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DISSERTATION

# Morphological alterations in human mesial temporal lobe epilepsy and the pilocarpine treated chronic epileptic rat

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# 1. Abstract

Temporal lobe epilepsies (TLE) are frequently drug-resistant focal epilepsies. They exist in two forms: TLE with hippocampal sclerosis (HS) and without HS dependent on the differences in cell loss in the hippocampus and entorhinal cortex. An accepted animal model of TLE with HS is the pilocarpine model of TLE where spontaneous seizures with varying incidence rate develop after an initial status epilepticus following a single injection of pilocarpine. A popular hypothesis suggests that cell loss and subsequent reorganization leads to abnormal network properties with a lowered threshold for seizures and that seizure frequency is related to cell loss and subsequent reorganization.

In a first study I depicted aberrant connectivity in human resected epileptic hippocampus and in chronic epileptic rats treated with pilocarpine at the age of three to four month. The degree of reorganization was less obvious in non-HS TLE than in HS TLE. The reorganization in the DG was similar in pilocarpine-treated rat at an age of 5 weeks while reorganization within area CA1 was less obvious. To test whether the degree of reorganization affects seizure initiation threshold, slices from human hippocampus were exposed to different methods for induction of seizure-like events. We found it initially difficult to induce ictaform activity by common protocols, only elevation of extracellular potassium concentration was able to induce self-sustained ictaform activity. The threshold for induction of ictaform activity was lower in HS than in non-HS tissue. Subsequently we found that epileptiform activity in tissue of drug-resistant TLE patients was resistant to CBZ in 82% of patients. The effect of CBZ in tissue of TLE patient did not depend on the type of activity, hippocampal pathology, excitability of the tissue, or equilibration time of the drug.

We hypothesized that cell loss in TLE is related to the metabolic state of the tissue. Recordings of NAD(P)H fluorescence with simultaneous monitoring of extracellular potassium concentration ($[K^+]_0$) in the human dentate gyrus, CA3, CA1 and subiculum revealed strong alterations in coupling of neuronal and metabolic activity. In the last publication, we investigated whether the reduced threshold for seizure induction by acetylcholine agonists is related to expression of the ACh hydrolyzing enzyme acetylcholinesterase (AChE). Although the total activity of AChE was not altered, epileptic rats showed alternative splicing of AChE pre-mRNA transcript. These studies point to functional alterations as the main cause of epilepsy.

## 2. Introduction and aims

TLE is a focal form of epilepsy, which is in 70% of cases resistant to pharmacological treatment (67, 71). Pathological examination of resected tissue in patients with mesial temporal lobe epilepsy (TLE) often reveals hippocampal sclerosis (HS), also called mesiotemporal sclerosis or Ammon's horn sclerosis (AHS) (78, 70, see also 28). HS is characterized by severe neuronal degeneration and astrogliosis usually affecting CA1, CA3 and CA4, with relative sparing of CA2 and dentate gyrus granule cells (75, 54, 70). It was shown that such epilepsies are also characterized by cell loss in the entorhinal cortex (20). In the sclerotic hippocampus, neuronal degeneration and astrogliosis are accompanied by mossy fiber sprouting into the supragranular layer of the dentate gyrus (SGL) (53).

Mossy fiber sprouting (MFS) is a major anatomical reorganization seen in patients with TLE and in animal models of TLE (83, 84, 53 see also 44, 28). Mossy fibers originate from granule cells in the dentate gyrus that normally innervate interneurons, mossy cells and CA3 pyramidal cells. MFS indicates innervation of postsynaptic targets in abnormal locations, including the granule cell dendrites in the inner molecular layer (IML) of the dentate gyrus (86, 42, 84, 32, 5, 76) and basal dendrites of CA3 pyramidal cells in the hippocampus proper (72, 73). Mossy fiber sprouting is one form of axonal plasticity that might be an attempt of the brain to restore function following neuronal damage. There was a correlation between density of sprouting and severity of hilar cell loss both ipsilaterally and cotralaterally (14, 68, 64). Axonal growth and sprouting is a pathological hallmark of TLE and seems to be crucial for reorganization and dysfunction of the local hippocampal circuit (85, 44). It was hypothesized that formation of aberrant fibers is a necessary condition for chronic epilepsy and that it may also be related to pharmacoresistance. By using different morphological methods, I characterized cell loss and abnormal network formation and correlated them with sensitivity to high $K^+$-induced seizures as well as with pharmacoresistance to carbamazepine.

Evidence from different studies suggests increased expression of multidrug resistance proteins in human epileptic tissue (3, 87). These transporters are strongly dependent on sufficient supply with ATP. We therefore studied the tissue capability to

adapt neuronal metabolism to neuronal activity. ATP synthesis in neurons is mostly dependent on oxidative phosphorylation and thus on appropriate delivery of NADH and $FADH_2$ to the mitochondrial electron transport chain. NADH can be monitored due to its autofluorescence. It was previously shown that neuronal activation leads to a decline in NADH fluorescence followed by prolonged increases in the NAD(P)H signal.

Different models of status epilepticus-induced hippocampal sclerosis exist at present in which a subgroup or all animals develop spontaneous seizures after a 1-3 hr period of status epilepticus and a subsequent seizure free period of usually more than 2 weeks (49, 14, 31). In the rodent pilocarpine model of epilepsy, a single systemic injection of pilocarpine (a cholinergic agonist) could induce status epilepticus (SE) and cell damage that is similar in many respects to damage observed in TLE patients (89, 15, 45). In our studies, pilocarpine injection led to a status epilepticus after 38 min on average. Following a seizure-free interval (silent period) of about 2-3 weeks they began to develop recurrent spontaneous seizures.

