Examination of the interneuronal reorganization in the surgically removed hippocampi of temporal lobe epileptic patients.

PhD thesis

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INTRODUCTION

The neuropeptide substance P (SP) may play a crucial role in the regulation of hippocampal principal cell activity and in the generation of epileptic seizures. Treatment with SP enhances the self sustaining status epilepticus induced by perforant path stimulation or kainate. During status epilepticus principal cells express SP, which may modulate hippocampal excitability and contribute to the maintenance of epileptic seizures. There are many SP receptor (SPR)-positive inhibitory cells in the human hippocampus which could not be visualized by other markers and according to previous studies these cells showed plastic changes in epilepsy. Therefore we aimed to examine the alterations of SPR-positive cells in the CA1 region of the epileptic human hippocampus. The CA1 region is known to be the most vulnerable hippocampal subfield in temporal lobe epilepsy, and we were interested in alterations of an interneuron type in CA1 that was found to show plastic changes in response to epilepsy in the dentate gyrus. Alterations in the synaptic input of SPR-immunoreactive cells were quantified to see whether the recruitment of SPR-immunoreactive cells has changed in the reorganized network.

The fate of calretinin (CR)-containing cells was studied in models of epilepsy and in human epileptic patients. However, there is a contradiction in the literature. In most cases a loss of CR-positive cells was found in animal models and in the epileptic human dentate gyrus. However, the preservation or even an increase of the number of CR-positive cells has been observed by Blumcke et al. in human temporal lobe epilepsy patients. Previous studies described that in the rat CA1 region, CR-containing cells selectively terminate on interneurons. In the human hippocampus, CR-containing cells participate primarily in the innervation of other

interneurons, and to some extent principal cell dendrites as well. Therefore they are likely responsible for the synchronization of dendritic inhibitory cells, which is necessary for an efficient control of input plasticity of principal cells. We carried out detailed quantitative analyses of the number of CR-positive cells in all subfields of the hippocampus. Control hippocampi with different post mortem delays were examined to reveal whether the resulting differences in preservation can influence the density of CR-positive cells, and they were compared with surgically removed human epileptic hippocampi. In addition, the morphology of cells and synaptic target distribution of CR-positive terminals were examined to investigate the degree of synaptic reorganization of CR-containing cells in the CA1 region.

AIMS

Two non-overlapping interneuron populations – calretinin (CR)- and substance P receptor (SPR)-expressing cells - were studied in the surgically removed hippocampi of temporal lobe epileptic patients and compared with post mortem control samples.

Aims of the experiments:

- Examination of the distribution and morphology of SPR-positive inhibitory cells in the CA1 region of control and epileptic hippocampi
- Examination of the functional type of the SPR-positive cells in the hippocampal inhibitory network by colocalization experiments
- Examination of the synaptic input of SPR-positive interneurons in the control and epileptic CA1 region

- Examination of the distribution and morphology of CR-containing neurons in control samples with short and long post mortem delay and in epileptic samples
- Examination of the synaptic reorganization of the CR-positive interneurons in the epileptic human CA1 region

METHODS

The morphological changes of CR- and SPR-expressing cells were studied in the surgically removed hippocampi of temporal lobe epileptic patients (72) and compared with post mortem control samples (11). Control hippocampi were kindly provided by the Lenhossek Human Brain Program, Semmelweis Medical University, Budapest, Hungary. None of the control subjects had a record of any neurological disorders. Brains were removed 2–10 h after death. The study was approved by the ethics committee at the Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health (TUKEB 5-1/1996, further extended in 2005) and performed in accordance with the Declaration of Helsinki.

After surgical removal, the epileptic tissue was immersed into a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer. In the case of 9 samples from the 11 control hippocampi the procedure was similar. The other two of the control brains were removed from the skull after death (2 h and 4 h, respectively), both internal carotid and vertebral arteries were cannulated, and the brains were perfused first with physiological saline, followed by the fixative solution.

