The epileptic human hippocampal cornu ammonis 2 region generates spontaneous interictal-like activity in vitro

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The dentate gyrus, the cornu ammonis 2 region and the subiculum of the human hippocampal formation are resistant to the cell loss associated with temporal lobe epilepsy. The subiculum, but not the dentate gyrus, generates interictal-like activity in tissue slices from epileptic patients. In this study, we asked whether a similar population activity is generated in the cornu ammonis 2 region and examined the electrophysiological and neuroanatomical characteristics of human epileptic cornu ammonis 2 neurons that may be involved. Hippocampal slices were prepared from postoperative temporal lobe tissue derived from epileptic patients. Field potentials and multi-unit activity were recorded in vitro using multiple extracellular microelectrodes. Pyramidal cells were characterized in intra-cellular records and were filled with biocytin for subsequent anatomy. Fluorescent immunostaining was made on fixed tissue against the chloride–cation cotransporters sodium-potassium-chloride cotransporter-1 and potassium-chloride cotransporter-2. Light and electron microscopy were used to examine the parvalbumin-positive perisomatic inhibitory network. In 15 of 20 slices, the hippocampal cornu ammonis 2 region generated a spontaneous interictal-like activity, independently of population events in the subiculum. Most cornu ammonis 2 pyramidal cells fired spontaneously. All cells fired single action potentials and burst firing was evoked in three cells. Spontaneous excitatory postsynaptic potentials were recorded in all cells, but hyperpolarizing inhibitory postsynaptic potentials were detected in only 27% of the cells. Two-thirds of cornu ammonis 2 neurons showed depolarizing responses during interictal-like events, while the others were inhibited, according to the current sink in the cell body layer. Two biocytin-filled cells both showed a pyramidal-like morphology with axons projecting to the cornu ammonis 2 and cornu ammonis 3 regions. Expression of sodium-potassium-chloride cotransporter-1 and potassium-chloride cotransporter-2 was reduced in some cells of the epileptic cornu ammonis 2 region, but not to an extent corresponding to the proportion of cells in which hyperpolarizing postsynaptic potentials were absent. Numbers of parvalbumin-positive inhibitory cells and axons were shown to be decreased in the epileptic tissue. Electron microscopy showed the...
Epileptiform activity in the CA2 region

Temporal lobe epilepsy (TLE) is often associated with hippocampal sclerosis. Hippocampal sclerosis consists of a stereotyped and severe neuronal loss in the cornu ammonis (CA)1 and -3 areas of the hippocampus with a slight/moderate cell loss in the dentate gyrus and the CA2 region (Loup et al., 2000; Proper et al., 2000; Pirker et al., 2001; Andrioli et al., 2007). There seems to be little or no neuronal death in the subiculum of patients with classical hippocampal sclerosis (Andrioli et al., 2007). The pattern of cell loss in epilepsies of the temporal lobe is highly heterogeneous and several grading systems have been used by different groups to describe hippocampal sclerosis (Wyler, 1992; Proper et al., 2000; de Lanerolle et al., 2003; Wittner et al., 2005).

Work on excised human epileptic tissue has demonstrated considerable anatomical changes in preserved regions. In the dentate gyrus, which is the largest and best studied of these regions, there is an intense re-organization of both excitatory and inhibitory neuronal networks. Dentate granule cells are dispersed (Houser, 1990), certain inhibitory interneuron classes are selectively lost (de Lanerolle et al., 1989; Maglóczy et al., 2000; Wittner et al., 2001), and axonal sprouting occurs for surviving excitatory (Houser et al., 1990; Babb et al., 1991) and inhibitory cells (Maglóczy et al., 2000; Wittner et al., 2001).

In the CA2 region of epileptic patients, while there is only limited pyramidal cell loss, there is evidence for a major re-organization of perisomatic inhibition. The density of parvalbumin (PV)-containing perisomatic inhibitory cells in the CA2 region is considerably decreased (Andrioli et al., 2007). An inhomogeneous distribution of the axon terminals of axo-axonic cells has been described with a lack of staining in some regions and an increase in the complexity of many surviving terminals (Arellano et al., 2004). Furthermore, the identity of GABA A receptor subunits, contributing to perisomatic inhibition in the CA2 region was altered (Loup et al., 2000), suggesting perturbations at the subcellular level with possible functional consequences for GABAergic signalling.

This re-organization of inhibitory and excitatory circuits may contribute to the generation of epileptiform activity by surviving regions of hippocampal formation. In vitro studies of human tissue have shown epileptiform population activities in both the dentate gyrus and the subiculum. Interictal-like activity is generated spontaneously in the subiculum (Cohen et al., 2002; Wozny et al., 2005; Huberfeld et al., 2007). In the dentate gyrus, seizure-like events have been induced by electrical stimuli or by elevating extracellular potassium levels (Gabriel et al., 2004). Single cell records show profound changes in synaptic responses and cellular excitability in surviving regions. In the dentate gyrus, an impaired inhibition is correlated with the degree of mossy fibre sprouting (Franck et al., 1995). It probably contributes to hyperexcitable population responses to anti-dromic and ortho-dromic stimuli (Masukawa et al., 1989, 1992, 1996, 1997, 1999; Urano et al., 1994; Isokawa and Fried, 1996). Individual granule cells also discharge more intensely in response to current injection (Dietrich et al., 1999). In the CA2 region, hyperpolarizing inhibitory synaptic responses are weak or absent (Williamson and Spencer, 1994). In the subiculum, depolarizing as well as hyperpolarizing inhibitory synaptic potentials in pyramidal cells may contribute to spontaneous interictal-like activity (Cohen et al., 2002; Wozny et al., 2005; Huberfeld et al., 2007).

The deficit in GABAergic signalling in the human epileptic subiculum has been associated with a perturbed chloride homeostasis (Palma et al., 2006; Huberfeld et al., 2007). The internal chloride concentration is largely controlled by two cation–chloride cotransporters (Payne et al., 2003; Sipila et al., 2006). The Na–K–2Cl cotransporter NKCC1 and the K–Cl cotransporter KCC2 have functionally opposing effects. NKCC1 increases, while KCC2 was downregulated (Palma et al., 2007), resulting in elevated levels of internal chloride and depolarizing GABAergic events (Palma et al., 2006; Huberfeld et al., 2007).

