Carbamazepine-resistance in the epileptic dentate gyrus of human hippocampal slices

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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. Here we studied the effects of carbamazepine (CBZ) at therapeutically relevant concentration on epileptiform activity electrophysiologically recorded in acute hippocampal slices of patients with mesial temporal lobe epilepsy (MTLE; 28 patients, 49 slices) or extra-hippocampal tumours (tumour; 6 patients, 11 slices). Epileptiform activity was induced by hilar stimulation (0.067 Hz) during elevation of extracellular potassium concentration ([K+]o) and remained self-sustained in presence of 10–12 mM [K+]o. Quantitative analysis of data revealed that epileptiform activity in tissue of tumour-patients was predominantly suppressed by CBZ, indicating that the ‘epilepsy model’ used is CBZ-sensitive. In contrast, epileptiform activity in tissue of drug-resistant MTLE patients was resistant to CBZ in 82% of patients, partially suppressed in 11% and completely suppressed in 7%. The effects of CBZ in tissue of MTLE patients did not depend on the type of activity, hippocampal pathology, 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 MTLE 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.

Keywords: drug resistance; electrophysiology; epilepsy; hippocampus; human

Abbreviations: AED = anti-epileptic drug; BBB = blood brain barrier; CBZ = carbamazepine; HS = hippocampal sclerosis; high K-ACSF = high K+-containing artificial cerebrospinal fluid; MTLE = mesial temporal lobe epilepsy; SLE = seizure-like event


Introduction

Drug resistance is a crucial problem in the treatment of focal epilepsies. Many patients suffering from mesial temporal lobe epilepsy (MTLE) do not attain seizure control despite appropriate medical treatment, particularly in MTLE associated with hippocampal sclerosis (HS) (Semah et al., 1998). A selected group of MTLE-patients benefit from surgical resection of the epileptogenic tissue (Engel, 1996), suggesting that the removed tissue is characterized by hyperexcitability and drug resistance. Slices of both sclerotic and non-sclerotic hippocampal tissue can be successfully challenged to develop ongoing spontaneous epileptiform activity in the dentate gyrus (Gabriel et al., 2004). This provides an intriguing possibility to study effects of anti-epileptic drugs (AEDs) in human epileptic tissue of drug resistant patients (Remy et al., 2003a).

The nature of drug resistance is likely multifactorial. The most favoured concept suggests that ATP-dependent drug efflux pumps (Lee et al., 2001; Löschner and Potschka, 2005a),...
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which are up-regulated in epilepsy (Tishler et al., 1995; Lazarowski et al., 1999; Dombrowski et al., 2001; Marchi et al., 2005), impede the penetration of drugs into the epileptogenic tissue, thus keeping the concentration of drugs low at the cellular targets (transporter hypothesis) (Abbott and Romero, 1996; Abbott et al., 2002; Kwan and Brodie, 2005; Lösch and Potschka, 2005b, c). Drug efflux pumps are overexpressed at the blood brain barrier (BBB) (Aronica et al., 2003a, b, 2004; Calatozzolo et al., 2005) or in astrocytes around the microvasculature (Golden and Pardridge, 2000; Sisodiya et al., 2001, 2002). This would imply that direct application of the AED into the epileptic tissue overrides drug resistance at the BBB and block epileptiform activity.

Alternatively, drug resistance may result from functionally or genetically modified sensitivity of drug targets (target hypothesis) (Remy and Beck, 2006). This concept suggests that epileptic tissue does not respond to AEDs at therapeutically relevant concentration in the brain parenchyma. Additionally, drug transporters ectopically expressed in neurons and astrocytes of sclerotic hippocampal tissue (Aronica et al., 2004) pump the drugs out of the intracellular compartment. This may contribute to failure of the cellular drug response, provided the drug binds to an intracellular target site.

The present study has been undertaken to find out whether epileptiform activity in the dentate gyrus of epilepsy patients would be sensitive to AEDs which have direct access to the parenchyma. For this purpose we investigated acute hippocampal slices from resected tissue of patients operated on MTLE or extra-hippocampal tumours. We hypothesized that epileptic tissue does not respond to AEDs at therapeutic relevant concentration in the brain parenchyma. For this purpose we investigated acute hippocampal slices from resected tissue of patients operated on MTLE or extra-hippocampal tumours. We hypothesized that epileptic tissue does not respond to AEDs at therapeutic relevant concentration in the brain parenchyma.

Neuronal activity or hippocampal pathology.

Clinical diagnosis

Human hippocampi were surgically resected in order to cure medically intractable temporal lobe epilepsy (n = 28) or to remove tumours nearby (n = 6). Drug resistance has been assumed when seizure control could not be obtained by two or three AEDs increased to maximal tolerated dose within 2 years (Dlugos, 2001). The pre-surgical evaluation of MTLE was performed in the Epilepsy-Center of Berlin-Brandenburg according to the German and European guidelines for pre-surgical evaluation (Baumgartner et al., 2000; European Federation of Neurological Societies Task Force, 2000). Some of the tumour cases were diagnosed and prepared for surgical intervention in the Department of Neurosurgery. The study was approved by the Ethics Committee at the Charité (EA125/2001, EA1/042/04) and performed in accordance with the Declaration of Helsinki. A written informed consent for the study was obtained from every patient before surgery. All operations were done by one of the authors (T.-N.L.) guaranteeing the same dissection technique of the hippocampus.

