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The loss of Ivy cells and the hippocampal input modulatory O-LM cells contribute to the emergence of hyperexcitability in the hippocampus

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Abstract

Epilepsy is a frequent neurological disorder that affects directly 0.5–1.5% of the world's population. Despite advances regarding therapy, about 30% of patients cannot be relieved of seizures, mainly because the pathophysiological mechanisms are still not elucidated completely. Basket, axo-axonic, bistratified, *oriens-lacunosum moleculare* (O-LM) and Ivy cells exert spatially and temporary different inhibition on principal neurons. Our aim was to evaluate the alterations of these interneuron populations during epileptogenesis. We induced status epilepticus in male Wistar rats using intraperitoneal pilocarpine injection, which was followed, after a latency period, by spontaneous recurrent seizures (SRS). Nissl staining was used for the analysis of gross morphological changes, whereas triple immunofluorescent-labeled sections (parvalbumin, somatostatin, neuropeptide-Y) were used for differentiation of the selected interneuron types. Putative interneurons identified by their neurochemical contents were quantified, and the cell density was calculated. Although animals developing SRS showed similar behavior, the degree of hippocampal sclerosis was different. In animals with hippocampal sclerotic cell death pattern the density of perisomatic inhibitory neurons was higher, but not significantly. The dendritic inhibitory bistratified cells were preserved, whereas the number of O-LM cells showed a significant decrease. A substantial loss was observed in the number and density of Ivy cells. We suggest that the loss of hippocampal input modulatory O-LM cells, and overall excitation controlling Ivy cells, has a role in the emergence of hyperexcitability. In the same time, alterations of output controlling interneurons might contribute to the propagation of the pathological synchronization to the cortex.

Keywords: epilepsy, epileptogenesis, hippocampus, interneuron, Ivy cell.

Introduction

Epilepsy is a neurological disorder affecting directly approximately 0.5% to 1.5% of the world's population [1, 2]. It also should be noted that epilepsy in the 21st century is still a personal, social and economic burden [3–5] and the stigmatized patients have less educational and employment opportunities [6, 7].

The modern concept delineates epilepsy as a chronic disorder with spontaneous recurrent seizures and transitory behavioral modifications due to abnormal excessive or synchronous neuronal activity in the brain [8]. Although much progress has been made in the identification of causes and pathomechanisms involved in epilepsy, about 40% of epilepsies still have an unknown etiology [9]. Furthermore, at least a quarter of the patients with epilepsy cannot be relieved from the reoccurrence of seizures even with novel drugs [10]. Therefore, it is easy to understand the need for more research into the pathomechanisms, as well as the diagnosis, treatment and prevention of the epilepsy itself and its consequences [11].

It is considered that the limbic structures, the hippocampus, parahippocampal gyrus, amygdala are all epileptogenic regions [12, 13]. About 60% of all focal epilepsies are represented by the temporal lobe epilepsy (TLE), and the most common lesion described in drug-resistant forms is the hippocampal sclerosis [12, 14]. The hippocampus is of particular interest in neuroscience as it is involved in learning and memory, and displays a variety of synchronous oscillations under physiological or pathophysiological conditions, such as theta and gamma rhythms, ripples, and epileptic seizures.

Inhibitory interneurons, besides providing general inhibition, temporally regulate the activity of principal cells. Distinct interneuron types act in discrete time windows, and interact with excitatory inputs in a domain-specific manner. As the hippocampus has a major role in temporal lobe epilepsy [15], our aim was to quantify changes in the main interneuron populations in the pilocarpine model of TLE [16]: the *oriens-lacunosum moleculare* (O-LM) and bistratified interneurons that essentially modulate entorhinal cortical input, the basket cells that provide perisomatic inhibition, the hippocampal output controlling axo-axonic cells, and the overall excitation controlling Ivy cells population. The identification of numerical and/or structural alterations, as well as a possible resistance to epilepsy of certain inhibitory interneuron populations may elucidate the changes of the excitatory-inhibitory balance and emergence of widespread hyperexcitability and synchronization.