Little is known about the physiological and anatomical properties of the human epileptic CA2 region. Here, we show that an interictal-like activity is generated in the CA2 region in hippocampal slice preparations derived from TLE patients. We used...
Electrophysiological and anatomical techniques to clarify the properties and possible mechanisms of this epileptiform activity. Simultaneous multiple channel extracellular and intracellular records were made to describe excitatory and inhibitory signalling in the CA2 region, and to relate cellular responses to the synchronous population events. We explored the role of neuronal chloride homeostasis, which might be related to depolarizing GABAergic responses, by double immunofluorescence against the Cl-cotransporters KCC2 and NKCC1. Changes in the perisomatic inhibitory input were verified by light and electron microscopic analysis of PV-positive interneurons.

**Methods**

**Epileptic patients—control subjects**

Hippocampal tissue was obtained from operations on patients (age range, 28–52 years; seizures for 5–39 years) with pharmaco-resistant epilepsies of the temporal lobe. All patients gave a written consent, and our protocol was approved either by the Comité Consultatif National d’Ethique (France) or by the Hungarian Ministry of Health (Hungary). Control hippocampi (n=2, age 53 and 56 years) were kindly provided by the Lenhossek Human Brain Program, Semmelweis University, Budapest, Hungary. Control subjects died from causes unrelated to any brain disease and were processed for autopsies in the Department of Forensic Medicine of the Semmelweis University. The autopsy confirmed the absence of any neurological disorders. Brains were removed 2–4 h after death, and fixed by perfusion through the internal carotids and vertebral arteries, as described (Wittner et al., 2001, 2002, 2005). Both control brains (C10 and C11) have been used in previous studies (Wittner et al., 2001, 2002, 2005). The study was approved by the Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health (TUKEB 5-1/1996, further extended in 2005) and performed in accordance with the Declaration of Helsinki.

**Tissue preparation**

Tissue was transported from the operating room to the laboratory in a cold, oxygenated solution containing (in millimolar) 248 d-sucrose, 26 NaHCO3, 1 KCl, 1 CaCl2, 10 MgCl2 and 10 d-glucose, equilibrated with 5% CO2 solution containing (in millimole) 124 NaCl, 26 NaHCO3, 4 KCl, 35–37°C. After injection, slices were maintained for at least 1 h in the recording chamber. They were then fixed overnight in 4% paraformaldehyde with 15% picric acid in 0.1 M phosphate buffer (PB) at 4°C. They were resectioned at 70 µm and freeze-thawed above liquid N2 in PB containing 30% sucrose. Cells containing biocytin were revealed with the ABC reaction (Vector, 1.5 h, 1:250) using diaminobenzidine (DAB, Sigma, St Louis, MO, USA) as the chromogen. Sections were osmicated (20 min, 0.5% OsO4), dehydrated in ethanol and mounted in Durcupan (ACM; Fluka, Buchs, Switzerland). Cells were digitally reconstructed in three dimensions using the NeuroLucida system (MicroBrightField Inc. Williston, VT, USA).

**Immunohistochemistry**

Sixty-micrometre thick sections were cut from fixed tissue (see above), washed with 0.1 M PB and freeze–thawed over liquid N2 in PB containing 30% sucrose. Endogenous peroxidase activity was blocked with 1% H2O2. Non-specific staining was suppressed with 5% skim milk powder and 2% BSA in PB. KCC2 immunostaining used a polyclonal rabbit antibody (1:2000 dilution) (Payne et al., 1996), NKCC1 was stained with a monoclonal mouse antibody (T4, Developmental Studies Hybridoma Bank, Iowa City IA, USA, 1:2000 dilution) for 24 h at 4°C. The specificity of the KCC2 antibody has been detailed previously (Payne et al., 1996; Williams et al., 1999). The T4 NKCC1 antibody showed no staining in NKCC1 knockout mice (Chen et al., 2005). Alexa488-bound donkey anti-rabbit and Alexa594-bound horse anti-mouse fluorescent secondary antibody (3 h, 1:250; Invitrogen, San Diego, CA, USA) were used as secondary antibodies. Images of fluorescent immunostaining were made with a confocal microscope (SP2; Leica, Nussloch, Germany). The specificity of immunostaining was verified according to the following criteria: (i) incubating sections without primary antibody gave no specific staining; (ii) somatic or perisomatic dendritic membrane was clearly stained; and (iii) membrane staining could be distinguished from non-specific lipofuscin autofluorescence.
A monoclonal mouse antibody was used against PV (1:5000, Sigma, St Louis, MO, USA) for 24 h at 4°C. The specificity of the antibody was tested by the manufacturer. For visualization of immunopositive elements, biotinylated anti-mouse immunoglobulin G (1:300, Vector) was applied as secondary antiserum followed by avidin–biotinylated horseradish peroxidase complex (ABC; 1:300, Vector). The immunoperoxidase reaction was developed by DAB dissolved in Tris buffer (TB, pH 7.6) as a chromogen. Sections were dehydrated as described above. Dark, specific staining due to the antibody could be clearly distinguished from the light non-specific osmium staining of lipofuscin.

**Electron microscopy**

After light microscopic examination, areas of interest were re-embedded and sectioned for electron microscopy. Ultrathin serial sections were collected on Formvar-coated single slot grids, stained with lead citrate and examined with a Hitachi 7100 electron microscope. For the analysis of synaptic input to CA2 pyramidal cells, blocks were re-embedded from one control (C11) and three sclerotic epileptic hippocampi, containing the entire width of the pyramidal layer (one block from each patient). About 15–25 cells were digitized from each subject or patient (each from one 55 nm thick section). In one specimen (Patient P960830) numerous degenerating pyramidal cells were found. These cells were not included in the analysis. The perimeter of pyramidal cell somata and the contact length of all afferent synaptic boutons made with the soma were measured using the ImageJ program (Wayne Rasband, National Institutes of Health, USA). The synaptic coverage of each soma was measured as the ratio of the total length of afferent terminals divided by the somatic surface perimeter (Wittner et al., 2001, 2005).

