Changes of CB1 cannabinoid receptor distribution in temporal lobe epilepsy

PhD thesis

Mária Rita Karlócai

János Szentágothai School of Neurosciences
Semmelweis University

Supervisor: Zsófia Maglóczky PhD

Official reviewers: József Takács MD, PhD
Bence Rácz PhD

Members of the Final Examination Committee:
József Kiss MD, PhD, DSc (chair)
Andrea Székely MD, PhD
Hajnalka Ábrahám MD, PhD

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Introduction

According to the definition of WHO epilepsy is a chronic neurological disorder with various etiologies that affects people of all ages and is characterized by recurrent seizures. Approximately 5 million people worldwide have epilepsy. Seizures can vary from the briefest lapses of attention or muscle jerks, to severe and prolonged convulsions (i.e. violent and involuntary contractions, or a series of contractions, of the muscles). Seizures can also vary in occurrence, from less than one per year to several times per day.

At the neuronal network level it manifests as a state of pathological hyperexcitability and hypersynchronous activity. Imbalanced synaptic inputs may cause excessive neuronal activity, eventually leading to neuronal death and synaptic reorganization. Though, nowadays a high variety of antiepileptic drugs are available, a significant number of patients are pharmacoresistant (cannot be cured with medication). In case of therapy resistant patients with severe clinical consequences, and where the epileptic focus can be precisely localized, epilepsy surgery is a possible solution. The aim of the surgery is ending seizures by lesioning the epileptogenic focus. Approximately 85% of partial epilepsies originate from the temporal lobe, thus the surgery most often carried out is anterior temporal lobectomy.

The endocannabinoid system

Activation of the endocannabinoid system is involved in numerous physiological functions like food intake, pain sensation and memory formation. In the brain, the endocannabinoid system is responsible for retrograde synaptic signaling via activation of cannabinoid receptors type 1 (CB1-Rs). Endocannabinoids are released from the postsynaptic neurons in an activity-dependent manner, and bind to presynaptically located CB1-Rs, thereby suppressing transmitter release from axon terminals.

In addition to its physiological roles, this system was found to be affected in pathological processes as well. Controversial data were published regarding the effects of cannabinoids in epilepsy. On one hand, in an animal model of temporal lobe epilepsy (TLE), CB1-R agonists displayed anti-epileptic effects, which were proposed to be mediated via attenuating glutamate release. On the other hand, proconvulsive effects of CB1-R agonists were described as well. Moreover, a CB1-R antagonist was shown to prevent the long-term increase in seizure susceptibility, when applied in a certain time-window.

Moreover, human studies showed that recurrent seizures may lead to an adverse reorganization of the endocannabinoid system and to the impairment of its protective effect. CB1-Rs located at inhibitory synapses can be upregulated in the dentate gyrus, whereas downregulation of CB1-Rs located at excitatory synapses may occur in the inner molecular layer of the dentate stratum moleculare.

Aims

Specific questions we wished to answer:

-How does the strength of acute seizures correlate with later cell death and reorganization?

-What kind of anatomical changes can be found in the endocannabinoid system in the acute, the latent and the chronic phases of epilepsy at the light and electron microscopic levels?

-What kind of changes can be seen in the function of the endocannabinoid system in the acute phase using *in vitro* slice preparations?

-Does the seizure susceptibility in CB1-R knock-out animals differ from controls?

-Is the target distribution of CB1-R immunopositive elements altered in the mouse model of epilepsy and in human TLE patients?

Materials and methods

The pilocarpine model of epilepsy

For this animal model 20-30 g male CD1 mice and CB1-R knock-out mice were used. Animals were assigned to control and experimental groups. Age-matched control mice were injected with physiological saline. Experimental mice were injected with intraperitoneal Pilocarpine hydrochloride (340 mg/kg) to induce status epilepticus (SE) Scopolamine methyl nitrate (5 mg/kg) was injected 30 minutes in advance to reduce peripheral cholinergic effects of pilocarpine. After injection the behavioral signs of seizures were monitored and scored. Seizures were classified by using the modified Racine's scale (1-5), animals were separated into "weak" and "strong" groups according to their seizure activity. Seizure activity was defined for every animal by using the maximal value of Racine -scale reached more than once.