Materials and Methods

Animals

Male Wistar rats (100–250 g) were used for all experiments. The animals were housed under standard conditions: constant temperature (21–24^{0}C) and humidity,

12 hours light/dark cycle and free access to food and water. All procedures involving animals were carried out in accordance with *EU Directive 2010/63/EU* and national and local guidelines and policies (Ethical Committee approval number CEC 27/2011). Twelve, 5–6-week-old, rats were used for status epilepticus induction as well as six age- and batch-matched control animals. The use of younger animals is important as (1) the mortality is reduced [17] and (2) they are expected to develop spontaneous recurrent seizures (SRS) after a longer latency period than adults, thus there is a better homology with the human disease [18].

Pilocarpine-induced status epilepticus

Rats were randomly assigned to either control or pilocarpine-treated group. The later received a single injection of methyl-scopolamine (1 mg/kg, s.c.) 30 minutes prior to pilocarpine (350 mg/kg, i.p.) injection, and their behavior was observed by experimented researchers for at least five hours afterwards. Within the first 15–25 minutes after pilocarpine injection, 10 of the 12 (83%) rats developed seizures that evolved into status epilepticus (SE), which was interrupted two hours later by administration of Diazepam (5 mg/kg, i.p.). This time interval was established taking into consideration the following: (1) 30 minutes of recurrent seizures is the minimal time interval accepted to define SE, this is usually enough to produce changes characteristic to the pilocarpine model [19], however longer periods are generally preferred; (2) for better adherence of the model to the clinical history of TLE patients, as well as in order to reduce mortality a reduction of seizure period is advised [18]. Three rats that received pilocarpine died during induction or within 1–2 days, which is in accordance to literature data [18]. Therefore, the data obtained from seven epileptic rats was compared with six control animals.

Continuous video monitoring

All surviving rats were continuously video monitored during and after SE for a period of 120–150 days. Night-vision motion-capture camera with I-Catcher software (iCode Systems Ltd., Hampshire, England) was used for video recording. The observed spontaneous seizures were classified according to the modified Racine scale [20–22]. Video recordings were analyzed by several researchers, however a single person reanalyzed all putative SRS found. Animals that did not recover fully (no increase in body weight after the first week after induction with pilocarpine), or did not present SRS, were excluded from the study.

Tissue preparation and quantitative analysis

Histological processing was performed according to well-established and already published protocols [23–25]. After the monitoring period the surviving, epileptic, non-excluded animals, as well as the age- and batch-matched controls were deeply anaesthetized with a mixture of Ketamine (100 mg/kg, i.p.) and Xylazine (5 mg/kg, i.p.) and were transcardially perfused for one minute with ice-cold saline and 25 minutes with ice-cold fixative containing 4% (*w/v*) paraformaldehyde and 15% (*v/v*) picric acid made up in 0.1 M phosphate buffer (PB). Brains were removed from the skull and postfixed for one day at 4^0C in the same fixative. Afterwards, 60 μm thick coronary sections were cut using a Vibroslice (World Precision Instruments Inc., Saratoga, USA).

Every tenth section obtained was stained with cresyl violet according to the well-known Nissl staining protocol in order to determine the general histological characteristics along the fronto-caudal extent, and in order to evaluate both the degree of hippocampal sclerosis and the overall cellular death. Sections adjacent to ones chosen for immunohistochemistry were also stained with cresyl violet in order to assess pyramidal cell death along the rostro-caudal axis.

