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DISSERTATION

A new model of pharmacoresistant seizure like events and age specific effects of antiepileptic drugs

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List of abbreviations

$[K^+]_o$	Extracellular concentration of potassium
4-AP	4-Aminopyridine
ACSF	Artificial cerebrospinal fluid
AEDs	Antiepileptic drugs
ECm	Medial entorhinal cortex
FP	Field potential
GABA	γ-Aminobutyric acid
HFS	High frequency electrical stimulation
MEM	Minimal essential medium
Mg^{2+}	Magnesium
OHSCs	Organotypic hippocampal slice cultures
P14 – P19	Postnatal 14 - 19 day old rat
P3 – P10	Postnatal 3 - 10 day old rat
P3 – P5	Postnatal 3 - 5 day old rat
PAD	Primary afterdischarge
RSDs	Recurrent short clonic-like discharges
SLEs	Seizure like events

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Summary

Background and purpose: Drug resistance of epilepsy is an important clinical problem that affects around 75% of mesial temporal lobe epilepsies patients. Previously a number of reports have shown that in organotypic hippocampal slice cultures (OHSCs) the *in vivo* morphology and the basic intrinsic connection are retained. However, some reorganization of axonal connections also occurs which is comparable to that observed in patients with epilepsy and in animal models of temporal lobe epilepsy. It was hypothesized that this reorganization of neuronal networks in OHSCs may provide *in vitro* models of epilepsy. In this PhD thesis, the suitability of OHSCs as *in vitro* models of epileptiform activities was explored. Age specific effects of antiepileptic drugs (AEDs) were investigated using acute slices prepared from different age groups of rat. The mechanism of pharmacoresistance in immature rat brain was also investigated.

Experimental approach: OHSCs were prepared from 2-12 day old rats. Field potentials (FP) and extracellular potassium concentration ($[K^+]_o$) in area CA3 and CA1 were measured. AEDs were tested against seizure like events (SLEs) induced by low magnesium (Mg^{2+}) or 4-aminopyridine (4-AP). The effects of glutamate receptor antagonists, $GABA_A$ and $GABA_B$ receptor agonists, a GABA uptake blocker, a neurosteroid and taurine on low Mg^{2+} induced SLEs were also tested. AEDs were also tested against high frequency electrical stimulation (HFS)-induced primary after discharges (PADs) in OHSCs. Acute hippocampal-entorhinal cortex slices were prepared from 3-19 days old rats. FP and the $[K^+]_o$ were measured in CA3 and medial entorhinal cortex and the effects of AEDs, a NKKC1 blocker and a carbonic anhydrase blocker against 4-AP induced SLEs were studied.

Key results: Except neurotoxic dose of phenobarbital (200 µM), all the AEDs were unable to block SLEs induced either by low Mg^{2+} or 4-AP in OHSCs. The pathophysiological relevance of SLEs in OHSCs was demonstrated by reversible suppression of SLEs by glutamate receptor antagonists, $GABA_A$ receptor agonists, a GABA uptake blocker and a neurosteroid. In contrast to low Mg^{2+} or 4-AP model, HFS-induced PADs in OHSCs were suppressed by the AEDs. In the acute hippocampal-entorhinal cortex slices, SLEs around first postnatal week were more resistant to AEDs as compared to SLEs after second postnatal week. The NKCC1 blocker suppressed SLEs efficiently during the first postnatal week and had no major effects after the second week. The carbonic anhydrase inhibitor has similar age specific effect as other AEDs.

Conclusions: It is proposed that OHSCs can be used as either a pharmacoresistant or a pharmacosensitive epilepsy model depending on how seizures are induced. 4-AP induced SLEs in the acute temporal cortex slices showed strong pharmacoresistance only around first postnatal week and the NKCC1 cotransporter most likely contributes to this pharmacoresistance.

1. Introduction

Epilepsy is one of the most common serious neurological disorders responsible for substantial morbidity and mortality with seizure and medications. In adult, about 75% of patients with mesial temporal lobe epilepsies have pharmacoresistant seizures [31]. In childhood about 20-30% of patients with partial epilepsies and more than 50% with Lennox-Gastaut syndrome are pharmacoresistant [2]. The condition is more complicated in certain brain abnormalities, for example, when hippocampal sclerosis is combined with focal dysplasia or similar developmental alterations the chances of pharmacoresistance may reach more than 90% [37]. Surgery is possible in only a small proportion of pharmacoresistant patients. Therefore, it is very important to understand the mechanisms of pharmacoresistance, and there is a need for suitable epilepsy models to develop new medicines for pharmacoresistant patients [37].