Acute phase was examined 2 hours after pilocarpine injection. The period examined 1-3 days after the injection was regarded as the latent phase. EEG recordings were carried out 1 month after pilocarpine treatment in the chronic phase At this time point recurrent seizures occurred in most of the strong epileptic animals.

In vivo electrophysiology

Mice were anesthetized with a 1-1.5 % halothane-air mixture and secured in a stereotaxic frame. Stainless steel wire electrodes were placed on the skull and covered with conductive paste to decrease the impedance. The electrodes and the connector were embedded in dental acrylic cement. EEG activity was filtered at 1 Hz to 70 Hz and amplified (20 k). Sampling rate was 500 Hz , amplification 20 μV/mm, filter: LP: 70Hz, HP: 1Hz.

All EEG recordings were evaluated by three independent and experienced researchers to determine the occurrence of seizures or interictal spikes. EEG recordings were conducted out in 16 mice (6 controls, 4 weak and 6 strong epileptic) in the chronic phase. In 4

animals (2 controls, 2 chronically epileptic ones) 24-hours EEG monitoring was carried out.

Human tissue

Hippocampal samples were obtained from patients (n=44) with therapy-resistant temporal lobe epilepsy. Patients with intractable temporal lobe epilepsy underwent standard anterior temporal lobectomy; the anterior third of the temporal lobe was removed together with the temporomedial structures.

Control brain tissue (N=4) was obtained from 53-65 years old subjects with 2-4 hours post mortem delays. None of the control subjects had any record of neurological disorders. Brains were removed, the dissection was performed at the Forensic Pathology Department of the Semmelweis University Medical School. After surgical removal, the epileptic tissue was immediately dissected to 3-4 mm thick blocks, and immersed into a fixative.

Immunocytochemistry

Brains were removed from the skull and 60 μm thick vibrotome sections were cut from the blocks. Following washing in PB, sections were cryoprotected in 30% sucrose for 1–2 days, and three times frozen over liquid nitrogen. Sections were processed for immunostaining against CB1-R, Neuronal Specific Nuclear Protein (NeuN) and Heat Shock protein 72 (HSP72) as follows. After H_2O_2 treatment non-specific immunostaining was blocked and sections were incubated in the antibody for 2 days. For visualization biotinylated secondary antiserum was used followed by ABC. The immunoperoxidase reaction was developed by DAB as a chromogen. The control sections were processed in the same way.

Ultrathin serial sections were collected on Formvar-coated single slot grids, stained with lead citrate, and examined with an electron microscope.

Gallyas silver impregnation

The steps of this staining are the following: 2x5min in 2% NaOH and 2.5% NH_4OH), 10 min in 0–0.8% NaOH, 2.5% NH_4OH, 0.5% $AgNO_3$, 3x5 min in 0.5% Na_2CO_3 and 0.01% NH_4NO_3 in 30% ethanol, 1 min in 0.4–0.6% formaldehyde and 0.01% citric acid in 10% ethanol, pH 5.0 –5.5, 3x10 min wash in 0.5% acetic acid.

Nissl staining

2 mins in xylene, 3 mins in ethanol abs., 3 mins in 90% ethanol, 3 mins in 70% ethanol, 3 mins in 50% ethanol, 5 mins in cresyle violet, rinse in 70% ethanol, rinse in 70% ethanol+drops of acetic acid for fixation, rinse in 90% ethanol, 3 mins in ethanol abs., 2x3 mins of xylene.

Quantitative electron microscopic analysis

Ratio and number of symmetric and asymmetric synapses

The quantity of symmetric and asymmetric synapses established by CB1-R-immunopositive axon terminals was examined in the stratum moleculare of 3 control, 3 chronic epileptic and 3 acute epileptic hippocampi. Serial sections were made from the blocks reembedded from stratum moleculare and examined using an electron microscope. CB1-R stained terminals were analyzed in every 10^{th} section in order, following the rules of systematic random sampling. Significance was tested using Mann-Whitney U-test (Statistica 6.0).

Target distribution of CB1-R immunopositive terminals

Target distribution of CB1-R-immunopositive elements was studied in the stratum moleculare of the dentate gyrus in control and sclerotic subjects, where the highest fiber density was observed. Both in controls (N=105, two subjects) and in epileptic patients (N=175, 3 subjects) the postsynaptic target of CB1-R-immunopositive terminals establishing symmetric synapses were

examined (photographed and quantified) at the electron microscopic level.

