British Journal of Pharmacology (2008) 154, 709–724

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Standard antiepileptic drugs fail to block epileptiform activity in rat organotypic hippocampal slice cultures

K Albus, A Wahab and U Heinemann

Institut für Neurophysiologie, Johannes Müller Center of Physiology, CCM, Charité-Universitätsmedizin Berlin, Berlin, Germany

Background and purpose: Earlier studies had demonstrated that tonic–clonic seizure-like events (SLEs) resembling electrographic correlates of limbic seizures in animals and humans can be induced in organotypic hippocampal slice cultures (OHSCs). We have explored OHSCs for their suitability to serve as *in vitro* models of limbic seizures for studying seizure mechanisms and screening new antiepileptic compounds.
Experimental approach: OHSCs were cultivated according to the interface method. Neuronal activity and extracellular potassium concentration were recorded under submerged conditions. SLEs were induced by lowering magnesium concentration or by applying the potassium channel blocker 4-aminopyridine. The effects of standard antiepileptic drugs (AEDs), carbamazepine, phenytoin, valproic acid, clonazepam, diazepam and phenobarbital sodium on SLEs were analysed.
Key results: In more than 93% of OHSCs, AEDs did not prevent the induction of SLEs or stop ongoing seizure activity even when toxic concentrations were applied. This pharmacoresistance was independent of the method of seizure provocation, postnatal age at explantation (P2–P10) and cultivation time *in vitro* (2 months). SLEs were reversibly blocked by glutamate antagonists or the $GABA_A$-agonist muscimol.
Conclusions and implications: We present a simple to establish *in vitro* model of tonic–clonic SLEs that is *a priori* pharmacoresistant and thus has an advantage over animal models of pharmacoresistant seizures in which responders and non-responders can be sorted out only after an experiment. OHSCs could be suitable for exploring mechanisms of pharmacoresistant seizures and be used for the identification of new anticonvulsive compounds eventually effective in drug refractory epilepsy.
British Journal of Pharmacology (2008) **154,** 709–724; doi:10.1038/bjp.2008.112; published online 14 April 2008

Keywords: hippocampal slice cultures; pharmacoresistant epileptiform activity; antiepileptic drugs; carbamazepine; phenytoin; valproic acid; clonazepam; diazepam; phenobarbital sodium

Abbreviations: 4-AP, 4-aminopyridine; AED, antiepileptic drug; CBZ, carbamazepine; CLO, clonazepam; DIV, days *in vitro*; DZP, diazepam; MEM, minimal essential medium; OHSCs, organotypic hippocampal slice cultures; P, postnatal day; PHB, phenobarbital sodium; PHT, phenytoin; SLE, seizure-like event; VPA, valproic acid

Introduction

About 70% of adult patients diagnosed with mesial temporal lobe epilepsy have drug refractory epilepsy (Schmidt and Loscher, 2005). In these patients, antiepileptic drugs (AEDs) at present in use do not result in persistent suppression of seizures in spite of their modulator effects on the incidence and properties of seizures. A change in the therapy regimes of such patients, either to alternative monotherapy or polytherapy by combining standard AEDs such as phenobarbital, phenytoin (PHT) or carbamazepine (CBZ) with one of the newer AEDs developed over the last 10 years, has resulted in only moderate improvements (Reynolds, 2006). Epilepsy in childhood poses similar problems. Between 20 and 30% of symptomatic/cryptogenic partial epilepsies and more than 50% of patients with Lennox–Gastaut syndrome are classified as intractable (Arts *et al.*, 2004). This short scenario already reveals that the management of refractory epilepsies in adults and children is still unresolved and thus a challenging task for experimental and clinical research.

Pathomechanisms involved in pharmacoresistance may be classified into three general categories: disease-related mechanisms, genetics and drug-related mechanisms (Schmidt and Loscher, 2005). Two key hypotheses have been proposed as disease-related mechanisms. The target hypothesis postulates alterations in drug targets (Remy and Beck,

Correspondence: Professor Dr K Albus, Institut für Neurophysiologie CCM, Charité-Universitätsmedizin Berlin, Tucholskystr.2, Berlin 10117, Germany.
E-mail: klaus.albus@charite.de
Received 17 December 2007; revised 19 February 2008; accepted 28 February 2008; published online 14 April 2008

2006), whereas the transporter hypothesis postulates that overexpression of multidrug transporters could reduce the concentration of AEDs in the epileptogenic brain regions (Loscher and Potschka, 2005). Genetic alterations due to, for example, polymorphisms in drug transporters may also contribute to pharmacoresistance (Remy and Beck, 2006). Finally, tolerance as a drug-related mechanism may be involved in reduced drug efficacy (Loscher and Schmidt, 2006).

There are still inconsistencies and limited proof with all of these hypotheses (Schmidt and Loscher, 2005), and therefore the mechanism of pharmacoresistance needs to be investigated further. In addition to studies on brain tissue from patients with drug-resistant seizures (Jandova *et al*., 2006; Oby *et al*., 2006), an important element of this research is regarding experimental models of drug-resistant epilepsy. Different types of spontaneous seizures in epileptic dogs, spontaneous seizures in amygdala-kindled rats and post-status epileptic seizures after prolonged electrical stimulation of the basolateral amygdala have all been shown to be pharmacoresistant in subgroups of the experimental animals (Loscher, 1997; Brandt *et al*., 2004). Some chemical seizure models are refractory to many AEDs, but their predictive value for the development of therapeutic strategies for refractory epilepsy has been questioned (Loscher, 1997). All these models are *a posteriori* models in that responders and non-responders can be sorted out only after completion of the experiment. Taken together, experimental models of refractory epilepsy in adult patients are rare and the situation might be even less favourable in the area of paediatric epilepsy (Jensen, 2006).

In contrast to animal models, *in vivo* pharmacoresistance may be reliably predicted *in vitro*. The respective models were detected when working out *in vitro* models of seizure-like activity in acute slices of the neocortex or hippocampus and entorhinal cortex. In these tissues, different types of seizure-like events (SLEs) can be induced by increasing the extracellular potassium concentration (Leschinger *et al*., 1993), lowering the magnesium (Mody *et al*., 1987) or calcium concentration (Jefferys and Haas, 1982; Yaari *et al*., 1983), applying a potassium channel blocker such as 4-aminopyridine (4-AP) (Avoli *et al*., 1993) or by applying toxins such as veratridine (Tian *et al*., 1995). Most of these SLEs respond to the clinically used AEDs, the notable exception being the pharmacoresistant recurrent discharges, which are induced by combined application of bicuculline and 4-AP (Bruckner and Heinemann, 2000) or by the development during later stages of low magnesium-induced seizure-like activity (Heinemann *et al*., 1994). Recently, pharmacoresistant tonic–clonic SLEs have been described in an intact immature corticohippocampal preparation of 1-week-old rats (Quilichini *et al*., 2003).

Many of the *in vitro* models, including the low magnesium model of late status epilepticus, now form part of the preclinical evaluation of the anticonvulsant efficacy of new antiepileptic compounds. However, the use of pharmacoresistant *in vitro* seizure models is limited. The intact corticohippocampal preparation is functional only until the end of the first postnatal week. Late recurrent discharges in acute slices of the entorhinal cortex resemble electrographic patterns observed during the late stages of status epilepticus and thus represent a special case of pharmacoresistance. In addition, neither model is suitable for screening purposes, as the respective brain tissues are viable for restricted time periods only, and hence the number of animals used would be high. Therefore, we have explored the suitability of organotypic hippocampal slice cultures (OHSCs) as model(s) for pharmacosensitive and/or pharmacoresistant SLEs. Slices of CNS tissue prepared from young rodents would be ideally suited for screening purposes because they can be maintained in culture for many weeks to months, and it has been shown that under optimal conditions, nerve cells continue to differentiate and develop a tissue organization that closely resembles that observed *in situ* (Gahwiler *et al*., 1997).

We have used a decreased magnesium concentration, a situation met in eclampsia (Gulaboglu *et al*., 2007), and 4-AP to induce seizure-like activity in OHSCs. With this model, we confirmed and extended earlier findings (Gutierrez *et al*., 1999) that in OHSCs, SLEs resembling electrographic correlates of limbic seizures recorded with a depth electrode in animals (Lothman *et al*., 1989; Walton and Treiman, 1991) could be reliably induced. In addition, we found that in contrast to acute slices of the entorhinal cortex, in OHSCs not only short recurrent discharges but also tonic–clonic SLEs were refractory to standard AEDs. Some of theses findings have been published in abstract form (Albus *et al*., 2007).

Methods

Test systems

Organotypic hippocampal slice cultures were prepared according to the interface culture method (Stoppini *et al*., 1991); culture media optimal for preparing and culturing neurons from postnatal rodent hippocampus was used (Stoppini *et al*., 1991; Brewer *et al*., 1993; Noraberg *et al*., 1999). Wistar rats (2- to 10-day-old) were administered $4\,mg\,kg^{-1}$ xylazin (Rompun) and decapitated. The whole hippocampus was isolated and transverse slices (400 μM) were prepared with a McIlwain tissue chopper. The cut tissue was placed in a preparation medium (approximately 5 °C, pH 7.15) under low ambient CO_2 concentration consisting of Hank's balanced salt solution (HBSS), 10 mM HEPES, 880 μM $NaHCO_3$ and 30 mM D-glucose. Slices were separated from each other with fine tweezers and a spatula. In slices from the ventral hippocampus, the entorhinal cortex was left in place. One to three slices were placed on a Millicell culture plate insert (Millipore, Billerica, MA, USA; PICM 03050, 0.4 μm, 30 mm diameter) and transferred to culture plates with six wells. Each well contained 1.1 mL medium composed of 25 mL Hank's balanced salt solution, 50 mL Opti-MEM and 25 mL heat inactivated horse serum. In this medium, the concentration of $NaHCO_3$ was approximately 20.3 mM and that of D-glucose 30.1 mM. The culture plates were placed in an incubator at 36.5 °C with 5% CO_2. After 3 days, the medium was replaced with 1.1 mL of a chemically defined serum-free medium (Neurobasal A) supplemented with 1 mM L-glutamine and B 27, which contains several

hormones, fatty acids and free radical scavengers (Brewer *et al*., 1993). This medium was changed every second day.

Measurements

In acute experiments, slice cultures were continuously superfused (4 mL min^{-1}) with prewarmed (35.5 ± 0.2 °C) and oxygenated (95% O_2/5% CO_2) minimal essential medium (MEM) containing, in addition to amino acids and vitamins (in mM): NaCl 105, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1.8, $CaCl_2$ 1.6, glucose 10, $NaHCO_3$ 26.3 mM (pH 7.40, osmolality 265 mosm kg^{-1}). Concentrations of NaCl and $NaHCO_3$ and the osmolality match the respective values in Neurobasal A. SLEs were induced by superfusing the slice with MEM containing no magnesium ions and 5 mM KCl, referred to as low magnesium MEM. In this condition, the concentration of magnesium ions might still reach 0.08 mM owing to contamination by magnesium of the other constituents of the MEM (Mody *et al*., 1987). Increasing the potassium concentration from 3 to 5 mM resulted in a faster onset of SLEs, particularly during the first 2 weeks *in vitro*, and was therefore used for all OHSCs. The reliable induction of SLEs and pharmacoresistance were confirmed in control experiments ($n = 5$) run with 129 mM NaCl, 3 mM KCl and an osmolality of 303 ± 3 mosm kg^{-1}. The extracellular concentration of potassium $[K^+]_o$ in the pyramidal cell layer of CA3 was measured with a double-barrelled K^+-selective/reference glass microelectrode containing Fluka ionophore 60031 backfilled with 100 mM KCl/150 mM NaCl, respectively, prepared and tested as described previously (Lux and Neher, 1973). The reference electrode recorded the d.c. potential and population spikes in the pyramidal cell layer in CA3 in d.c. mode. To collect data for studies of neuronal interactions in OHSCs (data not shown), single unit and multiunit activities were also recorded (a.c. coupled) with tungsten-in-glass microelectrodes positioned in the dentate gyrus (granular layer), in the pyramidal cell layers of area CA3 and CA1 and, if present, in the medial entorhinal cortex (either layer III or V). Neuronal activities and $[K^+]_o$ were continuously recorded using Spike 2 (V 5.03, CED, Cambridge, UK) with sample rates of 20 kHz for field potentials and single units (filter cutoff 3 kHz) and 1 kHz for potassium signals (filter cutoff 0.3 kHz). Bipolar stimulation electrodes (tungsten wires with sharp tips, tip separation 80–200 μM) were positioned at the hilus/CA3 border to assess slice viability over time. For that population to spikes in the areas recorded from were evoked every 15–20 min by electrical stimulation (0.1 ms, 3–15 V) at half-maximal and maximal stimulus strength. Slices displaying a decrease of >20% in population spike amplitude in the course of an experiment were discarded from the analysis.