As a part of my PhD project, the suitability of organotypic hippocampal slice cultures (OHSCs) as a model of epileptiform activities was tested [1]. We selected this preparation, because from our lab and other labs, it has been shown that in OHSCs most cell types and their connections are preserved, but some reorganization of network also occurs which is comparable to that observed in tissues from patients with epilepsy [13,14,16] and in animal models of temporal lobe epilepsy [24]. In the present study using OHSCs, three models of epileptiform activities were investigated: the low magnesium (Mg^{2+}) model, the 4-aminopyridine (4-AP) model and the high frequency electrical stimulation (HFS) model.

Pharmacoresistance in early childhood may depend on physiological immaturities in ion homeostasis and other developmental characteristics. Therefore, the age specific effects of antiepileptic drugs (AEDs) were also studied in acute hippocampal-entorhinal cortex slices prepared from 3-19 day old rats, and the possible contribution of excitatory effects of GABA in pharmacoresistance of seizure like events in immature rat brain were evaluated [38].

Aims:

The aim of my PhD project was to develop new *in vitro* models of epileptiform activities, to explore the age dependent effects of AEDs and to investigate the mechanism of pharmacoresistance in immature brain. To fulfill this aim investigations were carried out (i) to test the suitability of OHSCs as a model of epileptiform activities using low Mg^{2+}, 4-AP, and HFS (ii) to characterize these models by testing different AEDs (ii) to find out the role of compounds that enhance GABA-mediated actions in pharmacoresistance of OHSCs (iii) to determine at which age in rats the seizure like events (SLEs) are pharmacoresistant preparing acute hippocampal-entorhinal cortex slices from different age groups (iv) to explore the role of inhibitors of carbonic anhydrase and NKCC1 in pharmacoresistance of SLEs in immature rat.

2. Methods

2.1. Preparation of organotypic hippocampal slice cultures (OHSCs)

OHSCs were prepared according to the interface culture method using culture media optimal for preparing and culturing neurons as described by Stoppini et al., 1991 [32]. 2-12 day old Wistar rats were decapitated and transverse hippocampal slices (400 µM) were prepared and placed on Millicell culture plate inserts which were transferred to culture plates with 6 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. The culture plates were placed in an incubator. After 3 days the medium was replaced with 1.1 ml of serum-free medium (Neurobasal A) supplemented with 1 mM L-glutamine and B27. This medium was changed every second day. Most slices were incubated for 5-35 days before experiment. However, in few slices incubation time was up to 64 days [1,35].

2.2. Recordings from OHSCs

All the recordings in OHSCs were carried out in submerged condition. OHSCs were superfused with 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. Concentrations of NaCl, and $NaHCO_3$, and the osmolality match the respective values in Neurobasal A. Seizure like events (SLEs) were induced by MEM containing no Mg^{2+} ions and 5 mM KCl, referred to as low Mg^{2+} MEM, or by 4-AP [1,35]. A hippocampal primary afterdischarge (PAD) in CA1 and CA3 was elicited by stimulating either the Schaffer collaterals or the hilus/CA3 border with bipolar tungsten electrodes using a 100 Hz stimulus train (0.1ms, 1s) [36]. The drug of interest was tested in these models. The DC-potential and population spikes and the extracellular concentration of potassium ($[K^+]_o$) in the pyramidal cell layer of CA3 was measured with a double barreled K^+-selective/reference glass microelectrode, prepared and tested in our laboratory as described by Lux and Neher, 1973 [21]. In addition single unit and multiunit activities were recorded (AC-coupled) with tungsten-in-glass microelectrodes positioned in the pyramidal cell layers of area CA3 and CA1 [1,35,36].

2.3. Preparation of acute hippocampal-entorhinal cortex slice

The experiments were performed as described previously [8] on horizontal hippocampus-entorhinal cortex slices (400 µm) prepared from 3 to 19 day old Wistar rats. After removing, brains were bathed in ice-cold and carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 129, KCl 3, $MgSO_4$ 1.8, $CaCl_2$ 1.6, NaH_2PO_4 1.25, $NaHCO_3$ 21, and glucose 10.

Slices were cut and immediately transferred into an interface-type recording chamber continuously perfused with ACSF [38].