Three to four consecutive sections of dorsal hippocampus were chosen for quantitative analyses. Triple immunofluorescent labeling was performed according to standard protocols [23, 25], using the following antibodies: polyclonal guinea pig anti-parvalbumin (PV) antibody (Synaptic Systems, code 195004), monoclonal mouse anti-somatostatin (SOM) antibody (GeneTex, code GTX71935, clone SOM-018), polyclonal rabbit anti-neuropeptide-Y (NPY) antibody (ImmunoStar, code 22940). The primary antibodies were visualized using donkey anti-rabbit secondary antibody conjugated with Cy3 fluorophore (Cy3-conjugated AffiniPure Donkey Anti-Rabbit IgG, Jackson ImmunoResearch, code 711-165-152, batch 104528), donkey anti-mouse conjugated with Alexa488 (DyLight 488-conjugated AffiniPure Donkey Anti-Mouse IgG, Jackson ImmunoResearch, code 715-165-151, batch 84337), donkey anti-guinea pig conjugated with Cy5 or Alexa647 (Cy5 conjugated AffiniPure Donkey Anti-Guinea Pig IgG (H+L) Jackson ImmunoResearch, code 706-175-148, batch 80522 and AlexaFluor 647-conjugated AffiniPure Donkey Anti-Guinea Pig IgG, code 706-605-148, batch 104228), respectively. All antibodies were previously tested by several groups [25], alone and in combinations, no cross reactivity or autofluorescence was noticed; nevertheless with each new batch we also performed negative controls with the primary and the secondary antibodies separately. The triple immunofluorescent labeling allowed us to differentiate the dendritic and perisomatic GABAergic interneuron types by their neurochemical content: PV+, SOM- and NPY- basket and axo-axonic cells, the PV+, SOM+ and NPY+ bistratified cells, PV+ SOM+ and NPY- O-LM cells; the Ivy cells were identified as PV- SOM- and NPY+ cells [26–28]. Sections from pilocarpine-treated and control rats were always processed under the same conditions.

All sections were analyzed using a Leitz Wetzlar epifluorescence microscope mounted with a Hamamatsu Digital Camera (Hamamatsu Photonics Japan) and three band-pass filter cubes specifically designed for the fluorophors that we used (Chroma Technology Corporation, USA, codes 49011, 49004 and 49009, www.chroma.com). First, the whole hippocampal surface, filtered for PV labeling, was photographed with a 25× objective. The images obtained from all visual fields containing the hippocampal formation of a certain section were merged and the obtained detailed map was used to identify and delimit both the layers – *oriens* (O), *pyramidale* (P), *radiatum-lacunosum-moleculare* (R-LM) – and the regions (CA1, CA2, CA3) of the hippocampus. Quantitative

analysis was performed only in the CA1 region as the curvature of CA3 region makes it almost impossible to estimate the length of different layers, therefore normalization is not possible. For precision counting, a grid, dividing the optical field in smaller squares, was projected on the screen during analyses. Interneurons were identified and assigned to a certain interneuron type based on their neurochemical marker co-expression; this was assessed "online" by using/changing the microscope's filters. For identification and assignment of interneurons to a certain type, we scanned each visual field, initially with one filter, by manually changing the focal depth throughout the thickness of the section. Once a specific cell labeling was identified, the filters were changed without modifying the focal depth and visual field, to check for the co-localization of the fluorescence signal. All (single, double or triple labeled) interneurons identified by this method were drawn on the previously created detailed map for easier counting per area and region. Area and length of layers in CA1 region were measured after a prior micrometer to pixel calibration using ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

For testing if cellular alterations are significant, *F*-test for two samples of variances, and unpaired two-sample *t*-test assuming equal/unequal variances, accordingly, were performed. For statistical analysis, Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) and GraphPad InStat (GraphPad Software, San Diego, California, USA) was used. All tested samples showed normal distribution. We considered a difference statistically significant at a *p*-value of less than 0.05.

Results

Two of the surviving animals did not develop SE and had no SRS, therefore they were excluded from the study. Surviving, non-excluded rats presented SRS after a latent period lasting from one up to six weeks, with a mean time interval of 14±2.9 (Mean±SD) days. The seizure frequency showed a very diverse distribution with an average of 1.7 seizures/day.

In animals that did develop SE and SRS, we found sclerotic cell death pattern in 50% of cases and partial pyramidal cell death in the rest of the animals (Figure 1). Samples of animals not developing SE (*n*=2) showed similar histological (*i.e.*, normal) structure, as control animals.