Results

Reorganization of CB1-R expressing GABAergic axons in temporal lobe epileptic patients

The expression pattern of CB1-Rs present at inhibitory synapses was studied by immunocytochemistry in epileptic hippocampal tissue derived from intractable TLE patients (N=44). The antibody used in this part of the study stained CB1-R-containing axon endings forming symmetric synapses with their postsynaptic targets.

In the hippocampi of human TLE patients the pattern of cell loss was analyzed by light microscope in sections immunostained for different neurochemical markers labeling principal and non-principal cells. Based on anatomical alteration observed in the hippocampus patients could be classified into three different groups.

(i) Epileptic Type 1 (mild) (n = 6): similar to control, no considerable cell loss is present in the pyramidal cell layer in the CA1 region , layers are visible and intact.

(ii) Epileptic Type 2 (patchy) (n = 16): pyramidal cell loss is in patches in the CA1 pyramidal cell layer, but these parts are not atrophic. Interneuron loss is more pronounced.

(iii) Epileptic Type 3 (sclerotic) (n = 22): the CA1 region is shrunken, and atrophic, more than 90% of principal cells are missing and occasionally scattered pyramidal cells remain in the CA1 region.

In the present study the distribution and localization of CB1-R-immunoreactive elements was studied in controls, in non-sclerotic (Type 1 and Type 2) and sclerotic cases (Type 3)

Distribution of CB1-R expressing GABAergic fibers in control and epileptic human hippocampus

Immunostaining revealed numerous CB1-R-positive cell bodies of interneurons scattered in all hippocampal subfields. Dendrites remained unstained, but a dense meshwork of CB1-R.immunoreactive axons covered the entire hippocampal formation. The strongest axonal labeling was found in stratum moleculare of the dentate gyrus, mostly in their inner part. Strong staining appeared also in stratum pyramidale of CA1-CA3 containing numerous axon terminals. In contrast, no apparent labeling occurred in the neuropil in the hilus and str. lucidum.

In the non-sclerotic cases, the distribution of CB1-Rs in the dentate gyrus did not show any major changes compared to the normal post mortem controls. In contrast, a strong increase in CB1-R immunostaining was found in the dentate gyrus of epileptic patients with CA1 sclerosis, similar to what we have seen in the mouse model (see below). Immunopositive interneuron somata were preserved both in the dentate gyrus and in the CA1 area. The density of immunostained fibers increased in the dentate molecular layer and became inhomogeneous in the hilus forming dense arrays of boutons around the surviving mossy cells and interneurons.

Qualitative analysis revealed an increased density of immunostained axonal meshworks in the sclerotic stratum moleculare compared to the control or non-sclerotic cases. To quantify this increase, sections with fluorescent immunostaining were processed in 3 control and 10 sclerotic cases, and the density of immunolabeling was measured by confocal laser scanning microscopy. The results showed that the density of fibers had significantly increased in epileptic cases (type 3).

Ultrastructural analysis

Electron microscopic examination confirmed that the antibody used in this study visualized CB1-Rs only on terminals giving symmetric

synapses. The cellular and subcellular localization of CB1-Rs was similar in the epileptic and control cases. Target distribution of CB1-R-immunopositive elements was studied in the stratum moleculare of the dentate gyrus in control and sclerotic TLE subjects. Examination of immunogold terminals showed that CB1-Rs were localized in the membrane, outside the synaptic active zone. Both in controls (N=105, two subjects) and in epileptic patients (N=175, three subjects) the CB1-R-immunopositive terminals established symmetric synapses mostly on dendrites (75 v.72,5 %, spines 13,2 v. 15,5 %, and cell bodies 11,8 v. 13 %, in control and epileptic subjects, respectively).

Since changes affecting the endocannabinoid system described in the literature are quite confusing we used an animal model of TLE to study alterations of CB1-Rs in the acute, latent and chronic phases of epilepsy.