Experimental design

Recordings were commenced 1 h after the OHSCs had been transferred to the recording chamber. The experimental protocol used in most cases consisted of three periods with SLEs induced by low magnesium MEM, each lasting 20–40 min, separated from each other by recovery periods with normal MEM lasting between 90 and 120 min. During the first and third SLE periods, no AEDs were applied (control and washout, respectively), whereas the second SLE period was run with a given AED after a preincubation of 30–60 min. In addition, in a few OHSCs, we tested whether AEDs blocked ongoing SLE induced by the low magnesium MEM. A complete drug test including pre- and post-drug controls was denoted as a test run and lasted up to 5 h. To examine the dependence of pharmacoresistance on the mechanism used to induce the SLE, in nine OHSCs, SLEs were induced by application of 4-AP (100 or 200 μM). In some experiments, drugs were added to the culture medium for variable time periods of up to 4 weeks.

Data analysis and statistical procedures

To quantify the effects of AEDs on SLEs, we measured parameters that characterized the static and dynamic properties of tonic–clonic SLEs (Table 1). The values for these parameters of the first SLE in the pre-drug period were compared with the respective values of the first SLE during AED treatment and of the first SLE after washout of the AED. Comparisons were repeated for the second SLEs, then for the third SLEs and so forth. The number of SLEs used for comparisons per test run was 2–6. This procedure was chosen as it compensated for any minor changes in parameter values over time that were present in some OHSCs during the course of recurrent seizure activity.

Subsequently, for each SLE comparison, the mean of the sum of pre- and post-drug values was calculated as a reference value. Reference values and values during drug treatment of all SLE comparisons per AED and concentration were then compared by the paired *t*-test or in case of non-Gaussian distribution of values (D'Agostino and Pearson omnibus normality test) by the Wilcoxon-matched pair test (Prism V 5.00, GraphPad Inc., San Diego, CA, USA). Drug effects were presented as values during drug treatment normalized to reference values (set to 100%). In order to

Table 1 SLE parameters and SLE scores

Parameter	*Label*
Latency of 1. SLE (s)	A
SLE frequency (*N* SLEs h^{-1})	B
Duration of SLE (s)	C
Duration of tonic period (s)	D
Maximal extracellular potassium concentration (mM)	E
Maximal amplitude of negative potential shift (mV)	F
Amplitude of field potential transients during tonic period (mV)	G
Frequency of field potential transients during tonic period (s^{-1})	H
Duration of clonic period (s)	I
Frequency of clonic-like events (s^{-1})	K
Average duration of clonic-like events (s)	L
Amplitude of onset field potential of clonic-like events (mV)	M
Total SLE score (%)	B, C, E, F, G, H, K, L, M
Tonic period score (%)	D, F, G, H
Clonic period score (%)	I, K, L, M

Abbreviation: SLE, seizure-like event.

compare drug effects between slices, and in addition, anticonvulsant activity between different AEDs, for each slice and for each AED concentration, we calculated three composite scores: one representing total SLE, another representing the tonic period of the SLE and a third representing the clonic period of the SLE. These scores were the means of the sum of normalized parameter values B, C, E, F, G, H, K, L, M for total SLE; D, F, G, H for the tonic period; and I, K, L, M for the clonic period (Table 1).

Drugs, chemical reagents and other materials

Hank's balanced salt solution, HEPES, $NaHCO_3$, amino acids, vitamins and L-glutamine were obtained from Biochrom AG (12247 Berlin, Germany); neurobasal A, B 27, Opti-MEM from Invitrogen GmbH (76131, Karlsruhe, Germany); gabapentin, 4-AP, CBZ HBC complex, clonazepam (CLO), phenobarbital sodium (PHB), valproic acid (VPA) sodium salt, PHT sodium, bicuculline methiodide, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQ×), D-(−)-2-amino-5-phosphonopentanoic acid (DL-AP-5) from Sigma-Aldrich Chemie, GmbH (82824 Taufkirchen, Germany); diazepam (DZP) from Ratiopharm GmbH (89023 Ulm/Donautal, Germany); Rompun from Bayer GmbH (51368 Leverkusen, Germany). PHT was dissolved in 0.2 N NaOH and diluted to the final concentration in MEM. CLO was dissolved in dimethylsulphoxide and diluted in MEM to a final concentration of 0.01–0.2% dimethylsulphoxide. The other drugs were dissolved in distilled water, stored as stock solutions and then diluted (200–1000×) to their final concentrations in MEM.

Drug concentrations

The concentrations of AEDs (Table 2) were adjusted according to anticonvulsive plasma concentrations in rats (Loscher *et al*., 1991) and, in the case of gabapentin, to tentative therapeutic target ranges in humans (Neels *et al*., 2004). The extracellular fluid (ECF) concentrations of the AEDs employed in our study were calculated from ratios between ECF concentration in the brain and total plasma concentration (ECF/plasma ratio), which are with one exception (gabapentin) well below unity: 0.05 for phenobarbital (Walker *et al*., 1996; Potschka *et al*., 2002); 0.029 for VPA (Scism *et al*., 2000); 0.07–0.13 for PHT (Walker *et al*., 1996). If protein binding for CBZ is assumed to be 70–80% (Neels *et al*., 2004), then its ECF/ plasma ratio can be estimated to range between 0.08 and 0.11 (Rambeck *et al*., 2006). ECF concentrations for CLO and DZP were not available; the respective ECF/plasma ratios are supposedly low if it is assumed that protein binding for CLO is 86% and that for DZP 96% (Neels *et al*., 2004).

Table 2 Overview of experimental data

Drug	*Slices cultured/test runs* *Postnatal day* *Days* in vitro	*Induction of seizure-like events* (n, *slices cultured)*	*Drug concentration in vitro* (μM)	*Anticonvulsant plasma concentrations* (EC_{50}) *in rats* (μM) *(Loscher et al., 1991)*
Carbamazepine (CBZ)	27/33 P 2–10 DIV 7–56	Low Mg/K5 (21) Low Mg/K3 (1) 4AP 100–200 (5)	30–180	17.1–22.9
Phenytoin (PHT)	21/26 P 2–10 DIV 5–27	Low Mg/K5 (13) Low Mg/K3 (4) 4AP 100–200 (4)	40–100	13.8–23.6
Valproic acid (VPA)[a]	16/23 P 7–10 DIV 12–41	Low Mg/K5 (16)	800–2000	1420–1631
Clonazepam (CLZ)	4/7 P 7–9 DIV 7–21	Low Mg/K5	1–20	2.5–4.5
Diazepam (DZP)	10/12 P 6–9 DIV 8–40	Low Mg/K5 (9) Low Mg/K3 (1)	3.5–35	0.67–8.05
Gabapentin (GP)	2/2 P 6,7 DIV 9,10	Low Mg/K5 (1) Low Mg/K3 (1)	120–300	70–120[b]
Phenobarbital (PHB)[c]	12/13 P 7–8 DIV 6–36	Low Mg/K5	100–200	56–125
CNQX + DL-AP-5	6/6 P 8–9 DIV 15–32	Low Mg/K5 (5) 4 AP 200 (1)	CNQ× 5–30/DL-AP-5 5-50	
Muscimol	5/5 P 7–9 DIV 8–44	Low Mg/K5	10	

[a]Includes five OHSCs preincubated with 1 mM VPA (7–29 days).
[b]Target range in patients (Neels *et al*., 2004).
[c]Includes three OHSCs preincubated with 100 μM PHB (15–28 days).

On the basis of published data (Loscher *et al*., 1991) and by applying an ECF/plasma ratio of 0.2 for all AEDs used, to compensate for interindividual variability and/or species/strain differences, the following ECF concentrations representing minimal neurotoxic concentrations (in μM) were calculated: CBZ 10, PHT 16, VPA 300, phenobarbital 42, CLO 0.06, DZP 1.1. An exception is gabapentin with 0% protein binding and a toxic threshold of 120 μmol L^{-1} in the plasma and, therefore, possibly also in the ECF. If it was assumed that, owing to a slow equilibration time course (Sokolova *et al*., 1998), concentrations of AEDs in the slice culture might have been only 50% of the concentration in the superfusate, the estimated tissue concentrations in our experiments were (in μmol L^{-1}): CBZ 15–90.5, PHT 20–50, VPA 400–1000, phenobarbital 50–100, CLO 0.5–10, DZP 1.75–17.5, gabapentin 60–150. In most cases, therefore, we were using concentrations close to the minimal neurotoxic concentrations, or exceeding these by two to six times, and up to 16 times in the case of the benzodiazepine tests.

Results

Decreasing the magnesium concentration or application of 4-AP induces different patterns of seizure-like activities in OHSCs

Application of a low magnesium MEM or 4-AP to OHSCs induced SLEs that were characterized by a negative potential shift superimposed by high frequency field potential transients (tonic period), followed by a period in which clonic-like after-discharges occurred. SLEs were accompanied by a rise in $[K^+]_o$ of usually more than 2 mM. Between the SLEs, interictal discharges were seen. The SLEs appeared to be synchronous in the dentate gyrus, the CA3 and CA1 regions and in the entorhinal cortex (data not shown). The occurrence of SLEs was not dependent on the presence of the entorhinal cortex during the period of culture (5–56 days) or on the age of the animals at the time of preparation of the culture slices (P2–10).

Three different types of SLEs were observed. The majority of the low magnesium-induced SLEs (52%) and 22% of the 4-AP-induced SLEs consisted of a tonic period followed by a clonic period of limited duration (tonic–clonic SLEs; Figure 1A). The mean time of the total duration and the 95% confidence interval (of the mean total duration) of tonic–clonic SLEs was 114 s (100, 128) and of the tonic and clonic periods, 26 s (23, 28) and 88 s (74, 101), respectively. Slice cultures in which tonic–clonic SLEs were eventually replaced by recurrent short clonic-like discharges (RSDs) were characterized as mixed type SLEs (Figure 1B). Mixed types made up 23% of the low magnesium-induced and 11% of the 4-AP-induced SLEs. Finally, in a number of slices, RSDs started immediately after the first tonic discharge (Figure 1C). This latter type was characterized as RSD-SLE and represented the majority of the 4-AP-induced SLEs (67%) and 25% of the low magnesium-induced SLEs. SLEs in OHSCs were completely and reversibly blocked by glutamate antagonists (Figure 2A) or the $GABA_A$-agonist muscimol (Figure 2B; Wahab *et al*., 2007). Viability of OHSCs seemed to be unaltered even after longer periods of recurring seizure-like activity (up to 120 min tested), as indicated by stable time and amplitude characteristics of SLEs and single stimulus-evoked field potentials.

If acute hippocampal slices were challenged with the low magnesium artificial cerebrospinal fluid, tonic–clonic SLEs occurred only if slices were from animals between the ages of P7 and P15 (Gloveli *et al*., 1995). In hippocampal slices from older animals, treatment with low magnesium or 4-AP usually induced interictal discharges, and tonic–clonic SLEs were seen only in the entorhinal cortex. In OHSCs, tonic–clonic SLEs in the hippocampus could still be reliably induced after 2 months *in vitro*, confirming earlier findings (Gutierrez *et al*., 1999). The seizure pattern induced by the prolonged application of low magnesium MEM or 4-AP can be compared to status epilepticus *in vivo*, as one SLE follows the other and then eventually transforms into a state of continuous activity, the late recurrent discharges. Tonic–clonic SLEs could also be induced with low magnesium in the hippocampus of an intact cortico-hippocampal formation of the P1–7 rat (Quilichini *et al*., 2003) and P3–4 mouse (Moser *et al*., 2006). However, the mean seizure frequencies in the latter preparations were much lower (rat 4.5 h^{-1} at P7; mouse 9 h^{-1} at P3–4) than in OHSCs (19.2 h^{-1}).