Seizures induced by pilocarpine are triggered by muscarinic receptor activation and can also be produced by injection of inhibitor of the ACh hydrolyzing enzyme, acetylcholinesterase (AChE) (90, 91). Changes in muscarinic functions have been suggested to play a significant role in the pathogenesis and maintenance of TLE, however the details and possible role of cholinergic dysfunction in the pathogenesis of epileptic syndrome are not known. Interestingly, excess muscarinic activation results in long-lasting modifications of gene expression and protein levels of key-cholinergic proteins (38, 79, 58). Such changes involve a shift in alternative splicing of the AChE pre-mRNA transcript. This yields elevated levels of the ‘readthrough’ AChE-R mRNA transcript, translated into soluble AChE-R monomers rather than the primary ‘synaptic’ AChE-S membrane adhered tetramers (58, 96). These two AChE variants can both hydrolyse AChE with similar efficiency, yet their different C-terminal sequences affect their subcellular localization (79, 96). In the hippocampus, such alterations in cholinergic transmission are associated with enhanced muscarinic receptor-mediated responses (57). I therefore also became interested in testing for functional alterations in cholinergic signaling in TLE structures.

# 3. Methods

## 3. 1. Hippocampal tissue from patients

Hippocampal tissue of pharmacoresistant temporal lobe epilepsy patients as determined by the Epilepsy Center of Berlin-Brandenburg according to the German and European Guidelines for presurgical evaluation (6, 24) were investigated in these studies. These studies were approved by the ethics committee of the Charité-Universitätsmedizin Berlin (joint medical faculty of the Humboldt University and of the Free University of Berlin, Germany). A written informed consent was obtained from each patient. All operations were done by Dr. Thomas-Nicolas Lehmann, guaranteeing the same dissection technique of the hippocampus.

## 3. 2. Pilocarpine animals

I used the post-pilocarpine status model (15). Rats (initially at an age of 12-14 weeks and later of 5 weeks in order to reduce mortality) were subjected to systemic treatment with pilocarpine (i.p.; 320 mg/kg, Sigma, St. Louis, MO) after a pretreatment with methylscopolamine (s.c.; 1 mg/kg, Sigma, St. Louis, MO) 30 min before pilocarpine application in order to reduce peripheral cholinergic effects. All rats developed a generalized convulsive status epilepticus (SE) after an average of 38 min. To terminate SE, rats were injected with diazepam (i.p.; 10 mg/kg, Ratiopharm, Ulm, Germany) after 1 hr and 20 min of sustained SE. Rats were then subcutaneously injected with ACSF supplemented with lactate (27.2 mM) and sodium hydrogen carbonate (71 mM) 90 and 210 min after diazepam injected in order to improve survival. Control animals were treated with the same protocol but instead of pilocarpine, saline was injected. Rats had free access to food and water.

## 3. 3. Cresyl violet staining

Paraformaldehyde (PFA, 4% in 0.1 M phosphate buffer)-fixed slices from humans and rats were incubated in 30% sucrose solution (0.1 M phosphate buffer) for 24 h and cut using a freezing microtome (Leitz, Leica Microsystem, Wetzlar, Germany).

Sections of 30 µm thickness were exposed to an ethanol series (3 min for each step): absolute ethanol, 96%, 90%, 70%, 50%, 30%, distilled water, then were immersed in cresyl violet solution (0.5% cresyl violet in distilled water) for 1 min 30 sec, briefly in distilled water and exposed in differentiation solution (50% ethanol and 3 drops of acetic acid 100% for 200 ml solution). Afterwards, sections were exposed again to another ethanol series: 70%, 90%, 96%, absolute ethanol, 2 times 2-isopropanol, 2 times xylol, embedded and coverslipped with depex (DePeX, Serva Electrophoresis GmbH, Heidelberg, Germany).

### 3. 4. Dextran-amine staining

Two hours after preparation without further manipulation, acute slices were marked with the fluorescent tracers fluorescein (fluoro-emerald®) and/or tetramethylrhodamine (rhodamine, fluoro-ruby®; Molecular Probes, Europe BV, Leiden, The Netherlands) with molecular weight 10 000. Small crystals were briefly prepared in advance. Using a 27-gauge cannula, a lesion was made in the stratum radiatum of area CA1 by vertical movement of the cannula under microscopic control (25 x magnifications). Immediately after this, a single crystal of the dye was inserted into the lesion. In order to permit good anterograde and retrograde labeling of neurons, slices were left for 8 h in the chamber and subsequently immersed into the fixation solution. For further processing, fixed slices were impregnated with 30% sucrose (0.15 M phosphate buffer) solution overnight and sections of 30 µm (for cell counting) and 50 µm thickness were cut using a freezing microtom (Leitz, Leica Microsystems, Wetzlar, Germany). All sections of 50 µm thickness were mounted on gelatin-coated slides, air-dried and coverslipped with non-fluorescent mounting media (Citifluor AF-1, W. Planet GmbH, Hamburg, Germany).

The dextran-amine fluorescent sections were studied with epifluorescent microscopy (Zeiss Axioscope, Zeiss, Oberkochen, Germany; excitation wavelength 495 nm with filter set 09 for fluorescein; excitation wavelength 550 nm with filter set 15 for rhodamine). After digital imaging with a microscope-mounted camera (DX30, Kappa opto-electronics, Gleichen, Germany) the images were processed with the software Kappa ImageBase Control® (Kappa opto-electronics, Gleichen, Germany).

