Changes in the numbers and distribution of calretinin in the epileptic rat hippocampus

Mutasem M. Abuhamed, *MD, PhD,* ***Xiao Bo,*** *MD, PhD,* ***Walid A. Alsharafi,*** *M.Med,* ***Li Jing,*** *MD, PhD,* ***Lili Long,*** *MD,* ***Wu Zhiguo,*** *MD,* ***Song Zhi,*** *MD, PhD.*

ABSTRACT

الأهداف: فحص الكلرياتين (CR) المحتوي على عصبونات التي تنحل في منطقة الحصين بعد حالة الصرع الفئران (SE) في أوقات زمنية مختلفة.

الطريقة: أجريت هذه الدراسة بجامعة وسط الجنوب – مستشفى الشانياه – الصين خلال الفترة من سبتمبر 2008م إلى يناير 2010م. تم اختيار البايلوكاربين لتوليد حالة الصرع كنموذج الصرع المزمن للفئران. لتحديد ما إذا كانت خلايا الحصين العصبية ممكن أن تتأثر بنوبات الصرع الحصيني. أجريت الفحوصات المناعية النسيجية في أقسام الدماغ التي تم الحصول عليها من المجموعة الضابطة المماثلة بالعمر كعينة مراقبة (n=50) والفئران المصابة بالصرع (n=170). استخدمت لطخة نيسل لمراقبة التغيرات النسيجية في الحصين.

النتائج: أظهرت نتائجنا عن فقدان شديد للخلايا في المناطق السرة، والحصينCA1، وCA3 في جميع الفئران المصابة بالصرع. أظهر التحليل الكمي عن اختلافات كبيرة بين المجموعة الضابطة، والفئران المصابة بالصرع في عدد العصبونات الإيجابية CR. وُزعت هذه العصبونات في السرة، ومناطق الحصينCA1، و CA3، و في التليف المسنن لفئران المجموعة الضابطة، ومجموعة الفئران المصابة بالصرع، لوحظ وفرة في الحصين في فئران مجموعة التحكم. كما لوحظ زيادة عابرة كذلك للعصبونات الإيجابية CR، في CA1 بين 7 و 15 يوم بعد الإصابة بالصرع. تقع معظم عصبونات CR في السرة، وCA1 للفئران المصابة بالصرع، وفي السرة لمجموعة التحكم.

خاتمة: تشير البيانات المتوفرة لدينا إلى أنه تمت ملاحظة نسبة مختلفة من العصبونات المثبطة في منطقة الحصين للفئران المصابة بالصرع، وتختلف أرقامها عن مجموعة التحكم. تُشير هذه النتائج إلى أن الدوائر المثبطة في الحصين، قد تُمثل استجابة تعويضية لتحقيق التوازن بين الداخلات المحفزة المساندة في المنطقة.

Objectives: To examine calretinin (CR)-containing interneurons that degenerate in the hippocampus in post status epilepticus (SE) rats at different time points.

Methods: This study was conducted at the Central South University, Xiangya Hospital, Hunan Province, P.R. China between September 2008 and January 2010. Pilocarpine-induced SE was chosen as a model to generate chronic epileptic rats. To determine whether hippocampal neuronal populations are affected by hippocampal seizures, immunohistochemical assays were performed in brain sections obtained from age-matched control (n=50) and epileptic rats (n=170). Nissl stain was used to observe pathological changes of the hippocampus.

Results: Our results revealed the most dramatic cell loss to be in the hilar, cornu Ammonis (CA)1, and CA3 areas in the epileptic rats. Quantitative analysis revealed significant differences between control and epileptic rats in the number of CR-positive interneurons. These interneurons were distributed in the hilar, CA1, and CA3 areas and in the dentate gyrus of both control and epileptic rats, but was more numerous in the hippocampus of normal rats. However, a transient increase of CR-positive interneurons was observed in the CA1 between 7 and 15 days post SE. The CR interneurons were mostly located in the hilar and CA1 for epileptic rats, and in the hilus for control rats.

Conclusions: Our data suggest that a different proportion of inhibitory interneurons was observed in the epileptic rat hippocampus, as their numbers differ from controls. These results indicate that the inhibitory circuits in the hippocampus may represent a compensatory response with a role to balance the enhanced excitatory input in the region.