Changes of CB1-R immunostained fibers in the mouse model of TLE

Epilepsy related alterations found in the human tissue were confirmed by examining changes in the same features in the mouse model (which shall be described in details later in the study).
The upregulation of CB1-Rs linked to symmetric synapses was seen in the mouse model as well, similar to that in human patients.
Quantification of the target distribution of CB1-R-immunopositive elements was carried out in the mouse model to compare it to those obtained from human tissues. Stratum moleculare of the dentate gyrus was studied in control and sclerotic animals. Similar to controls and human tissue, CB1-Rs were localized in the membrane, outside the synaptic active zone. Both in controls (N=53) and sclerotic cases (N=48) the target distribution of CB1-R immunopositive fibers was examined. Stained terminals established symmetric synapses mostly on dendrites (77.4 v.77.1%, spines 22.6

v.20.8% and cell bodies 0 v. 2.1 %). Thus, target distribution was unchanged, similar to results observed in human tissue.

The pattern of cell loss in pilocarpine induced epilepsy

In the next part we examined our animal model in more details. Based on the behavioral signs during the acute seizures, animals were classified as "weak" or "strong" epileptic animals using the modified Racine scale. Mice showing seizure intensity from Racine 1 to Racine 4 (shaking, chewing, nodding, forelimb clonus, rearing but no tonic-clonic seizures) were assigned to the weak group. However, all animals in the strong group exhibited strong tonic-clonic seizures (Racine 5) with other less severe behavioral manifestations. We examined the cell loss pattern in the hippocampi at different survival times using NeuN-immunostaining, GluR2/3-immunostaining and Nissl staining.

In the acute phase (2 hours) no cell loss was observed neither in the weak nor in the strong animals. In the latent phase (1-3 days post-pilocarpine) loss of sensitive interneurons and few principal cells could be seen in the CA1 and CA3 regions. In the chronic phase patchy cell loss was found infrequently in "weak" animals, but none of their hippocampi showed sclerosis. In contrast, hippocampi of the "strong" mice showed sclerosis in most cases (70%) meaning that CA1 and CA3 regions were shrunken, atrophic with more than the half of the cells missing.

Based on the severity of cell death, the degree of damage affecting different subregions (CA1, CA3, DG) the following classification was used (with a semiquantitative scale): type 1 (mild cell loss<10%), type 2 (patchy cell loss between 11-50%) and type 3 (sclerotic, (cell loss over 50%,). Statistical analysis revealed a significant correlation between the degree of cell loss and seizure strength in Racine scale values (105 animals; $p<0.05$; Pearson correlation). *Thus, behavioral signs of the acute seizures could be used to predict the degree of cell loss in the chronic phase*.

In vivo electrophysiological recordings

EEG recordings were carried out in 16 mice (6 controls, 4 weakly and 6 strongly epileptic mice) in the chronic phase. On the basis of these recordings, the EEG activity of pilocarpine treated mice differed from controls. strong synchronization and numerous interictal spikes were seen separately or in clusters. In weak animals recurrent seizures were rarely seen (in 1 mouse out of 4), while interictal spikes (incidence 2.2±2.26Hz, amplitude 581±160 μV) occurred in all cases. In members of the strong group recurrent seizures appeared (ictal spikes with a frequency of 5.9±2.3Hz, amplitude 546±112 μV) in all but one animals (5 out of 6).

In 2 controls and 2 strong mice 24 hours long EEG monitoring was carried out to investigate nocturnal epileptic activity. No animals showed exclusively nocturnal seizures, but they exhibited recurrent seizures during the whole day. Seizure incidence was the highest in the afternoon compared to other periods of the day.

Anatomical changes in the latent phase of epilepsy (1, 3 days post pilocarpine)

Immunostaining against Heat Shock Protein 72 (HSP72) was carried out to visualize cells suffering from excitotoxic damage, since the presence of this protein confirms the emergence of abnormal proteins due to various damaging effects.

In control hippocampi and in the acute phase of epilepsy immunostained cells were never seen, only light background staining appeared occasionally in the layers of principal cells. In the latent phase positive cells were not seen in weak epileptic animals, however, in members of the strong group robust changes occurred 1 day after the induction of the seizures. Principal cells with Golgi-like HSP72-staining appeared occasionally in mossy cells of the hilus in the str. pyramidale of the CA3, and among the granule cells.