### 3. 5. Neo-Timm staining

Two horizontal combined entorhinal-hippocampal slices per hemisphere were taken from each animal. The first set was obtained at a ventral level range -6.8 up to -7.6 from bregma (66) and two were from middle level, bregma -5.3 up to -6.6. Slices were immediately submerged in reactive solution (0.238 g $NaH_2PO_4$ and 0.234 g $Na_2S$ in 20 ml distilled water) for 45 min, 0.25% glutaraldehyde (0.15 M phosphate buffer) for 15 min, then in 70% ethanol (0.15 M phosphate buffer). Slices were incubated in 30% sucrose solution for 1 h prior to sectioning. Subsequently, 30 µm sections were cut using freezing microtome (Leitz, Leica Microsystem, Wetzlar, Germany). Sections then were immersed in the developing solution consisting of 80 ml aqueous solution containing 5.1 g citric acid, 4.7 g sodium citrate, 3.4 g hydroquinone and 180 ml gum arabic, altogether supplemented by 0.57 g silver nitrate in 1 ml distilled water.

Timm staining results were analyzed using the scale of Tauck and Nadler (86) as follows: 0, no or only occasional mossy fiber-like staining in the supragranular layer (SGL); 1, scattered mossy fiber-like staining above all parts of granule cell layer; 2, patches of heavy mossy fiber (MF) staining interrupted with regions of sparser staining in some areas of SGL or along the SGL; 3, a dense continuous band of MF staining around the whole SGL.

### 3. 6. Karnovsky-Roots staining

I used Karnovsky-Roots staining to identify AChE-containing fiber tracts (37) in rat hippocampus-entorhinal cortex and in human hippocampus. Slices of 200 µm were incubated in the solution containing 1.65 mM acetyl-ß-(methyl)-thiocholine iodide, 0.1 M maleic acid disodium salt anhydrous pH 6.0, 0.05 M sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferricyanide, 0.05 mM tetraisopropyl pyrophosphoramide (iso-OMPA, specific blocker of butyrylcholinesterase) in double distilled water at $37^0$C for 2 hours, washed 2 times in 0.1 M PBS, fixed in 4% PFA for at least 24 h, impregnated with 30% sucrose (0.15 M phosphate buffer) solution and cut into 60 µm thickness section using a freezing microtome (Leitz, Leica Microsystems, Wetzlar, Germany). Sections were then mounted on object glass, air-dried and coverslipped with Citifluor (Citifluor AF-1, W. Planet GmbH, Hamburg, Germany).

### 3. 7. Cell counting

Cells were counted on optimized pictures (digital imaging with the microscope-mounted camera DX30, Kappa opto-electronics GmbH, Germany, at the level of highest cell density) usually containing all cells detectable when scanning stepwise along the optical axis of the section, except for cases where one large cell may cover up a congruent or smaller cell below. Pictures were taken from subiculum, stratum pyramidale of areas CA1 and CA3, hilus with x20 objective (0.4 numerical aperture; x400 magnification; nominal depth of focus ∀4.2 µm) and from layers II, III, V/VI of MEC as well as LEC with x10 objective (0.3 numerical aperture; x200 magnification, depth of focus ∀7.6 µm). Using the software Kappa ImageBase Control® for positioning the grid all columns covering the target region (pyramidal cell layer of subiculum, CA1, or CA3) were numbered and 5 column numbers were randomly chosen. If a selected column had no field that permitted proper counting within the pyramidal cell layer the grid position was adjusted. Therefore, counting was performed in 5 randomly chosen 50 x 50 µm fields and each field had the same likelihood of being chosen. For the hilus 5 fields of 100 x 100 µm located between sectors 2 to 4 of the granule cell layer and the area CA3 were randomly chosen by a near similar procedure (instead of columns the fields were numbered). The number of neurons was determined by counting cells according to one or more of the following criteria: 1. presence of dendritic processes, 2. a cresyl violet positive cytoplasm in the soma, 3. a relative large nucleus, or 4. a clearly discernible nucleolus. Usually, all neurons that had about 2/3 of the visible soma within the field and somata overlapping the top or left margin of the field to near equal parts were counted. Furthermore, the maximal width of the DG granule cell layer was measured in 5 sectors of equal length (total length divided by 5). Although shrinkage was microscopically controlled using landmarks extending along the whole optical axis of the section, we did not correct for shrinkage because density of neurons will not be considered.

In order to characterize cell loss or the widening of the dentate granule cell layer, differences between the mean value for each section (n = 5 fields of 2500 µm$^2$) and a reference value were determined and expressed in percent of the reference value (cell loss = ((mean cell number – reference value) / reference value)* 100). The reference values were separately calculated as mean values for ventral, middle and dorsal sections of the

investigated middle part of the hippocampus from all control animals for a given region (CA1, CA3, … etc.). We emphasize that our counting methods and distance measures give only relative estimates and not absolute values per region and section. However, neuron counts were always performed in the same way and statistical differences between different groups of animals could be accurately determined.

### 3. 8. Statistical analysis

Cell counts, scores (graded Neo-Timm staining) and the cell loss (relative differences between control and pilocarpine-treated animals) were given as mean values with standard error of the mean (mean $\pm$ SE) with n for number of sections.

All “between groups” comparisons were performed using the independent sample t-test or one way analysis of variance (ANOVA) with post hoc test (Bonferroni or Dunnet’s T3, depending on whether equal variances could be assumed or not), and the Friedman test, respectively. Correlation was tested by determining the Spearman’s rho correlation coefficient. Statistical analysis was done using the SPSS software package (Release 12.0.1).