Effects of AEDs on low magnesium-induced seizure-like activity in OHSCs

In 86 (93.5%) of the 92 OHSCs explored, AEDs neither prevented the induction of SLEs nor stopped ongoing SLEs, and these results were not dependent on SLE type, method of SLE provocation, postnatal age at explantation, days *in vitro* (DIV) and presence of the entorhinal cortex. Although in most cases the AEDs were not able to block the SLEs, they did modify the time and amplitude characteristics of SLEs in a drug-specific and concentration-dependent way. As pharmacoresistance of RSD-SLEs in the hippocampus has been demonstrated previously (Yonekawa *et al*., 1995; Zhang *et al*., 1995; Dreier *et al*., 1998; Bruckner and Heinemann, 2000), we restricted the quantitative analysis of drug effects to tonic–clonic SLEs induced by low magnesium MEM.

Carbamazepine

The effects of CBZ on SLEs were analysed in 7 test runs, comprising 28 comparisons of tonic–clonic SLE (40 μM) and, in another 5 test runs, comprising 23 comparisons of tonic–clonic SLE (80 μM). In no case, even in an OHSC in which 180 μM CBZ were applied (data not shown), was the induction of SLEs prevented or ongoing seizure-like activity blocked. A typical experiment is shown in Figure 3. The most conspicuous effect was a concentration-dependent increase in the incidence of SLEs associated with a reduction of SLE duration. The amplitudes of field potentials and rises in $[K^+]_o$ were not significantly affected by CBZ. The concentration dependence and specificity of the effects of CBZ are evident when the results are depicted as a histogram as shown in Figure 6. The shortening of total SLE (C) and of both tonic (D) and clonic (I) periods strongly increased with concentration. With 80 μM CBZ, the clonic-like events (L) also became significantly shorter. Under both concentrations, the frequency of field potential transients (H) during

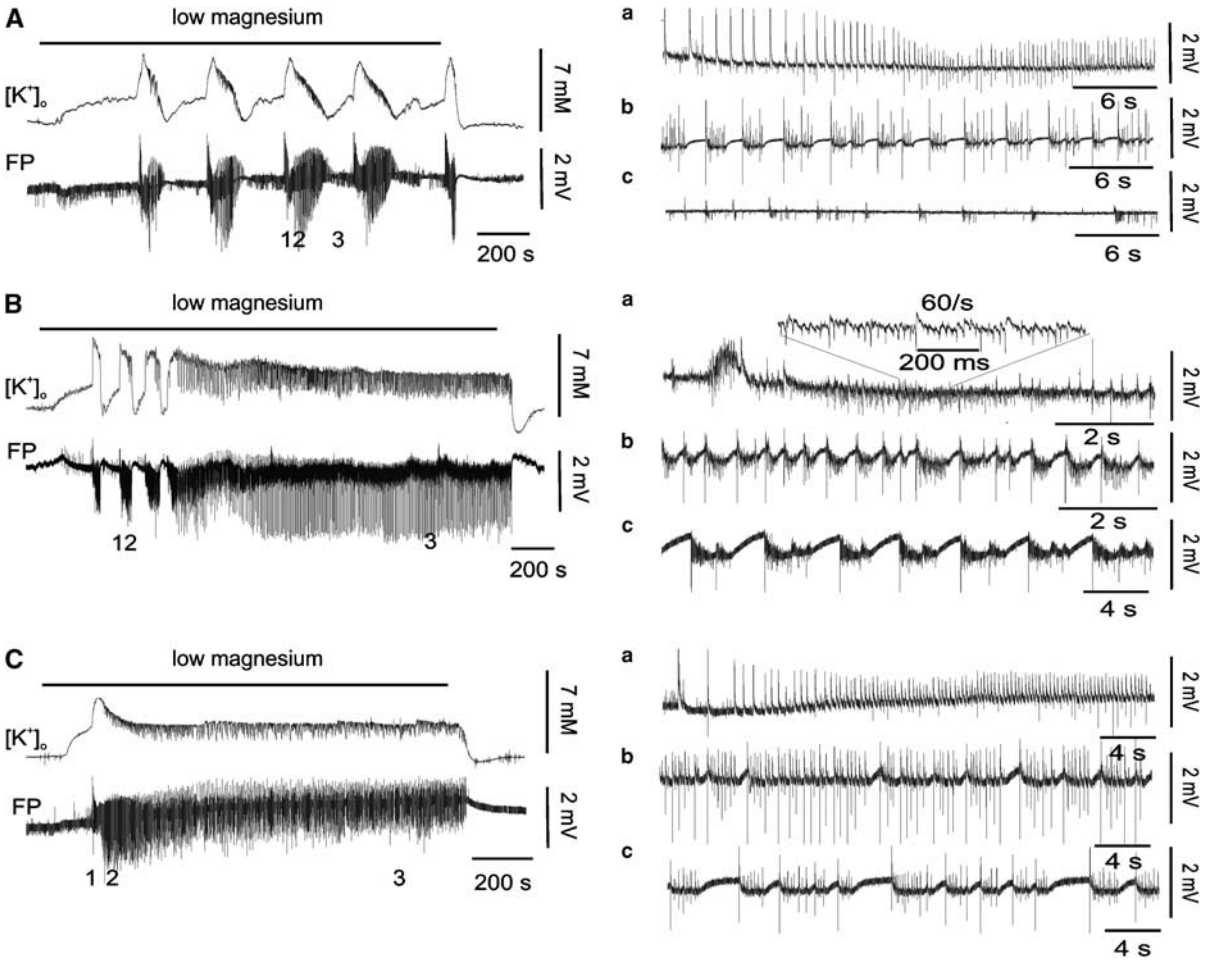

Figure 1 Types of SLEs in OHSCs recorded in the pyramidal cell layer of area CA3. Double-barrelled K sensitive microelectrodes were used to monitor d.c. field potentials (FP) and changes in extracellular potassium concentration ($[K^+]_o$). Epileptiform activity was induced by lowering the extracellular magnesium concentration. (**A**) Tonic–clonic SLEs consisting of a tonic period and a clonic period. Between the SLEs, interictal activity of low amplitude is recorded. Details of the field potential recordings labeled with 1, 2 and 3 are shown on an extended time scale on the right and represent tonic (a), clonic (b) and interictal (c) activity, respectively. (**B**) Mixed-type SLE changing after three tonic–clonic SLEs to recurrent short discharges (RSDs). Details of the field potential recordings labeled with 1, 2 and 3 are shown on an extended time scale on the right and represent tonic (a), clonic (b) and recurrent short discharge (c) activity, respectively. (**C**) RSD-SLE. Immediately after the first tonic discharge (1), RSDs (2) start, the frequency of which decreases over time (3). The parts of the field potential recordings labeled with 1, 2 and 3 are shown on an extended time scale on the right and correspond to a, b and c, respectively. The frequency of field potential transients during the tonic period in most recordings was less than 10 Hz. Occasionally, high frequency oscillations (>40 Hz) occurred (**B**a). OHSC, organotypic hippocampal slice culture; RSD, recurrent short clonic-like discharge; SLE, seizure-like event.

the tonic period was significantly reduced. The suppressive effects of CBZ were associated with concentration-dependent facilitatory effects on SLE incidence (B), the frequency of clonic-like events (K) and the amplitude of field potential transients during the tonic period (G).

Phenytoin

The effects of PHT on SLE parameters were similar at concentrations of 80 μM (8 test runs) and 100 μM (1 test run) and were therefore pooled for statistical analysis. When the effects of PHT were analysed, on the basis of 9 test runs comprising 27 comparisons of SLEs, the changes it induced were revealed to be almost identical to those exerted by 80 μM CBZ (Figure 6). The durations of total SLE (C) and of both tonic (D) and clonic (I) periods significantly decreased, whereas both SLE frequency (B) and frequency of clonic-like events (K) increased. A notable difference was that the frequency of field potential transients during the tonic period (H) decreased after the application of CBZ but remained unchanged in the presence of PHT.

Valproic acid

The effects of VPA on tonic–clonic SLEs were similar at concentrations of 0.8–1 mM (9 test runs comprising 24 comparisons of SLEs) and 2 mM (6 test runs comprising 15 comparisons of SLEs) and were therefore pooled for statistical analysis (Figure 6). VPA specifically reduced the negative potential shift amplitude (F), the amplitude of the $[K^+]_o$ transients (E) and the amplitude of field potential transients. The temporal parameters of SLEs were not altered. With the exception of one case, VPA failed to prevent the induction of SLEs or to stop ongoing seizure activity. According to clinical reports, VPA may exert therapeutic effects but only after some weeks (Neels *et al*., 2004). Hence, we investigated the effects of VPA in OHSCs after a preincubation period of 7–29 days with 1 mM VPA ($n=4$). However, VPA still failed to

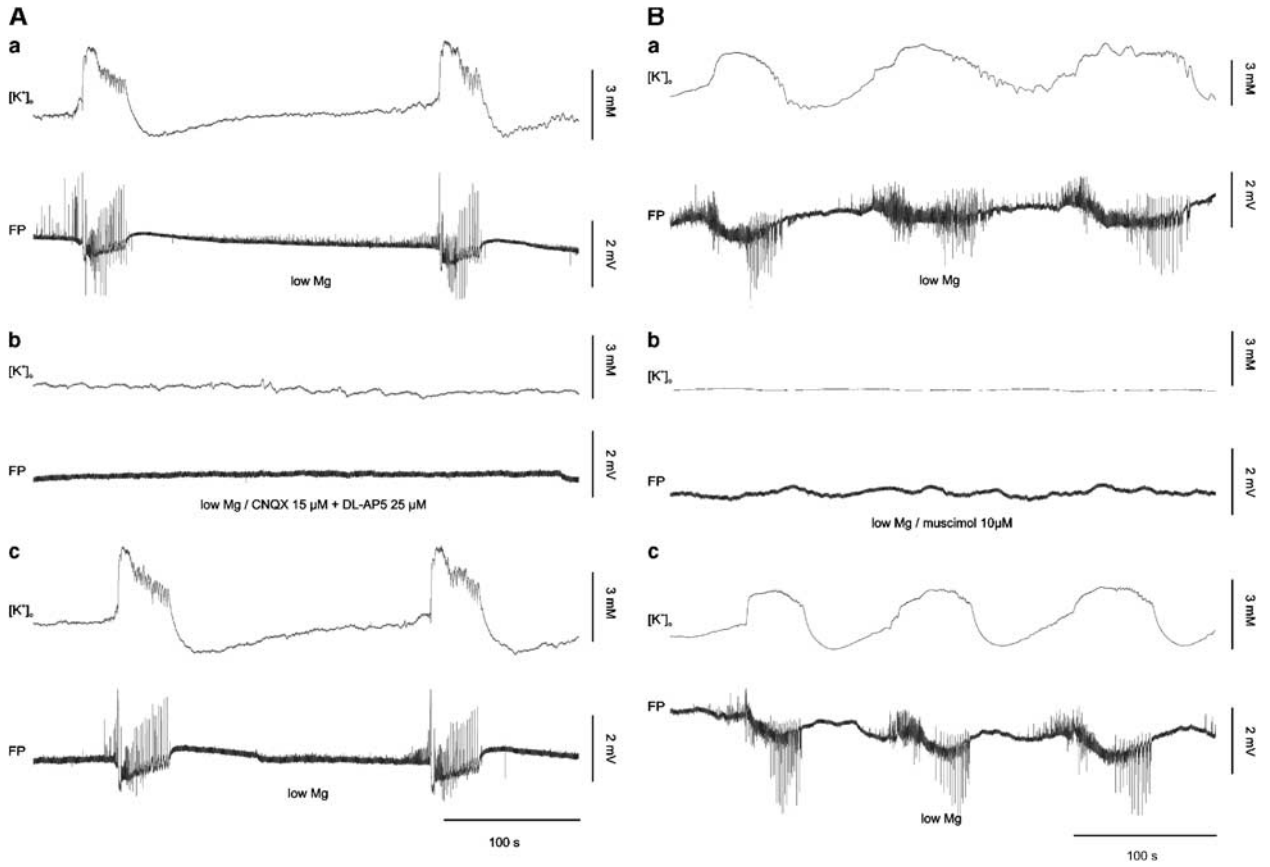

Figure 2 (**A**a–c) A combination of glutamate antagonists CNQX (5–30 μM) and DL-AP5 (5–50 μM) reversibly suppressed the low magnesium-induced tonic–clonic SLEs ($n = 6$). The OHSC was explanted at P8 and examined after 32 DIV; (**B**a–c) the $GABA_A$-agonist muscimol (10 μM; $n = 5$) reversibly suppressed the low magnesium-induced tonic–clonic SLEs. The OHSC was explanted at P8 and examined after 32 DIV. $[K^+]_o$ and field potentials (d.c. mode) were recorded in the pyramidal cell layer of CA3. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days *in vitro*; DL-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; OHSC, organotypic hippocampal slice culture; P, postnatal day; SLE, seizure-like event.

prevent the induction of SLEs by low magnesium and to stop ongoing SLEs in these OHSCs.

