm coronal paraffin sections were cut, mounted and stained by haematoxylin and eosin. The acidophilic neuron, identified by intense cytoplasmic eosinophilia accompanied by chromatin dispersion with loss of nuclear membrane integrity (Edwards *et al.*, 1995), was perceived as the marker for irreversible neuronal damage at the cellular level. The numbers of acidophilic neurons in different regions of the hippocampus were estimated on a 0-3 grading scale, 0 = none, 0.5 = slight (< 10%), 1.0 = mild (10-25%), 1.5 = mild-to-moderate (26-45%), 2.0 = moderate (46-54%), 2.5 = moderate-to-severe (55-75%), and 3.0 = severe (> 75%), as previously published (Fujikawa *et al.*, 1999; Fujikawa *et al.*, 2000). Boundaries of each region were shown in Fig. 1. Neuronal apoptosis cannot be ruled out in our study. Sections

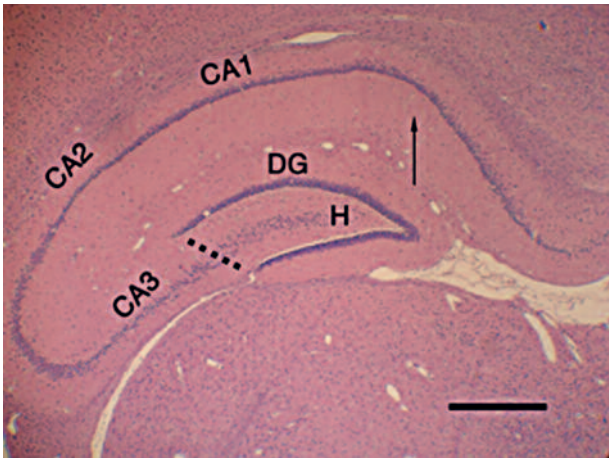
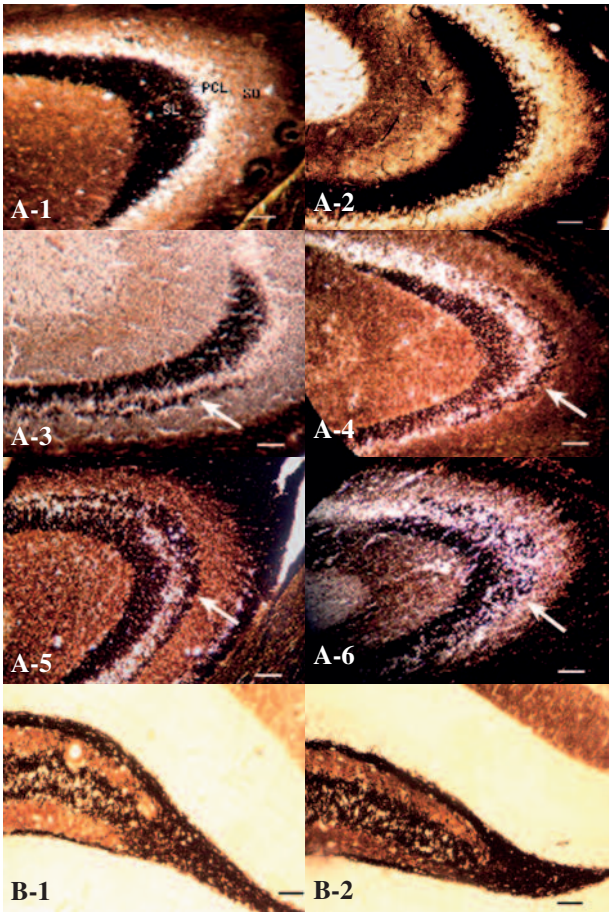