Occasionally few immunopositive cells were seen in the CA1 str. pyramidale as well.
Notably, three days after pilocarpine treatment a marked increase in HSP72 expression was found in CA1 and CA3 (mostly CA3c) pyramidal cells, and in the mossy cells of the hilus.
Irreversibly damaged cells were visualized with Gallyas silver impregnation, during this reaction silver binds to abnormal proteins; therefore, irreversibly damaged cells turn black in contrast to other living cells with red/orange staining.
One day or three days after the injection no visual changes could be seen in weak animals. In the strong animals 1 day post pilo a dark silver deposit was present in hilar neurons undergoing argyrophilic degeneration and occasionally in stratum oriens of the CA1 or CA3 pyramidal cells.
Three days after pilocarpine administration silver accumulation appeared in additional subregions; besides stratum oriens, numerous degenerated cells or dendrites were present in strata pyramidale and radiatum of the CA1 and CA3 moreover, several degenerating somata and dendrites appeared in the hilus.

Analysis of CB1-R distribution in different phases of pilocarpine-induced epilepsy

Distribution of CB1-Rs in control tissue
In control samples intense CB1-R immunostaining was found throughout the hippocampus. Immunopositive cell bodies of interneurons were seen in all hippocampal subfields, mostly in strata radiatum and lacunosum-moleculare of CA1 and CA3, as well as at the border of the hilus and in the inner molecular layer of the dentate gyrus. Intense staining of CB1-R-positive fibers was observed in stratum pyramidale of the cornu Ammonis and in the molecular layer of the dentate gyrus (DG). In contrast, less dense labeling was observed in the strata lucidum and granulosum.

Distribution of CB1-Rs in the acute phase of epilepsy

In the acute phase (i.e. two hours after seizure onset), epileptic hippocampi from members of the weak group showed control-like phenotype, namely no major changes were seen in the distribution and density of immunolabelled elements. In contrast, the tissue from the strong group exhibited a robust decrease in immunopositivity throughout the hippocampus, i.e. the dense axonal meshwork found in the strata moleculare, radiatum and oriens was substantially reduced. Moreover, CB1-R immunopositive boutons forming baskets in the principal cell layers could hardly be seen at light microscopic level. In this phase a global downregulation of the protein appeared.

Reversible changes in CB1-R expression in acute slices (2 hours post pilo)

To investigate the electrophysiological correlates of our anatomical findings observed in acute phase, we planned to record the effects of CB1–R activation on hippocampal function. *In vitro* slices were prepared from the hippocampi of control and strongly epileptic animals. As a first step, immunostaining for CB1-Rs was carried out on the slices at two time points: immediately fixed after the slice cut and after 2 hours of incubation in an interface-type holding chamber. Surprisingly, we observed that after 2 hours of incubation, slices from strongly epileptic animals showed control-like distribution of CB1-Rs. However, when slices from the same animals were immediately fixed after cutting, a similar decrease in CB1-R distribution was found as seen in perfusion fixed animals (compared to controls).

Increased mortality after seizures in CB1-R knock-out animals

We addressed the question how seizure susceptibility changes occurred in CB1-R knock-out mice. Therefore, 22 controls were

examined, 22 CB1-R knockout mice and their 21 wild type littermates were injected with pilocarpine in a different set of experiments. CB1-R knock-out animals developed very intense seizures (typical for strong epileptic animals) as they were more susceptible to epilepsy. Of the 22 animals only 3 animals had mild seizures , whereas, the 19 animals showing strong seizures died in 15 minutes.

Mice experiencing only mild or no seizures (members of the weak group), survived and later were sacrificed in the chronic phase. These animals showed no signs of anatomical alterations, NeuN and Gallyas labeling was similar to the staining in weak wild type littermates. These results demonstrate a protective role of CB1-R activation in epilepsy as it has been shown previously.

Ultrastructural changes of CB1-R expression in the acute phase of epilepsy

At the electron microscopic level degenerating profiles were observed occasionally throughout the hippocampus. They were mostly oedemic dendritic profiles, axon terminals and spines; however, they were not selectively CB1-R-positive. To quantify the changes, systematic random sampling was carried out. We could not take advantage of dissector method, since one cannot obtain accurate counts in a reasonable time, when structures of interest form a very small fraction. Moreover, we wished to minimize alterations caused by the sprouting of non-positive fibers in the examined area, therefore, we have examined a large area (>40.000 μm^2) in a single plane.