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From the Department of Neurology (Abuhamed, Bo, Alsharafi, Jing, Long, Zhiguo), Xiangya First Affiliated Hospital, and the Department of Neurology (Zhi), Xiangya Third Affiliated Hospital, Xiangya Medical College of Central South University, Changsha, Hunan Province, P.R. China.

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Address correspondence and reprint request to: Prof. Xiao Bo, Department of Neurology, The First Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, P.R. China. Tel. +86 (731) 4328197. Fax. +86 (731) 4328197. E-mail: xiaobo1515@yahoo.com

Temporal lobe epilepsy (TLE) is known as one of the most common forms of epilepsy.[1] Usually, it is associated with neuronal death and reactive gliosis of the hippocampus.[1-4] The hippocampus has been used in numerous studies investigating the mechanisms of epileptogenesis and short- or long-term consequences of epileptic cell death. Epileptic cell death reflecting selective neuronal vulnerability shows a characteristic pattern in the hippocampus with short-term survival periods after the injury.[5] The histopathological changes of the hippocampus in TLE have been studied for more than 10 decades, but the etiology and pathogenesis of these histopathological changes are disputed,[6,7] and their existence and direction of a possible precise relationship with the neuronal mechanisms has remained unidentified for most of the observed tissue abnormalities. The hippocampus is composed of several sub regions that exhibit a differential involvement in TLE. Dentate hilus and cornu Ammonis (CA)1 field sclerosis were found to be the most frequent gross morphological alterations in animal models,[8] and human cases,[3,9] whereas CA3 fields and the dentate gyrus (DG) show fewer alterations. The most striking morphological features of hippocampal sclerosis include severe loss of neurons. In addition, some populations of interneurons are also affected,[10,11] whereas other interneurons are well preserved in hippocampal sclerosis.[12-15] Surviving neurons show marked synaptic reorganization and axonal sprouting, and this may lead to aberrant neuronal circuitries.[3,16] Numerous studies of hippocampus tissue with different methods have already provided valuable data on the vulnerability of different cell types.[17-20] Detailed studies of the vulnerability of non-principal cells in the hippocampus started more recently. Gamma-aminobutyric acid (GABA) interneurons are known to play important roles in the regulation of behavior dependent network activity patterns in the hippocampus of patients with epilepsy. The GABA interneuron shows a compact relation to output-input closeness of epileptic network activity patterns in the hippocampus.[3] The calcium level within the cells is critical in maintaining the balance of cellular homeostasis and other processes. Calcium-binding proteins are thought to play an important role in buffering the calcium level within the cells.[21] Deregulation of calcium has been proposed to be engaged in TLE. Calcium plays an important role in the neuronal membrane excitability function of neurons.[21,22] The calcium signal is converted into the cellular response partly via intracellular calcium-binding proteins, which are believed to be engaged in the arrangement of cellular and many enzyme activities, in regulation of calcium, and in buffering. In the CNS, calcium-binding proteins have also been revealed to establish definite markers of neuronal subpopulations and neural pathways.[23] In a manner that attracts interest, alterations in the expression of different calcium binding proteins have been monitored in neurodegenerative disorders, so that a preserving role for some of these proteins in certain neuron subpopulations has been presumed.[24] Various calcium-binding protein markers have been found in specific neuronal populations in the nervous system. Among the many of these proteins, calretinin (CR) has been established abundantly in several types of neurons. Calretinin is a 29 kDa calcium-binding protein that is 58% homologous to calbindin D28K.[25,26] Calretinin has been found in the hippocampus of various mammals, including humans. The interneurons expressing CR usually synapse mainly on hippocampal GABAergic neurons.[27] They have been demonstrated to regulate synchronous activity of principal hippocampal neurons,[28,29] and the absence or decrease of hippocampal CR expression has been accompanied with meaningful interruption of long-term potentiation induction. Calretinin has been found in the hippocampal interneurons.[30,31] The vulnerability of CR-positive cells and CR-containing cells was examined in epilepsy,[32,33] and ischemia,[34] and their extreme vulnerability to both epileptic and ischemic insults was described. However, very little is known about CR-containing cells in the epileptic hippocampus. The rat hippocampus, after injection of pilocarpine, presents a well-characterized model of neurodegeneration, with a clear pattern of neuronal necrosis without substantial demyelination, associated with a marked gliotic response. Histological changes are also accompanied with neurobehavioral alterations, and pilocarpine has also been considered as a potential tool for the study of hippocampal dysfunction in animal models. The aim of the present study was to examine immunohistochemical changes in the expression and cellular distribution of CR at different time points in this animal model of TLE.