Neuro pathological diagnosis

Neighbouring sections of the tissue investigated for effects of CBZ were analysed by the Department of Neuropathology at the Charité for diagnostic purposes. As already described in detail (Gabriel et al., 2004; Kann et al., 2005), removed hippocampi were divided into two groups according to the degree of nerve cell loss, using the Wyler classification (Wyler et al., 1992). In short, Wyler grades 1 and 2 were grouped to ‘nonHS’ (HS for hippocampal sclerosis), while grades 3 and 4 were grouped to ‘HS’. As the degree of sclerosis varies throughout the hippocampus (Masukawa et al., 1995), we always repeated the Wyler grading on cresyl violet stained sections from the tissue investigated in our laboratory. If different grading results were obtained (n = 2), we used the grading of the tissue studied for the effects of CBZ.

Tissue transport, preparation and maintenance

Two to three coronal sections of ~4 mm thickness were cut from the hippocampal head and body in the operation theatre and immediately incubated in cold (1–4°C) carbogenated transport solution containing (in mM) KCl 3, NaH2PO4 1.25, glucose 10, MgSO4 2, MgCl2 2, CaCl2 1.6, NaHCO3 21, sucrose 200 and (±) α-tocopherol 0.1 (pH 7.4, osmolality 303 mosmol/kg, 0.005 v/v% ethanol) (Gabriel et al., 2004; Kann et al., 2005). Transport to the laboratory was performed in an airtight cooling receiver within 30 min. At arrival in the laboratory the temperature of the solution was still <4°C and partial pressure of oxygen was >580 torr.

Subsequently, the tissue was coronally dissected into slices of 500 μm thickness using a vibratome (Campden Instruments Ltd, Leicester, UK). The slices were immediately transferred into interface chambers, perfused at a rate of 1.7 ml/min with pre-warmed (34.5 ± 0.5°C) carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 129, KCl 3, NaH2PO4 1.25, glucose 10, MgCl2 2, CaCl2 1.6, NaHCO3 21 (pH 7.4, osmolality 300 mosmol/kg). Recordings commenced 4 h after preparation of slices to permit optimal recovery after surgery and transport in sodium free solution.

Electrophysiological recordings and stimulation

Extracellular recordings as well as hilar stimulation were performed as previously described (Gabriel et al., 1998). The recording electrode was placed into the dentate granule cell layer, opposite to area CA1. We used double-barreled K+-selective/reference micro-electrodes (containing Fluka 60031 ionophore backfilled with 100 mM KCl/150 mM NaCl, respectively) which were prepared and tested as described by Lux and Neher (1973). All electrodes
responded to a 10-fold change in K⁺ concentration with a potential shift of 60 mV. Field potential and extracellular potassium concentration (K⁺;o) were continuously recorded using Spike2 (version 4.01, CED; Cambridge, UK) with sample rates of 5 kHz for field potentials (filter cut-off 1 kHz) and 100 Hz for [K⁺;o] (filter cut-off 1.6 Hz).

Bipolar stimulation electrodes (platinum wires of 20 μm diameter, tip separation 40–80 μm) were positioned at the hilus/CA3 border. In order to confirm slice viability and to assess similar stimulation efficacy with respect to the level of neuronal activation in all slices, paired stimuli (0.1 ms, 1–30 V, 50 ms interval) with intensities eliciting similar proportions of the maximal population spike amplitude in different slices were applied every 20 s, in the beginning and at the end of each experiment. Slices displaying maximal population spike amplitudes of <1.5 mV in the beginning or loss of >20% of the maximal amplitude at the end of the experiment were excluded from the analysis.

**Experimental protocol**

Epileptiform activity was induced by continuous paired hilar stimulation (0.067 Hz, intensity eliciting population responses with 80% of the maximum amplitude) and perfusion with ACSF containing 10–12 mM K⁺ (high K-ACSF). When epileptiform events occurred independently of stimulation, the stimulation was turned off. Then, each experiment was divided in three subsequent periods:

First, we waited for another 5–15 min until the epileptiform activity had stabilized with respect to event rate and amplitude (control).

Second, we changed the perfusion medium to CBZ-high K-ACSF, with CBZ (RBI/Sigma, Deisenhofen, Germany; pre-dissolved at 100 mM in dimethylsulfoxide) at a final concentration of 50 μM for at least 20 min (CBZ).

Third, we changed the perfusion medium back to high K-ACSF in order to wash out CBZ for 20–60 min (wash).

In some experiments, the drug perfusion was prolonged for another 20 min, applying CBZ at concentrations of either 50 or 100 μM. A concentration of 50 μM CBZ corresponds to upper therapeutically relevant serum levels (Strandjord and Johannessen, 1980; Semah et al., 1994; Neels et al., 2004), while 100 μM CBZ is in the supra-therapeutic range.

**Procedures of data analysis**

**Categorization of epileptiform activity**

As recently reported, four types of epileptiform events occurred in the dentate gyrus (Gabriel et al., 2004). They were categorized as (i) tonic–clonic seizure-like events (tonic–clonic SLEs) when slow negative field potential shifts of >5 s duration were super-imposed by a high frequency fluctuation of small transients and subsequent low frequency clonic-like discharges; (ii) ictal spiking when very short negative discharges with event rates >40/min (duration of 1–10 s) occurred; (iii) inter-ictal spiking when negative field potential transients corresponding to inter-ictal spikes or spike-wave complexes with event rates <40/min appeared; and (iv) tonic SLEs when low frequency negative field potential shifts of >5 s duration without superimposed clonic-like discharges were recorded.

**Quantification of drug effects on epileptiform activity**

The aim of this analysis was to quantify the changes of epileptiform activity following perfusion with CBZ-high K-ACSF (Fig. 1). At least 3, and up to maximum of 15 epileptiform events from the last 3 min epoch of each experimental period (control, CBZ, wash) were analysed.