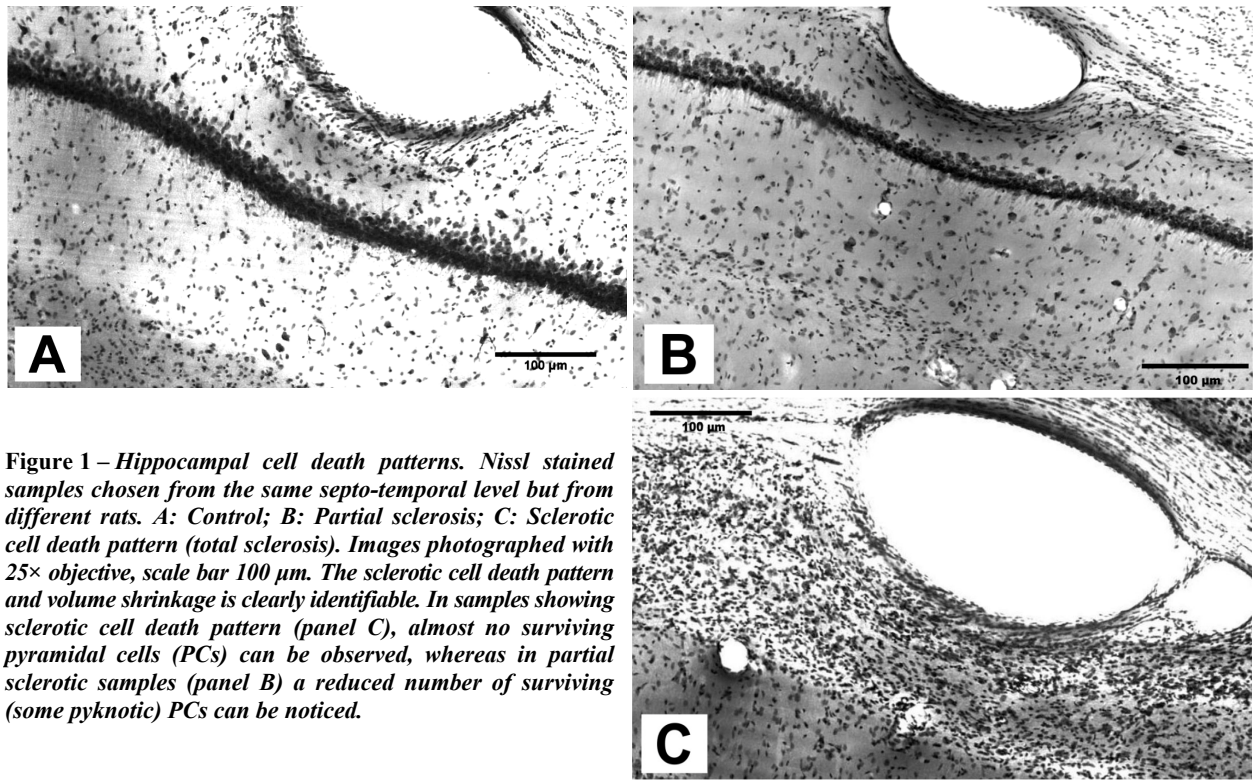

Figure 1 – ***Hippocampal cell death patterns. Nissl stained samples chosen from the same septo-temporal level but from different rats. A: Control; B: Partial sclerosis; C: Sclerotic cell death pattern (total sclerosis). Images photographed with 25× objective, scale bar 100 µm. The sclerotic cell death pattern and volume shrinkage is clearly identifiable. In samples showing sclerotic cell death pattern (panel C), almost no surviving pyramidal cells (PCs) can be observed, whereas in partial sclerotic samples (panel B) a reduced number of surviving (some pyknotic) PCs can be noticed.***

Layer thickness of *stratum oriens* and *radiatum-lacunosum-moleculare* decreased significantly (by 43.75% and 48.64%, respectively, $p<0.01$ in both cases), quantitatively sustaining the extent of volume decrease. The decrease of the pyramidal layer thickness was 18.51%, a difference that was found to be not significant ($p>0.05$). Layer length was also decreased by 25.98% ($p<0.01$), when compared to control.