Methods. *Animals.* This experimental study was conducted at the Central South University, Xiangya Medical College, First Xiangya Hospital, Hunan Province, P.R. China from September 2008 to January 2010. The Central South University Xiangya Hospital Ethics Committee approved the research protocol. All experiments were carried out in accordance with Chinese regulations governing animal welfare and protection. Protocols were also reviewed and approved by the local animal care committee, in accordance with the guidelines of the Principles of Laboratory Animal Care. Extreme care was taken to ensure that animals were always treated in a humane manner. Every effort was made to minimize animals used and their suffering. Two hundred and twenty adult male Sprague-Dawley rats weighing 250-300g at the beginning of the study were initially selected. Out of 220 rats, 170 rats were used in the epileptic group (epileptic preliminary

experiment rats n=50, and epileptic rats n=120) and 50 rats in control group. Rats were maintained in controlled conditions consisting of 12-hour light/dark cycle with food and water. All experiments were performed at the same time of the morning to minimize possible effects of circadian variation. The animals were assigned to control and epileptic (latent period and chronically epileptic) groups. The method proposed by Glien et al[35] was used in the preliminary experiment. The other epileptic animals (120) were obtained using the pilocarpine model of epilepsy,[35] with some modifications. Briefly, rats were injected intraperitoneally (i.p.) with a single dose of lithium chloride 125mg/kg i.p. 18-24 hours prior to pilocarpine administration to reduce the pilocarpine dose required to induce seizures. Pilocarpine hydrochloride (Sigma, St. Louis, MO, USA) was then i.p. injected, followed by 10mg/kg at 30 minute intervals until SE ensued. The pilocarpine model of seizures was scored using the following scale, modified by Racine:[36] 1 = myoclonic jerks of forelimb; 2 = mild forelimb clonus (+ mouth and facial clonus); 3 = severe forelimb clonus; 4 = rearing in addition to severe forelimb clonus; 5 = rearing and falling in addition to severe forelimb clonus. Convulsive manifestations of SE were reduced by i.p. injections of chloral hydrate (0.5-1ml/kg) administered one hour after SE onset. Antiepileptic drug administration significantly increased survival of animals and also served to standardize the amount of seizure-triggered lesions.[37-39] Pilocarpine-treated rats were placed individually in transparent Plexiglas cages and monitored for 10-12 hours/day during weekdays to define seizure frequency. Animals exhibiting SE remained asymptomatic for a period of 2-14 days (latent period) before exhibiting spontaneous recurrent seizures (3-5 per week) as previously shown.[40] It is important to indicate that the method of observing for spontaneous seizures was based on behavioral monitoring during the daytime. Animals exhibiting 3-5 seizures per week were aligned to the chronically epileptic group. Animals showing lower or higher seizure rates were excluded from the study. A control group was assigned with age and weight-matched rats injected with 0.9% sodium chloride (NaCl) (125 mg/kg) instead of pilocarpine. In the present study, we interrupt the behavioral manifestation of SE at one hour after onset (by using 10% chloral hydrate), which is within the time range needed to induce chronic epileptogenesis.[38] Additionally, we monitored the seizure frequency of pilocarpine-treated epileptic rats to ensure that the animals chosen for the chronically epileptic group experienced seizures at approximately the same rate.