**Results**

**Hippocampal anatomy**

In this study, we examined population activities of the human epileptic CA2 region and cellular properties of CA2 neurons in slice preparations from 13 TLE patients (11 with hippocampal sclerosis and 2 without hippocampal sclerosis). Fixed tissue was used for anatomical analysis: the expression of the chloride-cotransporters NKCC1 and KCC2 by neurons of the CA2 region was studied in seven patients (six hippocampal sclerosis, one non-hippocampal sclerosis), and the perisomatic input of CA2 pyramidal cells in PV-stained sections was examined in four patients (three hippocampal sclerosis, one non-hippocampal sclerosis).

We determined the boundaries of the CA2 region of the human hippocampal formation from previous descriptions (Lorente de No, 1934; Rosene and Van Hoesen, 1987). TLE is often associated with hippocampal sclerosis consisting of a severe atrophy of the CA1 and CA3 regions, whereas the subiculum, the dentate gyrus and the CA2 regions are relatively spared—although the degree of cell loss may vary in all regions (Sommer, 1980; Proper et al., 2000; Pirker et al., 2001; Andrioli et al., 2007). Our sclerotic samples showed classical signs of hippocampal sclerosis with a relatively homogeneous pattern of principal cell loss. The CA1 region was very atrophic, medium cell loss was observed in the CA3a/b subregions, and many neurons were lost in the CA3c/hilar region. The CA2 region was demarcated by the absence of CA1 pyramidal cells and a reduced thickness of the CA3 pyramidal cell layer (PCL) (see Supplementary Fig. 1). Although the CA2 region was clearly much better preserved than the CA1 and CA3 regions, it remains possible that a quantitative analysis would reveal a slight/moderate cell loss. In the patients with no hippocampal sclerosis, the border between the CA2 and CA1 region was determined by the thickness of the cell layer: CA2 pyramids form a densely packed, relatively thin layer, whereas the PCL in the CA1 and CA3 regions is thicker and cell bodies are more dispersed. Thickness of the cell body layer and borders of the CA2 region were visible in the unstained slices from which recordings were made. Electrophysiological recordings were made with both extra- and intra-cellular electrodes placed in the middle of the CA2 region to avoid either CA1 or CA3 cells. In fixed and immunostained tissue, we used additional characteristics to determine the boundaries of the CA2 region. Somata of CA2 pyramidal cells are larger than those of either CA3 or CA1 pyramidal cells, they are strongly stained with calbindin (Seress et al., 1992), and they usually contain more lipofuscin. Both immunostaining procedures used (fluorescence and peroxidase reaction combined with osmication) showed a non-specific lipofuscin staining clearly distinguishable from the specific stainings (see Methods section).

Based on a combination of these features, we could distinguish CA2 region from the two neighbouring hippocampal subfields. Quantitative anatomical experiments, including cell counts (KCC2 and NKCC1) and electron microscopy of CA2 pyramidal cells were also made from the middle of the CA2 region avoiding the region boundaries.

**Spontaneous interictal-like activity is generated in the CA2 region in vitro**

Interictal spikes detected on scalp electroencephalographic (EEG) recordings are a hallmark of focal epilepsies. Such events are absent in healthy subjects and represent one of the most important diagnostic signs of focal epilepsies. Interictal discharges in both human and experimental focal epilepsies are characterized by high amplitude (>50 μV), fast EEG transients or spikes, usually followed by a slow wave lasting several hundreds of milliseconds (for review see de Curtis and Avanzini, 2001). In human TLE, the use of intra-cranial electrodes reaching the parahippocampal gyrus confirmed the presence of interictal spikes in the deep structures and showed that they were more active than the neocortex (Clemens et al., 2003). In vivo intra-hippocampal (Ulbert et al., 2004b; Fabó et al., 2008) recordings with microelectrodes and high frequencies of data acquisition showed that an increased neuronal firing accompanied the field potential deflection of interictal spikes in the subiculum of patients with TLE.

Spontaneous synchronous discharges were generated by slices prepared from the subiculum of patients with TLE (Cohen et al., 2002; Wozny et al., 2005; Huberfeld et al., 2007). In vitro synchronous population events show similarities to interictal spikes recorded in vivo, consisting of rhythmically recurring fast field potential deflections associated with an increased neuronal firing. Caution is needed in comparing these population events with interictal spikes generated in vivo. Since neither in vivo...
intra-hippocampal recordings nor hippocampal tissue from healthy humans are available, we know very little about single cell activity or population oscillations that a healthy hippocampus generates. Based on the resemblances that we have noted, we will describe the synchronous population bursts observed in vitro as interictal-like activity.

The dentate gyrus, the CA2 region and the subiculum of the human hippocampal formation resist the severe cell loss associated with TLE. The subiculum, but not the dentate gyrus, generates an interictal-like activity in tissue slices from epileptic patients (Cohen et al., 2002; Wozny et al., 2005; Huberfeld et al., 2007). We asked whether a similar activity is generated by the CA2 region of epileptic human hippocampus.

Spontaneous interictal-like activity was observed in the CA2 region in vitro, in physiological bathing solution in 15 of 20 slices, derived from 10 of 13 TLE patients. The frequency of the synchronous events varied between 0.1 and 1.5 Hz, with a mean of 0.50 ± 0.42 Hz (mean ± SD, n = 12 slices). In the majority of the slices (n = 13), interictal-like activity region was generated repeatedly with a similar interval between events (Fig. 1A1), but in two slices, from two different patients, interictal-like events tended to occur in groups separated by long silences (Fig. 1A2 and 1A3). In 10 slices, field potentials were recorded with two or three electrodes located separately in the pyramidal layer. These records showed that interictal activities were synchronized within the region.