A total of 298 terminals were digitized (169 of control tissue, 129 of strong epileptic tissue) and analyzed. First, we compared the ratio of immunolabelled axon terminals establishing symmetric versus asymmetric synapses in the same sample of 298 terminals. The percentages of CB1–R immunopositive axon endings forming symmetric and asymmetric synapses found in acute epileptic

samples were calculated and compared to the percentage observed in controls. The analysis revealed no significant change in these ratios (control: 79.6 ± 7.6 % in case of asymmetric and 20.4 ± 7.6 % in case of symmetric synapses; strong epileptic: 80 ± 4 % in case of asymmetric and 20 ± 4 % in case of symmetric synapses, $p>0.05$; Mann-Whitney Utest). To uncover changes in the overall number of stained terminals, we counted every stained terminal in a given area of str. moleculare, and normalized the results to unit area (40 000 μm^2). Compared to control tissue, a significant decrease was found in the number of labeled asymmetric and symmetric synapses (control asymmetric: 18.9 ± 2.3; symmetric: 6 ± 0.2; strong epileptic asymmetric: 11.8 ± 3.7, symmetric: 3.2 ± 0.7; $p<0.05$; Mann-Whitney test).

In the next step we counted the number of gold particles in the membrane of CB1-R-stained terminals and normalized it to the perimeter of the terminal membrane (particle /1 µm) to uncover any changes occurring on a smaller scale. No difference was found in the normalized quantity of gold particles either in case of symmetric (control: 0.69±0.29, strong epileptic: 0.59±0.34) or in case of asymmetric synapses (control: 0.64±0.27, strong epileptic: 0.63± 0.33). However, when measuring the perimeter of immunopositive terminals an increase occurred in case of symmetric synapses indicating enlargement of these terminals. Compared to controls a significant increase in perimeter ($p<0.05$; Mann-Whitney U test) was found in case of terminals forming symmetric synapses (control: 2±0.67 µm, strong epileptic: 2.63±0.87 µm) however, no such difference was observed among stained terminals forming asymmetric synapses (control: 1.9±0.76 µm, strong epileptic: 2.12±0.9 µm). Taken together, the results imply that mechanism(s) other than axon terminal degeneration could account for the loss of CB1-R staining in the acute phase of epilepsy.

To understand further changes in CB1-R expression we examined other phases as well.

Distribution of CB1-Rs in the latent phase of epilepsy (1 and 3 days post pilo)

One day after pilocarpine injection CB1-R levels were control-like both in weak and strong animals. Three days after pilocarpine injection upregulation of CB1-R immunoreactivity occurred in some animals of the strong group. There was a gradual recovery in CB1-R intensity 1 and 3 days post pilo. 3 days after status epilepticus principal cells began to degenerate and HSP72 staining was more extended referring to excitotoxic damage caused by the initial seizures. However, in the latent phase the decreased intensity of the receptor staining was no longer observed. CB1-R staining proved to be control-like or moderately increased.

Distribution of CB1-Rs in the chronic phase of epilepsy (1 and 2 months)

To study the long-term changes in CB1-R levels, their distribution was examined one month after pilocarpine injection, in the chronic phase. In general, similar alterations in CB1-R staining were found in these animals as we observed earlier with antibody recognizing only CB1-R at inhibitory terminals (Magloczky et al., 2010). In epileptic animals of the weak group the distribution of CB1-Rs was mostly control-like. Occasionally, a highly restricted upregulation appeared in str. pyramidale of CA1. In the sclerotic samples the general CB1-R immunostaining was enhanced throughout the hippocampus. The density of immunostained fibers in CA1 increased heavily in surviving elements of strata pyramidale and radiatum. Similarly, in DG a dense CB1-R-positive axonal plexus was found in strata moleculare and granulosum. Immunopositive interneuron somata were present both in the dentate gyrus and in the CA1 and CA3 areas.

Ultrastructural analysis of CB1-R distribution in the chronic phase

At the electron microscopic level, numerous glial elements and occasionally degenerating profiles were seen, changes that are typical for epileptic tissues, however glial elements were not positive for CB1-R staining. The general ultrastructural features of CB1-R-positive elements were unchanged; nevertheless, the number of stained terminals increased significantly ($p<0.05$). Changes in the ratio of stained terminals establishing symmetric versus asymmetric synapses were analyzed (169 controls, 178 strong epileptic). In epileptic animals the ratio was significantly changed; the mean percentage in control tissue was 79.6 ± 7.64% in case of asymmetric and 20.4 ± 7.6% in case of symmetric synapses, in strong epileptic tissue 51.4 ± 3.9% of the examined terminals proved to be asymmetric, whereas 48.6 ± 3.9% was symmetric ($p<0.05$; Mann-Whitney test). These results may suggest either a loss of stained asymmetric synapses or an increase of symmetric synapses, or both.