Immunohistochemistry. Animals were killed 2 hours, 6 hours, one day, 3 days, 7 days, 15 days, 30 days, and 2 months after pilocarpine-induced SE. Experimental animals were paired with control animals and killed simultaneously. Experimental rats and control rats were perfused and processed for immunohistochemistry. Briefly, rats were deeply anesthetized using a 10% chloral hydrate (3.5ml/kg) and then perfused transcardially with 200ml 0.9% NaCl followed by 350-450ml 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the brains were removed and postfixed in the same fixatives overnight at 4°C followed by cryoprotection in 30% sucrose in PBS. Coronal brain sections were sectioned with a cryostat (HM500 Microm by AO company, Mountain View, CA, USA) at 20mm and stored in 0.1M PBS, pH 7.4. Several 1-in-4 hippocampal sections were collected and mounted on polylysine-coated slides, air-dried, and preserved in the -80°C refrigerator. Some sections were stained with Nissl's staining to determine the general histological characteristics of the hippocampal region. Nissl's staining of homologous hippocampal sections was routinely performed to evaluate the amount of neuronal damage in the hippocampal formation. Sections obtained from experimental and control rats were processed using immunohistochemical techniques. The protocols for immunohistochemistry were based on the description by Kobayashi and Buckmaster[39] with some modifications. Sections were incubated for 25 minutes in 0.3% H_2O_2 at room temperature to inhibit endogenous peroxidase activity, and then incubated with 0.03% TritonX-100 in 0.01M PBS (pH 7.4) at 37°C for 25 minutes. The slides were rinsed again 3 times for 10 minutes and non-specific staining was minimized by incubating slices in 5% normal goat serum in 0.01M PBS for 25 minutes at 37°C. Primary antibodies were diluted in normal goat serum. Sections were incubated overnight with primary antibodies (Polyclonal rabbit anti calretinin serum, 0.3:1500, T4103, Bachem, Switzerland) at 4°C. After incubation with primary antibodies, sections were rinsed 3 times in PBS (10 minutes each). Sections were incubated with their biotinylated secondaries diluted in 2% goat or house serum at 37°C for 3 hours at a dilution of 1:200; (goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA). After further rinsing 3 times in 0.01M PBS, sections were incubated in avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA, USA) for 2 hours and the reaction was revealed using 3-3'-diaminobenzidine (DAB, Invitrogen, Carlsbad, CA, USA) as a chromogen. The reaction was stopped by rinsing the sections in distilled water. In each experiment, some sections were incubated without the primary antibody to determine staining specificity (negative controls). Sections were mounted onto gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene, and cover slipped for further analysis.

Image analysis. Two investigators who were blind to the group's treatment carried out quantitative analysis

of the number of Nissl-stained neuron and CR-positive interneurons. Starting from a random position, the number of sections were analyzed per rat per time point. Similar level cross-sections in control groups were selected. Interneurons were counted under the 20 × objective within a probe volume defined by the counting frame and the dissector height. For quantitative analysis of profiles in the hilus of the DG, a contour of the hilus was drawn along the outer border of the dentate granule cell layer, while straight lines were drawn from the ends of the granule cell layer to the proximal end of the CA3 pyramidal cell layer.[39] In the hilus, CR-positive interneurons were directly counted. The lateral borders of the CA1 were revealed by straight lines perpendicular to the pyramidal cell layer at the limit with CA3 and the subiculum. In the CA1 area, 4 nonoverlapping visual fields were randomly sampled to count CR-positive interneurons in each field. Only neurons within the field or overlapping the right or superior border of the field were counted. In the CA3 area, 4 nonoverlapping visual fields were randomly sampled to count interneurons. To estimate the number of Nissl-stained neurons, 4 regions of each hippocampus were revealed: the stratum pyramidale, which included the pyramidal cells in the CA1 area; the stratum pyramidale (CA3 area); the granule cell layer in the DG; and the hilus. Five nonoverlapping visual fields were randomly sampled to count Nissl-stained neurons in each region under 40 × objectives. The average number of Nissl-stained neurons and CR-positive interneurons counted per visual field was calculated. The numbers of labeled neurons were estimated by using the neurons±SEM for each group of control and experimental rats. Using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 12, the data were analyzed statistically with a mixed model analysis of variance (ANOVA) and Student's t-test to determine significant differences in the number of neurons per area between groups (control and pilocarpine-treated rats) in each region and each interval (2 hours, 6 hours, one day, 3 days, 7 days, 15 days, 30 days, and 2 months).