We examined the activity of the dentate gyrus in extracellular recordings made from the granule cell layer in 6 of 10 patients where an interictal-like activity was detected in CA2. In all cases, we detected a spontaneous MUA in the granule cell layer. Synchronous events, characterized by an acceleration of MUA, together with a field potential, were not detected in the dentate region in any of these slices (n = 6, Supplementary Fig. 2). Intra-cellular records from dentate granule cells revealed a spontaneous discharge of single action potentials and an absence of events indicative of population synchrony (n = 3, Supplementary Fig. 2).

We asked whether there was a temporal relation between synchronous activity generated in the CA2 region and that recorded in the same slice from the subiculum. We performed simultaneous recordings from CA2 and the subiculum (n = 6 slices), in slices where both regions generated an interictal-like activity. The occurrence of synchronous events in the CA2 region was independent of the subicular activity (Fig. 1B). Furthermore, interictal-like events were generated independently in the CA2 region in slices containing the hippocampus proper and lacking the subiculum (n = 4 slices). When interictal-like events were observed both in the CA2 region and the subiculum, the frequency of synchronous events in the CA2 region was usually lower (0.51 ± 0.50 Hz) than that in the subiculum (1.31 ± 0.79 Hz).

Figure 1 Spontaneous interictal-like activity emerged in vitro in the hippocampal CA2 region of TLE patients, showing either continuous rhythmicity (A1) or inhomogeneous incidence (A2 and A3). This interictal-like activity was independent of the subicular activity (B). Upper traces are wide band recordings, lower traces show field potential (band pass filtered, 1–50 Hz). Asterisks indicate interictal-like events. Single events, magnified on the right side, consisted of field potential deflections with elevated neuronal firing. From epileptic patients P250105 (A1), P180105 (A2 and A3), P050607 (B).
The shape of the interictal-like events varied between slices, but a field potential transient of \( \sim 30-100 \mu V \) amplitude, recorded in the PCL, associated with an increased frequency of multi-unit firing was observed in every case (Fig. 1). The field potential transient was either biphasic with a positive and a negative component, or showed only a positive deflection (Fig. 1, insets). Multi-unit firing usually increased during the rising phase of the field potential transient, and often continued during later phases (Fig. 1).

In two slices with interictal-like activity in the CA2 region, a laminar micro-electrode (see Methods section) was used to measure the LFPg (Fig. 2). The 24-channel electrode (distance between contacts: 150 \( \mu m \)) was placed perpendicular to the PCL, permitting recording from the entire width of the CA2 region, along the somatic–dendritic axis. Deflections in the LFPg signal were detected in both the pyramidal layer and proximal dendritic areas. MUA was visible in the cell body layer (Fig. 2A and B). CSD analysis shows an estimate of the transmembrane currents of the local neuronal population. Inward currents (sink) could reflect either an active, synaptically driven excitation or a passive return current. Outward currents (source) might represent an active inhibition, or the passive return current of a sink. During interictal-like activity, CSD analysis showed a sink in the stratum pyramidale and sources in dendritic regions of stratum radiatum and stratum oriens (Fig. 2C). This confirms that synchronous events are locally generated in the CA2 region, and suggests the presence of an excitation in the cell body layer. MUA analysis confirmed an increase in neuronal firing in the pyramidal layer during interictal-like activity (Fig. 2D).

### Cellular properties of human CA2 pyramidal cells

Intra-cellular records were made from 19 human CA2 pyramidal cells (Fig. 3, Table 1). Most of them fired in the absence of injected current \( (n=13/19) \). The mean resting membrane potential of six CA2 pyramidal cells that did not fire was \(-71.9 \pm 3.9 \) mV. Spontaneous firing consisted of single action potentials in all cells. Burst firing could be elicited by current injection in three cells (Table 1). The mean input resistance of CA2 pyramidal cells \( (n=17) \) was \(25.6 \pm 6.1 \) M\( \Omega \) and their time constant measured from responses to 200 ms duration, 0.5 mV amplitude negative current injections was \(15.5 \pm 7.1 \) ms. In the only previous study on CA2 pyramidal cells from human hippocampus with Ammon’s horn sclerosis, Williamson and Spencer (1994) noted that hyperpolarizing postsynaptic potentials (IPSPs) were weak or
Figure 3 The majority of the recorded human CA2 cells fired action potentials spontaneously (A). Spontaneous EPSPs (asterisks) were detected in all cells, and IPSPs (arrows) were observed in about one-third of the cells. Most of the CA2 pyramidal cells (n = 11/15) received depolarizing synaptic potentials simultaneously with interictal-like events (B), while the remaining cells were hyperpolarized during synchronous events (C). B1, C1 show single sweeps, B2, C2 show superimposed sweeps. Cells P041012_C11 (A and C), P050125_C4 (B).

Table 1 Cellular properties of human CA2 pyramidal cells

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rare at resting potential. Our data reinforce this observation. Spontaneous excitatory postsynaptic potentials (EPSPs) could be easily detected in all recorded cells (n=19) but hyperpolarizing potentials were evident in only six CA2 cells (Fig. 3A, Table 1).

**Behaviour of human CA2 cells during interictal-like activity**

During interictal-like events in the subiculum, the majority of pyramidal cells (~80%) hyperpolarize, while a minority of ~20% depolarize and sometimes discharge. We asked whether CA2 pyramidal cells also display different behaviours during synchronous events (n=15 cells). We found both hyperpolarizing and depolarizing responses of CA2 cells, in a rather different proportion to that recorded from the subiculum (Table 1). At resting membrane potential, large depolarizing synaptic potentials occurred simultaneously with interictal-like events in 73% of the cells (n=11/15), whereas a smaller group of cells (27%, n=4/15) showed a larger hyperpolarizing responses (Fig. 3B and C). At rest, the responses of a given cell to consecutive synchronous events were either depolarizing or hyperpolarizing. Mixed responses, or variable responses were not detected. The depolarizing or hyperpolarizing nature of the response in a given cell did not change as neuronal membrane potential varied between ~50 and ~70 mV.