2.4. Recordings from acute hippocampal-entorhinal cortex slices

All the recordings in acute hippocampal-entorhinal cortex slices were performed in interface system. Changes in the extracellular concentration of potassium ($[K^+]_o$) and field potentials in the pyramidal cell layer of CA3 and upper layers of the medial entorhinal cortex (ECm) were measured with double barrelled K^+-selective/reference glass microelectrodes. SLEs were induced by adding 100 µM 4-aminopyrdine (4-AP) to the ACSF. Testing of drugs was carried out against SLEs for 60 to 80 minutes [38].

2.5. Data analysis and statistical procedures

SLEs in OHSCs and acute slices were considered as blocked if they disappeared completely after wash in of a drug and reappeared during the wash out period. To quantify the effects of drugs on SLEs in cases where SLEs were not blocked we measured following properties of tonic-clonic SLEs: (i) onset of first SLE (s); (ii) maximal $[K^+]_o$ (mM); (iii) duration of SLE (s); (iv) duration of tonic-like period (s); (v) duration of clonic-like period (s); (vi) frequency of field potential transients during tonic-like period (/s); (vii) duration of clonic-like events (s); (viii) frequency of clonic-like events (/s); (ix) maximal amplitude of negative potential shift (mV); (x) duration of negative potential shift (s). During drug application in acute slices we measured the above mentioned 5 parameters (from i to v). Statistical significance was determined by Wilcoxon matched-paired rank test. To quantify the effects of drugs on HFS induced PADs, we measured its duration.

3. Results

3.1. Low Mg^{2+} or 4-aminopyridine (4-AP) induced seizure-like activities in OHSCs

Application of a low Mg^{2+} or 4-AP to OHSCs induced SLEs that were characterized by a negative potential shift superimposed by high frequency field potential transients (tonic-like period) followed by a period in which clonic-like afterdischarges occurred. The tonic-like phase is accompanied with maximum elevation in extracellular concentration of potassium ($[K^+]_o$), whereas clonic-like phase is accompanied with ripples and gradual decline back in $[K^+]_o$ to normal level. The recovery and undershoot of $[K^+]_o$ associated with the late phase of a SLE and the subsequent suppression of neuronal activities, respectively, were not different between pre- and post-drug controls, indicating that the function of the sodium/potassium-ATPase was unaffected by seizure activity [18]. Between the SLEs, interictal discharges were seen. The

occurrence of SLEs was not dependent on the presence of the entorhinal cortex or on the age of the animals at the time of preparation (P2 - P12). The majority of the low Mg^{2+}-induced SLEs consisted of a tonic period followed by a clonic period of limited duration (tonic-clonic SLEs). However, in a subset of slice cultures, tonic-clonic SLEs were eventually replaced by recurrent short clonic-like discharges (RSDs), which were termed as mixed type SLEs. Finally, in a number of slices, RSDs started immediately after the first tonic discharge [1,35]. This latter type was termed as RSD-SLE. In OHSCs, tonic-clonic SLEs in the hippocampus could still be reliably induced after 2 months of their preparation, confirming earlier findings [17].

3.2. Effects of antiepileptic drugs (AEDs) on seizure like activities in OHSCs

In 86 (93.5%) of the 92 OHSCs explored, AEDs could not completely block the low Mg^{2+} or 4-AP induced SLEs, and these results were not dependent on SLE type, method of SLE provocation, postnatal age at explantation, days in an incubator and presence of the entorhinal cortex. Although in more than 90% of cases, the AEDs were not able to block the SLEs but they modified the time and amplitude characteristics of SLEs in a drug-specific and concentration-dependent way [1].