Results. In the preliminary research, Glien et al's[35] protocol was used. Pilocarpine was injected at a dose of 30 mg/kg followed later by repeated application of low dose pilocarpine 10mg/kg at 30 minutes intervals. Thirty of 50 rats (60%) developed SE and 5 rats (10%) died, 15 rats (30%) did not enter SE and one rat that developed SE died during the procedure and, therefore, was excluded from the study. In the other group of rats (n=120), the pilocarpine was injected at an initial dose of 25mg/kg followed by repeated 10mg/kg, resulting in 8% mortality, and 85% SE. The experimental hippocampus shows a neuronal cell loss pattern with comparable subregions to the normal hippocampus as shown in **Table 1**. Light-microscopic analysis of Nissl-stained sections showed that treatment with pilocarpine produced varying degrees of neuronal cell loss throughout the hippocampus (**Figure 1**), which was especially marked in the CA1 (**Figure 1F, I, L**) and the CA3 (**Figure 1E, H, K, N**) pyramidal cell layers, and was light in granular cells in the DG of the hippocampus. At one-day post injection of pilocarpine, a dramatic loss of hilar neurons was shown in cresyl violet stained sections (**Table 1**). Extensive loss of the proximal part of the pyramidal cells of CA3 was evident (**Figure 1N**), while the CA1 and most of the CA3 pyramidal cells were unaffected (**Table 1**). After 7 days post-pilocarpine injection, striking neuronal loss became apparent in the hippocampal sub region (**Figure 1D-L**), with degeneration of CA1 and dispersion of DG granule cells. The degenerative and morphogenic changes induced by pilocarpine injection

Table 1 - Number of Nissl-stained neuron profiles per section per region of the hippocampus in control and pilocarpine-treated rats (number/visual field).

Variable	Rats	Hilus	Py of CA1	Py of CA3	GC of DG
One day	Epileptic	59.2±5.26*	95.60±55.24	58.2±10.16	290.8±54.16
	Control	69.6±20.68	112.8±10.42	63.4±12.86	305.8±66.52
7 days	Epileptic	51.8±14.26*	77±17.24**	50.6±13.04*	250.8±53.14*
	Control	62.6±15.68	111.8±20.42	60.4±13.86	300.8±76.56
30 days	Epileptic	41.8±10.25**	40.24±14.42**	38.6±12.08**	244.8±33.14**
	Control	64.6±16.8	114.8±16.22	62.2±11.76	310.4±14.52
60 days	Epileptic	31±10.54**	26.2±9.46**	27.6±13.64**	240.1±57.9**
	Control	65.2±12.86	116.2±21.08	65.2±13.04	291.6±59.78

*Significant difference between controls and post-SE rats at the same time point ($p<0.05$).
**Significant difference between controls and post-SE rats at the same time point ($p<0.01$),
Py - pyramidal layer, GC of DG - granule cell layer of dentate gyrus

gradually progressed, at which stage the pyramidal layer of the CA1 region was almost completely unrecognized (**Figure 1I, L**), and the pyramidal layer of the CA3 (**Figure 1H**) was much decreased but still recognizable, and the DG was slightly enlarged. The CR immunoreactivity (IR) showed distinct patterns of neuronal vulnerability in response to pilocarpine injection (**Figure 2**). At 24 hours post-pilocarpine, selective decrease of CR-positive interneurons was seen in the hilus of the DG (**Figure 2E**), confirming that mossy cells are among the neurons most vulnerable to pilocarpine injection, while a dramatic decrease of CR-positive interneurons was seen in the hilus at 30 and 60 days (**Figure 2K, N**). In contrast, a transient increase of CR-IR was observed in interneurons of the stratum oriens and stratum radiatum of the CA1 at 7 days post-SE (**Figure 2G**). Also, appreciable decreases of CR-positive interneurons in the stratum lacunosum and stratum radiatum of the CA3 (**Figure 2L, O**) and in the CA1 (**Figure 2J, M**) were detectable at 30-60 days post SE. A significant effect of pilocarpine treatment was detected by quantitative analysis of the neuronal subpopulation exhibiting CR immunoreactivity, the number of CR-IR cells being significantly lower in the various regions of the