Pathological alterations such as cellular loss involving structural changes (in our case hippocampal sclerosis) usually result in volume changes. Because the reduction of volume of the hippocampus on the septo-temporal axis cannot be precisely measured, we avoided counting cells by volume and instead measured density, corrected for the reduction. Our data showed that both the pyramidal layer thickness and length changes significantly. Thickness of the pyramidal layer cannot be measured precisely; the measurement of its length is possible, even if it is reduced. Therefore, we used the layer's length to define an unbiased correction factor. As layer length varies not only with the extent of volume loss, but also according to size of the brain, we defined the correction factor as the decrease of the individual layer length compared to age- and batch-matched controls' average. Therefore, in order to calculate

the corrected density, the number of interneurons was first divided by the length of the CA1 pyramidal cell layer, and then multiplied with the correction factor.

We first analyzed the extent of GABAergic interneuronal alterations separately for samples of animals showing sclerotic, respectively partial sclerotic pyramidal cell loss. Although an increasing trend can be observed in the case of the perisomatic inhibitory interneurons (basket and axo-axonic cells), the changes were not significant (p>0.05 for all four interneuron types, Figure 2); thus, in our experiments, we found no correlation between the degree of hippocampal sclerosis and interneuronal alterations.

Oriens-lacunosum moleculare (O-LM) cells were identified as PV+, SOM+ and NPY- interneurons with their soma localized mainly in *str. oriens* and in *str. pyramidale* (Figure 3). This cell type innervates exclusively neuronal processes in *stratum lacunosum-moleculare*. We observed a significant decrease (53.49% reduction, p<0.001) in the corrected density of the putative O-LM cells (Figure 4).

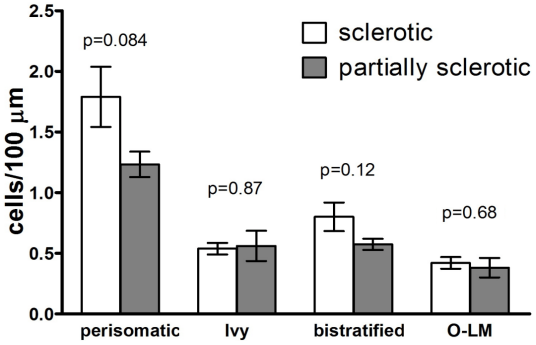

Figure 2 – ***Density (shown as corrected density, see Methods) changes of GABAergic interneuron populations in relationship with PC death pattern and structural alterations (Mean±SEM).***