In order to characterize different frequency components and amplitudes we determined six activity parameters: event rate (ev/min); slow field potential amplitude [sfp (mV), including the largest transients, superimposed on the sfp]; and event duration [duration (s), measured from start up to two-thirds recovery of the sfp]. Amplitudes and duration are illustrated in Fig. 1A, A1. The fifth parameter represents the mean frequency of transient potential fluctuations (with minimal size of 0.3 mV and a minimal time interval of 3 ms) superimposed on the sfp [frequency (Hz), see Fig. 1A, A2]. The last parameter, power of the high frequency band [P₁, 78–293 Hz (mV²), Fig. 1A, A3], estimates the high frequency component of epileptiform events. Its determination included: band pass filtering of field potential traces (80–300 Hz; Fig. 1A, A3A), computation of the power spectrum from the resulting high frequency event (Fig. 1A, A3B), and calculation of the sum of power from single frequency intervals (19.53 Hz) between 78 and 293 Hz. This analysis was performed using the available tools of the Spike2 program and additional homemade script files (Dr H. Siegmund, Institute of Neurophysiology, Charité).

Subsequently, the values taken from each event were separately averaged for each parameter and analysis epoch. In order to quantify the effects of CBZ the parameter values of the CBZ-epoch were normalized with respect to a reference value. At the time points of analysis, the high K-ACSF had been perfused for 20–40, 40–60 and 100–120 min. Irrespective of the presence of CBZ, all types of epileptiform activity displayed an ongoing change of parameter values during the course of recurrent spontaneous activity (Fig. 1B). Therefore, we calculated the reference value (100% ‘without CBZ’) as the mean value of control (e.g. 20 min in Fig. 1B) and wash epochs (e.g.100 min in Fig. 1B).

Effects of CBZ were normalized and expressed as percent of the reference value maintained after perfusion of CBZ ([CBZ value/reference value] × 100). In order to quantify the proportion of slices showing gradual different effects of CBZ, we averaged all normalized CBZ-parameters for each slice and graded the remaining averaged percentages of activity between 0 and 20%, 21 and 70% and >70% as suppressed, reduced and resistant, respectively. This categorization was derived from the bimodal histogram of the values with peaks at 0–10 and 90–100%, calculated for the whole sample of 60 slices as shown in Fig. 8B.

**Statistics**

Patients and slices were grouped according to clinical data, neuropathological diagnosis, and types of epileptiform activity observed during the experiments. Group data of ratio variables are displayed as mean ± SEM, throughout the paper. Data of nominal and ordinal variables are given as proportions of group members assigned to the categories. Statistical comparisons within and between groups were performed using the Wilcoxon matched-pair signed rank test (referred to as Wilcoxon test) and the Mann–Whitney U-test or parametric procedures like the dependent sample t-test and one-way analysis of variance including post hoc tests, respectively. Proportional differences between groups were evaluated applying the χ² homogeneity test. Relations between two parameters were indicated as Pearson correlation coefficient or Spearman rank correlation coefficient. All statistics were
**Results**

Data of experiments on 60 slices from 34 hippocampal specimens were analysed. The sample consisted of 49 slices of 28 patients with drug-resistant MTLE (MTLE-group) and 11 slices from 6 patients diagnosed to suffer from tumours (tumour group). All slices were studied between 6 and 24 h after surgical resection and responded to electrical stimulation at the hilus/CA3 border with one or more population spikes in the granule cell layer. Maximal population spike amplitudes were in the range of 1.5–10 mV in the beginning of the experiment and in the range of 1.3–9.2 mV at the end of the experiment. Here we describe the effects of bath-applied CBZ on epileptiform activity induced in hippocampal slices. Notably, drugs that are bath-applied directly enter the parenchyma and do not have to cross the BBB. The equilibration time necessary to reach the maximal target concentration of the lipophilic drug is not known, but our experiments with longer application time show that there is no large change of the drug effect with

**Fig. 1 Analysis of epileptiform activity.** The activity has been induced by paired hilar stimulation (0.067 Hz, intensity eliciting 80% of the maximal population spike amplitude) and elevation of [K+]o (10–12 mM). (A) Extraction of event parameters from an SLE. (A1) Determination of event amplitudes and event duration: sfp = maximal amplitude of the slow field potential shift; peak = peak amplitude (including maximal negative transients); duration = time from start of the slow field potential shift up to two-thirds of recovery. (A2) Determination of intra-event frequency by counting transients >0.3 mV per time of the discharge period (minimal time interval 3 ms). (A3) Determination of the power of the high frequency band (78–293 Hz) including band pass filtering of trace A1 (80–300 Hz), calculation of the power spectrum from trace A3A (fi = 19.53 Hz) and summation of power from frequency intervals between 78 and 293 Hz. (B) Time course of epileptiform activity (without drug application) showing progressive changes of amplitude and event rate on an example of ictal spiking. Based on these changes we determined the reference value as average of control value (e.g. derived from the 20 min trace) and the recovery value (e.g. taken from the 100 min trace).
prolonged equilibration time (40 min). We saw the first blocking effects already after 10 min in both sclerotic and non-sclerotic tissue. Therefore, we assume that effective concentration of CBZ might be already attained after 20 min.