Immunocytochemistry

60 µm thick Vibratome sections were cut from the blocks, and sections were processed for immunostaining. The following primary antibodies were used: policlonal rabbit-anti SPR, monoclonal mouse-anti CR, policlonal mouse-anti CR, monoclonal mouse-anti calbindin (CB), monoclonal mouse-anti parvalbumin, policlonal mouse-anti cholecystokinin, monoclonal rat-anti somatostatin. In case of DAB-reaction biotinylated secunder sera was applied for 2 hours, followed by avidin-biotinylated horseradish peroxidase complex (ABC) for 1.5 hours. In case of DAB-Ni-reaction Elite ABC was applied. Sections were developed by DAB and/or DAB-Ni chromogen. For double immunofluorescent staining CY3-, Alexa-488- és FITC-conjugated secondary sera were used.

Sections were treated with 1% OsO_4, dehydrated in ethanol (1% uranyl acetate was added at the 70% ethanol stage) and mounted in Durcupan. After light microscopic examination, areas of interest were reembedded and sectioned for electron microscopy.

Quantitative analysis

Density of SPR- and CR-positive cells

Two to four sections of the hippocampi were drawn by camera lucida from control (n=7) and epileptic (n=25) samples. The area of each region was measured by the NIH ImageJ program. The cell number was determined per unit area (mm^2) in a 60 µm thick section. In the case of the CA1 region the cell number was determined also per unit length (mm) of the subfield because of the radial shrinkage of the sclerotic CA1. Data were evaluated by the Statistica 6.0 program. The non-parametric Mann-Whitney U-test was applied to compare the data of the control and the epileptic groups ($p<0,05$).

Dendritic arborisation of the SPR-positive cells

Camera lucida drawings were made of all SPR-positive cells in segments of the CA1 region (control: n=2; epileptic: n=7). The total number of dendritic branchpoints of each cell was determined. Data were evaluated by the Statistica 6.0 program. The non-parametric Mann-Whitney U-test was applied to compare the data of the control and the epileptic groups ($p<0,05$) and the non-parametric Kruskall-Wallis ANOVA test to compare data of several groups ($p<0,05$).

Synaptic coverage of SPR-positive cells

The strata oriens, pyramidale and radiatum were reembedded from the CA1 region and sectioned for electron microscopy (control: n=2; epileptic: n=8). Each immunolabelled dendrite was photographed from single sections. The perimeter of the dendrites and the length of the synaptic active zones were measured by NIH ImageJ. The synaptic coverage was determined as total synaptic length (µm) per 100 µm dendrite perimeter. Data were evaluated by the Statistica 6.0 program. The non-parametric Mann-Whitney U-test was applied to compare the data of the control and the epileptic groups ($p<0,05$) and the non-parametric Kruskall-Wallis ANOVA test to compare data of several groups ($p<0,05$).

Postsynaptic targets of CR-positive interneurons

Strata oriens, pyramidale, radiatum and lacunosum-moleculare were reembedded from the CA1 region and sectioned for electron microscopy (control: n=2; epileptic: n=6). Photographs were taken of every CR-positive synaptic terminal in each section, and the distribution of the postsynaptic target elements of the CR-immunoreactive terminals was determined.

RESULTS

Dependence of the immunostaining on age, fixation and post mortem delay

The preservation of the post mortem perfused controls and the immersion fixed controls with short post mortem delay was comparable to the immediately fixed epileptic samples and perfusion fixed animal tissues. In a previous study CR-immunostaining was claimed to be sensitive to post mortem delay. Sensitivity of CR-containing interneurons in ischaemia was also demonstrated. On the other hand, CR-positive neurons were shown to be resistant in epilepsy in a study that used control samples with long post mortem delays. In the present study, we examined quantitatively the effect of post mortem delay on the number and distribution of CR-positive elements, and these control samples with different post mortem delays were compared to the epileptic samples.

Pathological classification of the epileptic samples

Epileptic patients were classified on the basis of principal cell loss and interneuronal changes examined at the light microscopic level as follows: Epileptic Type 1 (mild) (n=12): similar to control, no considerable cell loss in the CA1 region, layers are visible and intact, their borders are clearly identified. Epileptic Type 2 (patchy) (n=22): Pyramidal cell loss in patches in the CA1 pyramidal cell layer, but these segments of the CA1 region are not atrophic. There is a slight loss in certain interneuron types. Epileptic Type 3 (sclerotic) (n=38): the CA1 region is shrunken, atrophic, more than 90% of principal cells are missing. Only the stratum lacunosum-moleculare is present in the CA1 region as a distinct layer, the others could not be

separated from each other. Considerable changes in the distribution and morphology of interneurons can be observed in the samples of this group.