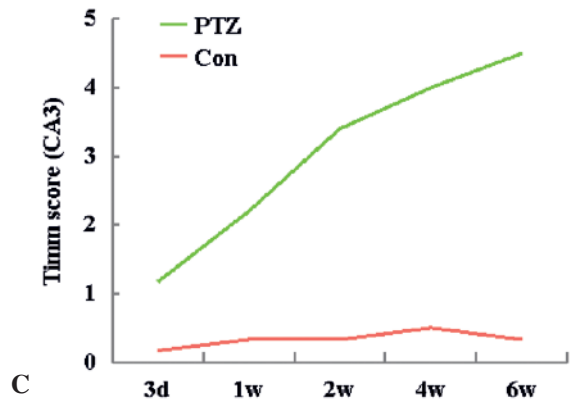
FIG. 1. — Example of a hippocampal region where extent of hippocampal injury was evaluated. CA3 region begins at the region bisected by an imaginary line connecting the two edges of the dentate granule cells to the CA2 region. CA1 extended from the CA2 region to an imaginary line drawn perpendicular to the crest of the dentate gyrus (DG; arrow). The hilus (H) was defined as the inner border of the granule cell layer (GCL) together with the area formed by two imaginary straight lines connecting the two tips of the GCL with the proximal end of the CA3c area. Scale bar = 100 μm.

were examined by a blinded investigator without knowledge of any other data on that animal.

2.5. STATISTICAL ANALYSIS

Since Timm score and H&E score are both rank material which do not follow Gaussian distribution, nonparametric test was used in our study. $P < 0.05$ was considered as statistically significant. All statistical analysis were two sided and were performed using Statistical Package for the Social Sciences (SPSS) version 16.0.





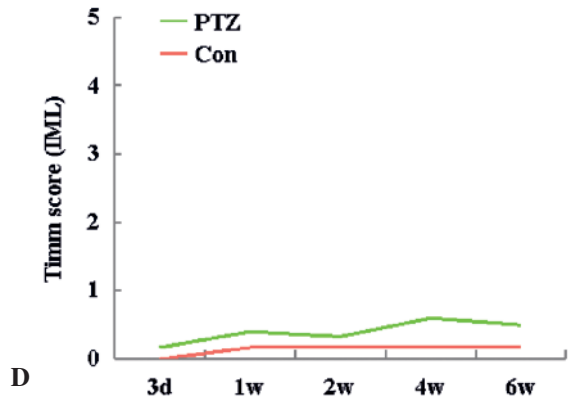


FIG. 2. — Changes of MFS in the hippocampus of PTZ kindling rats. The intensity of Timm granules increased with the development of kindling in area CA3 in the PTZ group (A, arrow). However, no obvious MFS was observed in the IML in both the control and PTZ group (B). C, D show the time-dependent-changes of Timm score in area CA3 and IML of the hippocampus respectively. There was significant difference in Timm score in area CA3 at every time point between the PTZ and control group ($P < 0.05$). Timm score in the IML in the PTZ group did not differ from that of the control at any time point (SL, stratum lucidum; PCL, pyramidal cell layer; SO, stratum oriens; IML, inner molecular layer; Scale bars = 100 μm).

3. Results

3.1. Behavioral and electrographical seizure activity

With the exception of a rat died at 1 week of persistent generalized tonic-clonic seizure and another one at 4 weeks died of SE after being kindled, the remaining of the PTZ treated group developed seizure activity of different degrees after continuous PTZ injections for 18~22 days. The seizure activity usually occurred 5~10 min after injection with duration of 5~30 min. SRS at grade 2-3 and typical epileptic waves in EEG records were detected in kindled rats. On the other hand, no epileptiform activity was observed either electrographically or behaviorally in the control rats.

3.2. Timm scores

There was significant difference in Timm scores in area CA3 at every time point between the PTZ group and control group ($P < 0.05$) (Fig. 1A, C). In addition, the degree of MFS in area CA3 progressed with the evolution of behavioral kindled seizures and reached the peak at 6 weeks. On the other hand, Timm scores in the IML were 0~1 throughout the experiment in the PTZ group, without significant difference from the control ($P > 0.05$) (Fig. 1B, D). There was no significant difference in Timm scores in area CA3 and the IML among each time point within the control rats ($P > 0.05$).