**Epileptiform activity in slices of the MTLE-group is resistant to CBZ irrespective of the type of activity**

In the MTLE-group, we induced epileptiform activity in the dentate gyrus of 34 slices from 19 sclerotic specimens (Fig. 2A, HS, top) and in 12 slices of 9 non-sclerotic specimens (Fig. 2A, nonHS, bottom). Four types of epileptiform activity/epileptiform events were observed (Fig. 2B): (i) tonic–clonic SLEs (first trace), (ii) ictal spiking (second trace), (iii) inter-ictal spiking (third trace) and (iv) tonic SLEs (fourth trace). Details of their characteristic activity parameters (reference values) are given in Table 1. Tonic–clonic SLEs exclusively appeared in sclerotic specimens while tonic SLEs only occurred in the non-sclerotic tissue ($P < 0.001$, $\chi^2$-test). The data so far were consistent with those recently reported by our group (Gabriel et al., 2004).

In the tumour-group, non-sclerotic slices developed tonic SLEs (90%) and inter-ictal spiking (10%) whereas ictal spiking was observed in one slice from a sclerotic specimen of a CBZ-sensitive patient.

As different types of epileptiform activity may be more or less sensitive to AEDs (Dreier and Heinemann, 1991), we first compared effects of CBZ between the four types of epileptiform activity in the MTLE-group. The results are displayed in the summary graph of Fig. 2C. After 20 min perfusion with 50 $\mu$M CBZ-containing high K-ACSF the mean activity parameters of all event types remained in a range of 76–108% of the reference value, clearly indicating resistance to CBZ. One-way-analysis of variance including post hoc tests (Bonferroni or Dunnett-T3 depending on homogeneity of variances) did not yield differences of the remaining activity between different events, suggesting similar effects of CBZ on the four types of epileptiform activity. Therefore, we were able to pool normalized data from different epileptiform activities in order to compare CBZ-effects between slices of different patient groups. We subdivided the MTLE-group according to hippocampal pathology and compared CBZ-effects between three slice groups: HS-MTLE, nonHS-MTLE and tumour (all nonHS except one sclerotic slice obtained from a CBZ-sensitive patient).

**Epileptiform activity is resistant to CBZ in slices of the MTLE-group, irrespective of the hippocampal pathology**

The groups consisted of 34 slices (19 patients with HS-MTLE), 15 slices (9 patients with nonHS-MTLE), and 11 slices (6 patients (5 nonHS-tumour, 1 HS-tumour)). In Figs 3A–C and 4 sample traces are shown for each group. In spite of CBZ application, the epileptiform activity persisted as long as high K-ACSF has been perfused, in both the HS-slice and the nonHS-slice from the MTLE group. The HS-MTLE slice (Fig. 3A) displayed tonic–clonic SLEs which were negligibly affected by CBZ. The tonic SLEs from the nonHS-MTLE slice (Fig. 3B) became smaller, the large amplitude events were reduced in number but additional small amplitude events occurred. In striking contrast,
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Table 1 Reference values of activity parameters for different epileptiform events in the dentate gyrus of MTLE-slices

<table>
<thead>
<tr>
<th>Event parameter (unit)</th>
<th>Tonic–clonic SLE (mean ± SEM)</th>
<th>Ictal spiking (mean ± SEM)</th>
<th>Inter-ictal spiking (mean ± SEM)</th>
<th>Tonic SLE (mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>10</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Event rate (events/min)</td>
<td>1.5 ± 0.24</td>
<td>61.0 ± 4.47</td>
<td>20.0 ± 2.91</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>sfp-amplitude (mV)</td>
<td>1.7 ± 0.38</td>
<td>0.9 ± 0.18</td>
<td>0.8 ± 0.14</td>
<td>3.8 ± 1.10</td>
</tr>
<tr>
<td>Peak amplitude (mV)</td>
<td>3.2 ± 0.58</td>
<td>1.2 ± 0.17</td>
<td>1.4 ± 0.26</td>
<td>5.3 ± 1.55</td>
</tr>
<tr>
<td>Duration (s)</td>
<td>24.7 ± 4.77</td>
<td>0.2 ± 0.04</td>
<td>1.5 ± 0.52</td>
<td>21.8 ± 7.66</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>6.9 ± 1.37</td>
<td>5.1 ± 4.06</td>
<td>6.6 ± 5.04</td>
<td>7.2 ± 4.58</td>
</tr>
<tr>
<td>Power (80–300 Hz) (10⁻³ mV²)</td>
<td>2.6 ± 1.11</td>
<td>0.5 ± 0.16</td>
<td>1.1 ± 0.55</td>
<td>1.5 ± 0.62</td>
</tr>
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</table>

In order to consider the time course of epileptiform activity in absence of drug application (see Fig. 1B) these reference values have been calculated as [(control value + wash value)/2].