Spontaneous IPSPs were detected exclusively in cells, where interictal-like events were associated with hyperpolarizing potentials (n=4). In cells with depolarizing responses, only spontaneous synaptic depolarizations, but no spontaneous hyperpolarizations, were detected (n=11).

**Morphology of human CA2 cells**

Three CA2 cells were fully recovered after biocytin injection. All were pyramidal cells, with a pyramidal shaped cell body, one thick apical dendrite traversing striatum radiatum and lacunosum-moleculare and several thinner basal dendrites in striatum oriens. Axon collaterals were well labelled for two of these cells and ramified in the CA2 region as in the rat hippocampus (Mercer et al., 2007). One of the cells projected to the CA3 region as well (Fig. 4). In both cases, axons arborized in both the striatum oriens and radiatum. Since our tissue samples were from epileptic patients, it is possible that this axonal distribution results in part from a reactive axonal re-organization.

**Cation–chloride cotransporters in the human epileptic CA2 region**

Huberfeld et al. (2007) suggested that different cellular behaviours during interictal-like events in the subiculum reflect differences in intra-cellular chloride homeostasis. We first used immunostaining techniques to search for possible differences in the expression of molecules that regulate Cl-homeostasis in CA2 neurons. Double immunofluorescent staining was used to examine the presence of the intra-cellular chloride accumulating NKCC1 and the chloride extruding KCC2 molecules in specimens from seven epileptic patients (Fig. 5, Table 2). As a control, we used hippocampal tissue derived from two healthy monkeys perfused in another study. While, we note that monkey data cannot be directly compared with human epileptic tissue, both species are primates and many anatomical features of the hippocampus are comparable (e.g. Chan-Palay et al., 1986; Nitsch and Leranth, 1991; Seress et al., 1991, 1993; Sloviter et al., 1991; Lavenex et al., 2009). A cautious comparison might then be realistic. In the monkey hippocampus, 99.4% of examined CA2 pyramidal cells were positive for NKCC1 (n=788/793) and 98.6% of the cells were positive for KCC2 (n=782/793). These results suggest that both chloride cotransporter molecules are present in nearly all pyramidal cells of the healthy primate CA2 region. Our electrophysiological results, where about two-thirds of CA2 cells showed depolarizing response to synchronous bursts, led us to expect a significant decrease in KCC2-positive cells in the CA2 region. However, we found that the proportion of the NKCC1-positive cells was lower, 87.5 ± 5.4% (n=1681/1892 cells from seven patients) than in the monkey CA2 region, and the proportion of KCC2-positive cells was slightly lower, at 93.8 ± 2.2% (n=1785/1892 cells). There was some variability in tissue from the different patients, but considerable numbers of NKCC1-negative pyramidal cells were always evident. Interestingly, the proportion of NKCC1+/KCC2− cells was similar in control monkey and epileptic human tissue.

**Figure 4 CA2 pyramidal cells project to the CA2 (n=2) and to the CA3 (n=1) regions. Axon collaterals were found in the strata oriens, pyramidale and radiatum. In this NeuroLucida drawing, cell body and dendritic tree are shown in red, axons in blue. Inset shows a photomicrograph from the same cell. P050125_C5.**
(1.01 and 1.05%, respectively). The ratio of double negative cells was elevated in the epileptic tissue (5.10% compared with 0.38% in control monkey CA2), suggesting that, in this region, reactive changes of the two chloride cotransporters in an epileptic brain could be linked.

### Somatic inhibitory input to human epileptic CA2 pyramidal cells

#### Light microscopy

The hypothesis that the strength of synaptic inhibition is reduced in an epileptic brain has been debated for many years. A decrease in the number of PV-positive interneurons has been described in the epileptic CA2 region, suggesting that inhibitory circuits are altered (Andrioli et al., 2007). An absence of spontaneous hyperpolarizing synaptic potentials in some CA2 pyramidal cells suggests that inhibitory systems in this region may be changed but that interneurons remain functional. The inhomogeneous distribution of perisomatic inhibitory axons reported in the CA2 region (Arellano et al., 2004) further suggests that structural changes in inhibitory systems might result in the preservation of inhibitory inputs to some, but not all cells. We examined this point by comparing PV-immunostaining of the CA2 region in tissue from two control subjects and five epileptic patients (four hippocampal sclerosis, one non-hippocampal sclerosis). In the control CA2 region, numerous PV-positive interneurons were present (see also Braak et al., 1991; Seress et al., 1993). The somata of

### Table 2 Proportions of NKCC1- and KCC2-positive pyramidal cells in control monkey and epileptic human CA2 region

<table>
<thead>
<tr>
<th></th>
<th>NKCC1+ KCC2+ (%)</th>
<th>NKCC1+ KCC2− (%)</th>
<th>NKCC1− KCC2+ (%)</th>
<th>NKCC1− KCC2− (%)</th>
<th>NKCC1+ (%)</th>
<th>KCC2+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey (n = 793)</td>
<td>98.3</td>
<td>1.0</td>
<td>0.3</td>
<td>0.4</td>
<td>99.4</td>
<td>98.6</td>
</tr>
<tr>
<td>Epileptic human (n = 1,892 cells from 7 patients)</td>
<td>86.4 ± 5.6</td>
<td>1.1 ± 0.4</td>
<td>7.4 ± 5.2</td>
<td>5.1 ± 2.3</td>
<td>87.5 ± 5.4</td>
<td>93.8 ± 2.2</td>
</tr>
</tbody>
</table>

Figure 5 Double immunofluorescent data show that the chloride accumulating cotransporter NKCC1 (green) and the chloride extruding KCC2 (red) are expressed by nearly all cells in the healthy monkey CA2 region (A). Arrowheads show the NKCC1+/KCC2+ cells. In the human epileptic CA2 region (B) both cotransporters are downregulated, although to different extents: 13% of CA2 pyramidal cells were NKCC1-negative (arrows), while only 6% were immunonegative for KCC2. Numerous double negative cells were observed in the epileptic CA2 region (asterisks). From Patient P050118.
the majority of the multi-polar inhibitory cells were located in the striatum pyramidale, with long dendrites traversing the entire width of the CA2 region, while other horizontal PV-positive cells were observed in the striatum oriens (Fig. 6A1). The axonal network in the pyramidal layer was homogeneous (Fig. 6A2). As in the previous studies (Arellano et al., 2004; Andrioli et al., 2007), we noted a strong decrease in PV-positive cells and axonal branches in the human epileptic CA2 region. Very few PV-positive cells were observed in epileptic tissue (Fig. 6B1). Axonal staining was inhomogeneous, with basket formations visible around the somata of some cells, but apparently absent from others (Fig. 6B2).