Changes in the distribution and morphology of SPR-immunoreactive cells

The SPR-immunolabelled cells of the human control hippocampus are interneurons on the basis of their morphology and localization. This cell population can be found in all regions of the hippocampus, and consists of morphologically diverse cells. The immunostaining labels only the soma and dendrites of the SPR-positive cells. The majority of the SPR-immunoreactive cells of the control samples have long and smooth dendrites in the CA1 subfield.

In the Ammon's horn SPR-positive interneurons are located mainly in the CA1 and CA3a,b regions with the largest numbers in the stratum pyramidale and radiatum. In the non-sclerotic CA1 region (mild and patchy) the numbers and distribution of SPR-immunolabelled interneurons were similar to the control (control: 12,5 ± 2,14 cell/mm^2, mild: 11,53 ± 1,01 cell/mm^2, patchy: 12,68 ± 1,84 cell/mm^2), whereas in the sclerotic samples significantly fewer positive cells were detectable (4,97 ± 1,25 cell/mm^2).

The morphology of the SPR-positive cells changed significantly in the epileptic CA1 region. Numerous, shorter, often beaded and distorted dendrites are typical of the epileptic hippocampi. The number of dendritic branchpoints is increased in the patchy epileptic samples compared to the controls. However in the sclerotic cases the extension of the dendritic tree is considerably decreased. Quantitative examinations proved that these changes are significant.

Colocalization of SPR and markers of functionally different interneuron types

SPR-immunocytochemistry does not result in axonal staining. Colocalization studies were carried out with known perisomatic-, dendritic- and interneuron specific inhibitory cell markers to reveal the functional type of these cells in the hippocampal inhibitory network. We could not demonstrate a considerable colocalization with these markers. The largest colocalization was seen with CB: 8.7 % of the SPR-immunolabelled interneurons were positive for calbindin in the CA1 region, whereas 20.8 % of the CB-positive interneurons have shown SPR-immunopositivity as well. The ratio of the CB-containing SPR-cells remained unchanged in the mild type (9.6 %), but increased in the tissues with patchy pyramidal cell loss and strong sclerosis (14.5 % and 16.9 %, respectively). Furthermore, the ratio of SPR-positive CB-interneurons increased in all epileptic samples.

Examination of the input characteristics of the SPR-immunoreactive cells

Ultrastructural features of the SPR-immunoreactive elements

The receptor is located on all parts of the somatic and dendritic membrane. The synaptic contacts were confined to the dendrites. The majority (~90 %) of the synaptic inputs were asymmetric (presumed excitatory) both in the control and epileptic samples. Occasionally, zonula adherentia-type contacts occurred between SPR-immunoreactive dendrites in epileptic cases. Degenerating immunopositive dendritic shafts were also often observed in the sclerotic cases, with degrading mitochondria and cytoplasmic matrix.

Synaptic coverage of SPR-positive dendrites in the CA1 region

To investigate whether SPR-immunoreactive cells are affected by synaptic reorganization, we examined the synaptic coverage of SPR-positive cells in the control and epileptic samples. The synaptic coverage was determined as total synaptic length (µm) per 100 µm dendrite perimeter. Our results show that the total synaptic coverage (including symmetric + asymmetric) remain unchanged in the epileptic samples (control, n=257: 6,02±0,6; mild, n=168: 6,07±0,9; patchy, n=377: 5,73±1,71; sclerotic, n=205: 7,1±1,8). Although the total synaptic coverage was not altered, the ratio of the symmetric synapses considerably increased in the sclerotic epileptic tissue (control: 0,48±0,07; mild: 0,81±0,24; patchy: 0,42±0,07; sclerotic: 1,02±0,21).

Number, distribution and morphology of CR-immunostained cells in the human hippocampus

CR-positive cells form a heterogeneous cell population, with large, multipolar cells in the hilus, spindle shaped cells in the strata moleculare and oriens, a population of small cells in the entire DG and multipolar cells, scattered through the layers of the CA1-3. In the CA1 region long, smooth, radially oriented dendrites could be seen which were often juxtaposed and ran parallel in close contacts. In these juxtaposed segments puncta adherentia could be observed in high power electron micrographs.