![Fig. 3 Effects of CBZ (50 μM, 20 min) on slices of different patient groups: MTLE-HS, MTLE-nonHS, and tumour-nonHS. (A) Tonic-clonic SLEs in an MTLE-HS-slice (A1) before perfusion with CBZ-high K-ACSF (control), (A2) at the end of the perfusion with CBZ-high K-ACSF and (A3) 20 min after removal of CBZ (wash). (B) Tonic SLEs in an MTLE-nonHS-slice (B1–3). (C) Tonic SLEs in a slice from the tumour-nonHS group (C1–3). Note that CBZ suppressed the activity only in the slice from the tumour-nonHS group. (D) Summary graph displaying the mean percentage of the reference values remaining after perfusion with CBZ-high K-ACSF for each parameter: ev/min = event rate; sfp = amplitude of slow field potential; peak = peak amplitude; duration = event duration; frequency = intra-event frequency of potential fluctuations (>0.3 mV, time interval >3 ms); P< sub>h</sub> high frequency power (f = 78–293). Columns and error bars represent mean ± SEM for slices of the MTLE-HS group (n = 34, white), the MTLE-nonHS group (n = 15, grey) and the tumour-nonHS group (n = 11, black), including the one sclerotic slice of a CBZ-sensitive patient (sample traces in Fig. 4). (E) CBZ-remaining averaged activity (mean ± SEM of averaged normalized parameter values per slice given in percent) in each group. (F) Proportion of slices maintaining the averaged activity at a level of 0–20, 21–70 or >70% in the three groups. After administration of CBZ, epileptiform activity persisted in both MTLE groups, independent of hippocampal pathology, but became suppressed in the tumour-group. Asterisks denote significant statistical differences (P < 0.05).]
the tonic events in the nonHS-tumour slice (Fig. 3C) completely disappeared already after 15 min perfusion with CBZ-high K-ACSF and reappeared after removal of CBZ within 40 min. This was also true in the sclerotic slice of a CBZ-sensitive patient belonging to the tumour-group (Fig. 4).

Results for all parameters and slice-groups are summarized in Fig. 3D. With CBZ applied for 20 min, the mean values for all parameters remained at 81–99% of the corresponding reference values in slices of the HS-MTLE group and in the order of 84–91% in slices of the nonHS-MTLE group. In contrast, slices of the tumour-group were highly sensitive to CBZ. The remaining percentage of activity parameters after CBZ application was in the order of 6–17% except for the power of the 78–293 Hz band (35%). One-way analysis of variance revealed significant differences between groups for all parameters. Additionally, the post hoc tests showed that the remaining activity in both MTLE groups was significantly different from that of the Tumour-group while the HS- and nonHS-group of MTLE-patients did not significantly differ from each other.

CBZ-induced changes in each of the six activity parameters were highly correlated to each other (Pearson correlation coefficients between 0.59 and 0.94, n = 60, all with P < 0.001) and there was no significant difference between them (n = 57, P = 0.38, Friedman test). Therefore, we averaged the normalized parameter values for each slice and classified activities remaining with CBZ at 0–20, 21–70 and >70% as suppressed, reduced and resistant, respectively. The mean level of the remaining activity was 89 ± 4.6% in the HS-MTLE-group, 87 ± 9.6% in the nonHS-MTLE-group, and with 14 ± 6.0% significantly lower in the tumour-group (Fig. 3E). Additionally, we have to note that the activity persisted in 94% of the HS-MTLE slices and in 87% of the nonHS-MTLE slices, but only in 9.1% of slices in the tumour-group (Fig. 3F, P < 0.001; χ²-test). In the remaining slices of each group, the activity was found to be suppressed.

Prolongation of CBZ application has insignificant effects on epileptiform activity

Although slices of the tumour-group responded to CBZ within <20 min, we tested whether epileptiform activity in MTLE-slices required a prolonged equilibration time to get more affected by CBZ (Fig. 5). We tested 18 slices of 12 MTLE-patients for the effect of 40 min treatment with 50 μM CBZ. The sample traces of a HS- and a nonHS-slice depicted in Fig. 5A and B show that the activity is ongoing after 40 min perfusion with CBZ-high K-ACSF albeit the sfp-amplitude was more reduced than after 20 min. We also tested whether the ability of slices to develop epileptiform activity could be changed by preconditioning the slices with 50 μM CBZ-containing normal ACSF (Fig. 6). In this set of experiments the slices were pre-equilibrated with CBZ-ACSF for 20 min before changing the perfusion to CBZ-high K-ACSF (n = 6, e.g. Fig. 6, upper traces) and compared the occurring epileptiform activity to a second trial of activity induced by high K-ACSF after intermittent washout of CBZ and high K-ACSF for 60–90 min (e.g. Fig. 6, lower traces). We recognized (i) that epileptiform activity occurred despite pre-equilibration with CBZ, and (ii) that the characteristics of this activity did not significantly differ from activity in the absence of CBZ.

In 16 out of 18 slices (88.9%, Fig. 5C) the activity persisted at a mean level of 92.9 ± 2.36% in comparison to 96.3 ± 3.25% after 20 min (Fig. 5D, P = 0.23, n = 16, Wilcoxon test). In such ‘resistant’ slices (91.7% of patients) there was no significant decline in any of the parameters analysed (Fig. 5E). In the remaining two slices the activity became suppressed and reduced (up to 30%), respectively.
Prolongation of CBZ application with increased CBZ-concentration significantly reduces amplitudes and high frequency power but does not suppress epileptiform activity

Next we investigated whether suppression of epileptiform activity requires a higher dose of CBZ. We tested 10 slices of six MTLE-patients for the efficacy of 100 μM CBZ during prolongation of the anticonvulsant treatment (Fig. 7).

MTLE- and tumour-patients are clinically and neuropathologically different

The two patient groups displayed different clinical data (Table 2). In comparison to the tumour-group, the MTLE-group was characterized by long duration epilepsy and drug resistance including resistance to CBZ/oxcarbazepine. At surgery, the MTLE-patients were more often treated with
of 20 min perfusion with high K-ACSF, after intermittent washout another 20 min. (B) Same slice at the end of a second trial of 20 min perfusion with high K-ACSF, after intermittent washout of activity was seen in tissue of four patients (partial efficacy group, 12%). In three out of the last four patients, the ‘reduction’ resulted from averaging data of both one slice displaying suppression of activity by CBZ and another one displaying resistance, possibly indicating that drug resistance may be focally expressed. One example is illustrated in Fig. 8C1 and C2. Together, about one-third of patients presented with high or partial efficacy of CBZ in the resected tissue. As patients showing high efficacy of CBZ in the resected hippocampus were mainly tumour-patients (71.4%) and patients showing low efficacy of CBZ were MTLE-patients (100%), the two sub-groups differ not only in the proportional distribution of tumour- and MTLE-patients (histogram Fig. 8B). Accordingly, the patients showing CBZ-remaining activities at corresponding levels have been classified as showing ‘high’, ‘partial’ and ‘low’ efficacy of CBZ, respectively.