Phenobarbital sodium

Phenobarbital sodium in a concentration of 100 μM (6 test runs comprising 15 comparisons of SLEs) did not prevent the induction of SLEs or stop the ongoing SLEs. This was also true in three additional OHSCs, which had been preincubated with 100 μM PHB for 15–28 days. However, 200 μM PHB (6 test runs comprising 16 comparisons of SLEs) almost completely suppressed SLEs in three out of six OHSCs (Figures 4A and B). The profile of effects of 200 μM PHB on SLEs differed from that of the other AEDs tested (Figure 6). The significant decrease of total SLE duration (C) was caused by a decrease of the duration of the clonic period (I) and not of the tonic period (D), which remained unaltered. These changes were accompanied by a reduction of the amplitude (G) and frequency (H) of field potential transients during the tonic period, a reduction of the amplitude of $[K^+]_o$ transients (E) during the tonic period and of the amplitudes of onset field potentials of clonic-like events (M). Additional changes consisted of an increase of SLE frequency (B) combined with a delayed onset of seizure-like activity (A).

Diazepam

Diazepam at all concentrations failed to prevent the induction of SLEs or to block ongoing SLEs. Low concentrations of DZP (5 μM; effects analysed in 6 test runs comprising 23 comparisons of SLEs) caused only minor changes of some SLE parameters, whereas 35 μM DZP (6 test runs comprising 21 comparisons of SLEs) strongly affected tonic–clonic SLEs (Figures 5 and 6). The most marked effects were an increase in SLE frequency (B) and a significant reduction of total SLE duration (C). The latter was caused mainly by a shortening of the clonic period (I), which was associated with a decrease of the frequency (K) and an increase of the duration (L) of clonic-like events. The significant reduction of the negative potential shift (F), which indicates a decrease in neural activation during the tonic period, was not accompanied by a reduction of either the $[K^+]_o$ (E) or the amplitude of field potential transients (G). In contrast, the latter parameter was increased.

Gabapentin and CLO

The effects of CLO on SLE parameters were similar at concentrations of 1–2 μM (4 test runs) and 20 μM (2 test runs) and were therefore pooled for statistical analysis (6 test runs

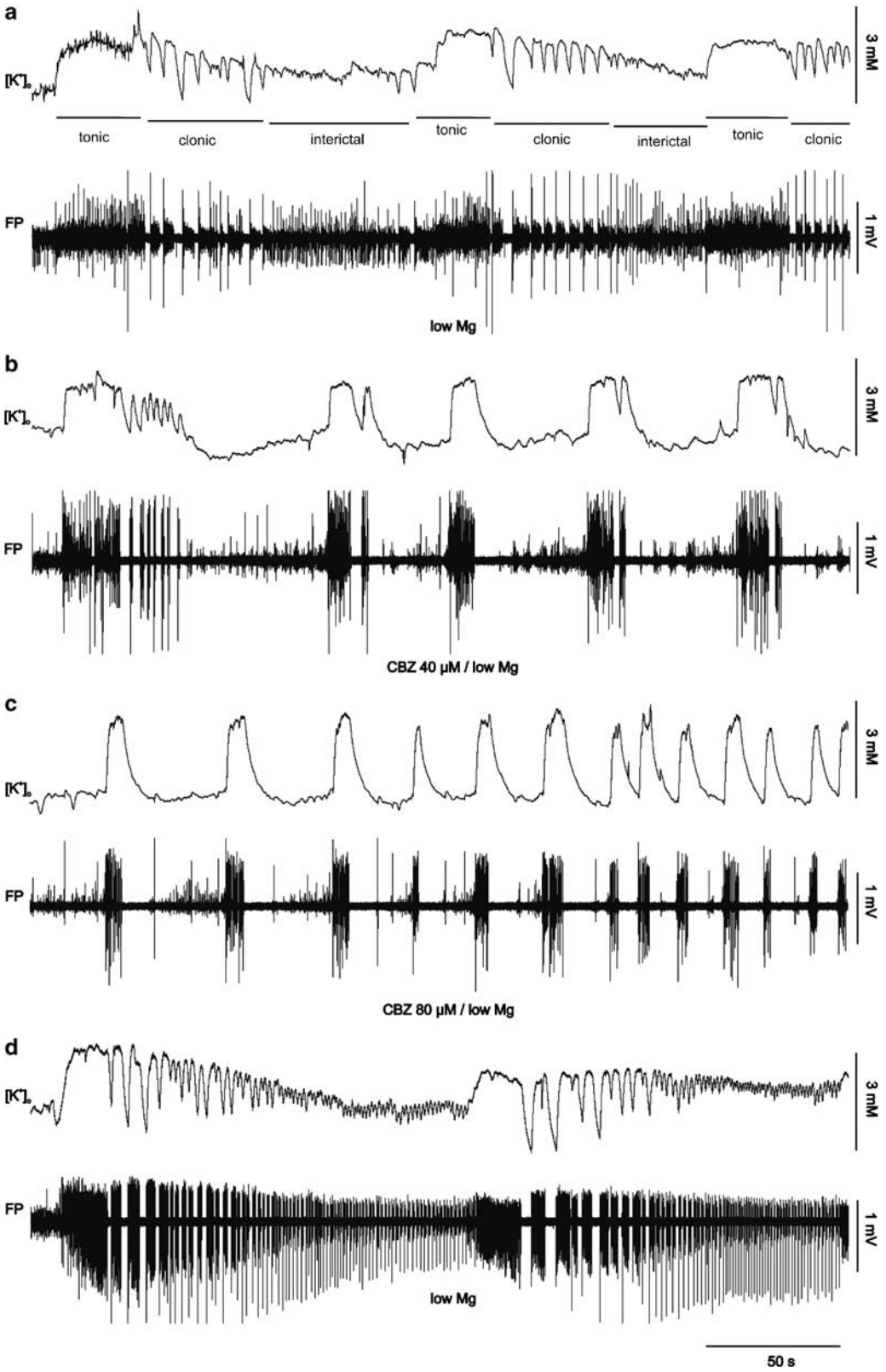

Figure 3 Shows the failure of CBZ to block induction of tonic–clonic SLEs induced by low magnesium MEM. Control with low magnesium MEM (a), CBZ 40 μM and low magnesium MEM (b), CBZ 80 μM and low magnesium MEM (c), after washout of CBZ with low magnesium MEM (d). The OHSC was explanted at P8 and examined after 38 DIV. Extracellular potassium concentration ($[K^+]_o$) and field potentials (FP; a.c. coupled) were recorded in the pyramidal cell layer of CA3. Note the strong reduction in the duration of the SLEs and the increase in their incidence. CBZ, carbamazepine; DIV, days *in vitro*; MEM, minimal essential medium; OHSC, organotypic hippocampal slice culture; P, postnatal day; SLE, seizure-like event.

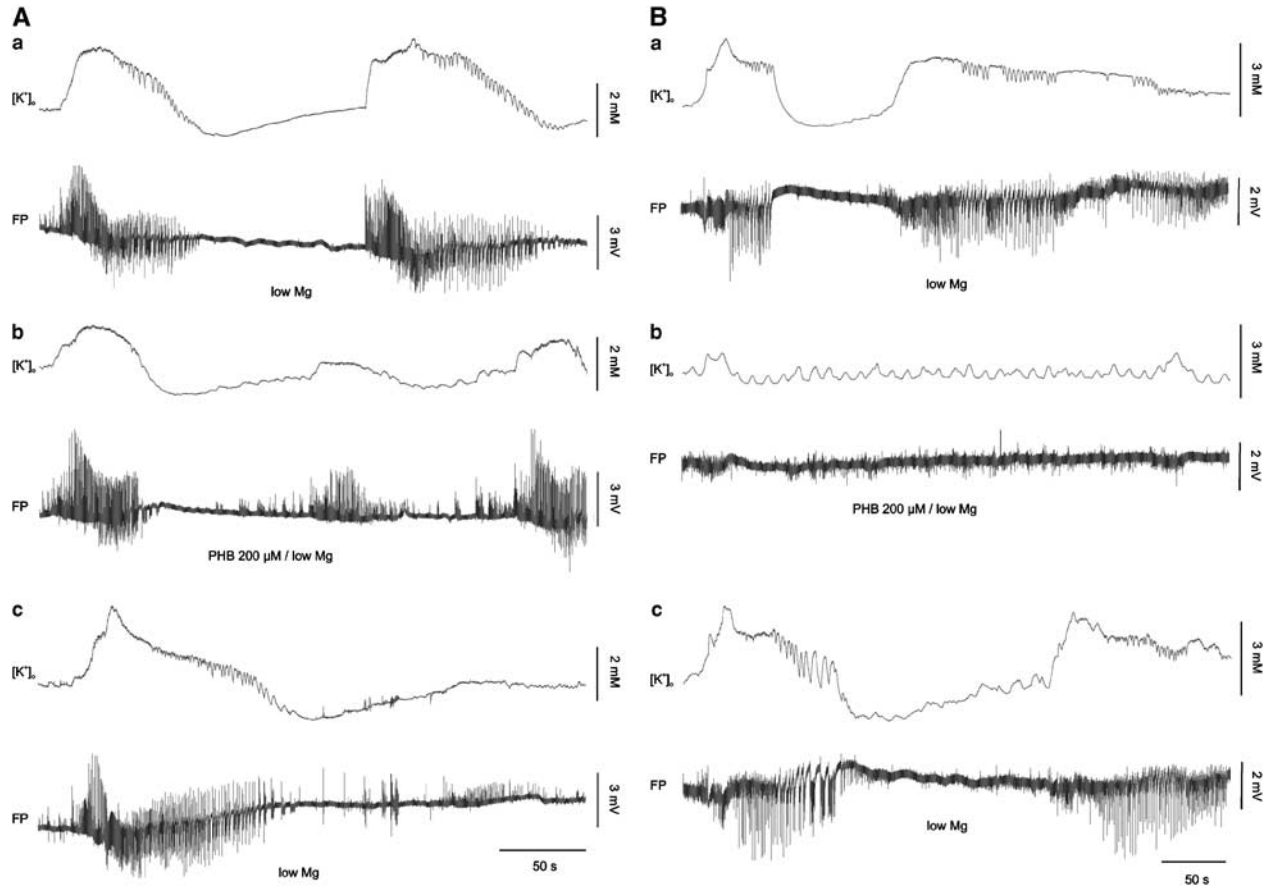

Figure 4 (**A**a–c) Shows that 200 μM PHB failed to block the induction of tonic–clonic SLEs induced by low magnesium MEM. The OHSC was explanted at P7 and examined after 17 DIV. (**B**a–c) PHB 200 mM markedly attenuated the seizure-like activity in an OHSC explanted at P8 and examined after 20 DIV. A short-lasting, low-amplitude tonic period associated with a small increase of $[K^+]_o$ was still present. $[K^+]_o$ and field potentials (d.c. mode) were recorded in the pyramidal cell layer of CA3. DIV, days *in vitro*; MEM, minimal essential medium; OHSC, organotypic hippocampal slice culture; P, postnatal day; PHB, phenobarbital sodium; SLE, seizure-like event.

comprising 27 comparisons of SLEs). CLO had similar effects on the temporal aspects of SLEs as high concentrations of DZP (Figure 6). It increased the frequency of SLEs and strongly reduced the total duration of SLEs. The latter effect was due to a selective shortening of the clonic period. The minor effects observed were a decrease in the $[K^+]_o$ transients and of amplitudes of the field potential transients (E) during the clonic period and the amplitudes of the initial field potential leading clonic-like events (M).