## 4. Results

### 4. 1. Fluorescent tracers in pilocarpine-treated rats reveal widespread aberrant hippocampal neuronal connectivity

In our control animals, injection of fluorescent dye into the hilus resulted in retrograde labeling of granule cells and anterograde labeling of the efferent mossy fiber tract. Hilar cells were visible together with their efferents, the commissural and associational fibers. When the fluorescent tracer was applied into the mossy fiber tract (CA3 stratum lucidum) of control animals, no fibers were labeled in the inner molecular layer (IML). By contrast, in pilocarpine-treated epileptic animals, injections of fluorescent dye into the mossy fiber tract stained a distinct fiber network in the IML of the dentate gyrus. This was observed in 64% of slices from epileptic animals. These findings confirmed

that expression alterations in Neo-Timm staining and other markers for mossy fiber sprouting are due to physical reorganization of the mossy fiber network.

I therefore also employed dextran-amine staining to detect aberrant connectivity in area CA1. In control animals, tracer injection into the stratum radiatum of area CA1 labeled a column of densely packed pyramidal cells with a vertical arrangement of parallel apical dendrites. Although Schaffer collaterals were sometimes visible at the proximal third of the pyramidal cell dendrites, few CA3 pyramidal cells were retrogradely labeled. Injection of a fluorescent tracer into stratum radiatum of area CA1 did not label pyramidal neurons remote from the injection site or in the subiculum.

In pilocarpine-treated animals, dye-injection into the stratum radiatum revealed that number and appearance of stained CA1 pyramidal neurons in slices varied among the animals. Some slices had only a few or no neurons labelled at the injection site. Sixty-four percent of the slices had a clear alteration of dendritic morphology such as dendritic pruning of slight to strong degree, loss of spines, distortion, swelling, gnarled shape, irregular nodulation of the dendritic shaft and leaf-like excrescences. Signs of aberrant connectivity were found in six of the 10 pilocarpine-treated animals, with the different hippocampal regions dye-injected. This reorganization involved back projection to area CA3 and increased coupling of CA1 pyramidal cells. Pilocarpine-treated animals showed 1-5 seizures within 40 h of observation time (5 x 8 h/week). The moderate frequencies (0.4-0.6/day, n = 5) were associated with the highest incidence of aberrant labeling and dendritic damage in area CA1. However, a staining of subicular pyramidal cells following tracer application into CA1 stratum radiatum was observed more often in the high frequency group (0.8-1.0/day, n = 3).

In follow up studies we did not find strong correlations between cell loss, reorganization and seizure frequency. The amount of cell loss was larger the older the animals were at time of status induction and abnormal connectivity correlated with cell loss but not with seizure frequency.

## 4. 2. Stimulus and potassium-induced epileptiform activity in the human dentate gyrus from patients with and without hippocampal sclerosis

Resected hippocampal tissue specimens from patients with TLE showed different degrees of mesiotemporal sclerosis. According to Wyler classification (93), we found no sclerosis in 4 specimens, mild sclerosis in 14 specimens, moderate sclerosis in 8 specimens, classical sclerosis in 12 specimens and total sclerosis in 24 specimens. Specimens with grades 3 (classical sclerosis) and 4 (total sclerosis) were considered to show HS, whereas grades 0-2 were classified as not having mesiotemporal sclerosis (non-HS). HS was accompanied by a lesion in 5 of 36 patients, whereas non-HS was associated with a lesion in the temporal lobe (tumor, cavernoma, dysplasia and cyst) in 17 of 26 patients.

We used fluorescent dextran-amine labeling in 50 specimens to disclose aberrant mossy fiber sprouting into the inner molecular layer (IML) of the dentate gyrus. Retrograde staining with fluoro-emerald or fluoro-ruby within the hilus revealed an extensive network of fluorescent mossy fibers in the IML of HS specimens, whereas ordered apical dendrites of granular cells dominated in the non-HS specimens. Neo-Timm staining for zinc usually present in mossy fiber terminals was performed in 43 of 62 specimens. Supragranular Timm staining in the IML was positive in 22 of 25 specimens with HS, whereas the Timm staining was negative or weak in 17 of 18 specimens with non-HS. In patients with HS, the granular cells were dispersed, whereas in patients without HS, the granule cell layer was dense and compact. Granule cell dispersion was found in 100% of the HS cases and in 8% of the non-HS cases ($\chi^2$ - test, $p < 0.001$). Based on the morphological results, we divided the present tissue sample into two groups: (1) the HS group characterized by Wyler grades 3 and 4, presence of mossy fiber terminals within the IML and granule cell dispersion; and (2) the non-HS group characterized by Wyler grades 0-2, negligible signs of MF sprouting and rare granule cell dispersion.

Subsequently, we tested for seizure susceptibility on these slices. Induction of epileptiform activity was most reliable with elevation in extracellular potassium concentration. The threshold for induction of seizure-like events was lower in HS than in non-HS tissue. Moreover, the induced patterns of epileptiform activity were also different. We could distinguish four different types of ictaform activity: (i) tonic-clonic seizure-like

events (SLEs) (first trace), (ii) ictal spiking (second trace), (iii) inter-ictal spiking (third trace) and (iv) tonic SLEs (fourth trace) (33). Tonic-clonic SLEs exclusively appeared in HS specimens while tonic SLEs only occurred in the non-HS specimens ($\chi^2$ - test, $p < 0.001$). The data so far were consistent with those recently reported by our group (28).