Resistance of activity was evident in tissue of 23 patients (low efficacy-group, 68% of patients in the whole sample). Suppression of activity was observed in tissue of seven patients (high efficacy-group, 20%). An apparent reduction of activity was seen in tissue of four patients (partial efficacy group, 12%). In three out of the last four patients, the ‘reduction’ resulted from averaging data of both one slice displaying suppression of activity by CBZ and another one displaying resistance, possibly indicating that drug resistance may be focally expressed. One example is illustrated in Fig. 8C1 and C2. Together, about one-third of patients presented with high or partial efficacy of CBZ in the resected tissue. As patients showing high efficacy of CBZ in the resected hippocampus were mainly tumour-patients (71.4%) and patients showing low efficacy of CBZ were MTLE-patients (100%), the two sub-groups differ not only in the proportional distribution of tumour- and MTLE-patients (histogram Fig. 8B). Accordingly, the patients showing CBZ-remaining activities at corresponding levels have been classified as showing ‘high’, ‘partial’ and ‘low’ efficacy of CBZ, respectively.

In vitro efficacy of CBZ coincides with cause of epilepsy, history of the patient’s CBZ-resistance, age at operation and duration of epilepsy

In order to analyse whether sensitivity to CBZ in resected hippocampal tissue is statistically related to clinical data, the normalized parameter values remaining after treatment with CBZ in each slice were averaged per patient, and subsequently averaged over all parameters. The resulting percentages of CBZ-remaining averaged activity in the data sample of patients (histogram Fig. 8A) were categorized as suppressed, reduced or resistant according to levels of 0–20, 21–70 or >70%, respectively, as already done with the slices (histogram Fig. 8B). Accordingly, the patients showing CBZ-remaining activities at corresponding levels have been classified as showing ‘high’, ‘partial’ and ‘low’ efficacy of CBZ, respectively.

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High efficacy of CBZ in resected hippocampal tissue coincided with clinically unproven drug resistance, CBZ-sensitivity or no treatment. By contrast, low efficacy of CBZ coincided with clinically proven drug resistance, including CBZ-resistance (Fig. 9A; \( P = 0.001, \chi^2\)-test). Besides, high efficacy of CBZ in the resected tissue was seen in patients of younger age (24.9 ± 4.71 versus 40.7 ± 2.54 years, \( P = 0.006 \)).
shorter duration of epilepsy (7.5 ± 4.47 versus 22.9 ± 2.90 years, \( P = 0.005 \)), and shorter treatment with CBZ (1.5 ± 0.71 versus 5.9 ± 0.80 years, \( P = 0.003 \)) (Fig. 9B). There was no difference between the two sub-groups with respect to age at onset of epilepsy, seizure type and seizure frequency, drug treatment at operation, and plasma concentrations of the AEDs (as far as normalized with respect to the maximum level for each drug detected within the sample of data). The third sub-group displaying partial efficacy of CBZ did not differ from each of the two other sub-groups. The proportional distribution of the three sub-groups of patients showing different efficacy of CBZ in the resected tissue only insignificantly changed with equilibration time of CBZ or even with higher concentration of CBZ during prolonged application (Fig. 9C).

**Sensitivity to CBZ and tissue excitability**

The \([K^+]_o\), at which stimulus-associated epileptiform discharges appeared [threshold \([K^+]_o\)] might reflect the degree of hyperexcitability in the epileptic tissue. This threshold \([K^+]_o\) was higher for patients whose slices responded to CBZ with suppression of activity than for patients who did not \([9.0 ± 0.52 \text{ range } 6.85–11.48 \text{ versus } 7.2 ± 0.28 \text{ range } 4.35–9.59 \text{ mM (} n = 30, \ P = 0.003, \text{ Mann–Whitney-test})]\). Moreover, there was a significant inverse correlation...
between the CBZ-remaining averaged activity and the threshold $[K^+]_o$-values (Spearman rho: $r = -0.44$, $P = 0.010$, $n = 34$; Fig. 9D; pooled sample of MTLE- and tumour-patients). Notably, when MTLE-patients were tested separately, the mean threshold $[K^+]_o$-values of patients showing high or low efficacy of CBZ in the slice did not significantly differ and the spread diagram of the remaining averaged activities and the threshold $[K^+]_o$-values (Fig. 9D, empty circles) revealed only a weak and insignificant correlation of the two parameters (Spearman rho $r = 0.29$, $P = 0.149$, $n = 27$). This may indicate that the correlation found in the whole population reflects rather a different distribution of both hyperexcitability and drug resistance between patients operated on MTLE and on extra-hippocampal tumours than a dependence of CBZ-effects on excitability of the tissue.