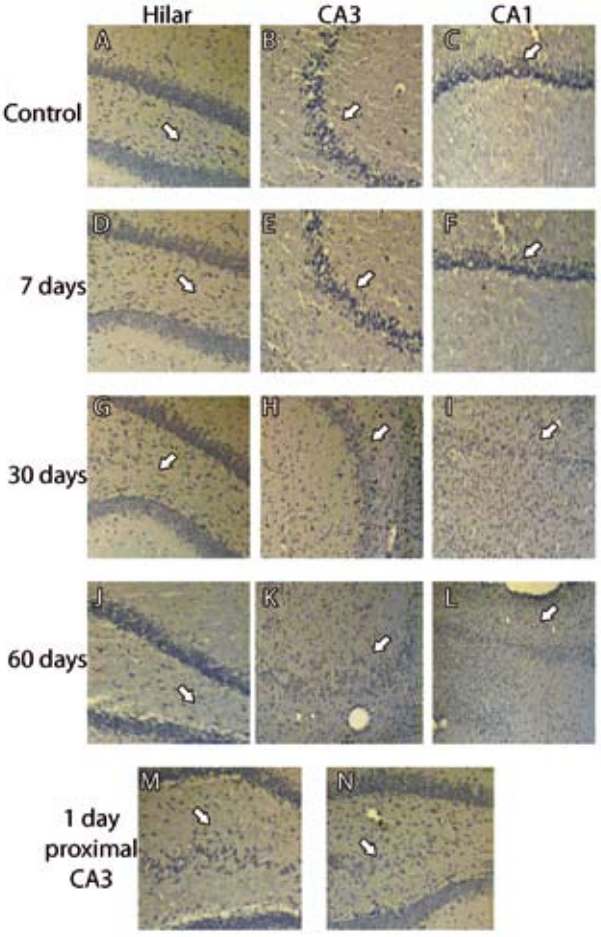

Figure 1 - Nissl-stained sections of the hippocampal formation of control (**A, B, C, M**) and pilocarpine-treated (**D, E, F, G, H, I, J, K, L, N**) rats. In controls, the cell bodies of principal cells are highly concentrated and form a continuous band in the pyramidal cell layer of the CA1 and CA3 fields and in the granule cell layer of the dentate gyrus. Many neurons are distributed in the CA1, CA3, and hilus of dentate gyrus. In the pilocarpine-treated rats with spontaneous seizures, the number of neurons is reduced in CA1 (**I, L**) and in the hilus of the dentate gyrus (**G, J**). A profound neuronal loss is also observed in regions of proximal CA3 (**N**), whereas the distal CA3 (**E**) is well-preserved. At 30d and 60d Dramatic neuronal loss is noted in the distal CA3 (**H, K**).