3.3. H&E scores

As opposed to kainic acid (KA) or pilocarpine kindling rat model (Fujikawa *et al.*, 1999; Fujikawa *et al.*, 2000), we rarely observed acidophilic neurons in different sectors of the PTZ-treated rats' hippocampus (Table 1), indicating that neuronal necrosis is more likely an outcome of acute injury rather than chronic damage. Despite lacking of evidence for PTZ-induced cell necrosis, our study found gliosis in area CA1 and hilus of the hippocampus in PTZ-treated rats (Fig. 3), suggesting the existence of neuronal loss, which was not examined in the current study.

4. Discussion

MFS is a typical pathologic finding in animals and humans with spontaneous seizures of temporal lobe origin (Dudek *et al.*, 1994), however, the question remains as to whether sprouting is present at the time when the first spontaneous seizures appear, or whether it develops as a consequence of recurrent seizures. Data obtained in the present study clearly showed that sprouting preceded the occurrence of spontaneous seizures, indicating that MFS is not the consequence, but more likely, the cause of SRS, probably via forming recurrent excitatory circuits (Lynch and Sutula, 2000; Cavazos *et al.*, 2003). However, its presence is not necessarily associated with the occurrence of spontaneous seizures (Nissinen *et al.*, 2001). With the presence of cyclo-

Table 1

Damage scores assessed by H&E stain

	Brain region			
	CA1	CA3	H	DG
Con (n = 6)	0.17 ± 0.11	0.17 ± 0.11	0.17 ± 0.11	0.17 ± 0.11
3d (n = 6)	0.17 ± 0.11	0.08 ± 0.08	0.08 ± 0.08	0.17 ± 0.11
1w (n = 6)	0.00 ± 0.00	0.08 ± 0.08	0.08 ± 0.08	0.00 ± 0.00
2w (n = 6)	0.17 ± 0.11	0.08 ± 0.08	0.08 ± 0.08	0.17 ± 0.11
4w (n = 6)	0.17 ± 0.11	0.17 ± 0.11	0.00 ± 0.00	0.25 ± 0.11
6w (n = 6)	0.13 ± 0.04	0.12 ± 0.04	0.10 ± 0.04	0.13 ± 0.04
P	0.757	0.304	0.703	0.259

The data represent means ± SEM of neuronal damage scores assessed by numbers of acidophilic neurons by H&E stain. $P < 0.05$ is considered as statistically significant.