The effects of gabapentin (120–300 μM) tested in 2 test runs comprising 5 SLE comparisons were not quantitatively evaluated. Neither CLO nor gabapentin prevented the induction of SLEs by low magnesium. Also, CLO failed to stop ongoing SLEs.

Summary of the effects of AEDs on low magnesium-induced seizure-like activity in OHSCs

The pharmacoresistance of SLEs in OHSCs was confirmed by analysis of the composite scores, which showed only minor changes under AED treatment. The total SLE scores (in%) ranged between 100 and 160, and the scores of tonic and clonic periods ranged between 80 and 158 (Table 3). Even in the presence of 200 μM PHB, where scores were calculated by including three OHSCs in which SLEs were almost completely suppressed, the total SLE score was reduced only to 90% and the scores for the tonic and clonic periods were 85 and 90%, respectively. The small changes of the SLE scores were due to the fact that a decrease in some parameters was compensated for by increases in other parameters (Figure 6). For example, the marked reductions in SLE duration caused by 80 μM CBZ, 80 μM PHT, 35 μM DZP or 200 μM PHB were compensated for by significant increases in SLE frequency. As a result, the total duration of seizure activity over time was much less affected than the duration of individual SLEs. Likewise, the preferred suppression of the clonic period over the tonic period with 35 μM DZP was not reflected in the respective scores. In contrast, the clonic period scored higher due to a strong increase in the duration of clonic-like events. With 80 μM CBZ and 80 μM PHT, both tonic and clonic periods were markedly reduced. However, in both cases, the clonic period scored higher because the frequency of clonic-like events had increased significantly, whereas parameters contributing to the tonic period score were only slightly increased or not affected at all.

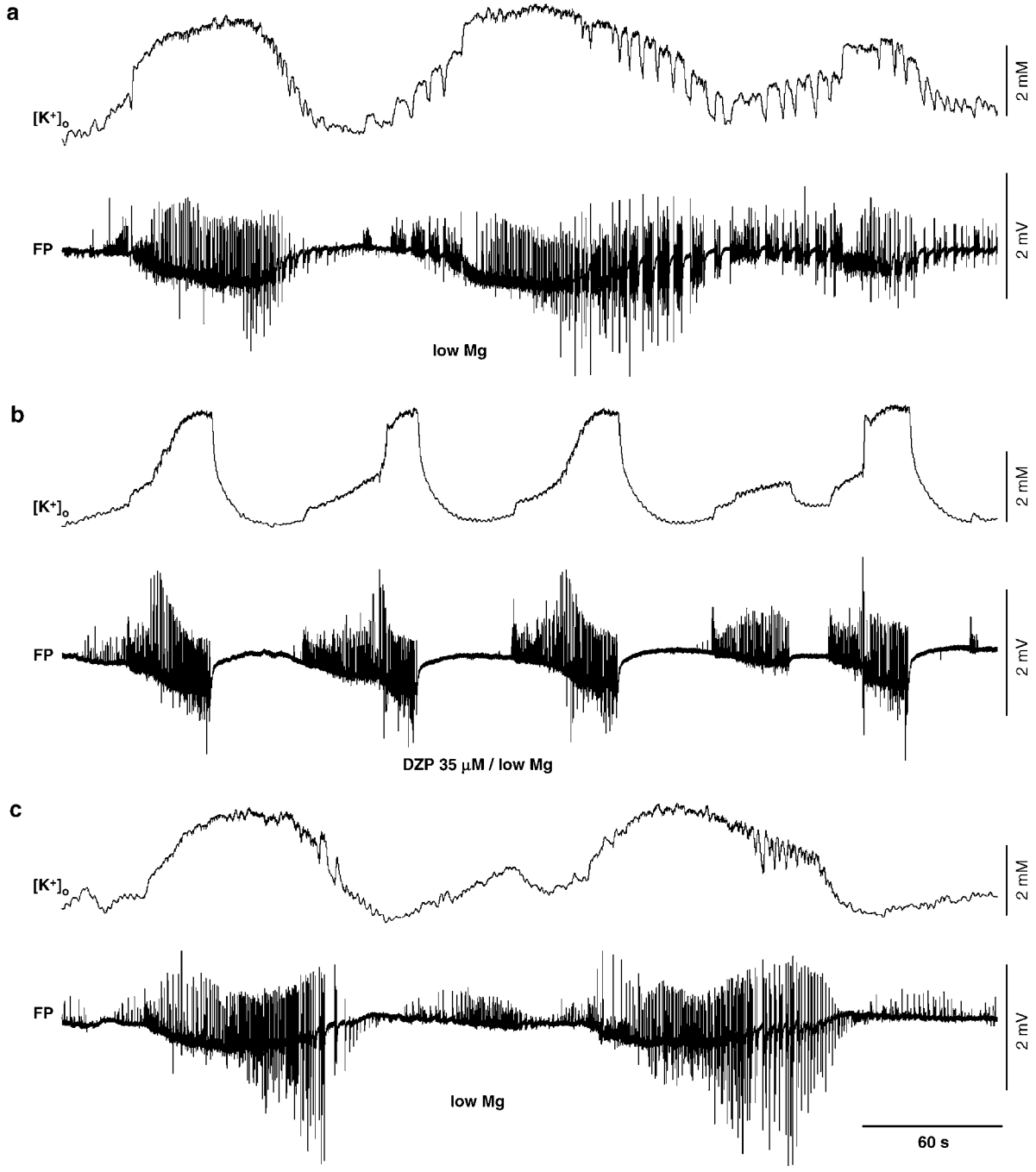

Figure 5 DZP (b) failed to block the tonic–clonic SLEs induced by low magnesium MEM (a: control, c: washout). The OHSC was explanted at P6 and examined after 17 DIV. $[K^+]_o$ and field potentials (d.c. mode) were recorded in the pyramidal cell layer of CA3. DIV, days *in vitro*; DZP, diazepam; MEM, minimal essential medium; OHSC, organotypic hippocampal slice culture; P, postnatal day; SLE, seizure-like event.

An almost complete, reversible suppression of low magnesium-induced spontaneous SLEs (SLE scores $<40\%$) occurred in five OHSCs, of which two were treated with 200 μM PHB, one with 2 mM VPA and one with 80 μM PHT. Nevertheless, tonic–clonic SLEs scoring $>60\%$ could be elicited in these OHSCs by a single electrical stimulus applied to the hilus. Another OHSC, in which the scores for total SLE and for tonic and clonic periods were 72, 57 and 45%, respectively, in the presence of 200 μM PHB (Figure 3b), was added to the group with complete SLE suppression.

Summary of the effects of AEDs on 4-AP-induced seizure-like activity in OHSCs

Nine of the 92 OHSCs treated with 4-AP demonstrated RSD-SLEs. Under CBZ (five OHSCs), the tonic period of the RSD-SLE became shorter and the frequency of field potential fluctuations decreased. In addition, the rise in $[K^+]_o$ in CA3 shortened and the fluctuations in $[K^+]_o$ associated with the recurrent clonic activity became smaller and shorter. PHT was tested in four OHSCs and in three of them caused a reversible change from RSD-SLEs to tonic–clonic SLEs. The

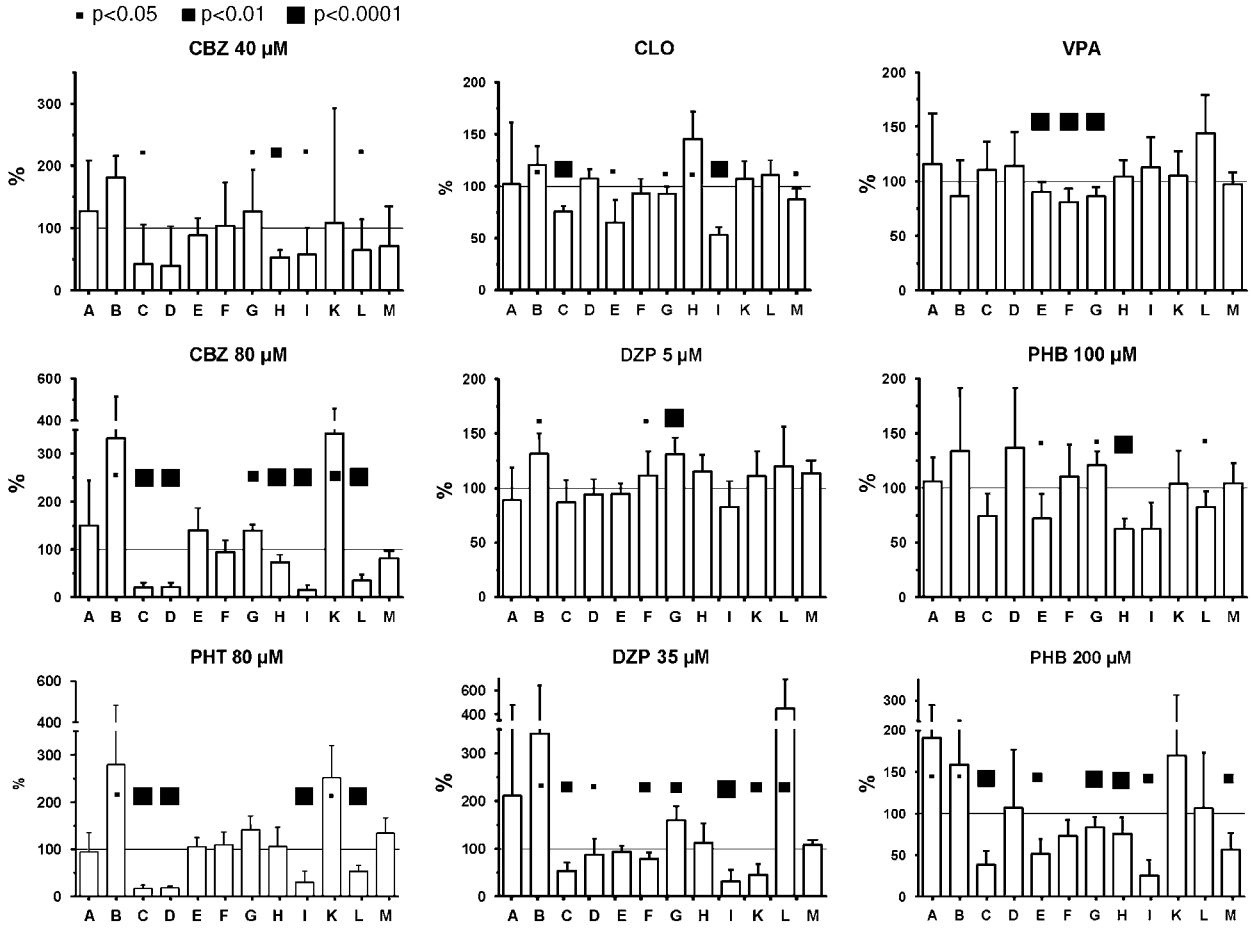

Figure 6 Effects of the AEDs on the amplitudes and time properties of tonic–clonic SLEs in OHSCs. Changes are presented as the value present during treatment with the AED normalized to the reference value. Each column represents the mean value + 95% confidence interval of the mean of one SLE parameter calculated on the basis of 6–15 test runs comprising 16–39 SLE comparisons. SLE parameters were labelled as follows (Table 1): (A) latency 1. SLE; (B) SLE frequency (*n* SLEs h^{-1}); (C) duration of SLE; (D) duration of tonic period; (E) maximal extracellular potassium concentration; (F) maximal amplitude of negative potential shift; (G) amplitude of field potential transients during tonic period; (H) frequency of field potential transients during tonic period; (I) duration of clonic period; (K) frequency of clonic-like events; (L) average duration of clonic-like events; (M) amplitude of onset field potential of clonic-like events. AED, antiepileptic drugs; OHSC, organotypic hippocampal slice culture; SLE, seizure-like event.

fourth OHSC tested was one of the two cases in which PHT (80 µM) completely suppressed SLEs (see above).