To address this question, we used systematic random sampling as described above and digitized every stained terminal in a given area of str. moleculare, and normalized the results to unit area (40.000 μm^2). Compared to control tissue a significant increase was found in the number of CB1-R-positive asymmetric and symmetric synapses (control asymmetric: 18.9 ± 2.3 symmetric: 6 ± 0.2; epileptic: asymmetric: 34.9 ± 10.9, symmetric: 32.3 ± 18.5, $p<0.05$; Mann-Whitney test). During the analysis of immunogold-labeled axon terminals, in certain terminals we noticed an increase in the number of immunogold particles in the hippocampi of chronically epileptic animals of the strong group.

To quantify these changes, immunogold particles were counted in 302 (125 controls and 177 strong epileptic) terminals. The number of gold particles attached to the membrane of CB1-R stained axon terminals forming symmetric or asymmetric synapses was counted and normalized to unit perimeter of the axon terminal membrane

(particle/1 µm). No difference was found between asymmetric synapses in control and epileptic tissue in the average quantity of gold particles (control: 0.64 ± 0.27, strong epileptic: 0.633 ± 0.46, $p>0.05$, Mann-Whitney test). In contrast, the number of immunogold particles significantly increased in axon terminals forming symmetric synapses (control: 0.69 ± 0.29, strong epileptic: 0.99 ± 0.49, $p<0.05$, Mann-Whitney test). Furthermore, the perimeter of immunopositive terminals establishing symmetric synapses significantly increased in strong epileptic animals in the chronic phase (control: 1.99±0.67 µm, strong epileptic: 2.7±0.9 µm). No such change was observed in terminals establishing asymmetric synapses (control: 1.89±0.8 µm, strong epileptic: 2.22±0.93 µm).

In summary, we found that the ratio of stained symmetric versus asymmetric synapses was unchanged in the acute phase, whereas a significant difference appeared in the chronic phase. The number of immunolabelled axon terminals forming asymmetric and symmetric synapses was decreased in the acute and increased in the chronic phase. In addition, the number of gold particles indicating the presence of CB1-Rs was increased on terminals forming symmetric synapses but only in the chronic phase (no such difference occurred in case of asymmetric synapses). These results may indicate a transient decrease of the receptor function in the acute phase leading to abnormally high transmitter release. In contrast, the elevation of CB1-R function in the chronic phase, may secure the balance of transmitter release, acting as an extremely powerful circuit-breaker on GABAergic and glutamatergic transmission.

Conclusions

In our model various aspects of epileptic cell loss and reorganization are similar to that seen in human TLE. In human TLE patients sclerosis is the most common cell loss pattern; therefore, alterations in animals with similar cell loss are likely to be relevant to human TLE.

On the basis of our results the strength of seizures in the acute phase can be used to predict future changes (e.g. cell loss) in the chronic phase. since the vulnerability of cells differs between “weak” and “strong” groups, as indicated by HSP72-expression in the latent phase. In case of CB1-R-staining, robust changes can only be found in animals with strong acute seizures. In animals with milder seizures and hardly any cell loss, changes in CB1-R distribution remains undetectable. In “strong” mice the expression of CB1-Rs related to the GABAergic and glutamatergic axon terminals is strongly decreased without specific degeneration in the acute phase. In addition, a significant increase can be seen in the number of CB1-Rs in a single terminal, but only in terminals forming symmetric synapses. The death of CB1-R knock-ou mice with strong acute seizures suggest that CB1-Rs may have a key role in the control of the first seizures, and thereby may prevent the seizures from reaching the “no-return” state. Thus, the decrease of CB1-Rs in animals with strong acute seizures may lead to elevation of glutamate release during acute seizures, as well as to the subsequent development of recurrent seizures, reorganization and cell loss. The increased density of CB1-Rs in the chronic phase may serve as a protective mechanism in most cases as proposed in earlier studies, and confirmed in the present study as well. The unchanged target distribution of CB1-R-positive elements indicate that the increased endocannabinoid tone is necessary on all cellular domains, thus; the greater input affects the somatic and dendritic regions as well.

PUBLICATION LIST

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