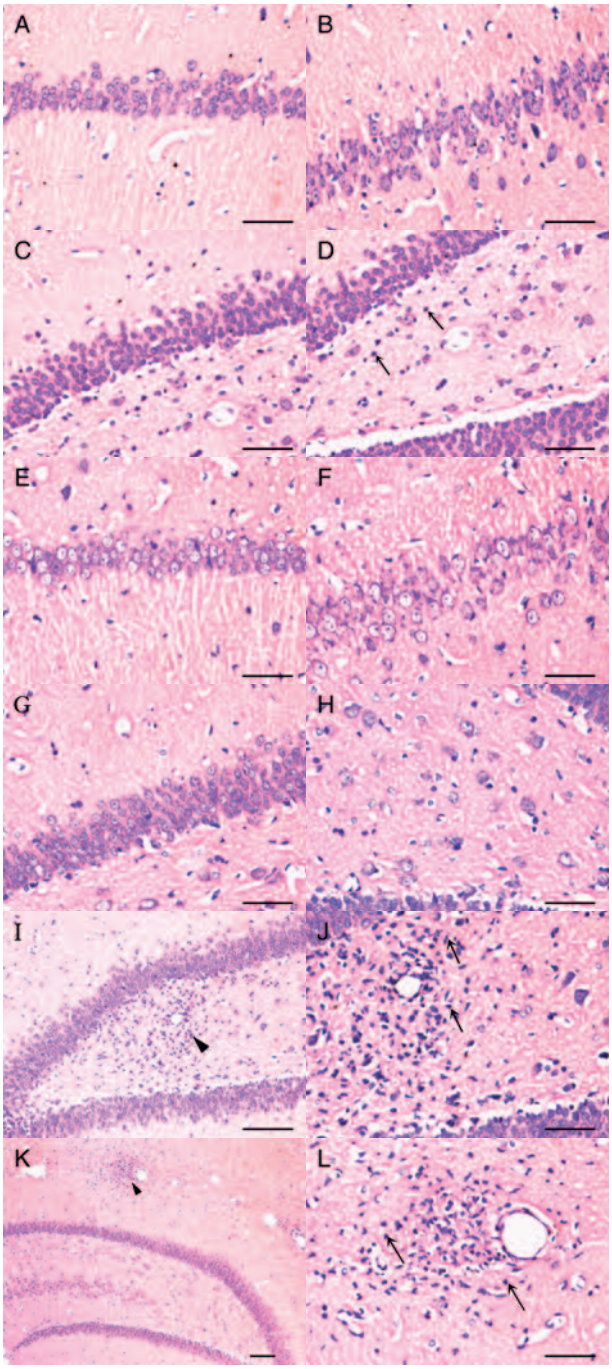


FIG. 3. — The morphology of hippocampal neurons in both the control and PTZ group. A-D show neurons in area CA1, CA3, DG, hilus in the control group from left to right, while E-H show neurons in corresponding areas in the PTZ group. No necrotic neurons (eosinophilic cytoplasm and large, round, basophilic chromatin clumps) are found in these areas. H&E stain clearly shows gliosis in hilus (I) and area CA1 (K) in the PTZ treated rats' hippocampus. J, L are magnifications of I and K respectively. Arrowhead point to gliosis. Arrows point to glial cells. Scale bars = 50 μm (A-H, J, L), 100 μm (I, K).

heximide, a protein synthesis inhibitor which is able to block MFS (triggered by pilocarpine-induced SE) and also gives relative protection against hippocampal neuronal death, animals still showed SRS (Longo and Mello, 1999). In addition, MFS can be induced experimentally without any epileptic seizures. For example, long-term potentiation induces MFS in the CA3 subfield of the hippocampus and in the IML of the dentate gyrus (Adams *et al.*, 1997). Also, lesions of the perforant path (Zimmer, 1974) or genetic mutations (prion protein null mouse, Colling *et al.*, 1997) induce sprouting without any seizure activity. One study in humans without a history of epilepsy also demonstrated MFS (Cassell and Brown, 1984).

Many published studies demonstrated CA3 MFS with or without aberrant sprouting into the dentate molecular layer (Holmes *et al.*, 1999; Cross and Cavazos, 2007), while in our study, no obvious MFS was observed in the IML of dentate gyrus, instead, an increased distribution of Timm granules was noticed in the CA3 region. This phenomenon probably attributes to: a. Difference in type, dose and severity of induced seizures by different convulsants. Pilocarpine (Kamida *et al.*, 2009) and KA (Tokuhara *et al.*, 2007) are more likely to induce SE which could lead to hippocampal neuronal death especially in area CA3. Even with the same convulsant, the distribution of Timm granules could differ among rats treated by different doses (Holmes *et al.*, 1999). b. Mild hippocampal damage in PTZ kindling model. Neuronal injury is believed to be the initial step of abnormal sprouting (Shetty *et al.*, 2005). It could lead to target cells deficiency in area CA3 and compensatory projection to the IML by sprouted mossy fibers. However, our study used moderate dose of PTZ to establish kindling model, SE rarely occurred throughout the experiment. And the extent of neuron injuries caused by generalized tonic-clonic convulsions and SRS was less severe than SE (Deshpande *et al.*, 2007). In spite of slight injury of CA3 neurons, sprouted mossy fibers could still find sufficient post-synaptic target cells in this area without turning back to the IML. Thus, the synaptic reorganization of the MF projection to CA3 was noted in the absence of overt neuronal loss in CA3 or other hipppocampal regions in our study. Besides, SRS occurred without obvious neuronal necrosis and loss in limbic system, suggesting lack of association between development of SRS and hippocampal damage (Brandt *et al.*, 2004).

In summary, our results showed that prominent MFS could be observed in area CA3 before the occurrence of SRS. Moreover, MFS and SRS developed in the absence of evident hippocampal

damage. These findings promote conclusions as below: (a) MFS is not the outcome of SRS. (b) Severe hippocampal damage is not required in the development of both MFS and SRS.

Acknowledgements

This work was supported by Foundation of Department of Education, Hunan Province, China.

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