Discussion

For our investigation, we selected AEDs clinically indicated for the treatment of partial or focal epilepsies, including temporal lobe epilepsy (CBZ, PHT, 1,4-benzodiazepines, PHB). VPA is effective in patients with all type of seizures, whereas gabapentin is approved for adjunctive management of partial seizures with or without secondary generalization.

Tonic–clonic SLEs in entorhinal–hippocampal slices prepared from adult animals, whether induced by elevation of potassium (Leschinger *et al*., 1993), lowering of calcium (Heinemann *et al*., 1985), lowering of magnesium (Dreier *et al*., 1998) or by application of 4-AP (Bruckner and Heinemann, 2000), usually responded to standard AEDs, whereas interictal discharges and RSD-SLEs were pharmacoresistant. In the present study, we demonstrated that in OHSCs, in addition to RSD-SLEs, tonic–clonic SLEs are also resistant to clinically relevant concentrations of AEDs and suggest that this preparation may serve as a model of drug refractory epilepsy.

In humans, total seizure suppression is required for full social and professional integration of a given patient. All patients with pharmacoresistance still have more or less frequent seizures, but seizure severity and incidence increases when the AEDs are withdrawn. Thus, pharmacoresistance in clinical terms is defined in a digital manner as either seizure-free or not. This does not exclude the fact that antiepileptic medication has a modifying effect on the disease.

Tonic–clonic SLEs in the hippocampus in vitro

In our study, we used two models of seizure induction in which synaptic inhibition is preserved: the low magnesium model and the 4-AP model. 4-AP blocks certain potassium ion channels, in particular those from the Kv1 and Kv3

Table 3 Effects of AEDs on seizure-like activity in OHSCs

AED/concentration	*Total SLE*	*Tonic period*	*Clonic period*
CBZ 40 μM	112	100.25	108.5
CBZ 80 μM	139.6	81.8	118.5
PHT 80 μM	133.1	93.8	117.5
VPA 1–2 mM	100.7	96.5	114.8
PHB 100 μM	159.7	107.3	88.5
PHB 200 μM	90.7	84.8	89.6
DZP 5 μM	112.9	113	107
DZP 35 μM	159.7	109	157.7
CLO 20 μM	99.9	109.7	89.5

Abbreviations: AED, antiepileptic drug; CBZ, carbamazepine; CLO, clonazepam; DZP, diazepam; OHSC, organotypic hippocampal slice culture; PHB, phenobarbital sodium; PHT, phenytoin; SLE, seizure-like event; VPA, valproic acid.

Numbers are composite scores (%) calculated from single parameter scores listed in Table 1. The total SLE score represents the mean of the sum of all single parameter scores (labelled B, C, E, F, G, H, K, L, M; see Table 1 and Figure 6) with the exception of the scores for the duration of the tonic and clonic period, respectively. The tonic period score represents the mean of the sum of scores of the duration of the tonic period, maximal amplitude of negative potential shift during the tonic period, amplitude of field potential transients during the tonic period and frequency of field potential transients during the tonic period (labelled D, F, G, H in Table 1 and Figure 6). The clonic period score represents the mean of the sum of scores of the duration of the clonic period, frequency of clonic-like events, average duration of clonic-like events and amplitude of onset field potential of clonic-like events (labelled I, K, L, in Table 1 and Figure 6). Control is 100%.

families. The availability of potassium ion channels from the Kv4 family with a low sensitivity to 4-AP has been found to be reduced in an animal model of temporal lobe epilepsy (Bernard *et al*., 2004). Changes in potassium current properties have also been found in human epileptic hippocampal tissue (Beck *et al*., 1996; Zuberi *et al*., 1999), in the kainate model of temporal lobe epilepsy (Zahn *et al*., 2007) and in heterotopic neurons, which lack functional A-type Kv4.2 potassium channels. It has been suggested that this abnormality contributes to the increased excitability and decreased seizure threshold associated with brain malformations (Castro *et al*., 2001). The reasons for seizure induction in the low magnesium model are the facilitated activation of NMDA receptors, an increased transmitter release and a reduced membrane surface charge screening (Mody *et al*., 1987; Mangan and Kapur, 2004). From pharmacological and genetic studies of NMDA receptors in diverse *in vivo* and *in vitro* models, it is generally agreed that NMDA receptor activation promotes limbic epileptogenesis (Mody *et al*., 1987; McNamara *et al*., 2006). Decreased magnesium concentrations are a critical factor in eclampsia where normalization of magnesium levels blocks seizures and improves outcome, as do PHT and DZP (Duley *et al*., 2003).

It has been shown previously (Kovacs *et al*., 2002) that in OHSCs, free radical-mediated cell damage and uncoupling between neuronal and metabolic activity appeared only after more than 1 h of continuous seizure-like activity and that cell damage was reduced by adding a free radical scavenger to the preparation. We restricted the application time of low magnesium MEM to $\leqslant$40 min, which rendered any significant cell damage unlikely. We found no difference (unpublished observations) between low magnesium-induced SLEs and SLEs induced by low magnesium MEM combined with supplement B27 (containing the free radical scavengers α-tocopherol and glutathione). In addition, the recovery and undershoot of $[K^+]_o$ associated with the late phase of an SLE and the subsequent suppression of neuronal activity, respectively, were not different between pre- and post-drug controls, indicating that the function of the sodium/potassium-ATPase was unaffected (Heinemann and Lux, 1975; D'Ambrosio *et al*., 2002). The pathophysiological relevance of low magnesium-induced seizure-like activity in OHSCs was further demonstrated by reversible suppression of SLEs with glutamate antagonists or $GABA_A$-agonists.

Susceptibility to seizures and pharmacoresistance

All major neuronal types are present in OHSCs and develop mature structural properties after 20 DIV (Heimrich and Frotscher, 1991; Zafirov *et al*., 1994; Frotscher *et al*., 1995). Also, the basic intrinsic connective organization *in vivo* is retained *in vitro* (Zimmer and Gahwiler, 1984; Frotscher and Heimrich, 1995; Gutierrez and Heinemann, 1999). If cut during the preparation, the axons will regenerate with correct specificity (Li *et al*., 1994; Frotscher *et al*., 1997). However, according to the rules of reorganization of nerve connection, additional connections are formed (Zimmer and Gahwiler, 1984; Gutierrez and Heinemann, 1999) that increase network excitability (Lindroos *et al*., 2005). Reactive collateral sprouting in OHSCs of rat (Li *et al*., 1994) and mice (Coltman *et al*., 1995) in 7- to 10-day-old animals started after 3–6 DIV. Most OHSCs used in our experiments were older than 13 days (explantation between P6 and P10 plus at least 7 DIV). Their increased propensity for seizure induction could, therefore, be related to aberrant axonal connections, which have been also noted in different animal models of temporal lobe epilepsy (Lehmann *et al*., 2001; Buckmaster *et al*., 2002) and in human epileptic tissue (Gabriel *et al*., 2004). SLEs induced with 4-AP or low magnesium in 8- to 23-day-old rat hippocampus without abnormal connectivity are blocked by standard AEDs (Fueta and Avoli, 1992; Fueta *et al*., 1995); this supports the notion that aberrant axonal connections in OHSCs, at least 2 weeks *in vitro*, contribute to pharmacoresistance.

Pharmacoresistance was also present in OHSCs less than 10 days old (explantation at postnatal day (P) 2 + 6–8 DIV) when aberrant connections are not yet fully developed. The mechanisms for this pharmacoresistance could be the excess recurrent collateral connectivity between CA1 and between CA3 pyramidal neurons still present in the 1-week-old hippocampus (Gomez-Di Cesare *et al*., 1997), an immature gate function of the dentate gyrus (Collins *et al*., 1983) and/or immaturity of receptors and channels (Collins *et al*., 1983; Grosse *et al*., 2000; Holopainen and Lauren, 2003; Brewster *et al*., 2007). An increased excitability of the immature hippocampus has also been attributed to slowed kinetics of the hyperpolarization-activated depolarizing current ($I(H)$) (Chen *et al*., 2001), delayed postnatal development of potassium channels (Heinemann *et al*., 1992; Klee *et al*., 1997; Lopantsev and Avoli, 1998), developmental changes in functions of the voltage-operated M-type potassium (Pena and avez-Perez, 2006) and depolarizing $GABA_A$

receptor-mediated responses in immature neurons (Luhmann and Prince, 1991; Wahab *et al*., 2007). Such depolarizing GABA responses are probably caused by an elevated intracellular chloride concentration maintained by the activity of the K^+-Cl^--cotransporter isoform 1 (NKCC1) (Rohrbough and Spitzer, 1996). Application of bumetanide, a blocker of NKCC1, has been shown to reduce seizure-like activity *in vivo* and *in vitro* in the high K^+- model (Dzhala *et al*., 2005) but not in other seizure models (Kilb *et al*., 2007). In accordance with these findings, we demonstrated that application of bumetanide (10–20 mM; 8 OHSCs explanted between P2–7; 6–10 DIV) did not affect either low magnesium-induced SLEs or the PHT resistance of low magnesium-induced SLEs (unpublished observations). The increased seizure susceptibility and pharmacoresistance in OHSCs less than 14 DIV is, therefore, most probably related to the special morphological and/or functional properties of the early postnatal temporal cortex. This view is supported by the findings that low magnesium-induced tonic–clonic SLEs in an intact 1-week-old corticohippocampal formation *in vitro* are also resistant to a number of AEDs, among them CBZ, PHT and gabapentin (Quilichini *et al*., 2003). However, some differences have been noted between both preparations. Benzodiazepines, PHB and VPA suppressed SLEs in the intact hippocampal preparation but not in OHSCs. The concentrations of PHB (300 μM) and VPA (3 mM) used were in a neurotoxic range and higher than those used in our experiments. Differences in the type of preparation and the postnatal age of the tissue might also contribute to differences in results. It should also be noted that seizure frequency is higher (see above) and therefore susceptibility to seizures is stronger in OHSCs than in an intact immature corticohippocampal formation.

Validity of our in vitro *model of pharmacoresistant tonic–clonic SLEs*

The seizure-like activity induced *in vitro* by low magnesium or 4-AP is a model of acute convulsions, and therefore the pharmacoresistance that we have described might be restricted to this seizure type and not pertain to seizures in animals and humans with epilepsy. However, the validity of our *in vitro* pharmacoresistant model is built not on the method of provoking SLEs but on tissue properties that compare with human epileptic tissue, such as abundance of axonal recurrent connections. Unlike acute slices of the hippocampus, OHSCs appear to qualify for screening anticonvulsive drugs that would be effective in pharmacoresistant epileptic tissue for the following reasons. Firstly, the abundance of axonal recurrent connections in OHSCs as compared with the normal hippocampus almost certainly contributes to the increased propensity of seizures induced in OHSCs and possibly also the pharmacoresistance of these seizures. It has been argued (Dudek and Sutula, 2007) that the continuous nature of the axonal sprouting and formation of recurrent excitatory connectivity, as a consequence of primary injuries, could account for aspects of the latent period and the progressive nature of epileptogenesis as well as the progression of intractability. The aberrant recurrent excitatory circuitry present in CA3 and the dentate gyrus of OHSCs resembles the results of such protracted processes *in vivo*. Secondly, the clinical evidence for *de novo* pharmacoresistance in many patients with temporal lobe epilepsy (Schmidt and Loscher, 2005) should be borne in mind, as this indicates that development of pharmacoresistance does not necessarily require a protracted history of epileptic seizures but rather can be related to the maintenance or, alternatively, the reappearance of aberrant excitatory connections. Thirdly, as the AEDs we have tested have molecular actions different from each other, it appears unlikely that a single mechanism detected in epileptic tissue or the immaturity of one particular neuronal or transmitter system is solely responsible for the pharmacoresistance in OHSCs. Research over the last decade has identified such cellular mechanisms as candidates of pharmacoresistance, namely changes in the expression of drug transporters, limiting the access of AEDs to epileptogenic foci (Kwan and Brodie, 2006; Sisodiya *et al*., 2006) and changes in molecular drug targets of AEDs and transmitter systems (Remy and Beck, 2006; Volk *et al*., 2006). These mechanisms have been identified in brain tissue exposed to epileptiform activity over a long period of time. Nevertheless, the contribution of such 'post-status' mechanisms to the *de novo* pharmacoresistance described here cannot be excluded.