**Electron microscopy**

These data suggest that perisomatic inhibition of CA2 pyramidal cells may be reduced. However, PV has been shown to disappear from cell bodies and dendrites in rat (Sloviter, 1991; Maglóczky and Freund, 1995) and non-human primate (Scotti et al., 1997a, b) models of epilepsy, as well as in the human epileptic hippocampus (Wittner et al., 2001, 2005). Therefore, we used electron microscopy to examine the profiles of synaptic terminals contacting the soma of CA2 cells from one control and three epileptic patients with hippocampal sclerosis. This procedure provides another view of inhibitory innervation that does not depend on the PV content of presynaptic axon terminals. We measured the soma perimeter of CA2 cells and the active zone lengths of all synapses they received. The synaptic coverage (micrometres of synaptic length/100 μm soma perimeter) (Wittner et al., 2001, 2005) was determined for each examined cell.

In control tissue, CA2 pyramidal cell bodies were innervated exclusively by symmetrical, presumably inhibitory synapses ($n = 28$ cells, Fig. 7A, Table 3). The percentage of PV-positive terminals was 17.5%, the proportion of perforated synapses (more than one active zone/axon terminal) was 10.7% ($n = 103$ boutons examined). The synaptic coverage of the cells varied between 0.14 and 1.49 μm synaptic length/100 μm soma perimeter, with a mean of 0.80 ± 0.08 (mean ± SEM, Fig. 7C), and showed a normal distribution (Kolmogorov–Smirnov normality test). The average length of active zones was 0.175 ± 0.006 μm (mean ± SEM).

In the dentate gyrus (Wittner et al., 2001) and CA1 region of human epileptic tissue (Wittner et al., 2005), numerous symmetrical, presumably inhibitory axon terminals innervate principal cell somata. Asymmetrical, presumed excitatory synapses terminated on cell bodies only occasionally. In the CA2 region of all three epileptic samples, many symmetrical synapses (Fig. 7B) terminated...
on pyramidal cell bodies. Unexpectedly, we also observed frequent contacts made by asymmetrical synapses (Table 3, Fig. 7C). Most presumed excitatory boutons showed similar characteristics to those of mossy terminals (Amaral and Dent, 1981). They were of large size, typically with a diameter >2 μm, densely packed with many round vesicles, some dense core vesicles and numerous mitochondria (Fig. 7B3). We therefore quantified symmetrical (inhibitory) and asymmetrical (excitatory) synaptic coverage separately for CA2 pyramidal cells (Table 3, Fig. 7C). In all three samples, we examined the somatic input of CA2 cells in a single plane (from one 55 nm thick section), to avoid biased sampling. With this method, asymmetrical synapses were detected on the soma of 50, 70 and 62% of CA2 pyramidal cells examined, while symmetrical synapses were present on all examined cells.

The mean symmetrical synaptic coverage of the cells in the epileptic CA2 region was similar to that of cells from control subjects (Table 3, Fig. 7C). However, the variability between cells was higher in epileptic tissue (from 0.11 to 2.75 μm synaptic length/100 μm soma perimeter). In one patient with numerous degenerating pyramidal cells in the CA2 region (P960830), the mean symmetrical synaptic coverage of the non-degenerating pyramidal cells was lower than in the others, but the difference was not significant (Kruskall–Wallis one-way ANOVA). The distribution of the symmetrical synaptic coverage of CA2 cells in epileptic tissue was comparable with that of control tissue, although it did not pass the normality test. The proportion of PV-stained axon terminals facing CA2 cell bodies was dramatically decreased: 6.25% of terminals were PV-positive in one patient (n = 80 axon

Figure 7. Electron micrographs show the somatic input of CA2 pyramidal cells in the control (A) and the epileptic (B) CA2 region. Pyramidal cell somata are shown on A1 and B1. PV-positive (A2 and B2) and PV-negative (A3 and B3) axon terminals formed symmetrical synapses on the cell bodies. Asymmetrical synapses including large mossy terminals (B4, MT) innervated CA2 cell somata (PC) only in the epileptic tissue. Symmetrical synaptic coverage (micrometre synaptic length/100 μm soma perimeter) was found to be similar in the control and epileptic tissue (C, mean ± SEM). From control C11; and epileptic Patients P960930 (B1–3), P020117 (B4), P960830.
in the CA1 region (for reviews see Cossart). However, it has been relatively neglected (Sloviter).

Table 3 Symmetrical (inhibitory) and asymmetrical (excitatory) synaptic coverage of CA2 cell somata in control and epileptic tissue