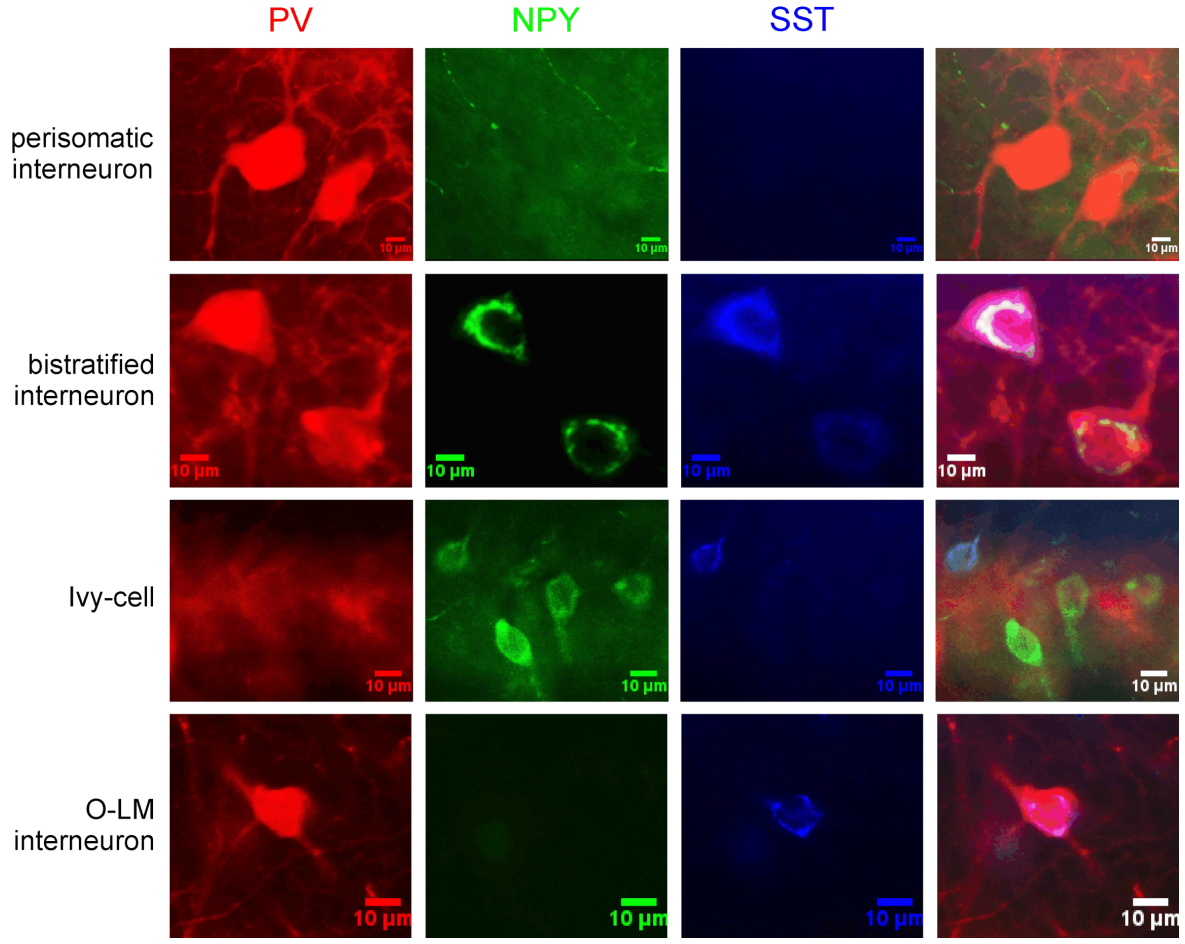

Figure 3 – ***Identification of interneuron types in the CA1 region. Single channel and composite images of the studied interneuron types. Perisomatic interneurons were identified as PV+ SST- NPY- cells, bistratified interneurons as PV+, NPY+, SST+ cells. Putative Ivy cells were identified as NPY+, SST- and PV- cells, whereas the O-LM interneurons are PV+, SST+ and NPY-.***

Bistratified interneurons have their axonal cloud stretching into *str. radiatum* and *oriens*, and were identified as PV+, SOM+, NPY+ cells (Figure 3). The corrected density of putative bistratified cells did not show significant alteration (p>0.5) reflecting the invulnerability of this interneuron population (Figure 4).

The perisomatic inhibitory interneuron group is formed by several types of cells, including the PV+, SOM- and NPY- basket cells and axo-axonic cells (Figure 3). We have not found any significant changes either in the number or in the corrected density (p>0.5) of perisomatic inhibitory interneurons (Figure 4).

The recently discovered GABAergic interneuron population of Ivy cells were identified as PV-, SOM- and NPY+ cells (Figure 3). We observed a significant (52.84% decrease, p<0.001, see Figure 4) change in the corrected density and number of putative Ivy cells in CA1 region compared to age and batch matched control animals.