### 4. 3. Carbamazepine resistance in the epileptic dentate gyrus of human hippocampal slices

Overexpression of drug efflux pumps at the blood brain barrier (BBB) has been suggested to be one important factor contributing to drug resistance in epilepsy. This would imply that resected brain tissue of drug-resistant patients is drug-sensitive in absence of the BBB. We therefore studied the effects of carbamazepine (CBZ) at therapeutically relevant concentration on epileptiform activity induced by elevation of potassium concentration in slices from clinically pharmacoresistant patients with TLE (28 patients, 49 slices) and pharmacosensitive patients with extra-hippocampal tumors (6 patients, 11 slices). The epileptiform activity in tissue of tumor-patients was predominantly suppressed by CBZ, indicating that the ictaform activity induced by $K^+$ elevation is not a priori pharmacoresistant. In contrast, epileptiform activity in tissue of drug-resistant TLE patients was resistant to CBZ in 82% of patients, partially suppressed in 11% and completely suppressed in 7%. In this last group, pharmacoresistance may be due to up-regulation of drug transporters at the BBB. The pharmacoresistance of TLE patients did not depend on the type of activity, excitability of the tissue, or equilibration time of the drug. Considering that CBZ has direct access to all compartments of the slice, our results suggest that CBZ-resistance mechanisms are located within the parenchyma of the dentate gyrus and contribute to drug resistance in the majority of TLE patients. BBB-located drug-resistance mechanisms *per se* may play a minor role in this region, because CBZ-sensitivity was only observed in 7% of CBZ-resistant patients (33). My next question was whether there are correlations between morphological alterations and pharmacoresistance in TLE patients.

TLE and tumor patients are clinically and neuropathologically different. In comparison to the tumor group, the TLE group was characterized by long-duration epilepsy and drug resistance including resistance to CBZ/oxcarbazepine. Relatively more patients in the TLE group had low frequency seizure rates and a higher trend to secondary

generalization. These TLE patients also showed a higher incidence of hippocampal sclerosis (HS: Wyler grades 3 and 4). In contrast, the patients in the tumor group (mainly extra-hippocampal dysembryoblastic neuroepithelial tumor, ganglioglioma WHO grade 1 and astrocytomas WHO grades 1 or 2) experienced only short periods of epilepsy and drug treatment. All six patients except the last one presented with low degrees of cell loss (non-HS: Wyler grades 1 and 2). However, in the TLE group tissue with Wyler grades 0-2 were as pharmacoresistant as tissue with Wyler grade 3-4. Thus the degree of cell loss and other signs of morphological alterations did not correlate with pharmacoresistance.

### 4. 4. Metabolic dysfunction during neuronal activation in the *ex vivo* hippocampus from chronic epileptic rats and humans

Our pilocarpine-treated animals showed neuronal cell loss predominantly in the medial entorhinal cortex and in the hippocampal formation. The hilus, CA3 and CA1 area in the hippocampal formation are significantly more affected than the subiculum and dentate gyrus. These findings correspond well to histopathological alterations of HS tissue from TLE patients (4, 11). We asked whether cell loss might relate to a reduced capability of the tissue to adapt neuronal metabolism to neuronal activity. Neural activation was elicited by electrical stimulation (10 s, 20 Hz) and resulted in transient increases in $[K^+]_0$ of up to 2.3 mM. The quantification of NAD(P)H fluorescent transients in normal samples showed a brief initial ‘drop’ that was followed by a prolonged ‘overshoot’ of up to several minutes. In chronic epileptic rats, the overshoot was smaller in area CA1 although increases in $[K^+]_0$ reflected substantial neuronal activation. However, the drops were similar to those of the control. In tissue from TLE patients, in the majority of experiments, we observed very small overshoots in both area CA1 and the subiculum that went along with large drops and comparable $[K^+]_0$ elevations. Moreover, these alterations of NAD(P)H transients were independent of the degree of sclerosis, because there were no significant differences when comparing HS and non-HS groups in both areas. This might suggest that the degree of cell loss is independent of disturbances in metabolism.

### 4. 5. Acetylcholine-induced seizure-like activity and cholinergic modified gene expression in chronically epileptic rats

To test for a potential role of ACh in induction of epileptiform activity, ACh or CCh were bath applied. We found a lowered threshold for induction of epileptiform activity in entorhinal cortex of pilocarpine- or kainate-treated epileptic animals. Previous studies showed that cortical injury and enhanced neuronal excitability are associated with modifications in the isoform composition of acetylcholinesterase (AChE), the enzyme that hydrolyzes acetylcholine (ACh). To investigate whether similar changes occur in chronic epileptic animals, we measured protein and mRNA AChE levels in cortices. Total AChE activity was similar in control and epileptic rats (11.11 $\pm$ 0.5 and 12.24 $\pm$ 0.6 µM/min x µg respectively, $p < 0.3$, Wilcoxon signed-rank test, $n = 7$ for both groups), indicating no massive down regulation of AChE. Also histochemical staining for AChE revealed diffuse staining of AChE throughout all layers in the EC from epileptic animals, predominantly in the layer II-III. In view of these findings and a lowered threshold for induction of epileptiform activity by cholinergic agonists and by ACh we looked for a possible isoform shift of the AChE-S protein, located in and near synaptic clefts, to the soluble AChE-R monomers, which may diffuse to extrasynaptic regions. To test this hypothesis biochemically, we applied hippocampal and temporal cortex homogenates to sucrose gradient centrifugation and measured AChE activity in the resultant fractions. Both analyzed areas exhibited a significantly marked increase in the globular, monomeric soluble AChE form, which by its wide tissue distribution may be less efficient in cleaving ACh at sites of release.