<table>
<thead>
<tr>
<th></th>
<th>Control 11 (n = 28 cells)</th>
<th>P960930 (n = 21 cells)</th>
<th>P020117 (n = 18 cells)</th>
<th>P960830 (n = 10 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetrical synaptic coverage (micrometre synaptic length/100µm soma perimeter, mean ± SEM)</td>
<td>0.80 ± 0.08</td>
<td>0.81 ± 0.12</td>
<td>0.83 ± 0.15</td>
<td>0.61 ± 0.12</td>
</tr>
<tr>
<td>Asymmetrical synaptic coverage (µm synaptic length/100µm soma perimeter, mean ± SEM)</td>
<td>–</td>
<td>0.46 ± 0.15</td>
<td>0.26 ± 0.11</td>
<td>0.48 ± 0.19</td>
</tr>
<tr>
<td>Cells receiving asymmetrical synapses (%)</td>
<td>–</td>
<td>62</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>Number of axon terminals giving synapses to soma</td>
<td>n = 103</td>
<td>n = 121</td>
<td>n = 69</td>
<td>n = 64</td>
</tr>
<tr>
<td>Ratio of boutons giving symmetrical synapses (%)</td>
<td>100</td>
<td>66.1</td>
<td>78.3</td>
<td>71.9</td>
</tr>
<tr>
<td>Ratio of PV+ terminals (%)</td>
<td>17.5</td>
<td>6.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ratio of boutons giving perforated synapses (%)</td>
<td>10.7</td>
<td>25.0</td>
<td>25.9</td>
<td>30.4</td>
</tr>
<tr>
<td>Average length of synaptic active zones (µm)</td>
<td>0.175</td>
<td>0.168</td>
<td>0.177</td>
<td>0.137±</td>
</tr>
<tr>
<td>Ratio of boutons giving asymmetrical synapses (%)</td>
<td>–</td>
<td>33.9</td>
<td>21.7</td>
<td>28.1</td>
</tr>
<tr>
<td>Ratio of boutons giving perforated synapses (%)</td>
<td>–</td>
<td>39.0</td>
<td>26.7</td>
<td>38.9</td>
</tr>
<tr>
<td>Average length of synaptic active zones (µm)</td>
<td>–</td>
<td>0.175</td>
<td>0.216</td>
<td>0.219</td>
</tr>
</tbody>
</table>

*P<0.01.

Discussion

Hippocampal tissue obtained during surgery from epileptic patients provides an excellent opportunity to explore cellular and network properties of the human hippocampus. This study focused on the CA2 region, which is resistant to the severe cell loss related to TLE (Sloviter et al., 1991). The CA2 region forms quite a large subfield in human hippocampus. However, it has been relatively neglected in work on epilepsy, in favour of studies on the robust synaptic re-organization in the dentate gyrus and the degenerative changes in the CA1 region (for reviews see Cossart et al., 2005; Maglóczky and Freund, 2005). One study has described electrophysiological properties of CA2 cells in slices of tissue obtained from epileptic patients (Williamson and Spencer, 1994), but network activity in the human CA2 region has not yet been investigated. Here, we report that the human epileptic CA2 region generates an independent epileptiform activity, similar in some respects to the interictal-like activity described in the subiculum.

Excitatory signalling was functional in epileptic CA2 pyramidal cells but hyperpolarizing inhibitory signalling was clearly present in only a minority of cells (cf. Williamson and Spencer, 1994). Immunostaining for Cl cotransporters suggested that chloride homeostasis of CA2 cells was considerably perturbed, although the reduced expression of NKCC1, and to a lesser extent KCC2, would be expected to strengthen hyperpolarizing inhibition. Immunostaining for the inhibitory cell marker PV, suggested that perisomatic inhibition of CA2 cells might be reduced. However, electron microscopic analysis instead revealed that somatic inhibitory inputs were preserved and showed that PV was absent from surviving interneurons. It also demonstrated aberrant somatic excitatory inputs.

Role of excitatory signalling

In the human epileptic subiculum in vitro, spontaneous interictal-like activity seems to depend on both glutamatergic and GABAergic signalling. Depolarizing GABAergic responses in a minority of pyramidal cells, with an altered chloride homeostasis, have been suggested to contribute to the generation of this interictal-like activity (Cohen et al., 2002; Huberfeld et al., 2007). The present study demonstrates that similar synchronous bursts, consisting of a field potential deflection with elevated neuronal firing, are generated in the CA2 region in vitro. As in the subiculum, pyramidal cells were either depolarized or hyperpolarized during interictal-like events. However, the proportion of the two responses differed. In the CA2 region about two-thirds of the pyramidal cells showed depolarizing responses, whereas in the subiculum only ~20% of the cells depolarize during interictal-like events. This result might be biased by the relatively small number of cells (n = 15) recorded in our study. However, the current sink in the cell body layer demonstrated by CSD analysis also indicates the importance of excitatory signalling in the generation of the synchronous events. Differences between the cellular composition, lamination and afferent connections in the CA2 region and the subiculum (Lorente de No, 1934; Sloviter et al., 1991; Seress et al., 1993) might also contribute to this dissimilarity. The combination of a functional excitation in all cells with the reduced inhibition suggests that the balance of excitation and inhibition is turned towards excitation, possibly favouring the emergence of epileptiform activity.
of synchronous population bursts. Our electron microscopic findings support previous data, that in the human epileptic hippocampus, mossy fibres sprout into the CA2 region (Houser et al., 1990) and form a novel somatic excitatory input to pyramidal cells. Synaptic excitation mediated by these aberrant inputs may contribute to the somatic current sink and the generation of interictal-like bursts in this region. This point could be tested further by asking whether the depolarizing cellular responses that accompany interictal-like events in most CA2 cells possess a GABAergic component, or whether they depend exclusively on glutamatergic signalling.

Although interictal-like events recorded in the CA2 region and in the subiculum differ in some respects, we should note the similarities between synchronous events recorded from the CA2 region in vitro and in vivo records of interictal spikes from the subiculum of epileptic patients. In vivo, two types of subicular interictal spikes display different CSD patterns (Fabó et al., 2008). Type 1 interictal spikes, like the synchronous events in the CA2 region in vitro, showed a current sink in the cell body layer, suggesting the presence of an active, perisomatic, excitatory mechanism (Fabó et al., 2008).

Role of GABAergic signalling

We detected spontaneous EPSPs and IPSPs in CA2 pyramidal cells suggesting that both excitatory and inhibitory synaptic circuits are functional in this region. Williamson and Spencer (1994) reported that inhibitory synaptic potentials were absent in most CA2 pyramidal cells from epileptic patients with hippocampal sclerosis. Inhibitory potentials were recorded in only 3 of 9 recorded cells and some events possessed slow kinetics, suggesting that they could have resulted from the activation of GABA_A rather than GABA_A receptors. In this work, spontaneous IPSPs with fast rather than slow kinetics were detected in 6 out of 19 recorded CA2 cells.