We have examined the number and distribution of CR-immunoreactive cells in control samples with different post mortem delays and in the hippocampi of epileptic patients with varying degrees of principal cell death. In control samples with short post mortem delays (2-4 hours) CR-containing cells were present in all regions of the hippocampus.

Significantly smaller number of CR-immunostained cells was present in every subregion in the case of control samples with long post mortem delay (str. granulosum+moleculare: 34.3%, hilus: 43.9%, CA1: 23.5%, CA3: 28% of control samples with short post mortem delay). In the non-sclerotic epileptic tissues the CR-positive cell number was similar to the control samples with short post mortem delay only the CA3 region and the hilus showed a significant reduction in the immunolabelled cell number (str. granulosum+moleculare: 74.9%, hilus: 65.4%, CA1: 66.7%, CA3: 60.5% of control samples with short post mortem delay). The number of CR-immunoreactive cells was significantly decreased in the sclerotic samples (str. granulosum+moleculare: 35,2%, hilus: 32.1%, CA1: 76.1%, CA3: 21.9% of control samples with short post mortem delay). The number of presumed Cajal-Retzius cells at the border of the stratum moleculare-hippocampal fissure was also significantly decreased in the control samples with long post mortem delay as well as in the sclerotic tissues and even in the non-sclerotic cases (24.8%, 46.4% and 56.8% of control samples with short post mortem delay, respectively).

In epileptic cases, the CR-positive cells were preserved in the non-sclerotic samples but their morphology showed considerable alteration. The dendrites were varicose, segmented and showed signs of degeneration. Contacts between CR-positive dendrites originating from different CR-containing cells were less frequently seen.

Electronmicroscopic examination of CR-positive interneurons

Electron microscopic features of CR-immunoreactive profiles

Ultrastructural features of CR-positive cells: the cytoplasmic rim around the nucleus was usually thin and contained a moderate number of

mitochondria. The majority of the synaptic inputs were confined to the dendrites. The synaptic input of the dendrites was moderate both in control and epileptic tissues. Zona adherentia-type contacts were often observed between juxtaposed CR-positive dendrites. In the sclerotic tissues several degenerating CR-positive cell bodies were seen with strongly infolded or partially segmented nucleus. The dendritic structure showed signs of degeneration even in the non-sclerotic samples. Zona adherentia-like contacts between CR-positive dendritic shafts were rarely seen in the epileptic cases.

Postsynaptic target distribution of CR-positive axon terminals

Electron microscopic studies were carried out to reveal whether the synaptic target specificities of CR-positive interneurons have changed in the epileptic tissues. Two types of CR-positive synaptic terminals were found**.** One of them formed symmetric synaptic contacts and was found in all layers of the CA1 region. The other type established asymmetric contacts and was confined to stratum lacunosum-moleculare. The former were the axon terminals of local CR-containing interneurons whereas the latter presumably originated from the thalamic nucleus reuniens. In the present study, we focused on the target distribution of local CR-containing interneurons giving symmetric synaptic contacts. The postsynaptic elements were classified according to their electron microscopic morphology or CR content, as pyramidal cell dendrites, unstained interneuron dendrites, CR-positive interneuron dendrites and spines. In control samples, the most frequent postsynaptic targets of CR-positive cells were CR-positive dendrites (23%) and pyramidal cell dendrites (22.4%). Unlabelled interneuron dendrites and spines were less frequently observed among the targets (7.6% and 2.12%,

respectively). However, in epileptic tissues there were significantly fewer CR-positive dendrites (5.13% in non-sclerotic and 3.16% in sclerotic cases) and pyramidal cell dendrites (10,3% in non-sclerotic and 0% in sclerotic cases) among the postsynaptic targets. Innervation of unstained interneuron dendrites increased (35,9% in non-sclerotic and 44,19% in sclerotic cases).