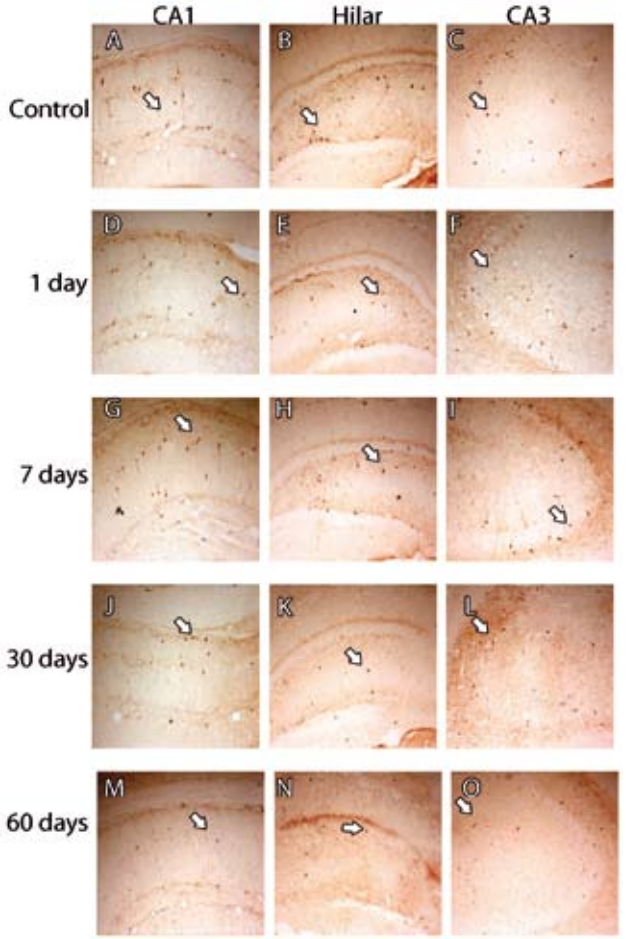

Figure 2 - Immunohistochemistry sections of the hippocampal formation of region-specific calretinin (CR) positive interneurons in the lithium pilocarpine treated rats compared with the control rats (**A, B, C**). **D, E, F** - One day post-pilocarpine treatment. Note the contrast between the decrease of CR positive interneurons in CA1, CA3, and hilar of the dentate gyrus. **G, I** - transient increase of CR staining in the CA1, and CA3 with preserved decrease CR staining in the hilar of dentate gyrus (**H**) at 7 days post-lithium pilocarpine treatment. **J, K, L** - 30 days and **M N, O** - 60 days post-lithium pilocarpine treatment, dramatic loss of CR staining in all hippocampal subfields.

Table 2 - Average of calretinin positive interneurons per section per region of the hippocampus in control and pilocarpine-treated rats (number/visual field).

Variable	Rats	Hilus	CA1	CA3
One day	Epileptic	40.5±6.30**	8.2±2.26*	3.6±1.40*
	Control	76.2±6.03	18.0±3.04	13.4±2.60
7 days	Epileptic	30.1±6.31**	10.8±4.76	5.6±4.22
	Control	80.4±6.91	17.2±1.60	11.44±4.28
15 days	Epileptic	44.6±8.16*	14.6±3.01	6.8±2.2
	Control	72.0±8.04	17.4±4.14	12.4±17.2
30 days	Epileptic	20.6±4.66**	7.4±1.12**	3.1±2.02**
	Control	74.8±4.70	16.4±1.28	12.4±2.6
60 days	Epileptic	19.5±8.64**	7.2±1.50**	2.16±1.50**
	Control	78.0±8.47	16.8±2.40	12.16±1.64

*Significant difference between controls and post-SE rats at the same time point ($p<0.05$),
**Significant difference between controls and post-SE rats at the same time point ($p<0.01$),
Py - pyramidal layer, GC of DG - granule cell layer of dentate gyrus

CA and in the DG of treated animals (**Table 2**). The effect on the hippocampal regions was significant. The comparisons showed a significant difference between the 2 experimental and control groups in the various subfields of the hippocampus at one day, 30 days, and 60 days.

Discussion. Analysis of the changes in the hippocampus in the pilocarpine rat model yielded 4 major novel results: 1) Modified Glien et al[35] protocol was practical for experimental purposes; 2) Pilocarpine injection was followed without delay by loss of neuronal cells in the hilar of the DG, along with loss of neuronal cell in the CA1 and CA3 areas; 3) Pilocarpine injection was followed without delay by decrease of CR interneurons in the hilus of the DG, along with an increase of CR interneurons in the CA1 and CA3 area; 4) The CR interneurons were markedly decreased in the chronic phase (30 and 60 days) in all hippocampus sub regions, and paralleled by a transient increase in CR positive interneurons in the CA1 and the CA3 (7 days). Administration of pilocarpine in animals can result in a chronic behavioral state that is similar to human TLE. Previous studies administered pilocarpine alone at a high systemic dose (320-400mg/Kg) to induce SE, but this experimental protocol, however, is associated with high mortality rate as described by Jope et al.[41] Later pilocarpine (30mg/kg), was administered in combination with lithium, which markedly potentiates the convulsant effect of pilocarpine, but the mortality rate was still high at 45%. Glien et al[35] administered lithium 24 hours prior to pilocarpine injection at 30mg/kg and repeated 10 mg/kg pilocarpine administration at 30-minute intervals. Status epilepticus was produced in approximately 61%, and the mortality rate was below 10%, which was significantly lower compared to previous studies. Thus, we used the method proposed by Glien et al[35] in the preliminary experiment, and 65% successfully developed SE, and the mortality rate was 12%. Though the mortality rate was reduced, the achievement ratio of inducing SE was not satisfactory. In an attempt to ameliorate such a low achievement ratio, we divided the pilocarpine treatments, injecting an initial dose of 25mg/kg, and then in additional 10mg/kg doses until production of SE (1-5 injections). This repeated administration of low-doses significantly increased the SE achievement ratio to 85%, while maintaining a low mortality rate (12%). A significant difference was observed compared to SE achievement ratio in the preliminary experiment. This approach was satisfactory as the achievement ratio of inducing SE was high, and the average delay from the first injection of pilocarpine to the onset of SE did not exceed 200 minutes, making this method practical for experimental purposes.