Partial effects of AEDs on tonic–clonic SLEs

The AEDs used in the present study have different binding sites and affect different parameters of neuronal activity. PHT and CBZ have similar properties in that they both promote the removal of sodium channel inactivation and induce a leftward shift of the steady state inactivation curve, both effects contributing to reduced action potential frequency (Ragsdale and Avoli, 1998; Catterall, 1999; Remy *et al*., 2003). Both these AEDs also reduce the presynaptic release of glutamate (Sitges *et al*., 2007). Accordingly, both AEDs had almost identical suppressive effects on the temporal properties of SLEs and did not alter the amplitudes of field potentials and $[K^+]_o$ transients. A notable difference was that the frequency of field potential transients during the tonic period decreased with CBZ but remained unchanged with PHT. PHT, in addition to sodium channels, also acts on high- and low-threshold calcium channels and potassium channels (Remy and Beck, 2006); this could induce an effect on the neuronal networks in OHSCs different from that caused by blocking sodium channels alone. VPA attenuated the amplitude of tonic parameters and did not affect the temporal structure of SLEs, effects that could be attributed to the inhibition of GABA metabolism and reuptake (Loscher, 1989). Similar effects of PHB (200 μM) were most likely due to a direct activation of $GABA_A$ receptors (Rho *et al*., 1996). This is also suggested by our observations that PHB, in addition to affecting amplitude parameters, concentration-dependently suppressed temporal parameters of SLEs and thus had similar effects on SLEs to low concentration $GABA_A$-agonists and GABA uptake inhibitors (Wahab *et al*., 2007). CLO and DZP at high concentrations selectively shortened the clonic period of the SLE as did 200 μM PHB but, in contrast, did not affect the amplitude parameter during the tonic period. 1,4-Benzodiazepines and phenobarbital are $GABA_A$ receptor

modulators, although with different actions on GABA-activated currents. DZP increases the frequency of channel openings, whereas barbiturates decrease the frequency of channel openings and increase the average lifetime of the open-channel (Study and Barker, 1981). The ineffectiveness of CLO and DZP at blocking SLEs cannot be attributed to a lack of receptors. At P10, the $GABA_A$ receptor subunits $\alpha1$ and $\alpha2$ in the rat hippocampus mediating the anticonvulsive actions of DZP (Kralic *et al*., 2002; Fradley *et al*., 2007) are present in concentrations only slightly higher ($\alpha1$) or lower ($\alpha2$) than in adults, and interneurons in the hippocampus strongly express the $\alpha1$ subunit (Yu *et al*., 2006).

Thus, our data demonstrate that the neuronal networks in OHSCs respond differently to AEDs. AEDs acting preferentially on sodium channels, similar to CBZ and PHT, reduce the durations of both tonic and clonic periods of SLEs, whereas high concentration AEDs interfering with the $GABA_A$ receptor (1,4,-benzodiazepines and PHB) only reduce the duration of the clonic period. Increasing the GABA concentration locally (VPA) attenuates the amplitudes of tonic phenomena, effects that are seen also with high concentrations of PHB.

Conclusions

Organotypic hippocampal slice cultures could be utilized as a simple to establish *in vitro* model of pharmacoresistant mesial temporal lobe epilepsy and to supplement data obtained from acute slices of the adult rat entorhinal cortex and hippocampus, an *in vitro* model already established as representative of the pharmacoresistant late stages of status epilepticus. OHSCs could also be used to screen drugs that have delayed onset actions and easily combined with safety and toxicology studies. As SLEs can also be induced in human tissue (Gabriel *et al*., 2004; Avoli *et al*., 2005; Jandova *et al*., 2006), it is now possible to transfer such testing directly to drug-resistant human tissue and perhaps thereby accelerate the development of agents that would be useful in the treatment of those patients who are drug refractory.

Acknowledgements

This research was supported by set (Stiftung zur Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen) and by DFG grant SFB-TR3 as well as by Epicure. We are grateful for helpful discussion with R Kovacs, O Kann and HJ Gabriel.

Conflict of interest

The authors state no conflict of interest.

References

Albus K, Wahab A, Heinemann U (2007). Standard antiepileptic drugs fail to block epileptiform activity induced by low magnesium or 4-aminopyridine in rat hippocampal slice cultures. Proceedings of the 7th Göttingen Meeting of the German Neuroscience Society. *Neuroforum Suppl* **13(1)**:183

Arts WF, Brouwer OF, Peters AC, Stroink H, Peeters EA, Schmitz PI *et al*. (2004). Course and prognosis of childhood epilepsy: 5-year follow-up of the Dutch study of epilepsy in childhood. *Brain* **127**: 1774–1784.

Avoli M, Louvel J, Pumain R, Kohling R (2005). Cellular and molecular mechanisms of epilepsy in the human brain. *Prog Neurobiol* **77**: 166–200.

Avoli M, Psarropoulou C, Tancredi V, Fueta Y (1993). On the synchronous activity induced by 4-aminopyridine in the CA3 subfield of juvenile rat hippocampus. *J Neurophysiol* **70**: 1018–1029.

Beck H, Blumcke I, Kral T, Clusmann H, Schramm J, Wiestler OD *et al*. (1996). Properties of a delayed rectifier potassium current in dentate granule cells isolated from the hippocampus of patients with chronic temporal lobe epilepsy. *Epilepsia* **37**: 892–901.

Bernard C, Anderson A, Becker A, Poolos NP, Beck H, Johnston D (2004). Acquired dendritic channelopathy in temporal lobe epilepsy. *Science* **305**: 532–535.

Brandt C, Volk HA, Loscher W (2004). Striking differences in individual anticonvulsant response to phenobarbital in rats with spontaneous seizures after status epilepticus. *Epilepsia* **45**: 1488–1497.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* **35**: 567–576.

Brewster AL, Chen Y, Bender RA, Yeh A, Shigemoto R, Baram TZ (2007). Quantitative analysis and subcellular distribution of mRNA and protein expression of the hyperpolarization-activated cyclic nucleotide-gated channels throughout development in rat hippocampus. *Cereb Cortex* **17**: 702–712.

Bruckner C, Heinemann U (2000). Effects of standard anticonvulsant drugs on different patterns of epileptiform discharges induced by 4-aminopyridine in combined entorhinal cortex-hippocampal slices. *Brain Res* **859**: 15–20.

Buckmaster PS, Zhang GF, Yamawaki R (2002). Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit. *J Neurosci* **22**: 6650–6658.

Castro PA, Cooper EC, Lowenstein DH, Baraban SC (2001). Hippocampal heterotopia lack functional Kv4. 2 potassium channels in the methylazoxymethanol model of cortical malformations and epilepsy. *J Neurosci* **21**: 6626–6634.

Catterall WA (1999). Molecular properties of brain sodium channels: an important target for anticonvulsant drugs. *Adv Neurol* **79**: 441–456.

Chen K, Aradi I, Thon N, Eghbal-Ahmadi M, Baram TZ, Soltesz I (2001). Persistently modified h-channels after complex febrile seizures convert the seizure-induced enhancement of inhibition to hyperexcitability. *Nat Med* **7**: 331–337.

Collins RC, Tearse RG, Lothman EW (1983). Functional anatomy of limbic seizures: focal discharges from medial entorhinal cortex in rat. *Brain Res* **280**: 25–40.

Coltman BW, Earley EM, Shahar A, Dudek FE, Ide CF (1995). Factors influencing mossy fiber collateral sprouting in organotypic slice cultures of neonatal mouse hippocampus. *J Comp Neurol* **362**: 209–222.

D'Ambrosio R, Gordon DS, Winn HR (2002). Differential role of KIR channel and Na(+)/K(+)-pump in the regulation of extracellular K(+) in rat hippocampus. *J Neurophysiol* **87**: 87–102.

Dreier JP, Zhang CL, Heinemann U (1998). Phenytoin, phenobarbital, and midazolam fail to stop status epilepticus-like activity induced by low magnesium in rat entorhinal slices, but can prevent its development. *Acta Neurol Scand* **98**: 154–160.

Dudek FE, Sutula TP (2007). Epileptogenesis in the dentate gyrus: a critical perspective. *Prog Brain Res* **163**: 755–773.

Duley L, Gulmezoglu AM, Henderson-Smart DJ (2003). Magnesium sulphate and other anticonvulsants for women with pre-eclampsia. *Cochrane Database Syst Rev* **2**: CD000025.

Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA *et al*. (2005). NKCC1 transporter facilitates seizures in the developing brain. *Nat Med* **11**: 1205–1213.

Fradley RL, Guscott MR, Bull S, Hallett DJ, Goodacre SC, Wafford KA *et al*. (2007). Differential contribution of GABA(A) receptor