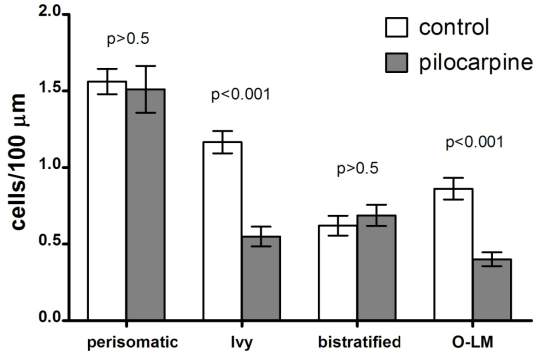

Figure 4 – ***Changes in corrected density of identified interneuron types. Data is presented as Mean±SEM. For descriptive statistics, see text.***

Discussion

The pilocarpine model of TLE is widely accepted as a homologue of human temporal lobe epilepsy and is frequently used in various studies. Studies that considered both seizure activity and histological alterations show very diverse results; this is confirmed in our study as well. Animals that developed epilepsy (spontaneous recurrent seizures) showed a diverse distribution of seizure frequency and histological alterations (structural changes, principal cells and inhibitory interneurons).

In animals with complete hippocampal sclerosis, the main excitatory (pyramidal) cells almost entirely disappeared and severe structural alterations occurred, such as reduction of layers *oriens* and *radiatum-lacunosum moleculare* to half of the initial thickness and length. Half of the animals showed milder hippocampal sclerosis and structural alterations, with a higher number of surviving pyramidal cells (seen between pyknotic cells). Earlier results in the literature have claimed that hippocampal sclerosis is the cause of epilepsy [29], however this view was disputed later [12]. In our study, rats developed SRSs even with milder hippocampal sclerosis; therefore, our data also suggests that severe histological alterations, which in humans are usually caused by brain trauma, hemorrhage, ischemia, infections, are not compulsory for epilepsy to evolve.

Earlier studies, in animal models and in human tissue [30–32], did not find a correlation between the extent of the interneuronal cell loss and the degree of hippocampal sclerosis. However, it must be noted that in these studies no attempt was made to differentiate between interneuron types. We tried to correlate the degree of hippocampal sclerosis with each identified interneuron type separately. Although the density of perisomatic inhibitory interneurons was higher in the sclerotic tissue (compared to sections with incomplete sclerosis), this change was not significant; the density of the other interneuron types did not change.

The cortical area with the least heterogeneous neuronal population and the smallest number of extrinsic inputs is probably the CA1 region of the hippocampus; therefore, it is well suited for studying both physiological and pathological processes [28]. The relatively uniform principal cells are supported by a rich diversity of GABAergic interneurons [33]. These interneurons provide general inhibition and temporally regulate pyramidal cell activity, therefore they have an important role in any, physiological or pathological, synchronous network activity. It must be noted however that there is a division of labor among inhibitory interneurons as distinct interneuron types subdivide the surface of pyramidal cells and act in discrete time windows. Our study tries to identify alterations of several interneuron types that may cause the pathological synchronization of the pyramidal cells.

Entorhinal cortical (EC) input is mainly modulated by O-LM interneurons, which exercise layer specific dendritic inhibition [26, 28, 34]. Our results show impaired dendritic inhibition; a significant part of the O-LM cells that modulate directly the entorhinal cortical input in *stratum lacunosum-moleculare* were lost. Bistratified interneurons modulate dentate gyrus and CA3 processed information from EC, as well as exercise inhibition in the *stratum radiatum* and *oriens*; it was suggested that they have role in the modulation of theta rhythm [35]. The lack of significant changes in density suggests the preservation of these cells in contrast to pyramidal cell loss. However, it is not known whether by synaptic reorganization the GABAergic inhibition increases on surviving pyramidal cells or the axon segments projecting to lost PC will degenerate.

The overall excitatory hippocampal activity and homeostasis may be controlled by NPY release, as suggested by *in vitro* patch clamp studies that showed a suppression of epileptiform activity with NPY [36]. Although the amount of *in vivo* evidence that exists currently is not sufficient, several recent studies hypothesized that Ivy cells [27, 37] may play a role in controlling excitability. In our study, we observed a robust reduction of putative Ivy cell population. Ivy cells are known to be NPY+ and show NOS and GABAARα1 immunoreactivity with the soma localized mainly in pyramidal layer. We identified these cells only by NPY immunopositivity and SOM and PV immunonegativity; however, no other NPY-positive and PV/SOM-negative cells are yet known in pyramidal layer [27].