There was a perfect correlation between cells in which hyperpolarizing spontaneous IPSPs were detected and those in which hyperpolarizing responses accompanied synchronous interictal-like bursts \((n=4)\). In contrast, spontaneous synaptic events were exclusively depolarizing in cells which exhibited depolarizing responses during interictal-like bursts \((n=11)\). These distinct cellular responses, together with the inhomogeneous distribution of perisomatic axon terminals revealed by PV immunostaining, could be consistent with a structural impairment of GABAergic inhibition in a large subset of CA2 cells. However, this hypothesis was not supported by the electron microscopic data which revealed no or little difference between the numbers of presumed inhibitory symmetric boutons contacting the soma of epileptic and control CA2 pyramidal cells. Similar to the findings in the CA1 region (Wittner et al., 2005), the symmetrical synaptic coverage was decreased only in samples with evidence for pyramidal cell degeneration (P960830). Changes in dendritic inhibition or alterations in the expression of GABA_A receptor subunits (Loup et al., 2000), neither of which we examined, might also contribute to the absence of spontaneous IPSPs in some pyramidal cells.

The decrease in PV-positive elements coupled with the preservation of somatic inhibitory contacts indicates a disappearance of PV from perisomatic interneurons, a reactive response also observed in the dentate gyrus and the CA1 region (Wittner et al., 2001, 2005). Similar results have been found in a non-human primate model of epilepsy, with a significant loss of perikaryal PV in the CA2 region of the Mongolian gerbil (Scotti et al., 1997a). In the human epileptic CA2 region, PV immunostaining is much decreased in perisomatic terminals as well as the cell body. This differs from data obtained from the dentate gyrus and the CA1 region (Wittner et al., 2001, 2005), where the ratio of PV-positive boutons terminating on axon initial segments and/or soma was unchanged or increased in the epileptic samples, compared to the control. The lack of PV modifies Ca^{2+}-buffering and so changes the functional properties of these interneurons (for review see Schwaller, 2009). Investigations in PV^{-/-} knockout mice showed that PV deficiency facilitated the depolarizing switch in the polarity of GABAergic responses induced by high-frequency stimulation. In the PV-KO mice, these depolarizing responses to GABA resulted in hypersynchronous firing and an increased susceptibility to seizures induced by pentylenetetrazol, a GABA_A receptor blocker (Schwaller et al., 2004). Similar mechanisms might operate in the human epileptic CA2 region, resulting in an absence of hyperpolarizing potentials in some cells even while somatic inhibitory inputs were preserved. In this way, the absence of PV might contribute to the generation of interictal-like activity in the CA2 region.

Chloride homeostasis

Two recent anatomical studies have suggested that the chloride accumulating NKCC1 cotransporter is upregulated and KCC2, the chloride extruding cotransporter, is downregulated in some cells of the epileptic human subiculum (Palma et al., 2006; Munoz et al., 2007). An increased expression of NKCC1 coupled with a reduced KCC2 should elevate internal chloride concentrations and favour depolarizing actions of GABA. Combined physiological and anatomical work revealed no immunopositivity for KCC2 in a proportion of cells in which depolarizing events accompanied interictal field potentials (Huberfeld et al., 2007). With a greater proportion of CA2 cells showing depolarizing interictal-like responses, we expected a considerable downregulation of KCC2, with a possible upregulation of the NKCC1 cotransporter. In contrast, we found that NKCC1 was absent from \(\sim 13\%\) of CA2 pyramidal cells, and KCC2 from only \(\sim 6\%\). While we noted a rather elevated proportion of cells in which both cotransporters were absent, this situation should tend to reinforce a hyperpolarizing GABAergic inhibition in the CA2 pyramidal cell population. Thus the discrepancy between our electrophysiological and immunofluorescent data indicates that changes other than expression of these cotransporters may account for the impaired GABAergic signalling in epileptic CA2 cells.

Conclusions

We have shown that interictal-like events are spontaneously generated in the CA2 region of the epileptic human hippocampus
in vitro. Their form was similar to those recorded from the subiculum, but our data suggest that the underlying mechanisms may differ. Instead of a modified chloride homeostasis, an aberrant perisomatic innervation of CA2 pyramidal cells by excitatory afferents may contribute to a perturbation of the balance between excitation and inhibition in this region. The importance of excitation is supported by the presence of a current sink in the pyramidal layer, a high proportion of cells that depolarized during interictal-like events and the existence of mossy fibre terminals on pyramidal cell somata. Some evidence seemed to suggest that a reduction in the efficacy of synaptic inhibition might also modify the balance between excitatory and inhibitory processes. It included both single cell electrophysiology and the loss of PV-immunopositivity both in somato-dendritic elements and axonal processes. However, electron microscopy revealed that somatic inhibitory inputs were preserved suggesting that PV expression in perisomatic interneurons was lost. Further, immunostaining for Cl cotransporters showed that NKCC1 was more strongly downregulated than KCC2. This modification is inconsistent with a depolarizing shift in basal Cl equilibrium potentials in CA2 cells. Thus, our results suggest that an aberrant excitatory mossy fibre input to pyramidal cell somata together with functional changes in the PV-positive perisomatic inhibitory network resulting in depolarizing GABAergic responses may underly the generation of interictal-like activity in the epileptic CA2 region.

Acknowledgements

We wish to thank Drs M. Palkovits, P. Sótonyi and Zs. Borostyankói-Baldau (Semmelweis University, Budapest) for providing control human tissue, and Ms K. Iványi, K. Lengyel, E. Simon and Mr Gy. Goda for excellent technical assistance. Thanks to R. Csercsa and A. Magony for the data analysis program SpikeSolution.

Funding

INSERM (Poste vert to L.W.); the Bolyai János Research Fellowship (to L.W.); French and Hungarian governments (NKTH-Fr38/2006, ANR; OTKA49122, ETT135/2006); and the European Union (FP7 EPICURE FP6 EC LSH-CT-2006-037315, NeuroProbes EU IP IST-027017).

Supplementary material

Supplementary material is available at Brain online.

References