Identity of interneuronal targets of CR-positive terminals in the CA1 region

CR-positive interneurons are known to be interneuron-selective inhibitory cells innervating mostly CB-positive interneurons in the CA1 region of the rat. In humans a part of them also belong to this functional group of interneurons. To investigate whether CB-containing interneurons - which mainly terminate in the dendritic region of pyramidal cells and large numbers of them survive in the epileptic hippocampus – are among the targets of CR-immunopositive interneurons in humans, we performed CR-CB double-immunolabelling in control and epileptic samples using DAB/DAB-Ni method at the light microscopic level. We found that the dendrites of CB-positive interneurons often received multiple contacts from CR-positive axon terminals both in control and non-sclerotic epileptic samples. These were presumably inhibitory contacts originating from local CR-positive interneurons. The examined CB-positive dendritic segments were located in the stratum oriens or radiatum close to the stratum pyramidale. A previous electron microscopic study proved that these layers were devoid of CR-positive terminals giving asymmetric synapses.

CONCLUSIONS

Our results suggest that the number of both SPR-and CR-positive cells is decreased in sclerotic epileptic hippocampus. In contrast, they are preserved in the non-sclerotic samples, but their morphology is considerably altered. They display plastic changes in epilepsy which correlate with the principal cell death. SPR cells show reactive changes in epilepsy including intense branching and growth of their dendritic tree, whereas dendrites of CR-positive cells are degenerated.

In the rat hippocampus, the direct action of SP on SPR-immunoreactive interneurons can enhance inhibitory synaptic input to pyramidal cells via increasing the excitability of these interneurons. Thus preservation of SPR-positive cells in the non-sclerotic epileptic human samples may be a compensatory mechanism aiming to increase the efficacy of dendritic inhibition. These observations support the hypothesis that an intense synaptic reorganization is under way even in the non-sclerotic epileptic CA1 region involving at least a part of the interneuron network, but without a massive pyramidal cell loss. The dramatic changes in the morphology of SPR-positive cells may suggest an altered function and enhanced efficacy of the SP system in the epileptic human hippocampus. Therefore, the SP system may prove to be a new drug target in the pharmacotherapy of epilepsy.

In contrast, CR-containing cells were proved to be highly vulnerable even in the non-sclerotic cases; they had segmented and degenerated dendrites. The degeneration of the interneuron specific inhibitory cells containing CR may result in impaired synchronization of dendritic inhibition and consequently, an abnormal potentiation of excitatory inputs to pyramidal cells. The degeneration of dendritic inhibitory cells containing CR may

further decrease the efficacy of dendritic inhibition. In addition the synaptic target specificity of CR-positive interneurons is altered even in the non-sclerotic samples: innervation of interneurons increased, whereas the innervation of pyramidal cell dendrites decreased which may also result in a decreased dendritic inhibition. This may partly explain why severe intractable seizures occur in non-sclerotic patients, where the majority of principal and non-principal cells are preserved. Other dendritic inhibitory interneuron populations, like somatostatin- and neuropeptid Y-containing interneurons were also shown to be vulnerable in epilepsy. The decreased dendritic inhibition caused by the above-mentioned phenomena may be partially compensated by the increased efficacy of the SP-system in the non-sclerotic cases.

These observations support the hypothesis that non-sclerotic patients have abnormal interneuronal networks with intact output from the CA1 region (with the preservation of most CA1 pyramidal cells), which may create a potent epileptogenic region.

LIST OF PUBLICATIONS

Publications connected with the dissertation

Toth K, Wittner L, Urban Z, Doyle WK, Buzsaki G, Shigemoto R, Freund TF, Magloczky Z

Morphology and synaptic input of substance P receptor-immunoreactive interneurons in control and epileptic human hippocampus.

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Brain. 2010 Sep;133(Pt 9):2763-77. Epub 2010 Jun 24.

Other publications

Zsófia Maglóczky, Kinga Tóth, Rita Karlócai, Sára Nagy, Loránd Erőss, Sándor Czirják, János Vajda, György Rásonyi, Anna Kelemen, Vera Juhos, Péter Halász, Ken Mackie, Tamás F. Freund

Dynamic changes of CB1 receptor expression in hippocampi of epileptic mice and humans

Epilepsia. 2010 Jul;51 Suppl 3:115-20.

Karlócai MR, Tóth K, Watanabe M, Ledent C, Juhász G, Freund TF, Maglóczky Z

Redistribution of CB1 cannabinoid receptors in the acute and chronic phases of pilocarpine-induced epilepsy

PLoS One. 2011;6(11):e27196. Epub 2011 Nov 4.