### Discussion

Our main results show that epileptiform activity in the dentate gyrus of drug-resistant MTLE-patients is predominantly CBZ-resistant, irrespective of the presence of hippocampal sclerosis and independent of the equilibration time of the drug. In contrast, epileptiform activity was CBZ-sensitive in slices of patients with extra-hippocampal tumours and with a short history of epilepsy. As CBZ, directly applied into hippocampal slices of resected tissue, has not to cross the BBB in order to reach cellular targets, our results indicate that the parenchyma of the dentate gyrus contains mechanisms of CBZ-resistance in most of the MTLE-patients. The results also suggest that drug transporters at the BBB are not determinants of dentate CBZ-resistance but might be responsible for drug resistance in two MTLE-patients (7%) whose slices were found to be highly sensitive to the drug.
Is the model of epilepsy used to test drug resistance in the slice CBZ-sensitive?

The first question is whether the high potassium-induced epileptiform activity might be per se pharmaco-resistant or in certain forms of activity less sensitive. Inter-ictal activity in areas CA3/CA2 (Rutecki et al., 1985; Korn et al., 1987; Colom and Saggau, 1994) and seizure-like events in area CA1 (Traynelis and Dingledine, 1988; Jensen and Yaari, 1997), induced by elevation of $\text{[K}^+]_o$ in normal rat slices, were blocked by the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Leschinger et al., 1993) and by AEDs, in particular CBZ (Leschinger et al., 1993; Arias and Bowlby, 2005). We already showed that stimulation-high $\text{K}^+$-induced self-sustaining epileptiform activity occurring in the dentate gyrus from human epileptic hippocampal tissue was suppressed by CNQX and by lowering extracellular calcium concentration (Gabriel et al., 2004). Now, we report that this synaptically mediated epileptiform activity was blocked by CBZ in 91% of slices from hippocampal tissue of patients with extra-hippocampal tumours. Additionally, in slices of MTLE-patients, each type of activity was found to be similarly affected by CBZ. Thus, data obtained from both the rat and human hippocampus provide arguments against the assumption that drug resistance is a property of high $\text{K}^+$-induced epileptiform activity.

Is CBZ actually a substrate of human drug transporters?

The fact that the epileptic dentate gyrus in slices of drug-resistant patients is predominantly CBZ-resistant renews the question, whether CBZ is a substrate of drug efflux pumps (Potschka et al., 2001, 2003). Other studies revealed that CBZ at therapeutically relevant concentrations did not interact with drug efflux pumps or inhibit those (Owen et al., 2001; Maines et al., 2005; Weiss et al., 2003). In MTLE-patients, the hippocampus to plasma concentration ratio of the primary active oxcarbazepine-metabolite 10-OHCBZ was found to be inversely correlated to the MDR1-mRNA.
levels (Marchi et al., 2005). This strengthens the assumption that P-glycoprotein (Pgp) which is up-regulated by MDR1-mRNA impairs access of oxcarbazepine to the focal epileptic tissue. Whether CBZ like oxcarbazepine would have reduced brain access in drug-resistant patients remains to be investigated. Therefore, suggesting BBB-located resistance mechanisms when dentate epileptiform activity in slices is suppressed by directly applied CBZ is still not conclusive.

The dentate gyrus of MTLE-patients contains CBZ-resistance mechanisms irrespective of the hippocampal pathology

In the majority of slices prepared from resected hippocampal tissue of MTLE-patients, the granule cell populations did not adequately respond to therapeutically relevant concentrations of CBZ, despite free access of the drug to the cellular targets. Therefore we propose that drug resistance mechanisms exist in the parenchyma and might be determined by several factors.

The results from whole cell patch clamp recordings in isolated dentate granule cells of MTLE-patients and from chronic epileptic animals showed that CBZ did not slow the recovery from inactivation of voltage-gated sodium channels and did not augment the use- and frequency-dependent block of these channels (Reckziegel et al., 1999; Remy et al., 2003a). Thus, we assume that reduced CBZ-sensitivity of voltage-gated sodium channels is one factor causing CBZ-resistance of epileptiform activity. However, epilepsy-related changes in the properties of sodium channels, which were recently reviewed (Remy and Beck, 2006), differ depending on the cell-type examined (Vreugdenhil et al., 1998 versus Remy et al., 2003a), on the AED studied (Remy et al., 2003a, b), and on the epilepsy model investigated (Vreugdenhil and Wadman, 1999 versus...
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Remy et al., 2003a). Therefore, our conclusions might be restricted to the dentate gyrus, to the drug CBZ, and to the high K+-model of epilepsy.

Another factor implicated in parenchymal CBZ-resistance might be drug efflux by transporter proteins expressed in relatively healthy neurons (MRP5) and astrocytes (MRP4, MRP5), for example in perilesional cortex (Nies et al., 2004), or overexpressed in neurons and/or astrocytes (Pgp, MRP1, MRP5) which reside in different epileptogenic pathologies (Sisodiya et al., 2002; Aronica et al., 2003a, 2004; Lazarowski et al., 2004; Vogelgesang et al., 2004). As already discussed, it is presently not clear whether and which efflux pumps transport CBZ. Nevertheless, if CBZ-resistance was significantly supported by parenchymal drug transporters ‘exclusively expressed in sclerotic hippocampi’ (Aronica et al., 2004) sclerotic tissue would be less sensitive to CBZ than non-sclerotic tissue. However, similar to effects of valproate on sodium currents in area CA1 (Vreugdenhil et al., 1998), the effects of CBZ on epileptiform activity in the dentate gyrus did not differ between sclerotic and non-sclerotic tissue, suggesting that dentate CBZ-resistance resembles CBZ-resistance of MTLE-patients, irrespective of the presence of hippocampal sclerosis. Additionally, it should be taken into account that CBZ, phenytoin and lamotrigine block sodium channels only when externally applied (Kuo, 1998). As the transporter pumps the drug out of the cell towards the extracellular side of the membrane, a contribution of drug transporters to failure of cellular CBZ-responses is not very likely, but remains to be experimentally tested.