3.3. Summary of the effects of AEDS on low Mg^{2+}-induced seizure-like activity in OHSCs

Carbamazepine 30-80 µM (*n*=21) and phenytoin 40-100 µM (*n*=17) were tested against low Mg^{2+} induced SLEs in OHSCs. Ongoing seizure like activities was never blocked in any case, even in an OHSC in which very high dose of carbamazepine (180 µM) was applied. The most noticeable effect of these two drugs in slice cultures in which SLEs were not blocked was a concentration-dependent increase in the incidence of SLEs associated with a reduction of SLE duration. The rises in $[K^+]_o$ were not significantly affected by carbamazepine and phenytoin. A notable difference was that the frequency of field potential transients during the tonic period decreased after the application of carbamazepine but remained unchanged in the presence of phenytoin. The effects of valproic acid (0.8-2 mM) on low Mg^{2+} induced SLEs were tested in 15 slice cultures. With the exception of one case, valproic acid failed to stop seizure activity. According to clinical reports, valproic acid may exert therapeutic effects but only after some weeks [26]. Hence, we investigated the effects of valproic acid in OHSCs after a preincubation period of 7–29 days with 1 mM valproic acid (*n*=5). However, valproic acid still failed to block SLEs in these OHSCs. Phenobarbital was tested at the concentrations of 100-200 µM against low Mg^{2+} induced SLEs (*n*=12). Phenobarbital 100 µM did not completely block SLEs. This was also true in three additional OHSCs, which were preincubated with 100 µM phenobarbital for 15–28 days. However, 200 µM phenobarbital almost completely suppressed SLEs in three out of six

OHSCs. The slices in which SLEs were not blocked by phenobarbital showed marked decrease in SLE duration but an increase in the incidence of SLEs. Diazepam 3.5-35 µM (*n*=10) and clonazepam 1-20 µM (*n*=4) could not block low Mg^{2+} induced SLEs in OHSCs. The most marked effects were an increase in SLE frequency and a significant reduction of total SLE duration. SLEs in OHSCs were completely and reversibly blocked by combined application of two glutamate antagonists CNQX + DL-AP-5 (*n*=7) [1].

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

4-AP induced RSD-SLEs in all nine OHSCs tested. With carbamazepine (*n*=5), the tonic period of the RSD-SLE became shorter and the frequency of field potential fluctuations was 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. Phenytoin was tested in four OHSCs and in three of them caused a reversible change from RSD-SLEs to tonic-clonic SLEs. In one OHSC phenytoin 80 µM completely suppressed SLEs [1].

The failure of AEDs and more specifically 1,4-benzodiazepines and therapeutic concentration of phenobarbital to block the SLEs in OHSCs prepared from immature rats [1] may be due to the impaired/altered GABA-ergic mechanisms based on the immaturity of receptors. To test the hypothesis of a possible contribution of altered GABA-mediated mechanisms to seizure susceptibility and pharmacoresistance in OHSCs, we have investigated the effects of compounds, which produce their actions on GABA system with mechanisms different than those of phenobarbital and 1,4-benzodiazepines.

3.5. Compounds that produce their actions on GABA system in OHSCs

The effects of muscimol and isoguvacine were analyzed in 32 test runs. Muscimol applied with 1 µM (*n*=5) did not block SLEs in any of the 5 OHSC tested, whereas at 5 µM it blocked SLEs in two out of five OHSC and at 10 µM (*n*=5) it reversibly blocked ongoing SLEs in all OHSCs tested. Isoguvacine at a concentration of 10 µM failed to block SLEs (*n*=6), and at a concentration of 50 µM blocked the SLEs in 2 out of 6 OHSCs, whereas, at 100 µM concentration it blocked the SLEs in all OHSCs (*n*=5). Concentrations of muscimol and isoguvacine ineffective in blocking SLEs nevertheless induced significant dose dependent changes in SLE parameters. Muscimol increased the latency of occurrence of the first SLE and strongly reduced the duration of SLEs and the duration of clonic-like events. Similar changes occurred under isoguvacine. The shortening of SLEs caused by muscimol and isoguvacine was mainly due to a strong reduction of the duration of the clonic-like period [35]. RSD-SLEs reversibly changed into tonic-clonic SLEs at an intermediate and the low concentrations of

muscimol and isoguvacine. A similar effect has been reported in acute hippocampal-entorhinal cortex slices [27], where late recurrent discharges were converted to tonic-clonic SLEs under 5-25 µM muscimol.