## 5. Discussion

In these studies I tested the hypothesis that cell loss and subsequent reorganization leads to abnormal network properties with a lowered threshold and increased incidence of seizures. Recent investigations showed that temporal lobe epilepsy is not a single pathological entity but rather might include different pathologies and thus may be associated with various pathophysiologies. Pathomorphological and physiological alterations that we have observed in our patient samples as well as in post-pilocarpine

status animals include aberrant network connectivity, cell loss, mossy fiber sprouting, metabolic dysfunction and cholinergic modified gene expression.

### 5. 1. Aberrant network connectivity in CA1 and CA3

My first interest was to see to what extent morphological alterations contribute to seizure threshold and seizure frequency. My studies revealed aberrant network connectivity in the DG and CA1 area in chronic epileptic hippocampi of pilocarpine-treated rats 3 months after approximately 2 h of status epilepticus. While the DG alterations were well comparable to other studies (12, 65) where a status epilepticus was used to induce a TLE, the morphological alterations in CA1 area were a new finding. This applied to back-projection from CA1 area to CA3, from the subiculum to CA1 and increased excitatory coupling within CA1 area. Other morphological alterations such as cell loss in CA1 area and dendritic abnormalities such as shortened dendrites, were in agreement with previous studies (Njunting et al., unpublished data). However, even in my population there was quite some variability. For example I noted a labeling of CA1 pyramidal cells by dye injected into area CA3. It is very unlikely that dendrites of labeled CA1 neurons took up the tracer in area CA3. Therefore, our results indicate the presence of CA1 pyramidal cell axon collaterals in area CA3. The observation was, however, only seen in 40% of the post-pilocarpine status animals. Also the back-projection from subicular cells to CA1 area was not seen in all animals.

A high degree of local excitatory connectivity in the longitudinal as well as in the transverse axis of the hippocampus seems to be a natural organization principle in CA3 (16, 59) but not in CA1 area (17). Finch et al. (25) and Amaral et al. (2) described a collateralization emerging from CA1 axons in normal animals predominantly within stratum oriens. Staining of the CA1 pyramidal cells outside the injection site in pilocarpine-treated animals indicates increased collateral connectivity in CA1 area, possibly due to axonal sprouting. Such collateral connectivity seems to be also present in CA1 area of human hippocampi with moderate neuron loss and gliosis (43) as well as in the kainate model of epilepsy (92, 23). This led us to suggest that sprouting of CA1 pyramidal cell axons is part of the hippocampal network reorganization during epileptogenesis.

The coincidence of axonal sprouting and dendritic damage in CA1 indicates that increased excitatory coupling within this area might be a compensatory mechanism for dendritic deafferentation. However, the degree of reorganization seemed to vary. When inducing status epilepticus in younger animals the extent of abnormal fiber connectivity outside CA1 area was reduced in spite of similar seizure frequencies, suggesting that the amount of reorganization is not related to seizure incidence and also indicating that the consequences of status epilepticus are age-dependent. Indeed, studies of consequences of status epilepticus in young rodents have suggested that relatively short periods of status epilepticus may not lead to large degrees of cell loss and reorganization. Thus it appears that cell loss and associated network reorganization is not a necessary condition for the development of epilepsy. This may not exclude that other symptoms of TLE such as difficulties in spatial orientation and formation of explicit memory traces are related to the abnormal connectivity.

### 5. 2. Cell loss in the hilus, CA3, CA1, entorhinal cortex, granule cell dispersion and mossy fiber sprouting

I confirmed cell loss in the hilus (80%), CA3 (40%), CA1 (40%) as well as in the medial (MEC, 50-60%), lateral entorhinal cortex (LEC) and granule cell dispersion in the chronic animals (Njunting et al., unpublished data). In HS patients, I observed the granule cells dispersion (100% of the cases), whereas in non-HS, the granule cell layer was dense and compact (8% of the cases, $\chi^2$ test; $p < 0.001$) (28). Previous studies showed that in hippocampal area of pilocarpine-treated animals, cell loss and mossy fiber sprouting is the most frequently observed alteration (56, 48, 46). This applies also to patients (47, 50, 8). Widening of the dentate granule cell layer is also characteristic for TLE patients with HS (22, 12, 65) as well as in post-pilocarpine status model possibly due to neurogenesis following seizure (77, 30).

Cell loss in the hilus and in MEC layer III has been established four weeks after status epilepticus (21, 60) and remained constant after 3-5 months (95, 31). In chronic kainate-treated rat which showed extensive cell loss in layer III of entorhinal cortex, 4-aminopyridine (4-AP) as a potassium channel blocker failed to induce seizure-like events (94). Progressive cell loss of both principal cells and calbindin (CB)-, calretinin (CR)-,

parvalbumin (PV)-immunopositive interneurons in layers II-III of LEC were also reported 2 months to 1 year post SE (52). Interestingly, the number of GAD65/67 positive interneurones remained unaltered (88). These studies indicate that the entorhinal cortex is importantly involved in the generation of TLE. Indeed studies from Spencer et al. (81) indicated that up to 50% of seizures commence outside the hippocampus proper. While neuron loss (with potential subsequent reorganization) is particularly large in these seizure regulatory regions, I showed in my study that cell loss is more related to reorganization rather than to seizure frequency.

To identify the MFS in human and pilocarpine-treated animals, I used Neo-Timm staining to visualize granule cell mossy fibers (5, 56). Our analysis of Neo-Timm staining in the pilocarpine treated rat (at the middle and dorsal hippocampal level) reveals a mean score of 2.71 $\pm$ 0.49 (n=7 animals) as compared to 0.0 $\pm$ 0.0 (n=4 animals) in the control group (Njunting et al., unpublished data). Based on the results one might argue that changes in zinc transport underlie the increased Timm staining scores. However, the direct demonstration of supragranular retrogradely labeled fibers and that of biocytin staining of axonal patterns (27) confirms the abnormal connectivity and makes this staining method a suitable tool to look for abnormal connectivity also elsewhere.