The third factor hypothetically involved in drug resistance is synaptic reorganization of excitatory and inhibitory neuronal circuits evident as mossy fibre sprouting, preservation of GABAergic inhibition, sprouting of perisomatic inhibitory fibres and loss or alteration of specific interneurons (Babb et al., 1989; de Lanerolle et al., 1989; Sutula et al., 1989, Houser et al., 1990; Lehmann et al., 2000; Magloczky et al. 2000; Wittner et al., 2001). In human MTLE, these changes are most pronounced in sclerotic tissue and partially present in non-sclerotic tissue. This suggests different degrees of hyper-excitation, hyper-inhibition, synchronization, impairment of dendritic granule cell inhibition, reduction of interneuron-selective inhibition and contribution to failure of GABAergic AEDs, when granule cells become activated (Magloczky and Freund, 2005). With focus on the present study, reorganization might support induction of epileptiform activity by mossy fibre activation (hilar stimulation) and neuronal depolarization [elevation of [K+]o]. The different levels of reorganization might be responsible for the expression of different types of epileptiform activity in sclerotic and non-sclerotic tissue (Gabriel et al., 2004). However, the result that effects of CBZ on epileptiform activity were similar with respect to the type of activity, the degree of sclerosis or the degree of excitability (K+-threshold) did not provide any hint on relations between CBZ-resistance and different levels of synaptic reorganization. Since reorganization of inhibitory circuits has not been investigated in the tissue tested for the efficacy of CBZ, the question whether it might be a factor of CBZ-resistance remains open.

The dentate gyrus of patients with extra-hippocampal tumours is CBZ-sensitive

We observed high efficacy of CBZ in the dentate gyrus of hippocampal slices in the majority of patients with primary extra-hippocampal tumours and only in a minority of MTLE-patients.

With respect to the tumour-patients we should consider that their CBZ-sensitivity in the dentate gyrus of the hippocampal slice has several clinical and experimental scenarios. The group comprised (i) a patient without epilepsy, without hippocampal pathology, and with the highest threshold [K+]o (11.5 mM at occurrence of stimulus-induced discharges), (ii) a drug-sensitive patient with HS and a lower threshold [K+]o (6.85 mM), and (iii) four patients with a short history of persistent seizures (not proven drug-resistant), non-sclerotic hippocampus, and a threshold [K+]o ≥8 mM. In combination, the high efficacy of CBZ and the high threshold [K+]o in hippocampal slices of patients with primary extra-hippocampal tumours reflect that the recordings were made in normal or mostly para-lesional hippocampus showing a low degree of hyperexcitability. Therefore, we assume that para-lesional hippocampus resected after short duration of epilepsy and treatment is still drug-sensitive. This might be supported by the finding of partial efficacy after 1.8 years of persistent seizures in the patient whose tumour partially infiltrated the hippocampus.

CBZ-resistance is rarely located at the hippocampal BBB

With focus on clinically proven CBZ-resistant MTLE-patients it is worth mentioning that high efficacy of CBZ was observed in only two specimens (one HS, one nonHS). As the serum concentration of drugs in the two patients was sufficiently high, the resistance mechanisms might be located at the BBB. At the BBB, drug transporters like Pgp and multidrug-resistance associated proteins (MRP1, MRP2, MRP4, MRP5) are present or overexpressed in capillary endothelial cells or in astrocytes around the lesonal microvasculature (Golden and Pardridge, 2000; Sisodiya et al., 2002, 2006; Aronica et al., 2004; Nies et al., 2004; Kubota et al., 2006). Whether overexpression of Pgp and MRP2 in microvessels of the hippocampus is actually restricted to sclerotic tissue (Aronica et al. 2004) or may be also found in non-sclerotic cases, as indicated by a reduced brain-to-plasma drug concentration ratio accompanied by increased expression of MDR1-mRNA in two out of four non-sclerotic specimens in the work of Marchi and
colleagues (Marchi et al., 2005), is presently not clear. Alternatively, it is conceivable that the dentate gyrus found to be highly or partially sensitive to CBZ in five MTLE-patients was not or only focally involved in drug resistance and seizure activity. This assumption is supported by the relatively high threshold \([K^+]_o\) in the slices of these patients.

**Outcome and further tasks**

Our findings reveal that the dentate gyrus of hippocampal tissue obtained from MTLE-patients with a long history of epilepsy is CBZ-resistant. This resistance is based on mechanisms located in the parenchyma, independent of hippocampal pathology. The CBZ-resistance in the slice resembles CBZ-resistance of the patient, likely to be explained by reduced target-sensitivity. Accordingly, our data supporting drug resistance by increased drug efflux at the BBB were disappointingly rare and not conclusive. The findings suggest that at least the dentate gyrus of MTLE-patients remains drug-resistant when drug transporters at the BBB could be blocked by new medicaments. Two things remain to be done. (i) To rule out that CBZ-resistance in the dentate gyrus is a regional particularity and possibly irrelevant for the patient’s drug resistance, the efficacy of several AEDs needs to be studied not only in the dentate gyrus but also in other regions involved in seizure activity and drug resistance (e.g. the temporal cortex). (ii) The function of drug transporters ectopically expressed in neurons and astrocytes of epileptic tissue (Marchi et al., 2004) has not been determined in relation to the cellular drug response. In order to estimate their contribution to parenchymal drug resistance the efficacy of AEDs should be tested in presence of drug transporter inhibitors.

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**References**


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