The effects of the neurosteroid alfaxalone were analyzed in 10 test runs. Alfaxalone at a concentration of 50 µM reversibly blocked ongoing SLEs regardless of seizure type. At a concentration of 20 µM it did not block ongoing SLEs but significantly reduced the duration of SLEs. Similar to muscimol and isoguvacine alfaxalone reversibly converted RSD-SLEs to tonic-clonic SLEs [35]. The $GABA_B$ receptor agonist (+)-baclofen was analyzed in 11 test runs. It neither prevented the induction of SLEs nor inhibited the ongoing SLEs at concentrations of 25 and 200 µM. However, at both concentrations (+)-baclofen shortened the duration of SLE and the durations of both clonic and tonic-like periods. At the concentration of 200 µM in addition the increase of $[K^+]_o$ and the duration of the negative potential shift during the tonic-like phase of an SLE were significantly reduced. Other enantiomers, (−)-baclofen (*n*=4) and (±)-baclofen (*n*=4), at concentrations of 25 and 200 µM were also tested and these were less effective on SLEs than (+)-baclofen [35]. The effects of nipecotic acid, a GABA uptake blocker, were analyzed in 21 test runs. At the concentration of 200 µM nipecotic acid did not block SLEs but strongly modified strength and duration of SLEs. At concentrations of 500 µM and 1 mM nipecotic acid blocked SLEs in all of the tested slices. However, in few slices in absence of SLEs single large amplitude population events associated with sharp transient peaks in $[K^+]_o$ occurred under nipecotic acid. Reversible changes of RSD-SLEs or mixed SLEs in tonic-clonic SLEs as seen with muscimol, isoguvacine and alfaxalone were also observed at 200 µM nipecotic acid. In addition to GABA-mimetics, taurine which is a glycine receptor agonist was also tested. Taurine at the concentration of 2 mM (4 test runs) did not block the ongoing seizure activity and induced only minor changes in seizure pattern [35].

3.6. High frequency electrical stimulation induced primary after discharges in OHSCs

High frequency electrical stimulation (HFS) induced primary after discharges (PADs) in OHSCs can be divided into three components. The first component was a low amplitude high frequency (> 20 Hz) tonic discharge, the second component was a high amplitude low frequency (< 20 Hz) tonic discharge and the third component was a clonic-like discharge, which was variable in duration, and sometimes absent. The mean of total duration and the 95% confidence interval (of the mean total duration) of PADs in CA1 in control was 19.7s [14.2;25.2]. The PADs appeared synchronously in the CA3 and CA1 and the duration of the PAD was independent of cultivation time *in vitro*, and the position of the recording electrode (CA3, CA1, dentate gyrus). The AEDs carbamazepine and phenytoin completely and reversibly blocked the HFS-induced

PAD. Carbamazepine 40 µM (*n=9*) and 80 µM reduced the duration of PAD by 96.6% and 99.5% of control respectively. Phenytoin 40 µM (*n = 11*) and 80 µM (*n = 8*) reduced the duration of PAD by 95.8% and 99% of control respectively [36].

Three major results can be drawn from experiments with OHSCs. (i) SLEs induced by low Mg^{2+} or 4-AP in OHSCs prepared from 2-12 days old rats and tested up to two months *in vitro* are refractory to standard AEDs (ii) the pharmacoresistance in these models most probably is not caused by the impaired/altered GABA-ergic mechanisms and (iii) PADs induced by HFS are blocked by standard AEDs in OHSCs with same experimental conditions as in pharmacoresistant seizure models. Hence, a single OHSC can express both pharmacosensitive and pharmacoresistant epileptiform activities.

There are previous reports that reorganization of network occurs during incubation time in OHSCs [6,7,16,40], therefore, it was difficult to say that whether pharmacoresistance of SLEs in low Mg^{2+} or 4-AP model is because of the developmental characteristics or reorganization of network that occurs during incubation time in OHSCs. Two possible ways to resolve this question were available. The first was to prepare OHSCs from animals older than 12 days and the second to investigate the pharmacosensitivity of induced SLEs in fresh tissue, i.e. in acute slices of temporal cortex. We and other laboratories as well so far have been unsuccessful to prepare OHSCs from older animals. Therefore, in the next project we tested effects of standard AEDs and other compounds on 4-AP induced SLEs in the acute hippocampal-entorhinal cortex slices prepared from P3 - P19 rats to uncover the role of developmental characteristics in pharmacosensitivity.

3.7. 4-AP induced seizure like activities in acute hippocampal-entorhinal cortex slices

In acute hippocampal-entorhinal cortex slices prepared from postnatal 3 - 19 day old rats (P3 - P19) application of 4-AP induced SLEs characterized by tonic like and clonic like activity in parahippocampal regions such as the medial entorhinal cortex (ECm) and in the hippocampus proper. In most slices tonic-clonic SLEs occurred independently from each other in CA3 and ECm. In a few slices with SLEs synchronized between CA3 and ECm seizure onset was changing between both regions. At postnatal age of 16 - 19 days (P16 - P19), 4-AP induced recurrent short discharges in the CA3 in a subset of slices which were not further analyzed. Unlike in treatments with low Mg^{2+} in the acute slices prepared from immature brain we rarely observed spreading depression when 4-AP was used to induce SLEs. In addition we did not observe any change in seizure pattern after prolonged exposure of 4-AP in immature slices. In P3 - P5 slices induction of SLEs was not always possible in ECm. Severity of SLEs was increased in ECm with age but not in area CA3 [38].