Our results showed a significant difference of MFS in non-HS vs. HS patients as well as in control vs. pilocarpine animals. However, it was reported that the frequency of spontaneous seizure is not necessarily associated with the degree of MFS (56, 14, 64) or the duration of SE (51, 63). Also in my studies, I found, neither for humans nor for animals, a correlation between degree of mossy fiber sprouting and seizure frequency. At present, we cannot exclude that reorganization contributes to generation of pharmacoresistance. Indeed, Albus et al. (1) showed that SLEs induced in rat organotypic hippocampal slice cultures are pharmacoresistant. In such preparation, aberrant axonal connections similar to that in human tissue have been reported. However, as I have mentioned before, there was a similar pharmacoresistance in the human material with and without HS.

We also tested the hypothesis that seizure threshold is dependent on reorganization. Using elevation of extracellular potassium concentration as a tool to induce seizure-like events, we found that seizure threshold was lower in TLE tissue with HS than in tissue without HS. However, not all methods to induce seizure-like events *in vitro* in

rodents work in the human tissue and it may be that some of the alterations in human tissue counteract epileptogenesis.

### 5. 3. Metabolic dysfunction

We used NAD(P)H fluorescence to monitor the capability of mitochondria to adapt metabolism to neuronal activity. NAD(P)H signals during neuronal activation were characterized by an early fluorescence dip followed by an overshoot due to activation of the tricarboxylic acid cycle and increased NADH and $FADH_2$ synthesis. In epileptic tissue we found unaltered drops but significantly smaller overshoots of NAD(P)H transients reflecting less effective $NAD(P)^+$ reduction in pilocarpine-treated rats. This difference was unlikely to be on account of less neuronal activation in chronic epileptic tissue, because increases in $[K^+]_0$ were virtually the same. The alterations were particularly obvious in CA1 area. This finding is in line with observations that CA1 area is much more vulnerable to seizure-induced neuronal damage than the subiculum (4, 56) and with the few reports that have described alterations of NAD(P)H transients in models of experimental epilepsy (41, 40).

In HS and non-HS tissue from TLE patients, we found very small overshoots of NAD(P)H transients. Together with the animal model data, we believe that this alteration is more a characteristic of the chronic epileptic human brain than of the human brain *per se*. In contrast to pilocarpine-treated rats, we observed no significant differences in alterations of NAD(P)H transients in both area CA1 and the subiculum when comparing HS and non-HS tissues. NAD(P)H has a central role in energy metabolism, but it is also a key element in a variety of intracellular signalling cascades (9) and is essential for biosynthesis and reduction of the cellular antioxidant, glutathione (19, 62). Our findings in this study showed significant alterations of NAD(P)H fluorescence transients during neuronal activation in acute hippocampal slices from chronic epileptic rats and humans, although mitochondria maintain a negative inner-membrane potential. These findings provide a cellular correlate for ‘hypometabolism’ as described for epilepsy patients and, thereby, suggest mitochondrial enzyme defects in TLE. Metabolic dysfunction in neurons and glial cells might significantly affect ATP homeostasis and excitability as well as intrinsic anti-oxidative

mechanisms. Under certain conditions, these disturbances might favour neuronal vulnerability and manifestation of seizures and status epilepticus (36).

### 5. 4. Cholinergic modified gene expression

Our results showed that reorganization is not likely to contribute to seizure incidence. Therefore, cellular functional and network alterations may be more important for seizure susceptibility than reorganization. I therefore looked for alterations in the cholinergic system, which is involved in regulation of attentional states (74). Our group found that in hippocampal-entorhinal cortex slices from chronic epileptic rats, low concentrations of ACh induced prolonged seizure-like and interictal-like activity and ACh-induced epileptiform activity is mediated via muscarinic receptors (96). The observation that kainic acid- and pilocarpine-treated animals showed a similar effect suggests that the enhancement of epileptiform activity by ACh is related to epileptogenesis and not to the fact that pilocarpine is a muscarinic agonist. In some slices from epileptic rats, but never from controls, inhibition of AChE induced rhythmic hypersynchronized activity, suggesting that endogenous spontaneous release of ACh may suffice to trigger ictal-like activity.

Additionally, specific changes in cholinergic transmission may occur in epilepsy. An increase in AChE alone would predict reduced efficacy of cholinergic transmission. However, our results in epileptic animals are in line with previous hippocampal recordings following repeated stressful stimulation (57) and suggest that shifts in the enzyme isoformal composition are associated with enhanced responses to cholinergic agonists. This study emphasizes the importance of the cholinergic system in the initiation and propagation of epileptic activity in TLE. Cholinergic hypersensitivity may explain the enhanced frequency of seizures in patients under psychological stress (55) as well as the cognitive disturbances frequently observed in patients (13).

## 6. Outlook

My investigation contributes to the increasing evidence that not only one single alteration or abnormality explains the production of seizures but rather a combination of morphological, physiological and neurochemical changes (see also Glass and Druganow

(29)). It revealed morphological alterations in patients as well as in the pilocarpine model, however these alterations are not correlated with seizure frequency.