subtypes to the anticonvulsant efficacy of benzodiazepine site ligands. *J Psychopharmacol* **21**: 384–391.
Frotscher M, Heimrich B (1995). Lamina-specific synaptic connections of hippocampal neurons *in vitro*. *J Neurobiol* **26**: 350–359.
Frotscher M, Heimrich B, Deller T (1997). Sprouting in the hippocampus is layer-specific. *Trends Neurosci* **20**: 218–223.
Frotscher M, Zafirov S, Heimrich B (1995). Development of identified neuronal types and of specific synaptic connections in slice cultures of rat hippocampus. *Prog Neurobiol* **45**: vii–xxviii.
Fueta Y, Avoli M (1992). Effects of antiepileptic drugs on 4-aminopyridine-induced epileptiform activity in young and adult rat hippocampus. *Epilepsy Res* **12**: 207–215.
Fueta Y, Siniscalchi A, Tancredi V, Avoli M (1995). Extracellular magnesium and anticonvulsant effects of valproate in young rat hippocampus. *Epilepsia* **36**: 404–409.
Gabriel S, Njunting M, Pomper JK, Merschhemke M, Sanabria ER, Eilers A *et al*. (2004). Stimulus and potassium-induced epileptiform activity in the human dentate gyrus from patients with and without hippocampal sclerosis. *J Neurosci* **24**: 10416–10430.
Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM (1997). Organotypic slice cultures: a technique has come of age. *Trends Neurosci* **20**: 471–477.
Gloveli T, Albrecht D, Heinemann U (1995). Properties of low Mg2+ induced epileptiform activity in rat hippocampal and entorhinal cortex slices during adolescence. *Brain Res Dev Brain Res* **87**: 145–152.
Gomez-Di Cesare CM, Smith KL, Rice FL, Swann JW (1997). Axonal remodeling during postnatal maturation of CA3 hippocampal pyramidal neurons. *J Comp Neurol* **384**: 165–180.
Grosse G, Draguhn A, Hohne L, Tapp R, Veh RW, hnert-Hilger G (2000). Expression of Kv1 potassium channels in mouse hippocampal primary cultures: development and activity-dependent regulation. *J Neurosci* **20**: 1869–1882.
Gulaboglu M, Borekci B, Halici Z (2007). Placental tissue iodine level and blood magnesium concentration in pre-eclamptic and normal pregnancy. *Int J Gynaecol Obstet* **98**: 100–104.
Gutierrez R, Armand V, Schuchmann S, Heinemann U (1999). Epileptiform activity induced by low Mg2+ in cultured rat hippocampal slices. *Brain Res* **815**: 294–303.
Gutierrez R, Heinemann U (1999). Synaptic reorganization in explanted cultures of rat hippocampus. *Brain Res* **815**: 304–316.
Heimrich B, Frotscher M (1991). Differentiation of dentate granule cells in slice cultures of rat hippocampus: a Golgi/electron microscopic study. *Brain Res* **538**: 263–268.
Heinemann U, Albrecht D, Beck H, Ficker E, von HD, Stabel J (1992). Delayed K+ regulation and K+ current maturation as factors of enhanced epileptogenicity during ontogenesis of the hippocampus of rats. *Epilepsy Res Suppl* **9**: 107–114.
Heinemann U, Dreier J, Leschinger A, Stabel J, Draguhn A, Zhang C (1994). Effects of anticonvulsant drugs on hippocampal neurons. *Hippocampus* **4**: 291–296.
Heinemann U, Franceschetti S, Hamon B, Konnerth A, Yaari Y (1985). Effects of anticonvulsants on spontaneous epileptiform activity which develops in the absence of chemical synaptic transmission in hippocampal slices. *Brain Res* **325**: 349–352.
Heinemann U, Lux HD (1975). Undershoots following stimulus-induced rises of extracellular potassium concentration in cerebral cortex of cat. *Brain Res* **93**: 63–76.
Holopainen IE, Lauren HB (2003). Neuronal activity regulates GABAA receptor subunit expression in organotypic hippocampal slice cultures. *Neuroscience* **118**: 967–974.
Jandova K, Pasler D, Antonio LL, Raue C, Ji S, Njunting M *et al*. (2006). Carbamazepine-resistance in the epileptic dentate gyrus of human hippocampal slices. *Brain* **129**: 3290–3306.
Jefferys JG, Haas HL (1982). Synchronized bursting of CA1 hippocampal pyramidal cells in the absence of synaptic transmission. *Nature* **300**: 448–450.
Jensen FE (2006). Pediatric epilepsy models. *Epilepsy Res* **68**: 28–31.
Kilb W, Sinning A, Luhmann HJ (2007). Model-specific effects of bumetanide on epileptiform activity in the *in-vitro* intact hippocampus of the newborn mouse. *Neuropharmacology* **53**: 524–533.
Klee R, Eder C, Ficker E, Heinemann U (1997). Age-dependent variations in potassium sensitivity of A-currents in rat hippocampal neurons. *Eur J Neurosci* **9**: 1970–1976.
Kovacs R, Schuchmann S, Gabriel S, Kann O, Kardos J, Heinemann U (2002). Free radical-mediated cell damage after experimental status epilepticus in hippocampal slice cultures. *J Neurophysiol* **88**: 2909–2918.
Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE, Morrow AL (2002). GABA(A) receptor alpha-1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacology* **43**: 685–694.
Kwan P, Brodie MJ (2006). Refractory epilepsy: mechanisms and solutions. *Expert Rev Neurother* **6**: 397–406.
Lehmann TN, Gabriel S, Eilers A, Njunting M, Kovacs R, Schulze K *et al*. (2001). Fluorescent tracer in pilocarpine-treated rats shows widespread aberrant hippocampal neuronal connectivity. *Eur J Neurosci* **14**: 83–95.
Leschinger A, Stabel J, Igelmund P, Heinemann U (1993). Pharmacological and electrographic properties of epileptiform activity induced by elevated K+ and lowered Ca2+ and Mg2+ concentration in rat hippocampal slices. *Exp Brain Res* **96**: 230–240.
Li D, Field PM, Yoshioka N, Raisman G (1994). Axons regenerate with correct specificity in horizontal slice culture of the postnatal rat entorhino-hippocampal system. *Eur J Neurosci* **6**: 1026–1037.
Lindroos MM, Soini SL, Kukko-Lukjanov TK, Korpi ER, Lovinger D, Holopainen IE (2005). Maturation of cultured hippocampal slices results in increased excitability in granule cells. *Int J Dev Neurosci* **23**: 65–73.
Lopantsev V, Avoli M (1998). Participation of GABAA-mediated inhibition in ictallike discharges in the rat entorhinal cortex. *J Neurophysiol* **79**: 352–360.
Loscher W (1989). Valproate enhances GABA turnover in the substantia nigra. *Brain Res* **501**: 198–203.
Loscher W (1997). Animal models of intractable epilepsy. *Prog Neurobiol* **53**: 239–258.
Loscher W, Fassbender CP, Nolting B (1991). The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. II. Maximal electroshock seizure models. *Epilepsy Res* **8**: 79–94.
Loscher W, Potschka H (2005). Drug resistance in brain diseases and the role of drug efflux transporters. *Nat Rev Neurosci* **6**: 591–602.
Loscher W, Schmidt D (2006). Experimental and clinical evidence for loss of effect (tolerance) during prolonged treatment with antiepileptic drugs. *Epilepsia* **47**: 1253–1284.
Lothman EW, Bertram EH, Bekenstein JW, Perlin JB (1989). Self-sustaining limbic status epilepticus induced by 'continuous' hippocampal stimulation: electrographic and behavioral characteristics. *Epilepsy Res* **3**: 107–119.
Luhmann HJ, Prince DA (1991). Postnatal maturation of the GABAergic system in rat neocortex. *J Neurophysiol* **65**: 247–263.
Lux HD, Neher E (1973). The equilibration time course of (K +) 0 in cat cortex. *Exp Brain Res* **17**: 190–205.
Mangan PS, Kapur J (2004). Factors underlying bursting behavior in a network of cultured hippocampal neurons exposed to zero magnesium. *J Neurophysiol* **91**: 946–957.
McNamara JO, Huang YZ, Leonard AS (2006). Molecular signaling mechanisms underlying epileptogenesis. *Sci STKE* **2006**: re12.
Mody I, Lambert JD, Heinemann U (1987). Low extracellular magnesium induces epileptiform activity and spreading depression in rat hippocampal slices. *J Neurophysiol* **57**: 869–888.
Moser J, Kilb W, Werhahn KJ, Luhmann HJ (2006). Early developmental alterations of low-Mg2+-induced epileptiform activity in the intact corticohippocampal formation of the newborn mouse *in vitro*. *Brain Res* **1077**: 170–177.
Neels HM, Sierens AC, Naelaerts K, Scharpe SL, Hatfield GM, Lambert WE (2004). Therapeutic drug monitoring of old and newer antiepileptic drugs. *Clin Chem Lab Med* **42**: 1228–1255.
Noraberg J, Kristensen BW, Zimmer J (1999). Markers for neuronal degeneration in organotypic slice cultures. *Brain Res Brain Res Protoc* **3**: 278–290.
Oby E, Caccia S, Vezzani A, Moeddel G, Hallene K, Guiso G *et al*. (2006). *In vitro* responsiveness of human-drug-resistant tissue to antiepileptic drugs: insights into the mechanisms of pharmacoresistance. *Brain Res* **1086**: 201–213.

Pena F, avez-Perez N (2006). Epileptiform activity induced by pharmacologic reduction of M-current in the developing hippocampus *in vitro*. *Epilepsia* **47**: 47–54.

Potschka H, Fedrowitz M, Loscher W (2002). P-Glycoprotein-mediated efflux of phenobarbital, lamotrigine, and felbamate at the blood-brain barrier: evidence from microdialysis experiments in rats. *Neurosci Lett* **327**: 173–176.

Quilichini PP, Diabira D, Chiron C, Milh M, Ben-Ari Y, Gozlan H (2003). Effects of antiepileptic drugs on refractory seizures in the intact immature corticohippocampal formation *in vitro*. *Epilepsia* **44**: 1365–1374.

Ragsdale DS, Avoli M (1998). Sodium channels as molecular targets for antiepileptic drugs. *Brain Res Brain Res Rev* **26**: 16–28.

Rambeck B, Jurgens UH, May TW, Pannek HW, Behne F, Ebner A *et al*. (2006). Comparison of brain extracellular fluid, brain tissue, cerebrospinal fluid, and serum concentrations of antiepileptic drugs measured intraoperatively in patients with intractable epilepsy. *Epilepsia* **47**: 681–694.

Remy S, Beck H (2006). Molecular and cellular mechanisms of pharmacoresistance in epilepsy. *Brain* **129**: 18–35.

Remy S, Gabriel S, Urban BW, Dietrich D, Lehmann TN, Elger CE *et al*. (2003). A novel mechanism underlying drug resistance in chronic epilepsy. *Ann Neurol* **53**: 469–479.

Reynolds E (2006). Treating refractory epilepsy in adults. *BMJ* **332**: 562–563.

Rho JM, Donevan SD, Rogawski MA (1996). Direct activation of GABAA receptors by barbiturates in cultured rat hippocampal neurons. *J Physiol* **497** (Part 2): 509–522.

Rohrbough J, Spitzer NC (1996). Regulation of intracellular Cl^- levels by Na(+)-dependent Cl^- cotransport distinguishes depolarizing from hyperpolarizing GABAA receptor-mediated responses in spinal neurons. *J Neurosci* **16**: 82–91.

Schmidt D, Loscher W (2005). Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. *Epilepsia* **46**: 858–877.

Scism JL, Powers KM, Artru AA, Lewis L, Shen DD (2000). Probenecid-inhibitable efflux transport of valproic acid in the brain parenchymal cells of rabbits: a microdialysis study. *Brain Res* **884**: 77–86.

Sisodiya SM, Martinian L, Scheffer GL, van der Valk P, Scheper RJ, Harding BN *et al*. (2006). Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathol Appl Neurobiol* **32**: 51–63.

Sitges M, Chiu LM, Guarneros A, Nekrassov V (2007). Effects of carbamazepine, phenytoin, lamotrigine, oxcarbazepine, topiramate and vinpocetine on Na+ channel-mediated release of [3H]glutamate in hippocampal nerve endings. *Neuropharmacology* **52**: 598–605.

Sokolova S, Schmitz D, Zhang CL, Loscher W, Heinemann U (1998). Comparison of effects of valproate and trans-2-en-valproate on different forms of epileptiform activity in rat hippocampal and temporal cortex slices. *Epilepsia* **39**: 251–258.

Stoppini L, Buchs PA, Muller D (1991). A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* **37**: 173–182.

Study RE, Barker JL (1981). Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of gamma-aminobutyric acid responses in cultured central neurons. *Proc Natl Acad Sci USA* **78**: 7180–7184.

Tian LM, Otoom S, Alkadhi KA (1995). Endogenous bursting due to altered sodium channel function in rat hippocampal CA1 neurons. *Brain Res* **680**: 164–172.

Volk HA, Arabadzisz D, Fritschy JM, Brandt C, Bethmann K, Loscher W (2006). Antiepileptic drug-resistant rats differ from drug-responsive rats in hippocampal neurodegeneration and GABA(A) receptor ligand binding in a model of temporal lobe epilepsy. *Neurobiol Dis* **21**: 633–646.

Wahab A, Albus K, Heinemann U (2007). Effects of GABA agonists and a GABA uptake inhibitor on pharmacoresistant epileptiform activity in hippocampal sloce cultures. *Abstr Soc Neurocience* **161**: 1.

Walker MC, Alavijeh MS, Shorvon SD, Patsalos PN (1996). Microdialysis study of the neuropharmacokinetics of phenytoin in rat hippocampus and frontal cortex. *Epilepsia* **37**: 421–427.

Walton NY, Treiman DM (1991). Motor and electroencephalographic response of refractory experimental status epilepticus in rats to treatment with MK-801, diazepam, or MK-801 plus diazepam. *Brain Res* **553**: 97–104.

Yaari Y, Konnerth A, Heinemann U (1983). Spontaneous epileptiform activity of CA1 hippocampal neurons in low extracellular calcium solutions. *Exp Brain Res* **51**: 153–156.

Yonekawa WD, Kapetanovic IM, Kupferberg HJ (1995). The effects of anticonvulsant agents on 4-aminopyridine induced epileptiform activity in rat hippocampus *in vitro*. *Epilepsy Res* **20**: 137–150.

Yu ZY, Wang W, Fritschy JM, Witte OW, Redecker C (2006). Changes in neocortical and hippocampal GABAA receptor subunit distribution during brain maturation and aging. *Brain Res* **1099**: 73–81.

Zafirov S, Heimrich B, Frotscher M (1994). Dendritic development of dentate granule cells in the absence of their specific extrinsic afferents. *J Comp Neurol* **345**: 472–480.

Zahn RK, Tolner EA, Derst C, Gruber C, Veh RW, Heinemann U (2008). Reduced ictogenic potential of 4-aminopyridine in the perirhinal and entorhinal cortex of kainate-treated chronic epileptic rats. *Neurobiol Dis* **29**: 186–200.

Zhang CL, Dreier JP, Heinemann U (1995). Paroxysmal epileptiform discharges in temporal lobe slices after prolonged exposure to low magnesium are resistant to clinically used anticonvulsants. *Epilepsy Res* **20**: 105–111.

Zimmer J, Gahwiler BH (1984). Cellular and connective organization of slice cultures of the rat hippocampus and fascia dentata. *J Comp Neurol* **228**: 432–446.

Zuberi SM, Eunson LH, Spauschus A, De SR, Tolmie J, Wood NW *et al*. (1999). A novel mutation in the human voltage-gated potassium channel gene (Kv1. 1) associates with episodic ataxia type 1 and sometimes with partial epilepsy. *Brain* **122** (Part 5): 817–825.