PV+ basket cells and axo-axonic cells are considered perisomatic interneurons. This group also includes two CCK+ (cholecystokinin) basket cell populations that are not discussed in our study, as we have not found any current data about changes of CCK cells in any model of epilepsy. Although in our study, it would be very important, PV+ basket and axo-axonic populations cannot currently be differentiated, except by electron microscopic examination of synaptic targets [26, 28]. In most of the before mentioned studies, perisomatic interneurons were identified as cells with PV immunoreactivity without differentiation of the two subgroups. Furthermore, previous quantitative studies did not exclude from the quantitative analysis other PV+ cells, such as O-LM (PV+ and SOM+) or bistratified (PV+, SOM+ and NPY+) cells. In our experiments, we differentiated these cells by using triple immunofluorescent labeling.

Perisomatic interneurons and output controlling axo-axonic cells (also called chandelier cells) did not show any alterations. Literature describes perisomatic inhibitory cells as resistant both in kainic acid and pilocarpine models [32, 38–40]. Other studies describe a slight loss in CA1, CA2 and subiculum, but relatively little difference was observed concerning these interneuron numbers in CA1, and even a relative increase in their density is noted [41].

In sclerotic CA1, PV-positive hypertrophic chandelier axon terminals and basket formations were described, which may reflect an enhanced perisomatic inhibition to the surviving CA1 pyramidal neurons [41]. Electron-microscopic examination of pyramidal and granule cell bodies in PV-immunostained sections of the epileptic human hippocampus also showed preservation of perisomatic innervation [42–44], which might compensate to some extent for the impairment of dendritic inhibition. An increase of GABA synthesizing enzyme GAD is also noted [43, 45]. The preservation and/or enhancement of perisomatic inhibition might reflect a compensatory mechanism of GABAergic inhibition of the hippocampal output, but also this enhanced and pathologically timed inhibition might allow the large-scale synchronization and propagation of the hyperexcitability to the neocortex [38, 40].

Besides cell loss, axonal sprouting is an important morphological change, which was described quite early, first at the level of granule cells [46–48]; later aberrant axon collaterals of CA1 pyramidal cells were characterized [49]. Furthermore, a wide range of evidence shows axonal sprouting of the GABAergic interneurons [50, 51]. Studies in chronic epileptic animals have indicated the occurrence of changes in the hippocampal network, including, but not limited to, sprouting. Such changes could be associated with enhancement of synaptic efficiency and may be important in epileptogenesis [52]. However, it is unlikely that any one structure, system or pathway alone is uniquely critical for epileptogenesis; instead, probably loss of selective types of interneurons, alteration of GABA receptor configuration, sprouting and other ultrastructural changes together could contribute to the development of spontaneous seizures [53]. In light of these discoveries, further studies are needed to assess not only the cellular density but also the relationship between fiber and cellular density. The lack of investigation of the ultrastructural changes that may be associated with the described cellular density alterations is a limitation of the present study.

Conclusions

The pilocarpine model of temporal lobe epilepsy shows a diversity regarding spontaneous recurrent seizure frequency and histological alterations. In our study, the occurrence of spontaneous recurrent seizures could not be correlated with the hippocampal sclerotic cell death pattern. In the GABAergic network, an impaired dendritic inhibition was observed accompanied with the preservation of perisomatic inhibition. The reduction in the number of the putative O-LM cells suggests that the modulation and temporal phasing of the entorhinal cortical input might be corrupted. General excitability controlling Ivy cell death extent is also severe. The surviving interneurons numerically might not be enough to exert the modulation of the remaining pyramidal cells. In this animal model, considered homologue of human TLE, the observed changes together may allow the large-scale synchronization that is followed by the propagation of the hyperexcitability to the neocortex, and thus the epilepsy can evolve to the recurring seizures state.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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