Morphological alteration may correlate with loss of inhibition, which is proposed by Sloviter (78). The preservation of GABAergic interneurons in epileptic hippocampus, together with reduction of GABA-mediated inhibition, has lead to the hypothesis that inhibitory GABAergic neurons in the hippocampus are dormant in TLE as a result of being disconnected from their excitatory inputs (7, 78). Loss of hilar neurons is a common denominator in TLE. Thus, this hypothesis suggests that early loss of hilar mossy neurons is the cause, rather than the result, of TLE (78). The loss of other neuronal populations, which varies markedly in different TLE sufferers, e.g., in CA1 and CA3, may then occur as a result of seizures caused by this initial loss of hilar mossy neurons and dormancy of GABAergic inhibition (78).

Another study by Stief et al. (82) about GABAergic interneurons at the border between stratum radiatum and lacunosum moleculare (SRL interneurons) which mediate feedforward inhibitory control of activity propagating from CA3 to CA1 (39, 18, 10) as well as generating rhythmic network activity (26, 10) showed that these SRL interneurons are synaptically disinhibited during TLE, increasing their spontaneous activity and, most probably, enhancing the efficacy feedforward inhibition of CA1 pyramidal cells. This increase in excitation-inhibition ratio is accompanied by morphological changes indicative of dendritic remodelling during TLE (81).

In pilocarpine-treated rat *in vitro* slice preparation, epileptic discharges from entorhinal cortex, as the site of origin for ictal discharges, propagate to the hippocampus via the perforant path (61). As ictal activity is associated with pronounced, long lasting depolarizations of entorhinal neurons (34, 35), NMDA-dependent $Ca^{2+}$ entry might occur and be a determinant factor in epilepsy-induced excitability and consequent cell damage (20, 80).

Taken together, comprehensive analysis of functional alteration such as network modelling needs to be done in order to understand the mechanisms involved in epilepsy as a seizure disorder, beside neurochemical and morphological aspects.

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# Statement of Contribution for Selected Publications

Contribution of Promovendus Marleisje Njunting to the following publications:

- Publication 1: Lehmann TN, et al., *Eur J Neurosci 2001; 14: 83-95.*
contribution approx. 10%

Prepared animal brain slices for an added experiment, applied fluorescent dextran-amine staining, fixed tissue specimens, cut tissue, prepared slides, obtained images using epifluorescence microscope with mounted camera, measured the dye spread, analysed data.

- Publication 2: Gabriel S, Njunting M, et al., *J Neurosci 2004; 24(46): 10416-10430.*
contribution approx. 50%

Applied fluorescent dextran-amine staining, fixed hippocampal specimen from patients, cut tissue, applied cresyl violet and Neo-Timm staining, prepared slides, obtained images using epifluorescence microscope with mounted camera, analysed data.

- Publication 3: Jandova K, et al., *Brain 2006; 129 (12): 3290-3306.*
contribution approx. 10%

Fixed hippocampal specimen from patients, cut tissue, applied cresyl violet and Neo-Timm staining, prepared slides, obtained images using microscope-mounted camera, analysed data.

- Publication 4: Kann O, et al., *Brain 2005; 128 (10): 2396-2407.*
contribution approx. 20%

Fixed hippocampal specimen from patients and pilocarpine animals, cut tissue, applied cresyl violet staining, prepared slides, obtained images using microscope-mounted camera, processed and analysed data.

- Publication 5: Zimmerman G, et al., *Eur J Neurosci 2008; 27(4): 965-975.*
contribution approx. 40%

Took part in planning the experiment, prepared animal brain, measured AChE activity, performed Karnovsky-Roots staining, fixed tissue specimens, cut tissue, prepared slides, obtained images using microscope-mounted camera, analysed data, corrected the manuscript.

Prof. Dr. Uwe Heinemann Marleisje Njunting

# Publications

In the following the publications are inserted according to their order of appearance in the previous page (page 30 “Statement of Contribution for Selected Publications)”.

# Curriculum Vitae

My curriculum vitae is not published in the electronic version of my thesis due to data privacy regulations.

# Publications

Selected publications for dissertation:

1. Lehmann TN, Gabriel S, Eilers A, **Njunting M**, Kovacs R, Schulze K, Lanksch WR, Heinemann U. Fluorescent tracer in pilocarpine-treated rats shows widespread aberrant hippocampal neuronal connectivity. Eur J Neurosci 2001; 14: 83-95. Impact Factor: **4.921**

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6. Lehmann TN, Gabriel S, **Njunting M,** Merschhemke M, Meencke HJ, Lanksch WR, Heinemann U. Aberrante Konnektivität im humanen epileptischen Hippokampus. Klin Neurophysiol 2002; 33: 200-206. Impact Factor: **0.321**

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## Poster Presentations

1. Njunting M, Gabriel S, Meencke H-J, Heinemann U, Lehmann T-N. *Altered fiber connections in human epileptic hippocampus – a dextran amine fluorescent tracer study.* 5th Meeting of the German Neuroscience Society and 29th Göttingen Neurobiology Conference, Göttingen, 2003.

2. Njunting M, Gabriel S, Schulze K, Jandova K, Heinemann U, Lehmann T-N. *Cell loss in the entorhinal cortex and hippocampus: relation to seizure frequency in the pilocarpine model of temporal lobe epilepsy*. Berlin Neuroscience Forum, Liebenwalde, 2004.

3. Njunting M, Gabriel S, Schulze K, Jandova K, Heinemann U, Lehmann T-N. *Relation of cell loss and seizure frequency in the entorhinal cortex and hippocampus in the pilocarpine model of temporal lobe epilepsy.* 6th Meeting of the German Neuroscience Society and 30th Göttingen Neurobiology Conference, Göttingen, 2005.

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## *Erklärung*

„Ich, Marleisje Njunting, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

**Morphological alterations in human mesial temporal lobe epilepsy and the pilocarpine treated chronic epileptic rat**

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 1 Juli 2008 Marleisje Njunting

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