The pattern and time course of neuronal cell loss in pilocarpine-treated rats showed a clear difference between the acute effects of pilocarpine seen after 24 hours, and the gradual morphological changes starting at 15 days post-pilocarpine. These results indicate that the impairment of neuronal cell function in the pilocarpine-treated hippocampus contributes to the onset of spontaneous recurrent seizures, whereas the chronic morphological changes may reflect a delayed effect of pilocarpine toxicity and/or distinct cellular responses to spontaneous recurrent seizures.

Pyramidal cells were preserved the in hilus and CA1, and were lost in the proximal part of CA3 at 24 hours

post-pilocarpine. The hippocampal morphology was still largely preserved up to 15 days post-pilocarpine. Destruction of a large population of pyramidal cells is therefore not required for the development of recurrent seizures, while the profound loss of pyramidal cells in the CA3 areas showed a major chronic (30, 60 days) effect of pilocarpine, suggesting that loss of pyramidal cells contributes to the development of chronic recurrent seizures. These findings have 2 major implications: 1) As the seizures occur despite the preserved CA1, the remaining CA3 pyramidal cells, and hilar of DG are sufficient for epileptogenesis in this model. Indeed, in the hippocampus, pyramidal cells in the CA3 have a high incidence of recurrent excitatory synapses and are the most vulnerable of the hippocampal principal cells to epileptiform activity,[42-45] while CA1 pyramidal cells and dentate granule cells induce negligible recurrent excitatory synapses and are more refractory to epileptiform events. These changes suggest an earlier starting and faster progression of the neuronal alterations in those hippocampal sub regions where the initial lesion was largest. 2) Morphological changes of CA1 pyramidal cells in the hippocampus appear as a consequence, and not a cause, of the epileptic process, as shown up to one year after kainic acid injection.[46]

Our data clearly shows that pilocarpine-treated animals with spontaneous recurrent seizures display, in the CA1, a significant increase in the number of CR-interneurons at 7-15 days, an increase, which is associated with major decreases in the numbers of CR neurons in the hilar of DG. Documentation of a profound expansion of CR-interneurons in the CA1 of human epileptic tissue suggests that sprouting of interneuron-selective interneurons also is accompanied with TLE, whereas CR positive interneurons were restricted in controls.[44] Sprouting in GABAergic circuitry is accompanied by the concurrent death of GABAergic interneurons, with some subfields inducing greater losses than others. Studies at different time points in the pilocarpine model showed an early loss of GAD-immunoreactive fibers in the supragranular field and outer two-thirds of the dentate molecular layer as well as GAT-1 and GAD immunoreactivities[45] in the hilus. This is consistent with the early partial loss of GABAergic interneurons in the pilocarpine model. Several results in this study supposed that the decrease in the number of these interneurons reflects neuronal death rather than a downregulation at the mRNA or protein/peptide levels. First, the loss of CR interneurons was associated with a drop in the total number of neurons. Many neuronal cell losses were observed in the hilus as soon as 24 hours after pilocarpine induced SE, and was still evident 7 and 15 days after injection. Furthermore, this neuronal cell loss was accompanied by the loss of CR containing interneurons in the hilus as soon as 24 hours after injection. In addition, our data shows that pilocarpine-treated animals with spontaneous recurrent seizures display an increase in the number of interneurons co-expressing CR in CA1 and CA3 at 7-15 days. The upregulation in peptide and/or calcium binding protein expression, in most neurons that in experimental conditions would show detectable levels, might contribute to these increased-cell numbers, however, it is more likely that these data reflect the changes of CR interneurons in epileptic animals.

In conclusion, in the present study the suggested modifications of lithium pilocarpine protocol may be used as an alternative approach to increase the achievement ratio of SE induction. Different CR-IR neuron expressions in different subfields at different time points have different vulnerability to injury produced by seizures, and hilar interneurons are the most vulnerable. Loss of GABAergic CR interneurons plays an important role in the generation of TLE. Axonal sprouting of inhibitory GABAergic interneurons, especially a transient increase of CR-IR neurons within hippocampus subfields, may constitute the aberrant inhibitory circuit and play a significant role in the generation and compensation of TLE.

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