3.8. Effects of AEDs on 4-AP induced seizure like activities in acute hippocampal-entorhinal cortex slices

SLEs in P3 - P10 slices and more specifically P3 - P5 slices were more resistant to standards AEDs as compared to SLEs in P14 - P19 slices (Fig. 1). SLEs in CA3 were more resistant to standard AEDs as compared to SLEs in ECm (Fig. 1). When SLEs in CA3 and ECm were not blocked by AEDs they were significantly modified by the compound. Carbamazepine (*n*=28) and phenytoin (*n*=27) in these cases prolonged SLEs in CA3 in P3 - P10 slices in spite of an almost complete suppression of the tonic period. They decreased the incidences of SLEs and peak in rise of $[K^+]_o$ associated with SLEs in CA3 in both P3 - P10 and P14 - P19 groups. In ECm both drugs reduced the duration and incidence of SLEs, and $[K^+]_o$.

Valproic acid and phenobarbital were tested against 4-AP induced SLEs in a total of 33 and 34 slices respectively. As with carbamazepine and phenytoin, the SLEs in P3 - P10 slices were more resistant to valproic acid and phenobarbital than the SLEs in P14 - P19 slices and the SLEs in CA3 were more resistant than the SLEs in ECm (Fig. 1E-H). SLEs not blocked by valproic acid and phenobarbital displayed the significant modifications. In contrast to carbamazepine and phenytoin tonic like activity was not suppressed by valproic acid and phenobarbital. Valproic acid 2 mM and phenobarbital 200 µM in P3 - P5 slices prolonged duration of SLEs in area CA3 by increasing the duration of both tonic and clonic like events. In P14 - P19 slices SLEs were reduced in duration or remained unchanged [38].

3.9. Bumetanide

As AEDs prolonged SLE duration during the first but not the second and third postnatal week, thus we hypothesized that this might be due to the differential Cl^- transmembrane gradients, thereby affecting the efficacy of inhibition. Depolarizing actions of GABA [4] in the first postnatal week were ascribed to a strong expression of the cation-chloride cotransporter NKCC1. It has been proposed that excitatory GABA contributes to enhanced excitability and ictogenesis in the developing rat hippocampus [34]. We therefore tested the effects of bumetanide, a loop diuretic blocking NKCC1 on 4-AP induced SLEs in 29 slices from rats between 3 and 19 days old. The anticonvulsant effects of bumetanide were region and age dependent. The postnatal age and region dependent action of bumetanide is confirmed in the summary histogram (Fig. 1I). The seizure suppressing effects of bumetanide in CA3 was restricted to P3 - P5 at this age SLEs were completely suppressed in 91% of the slices tested (*n*=11). At latter postnatal times SLE in area CA3 were not affected. In the ECm bumetanide showed blocking effects weaker than CA3. Hence, bumetanide showed the effects opposite to the effects of standard AEDs.

3.10. Acetazolamide

A possible contribution of bicarbonate mediated GABA dependent depolarization to 4-AP induced SLEs was investigated by applying carbonic anhydrase inhibitor azetazolamide (0.5 mM or 2 mM) to 34 slices. Azetazolamide at 0.5 mM failed to suppress SLEs in CA3 at all postnatal ages, whereas, it blocked SLEs in the ECm in 20% and 30% of slices at P3 - P10 (*n*=5) and P14 - P19 (*n*=7) respectively. Analysis of data with 2 mM azetazolamide forced us to break P3 - P10 group into two (P3 - P5 and P6 - P10) for this dose. We found that the proportions of slices in which SLEs were blocked in CA3 were 33% at P3 - P5 (*n*=6), 100% at P6 - P10 (*n*=6) and 80% at P14 - P19 (*n*=10). The respective proportions in the ECm were 83%, 100% and 90%. The slices in which acetazolamide did not block the SLEs it increased the duration but decreased the incidence of SLEs/10min [38]. Hence, the effects of carbonic anhydrase inhibitor acetazolamide in the acute hippocampal-entorhinal cortex slices were similar to the effects of other antiepileptic drugs but different than those of NKCC1 inhibitor bumetanide.

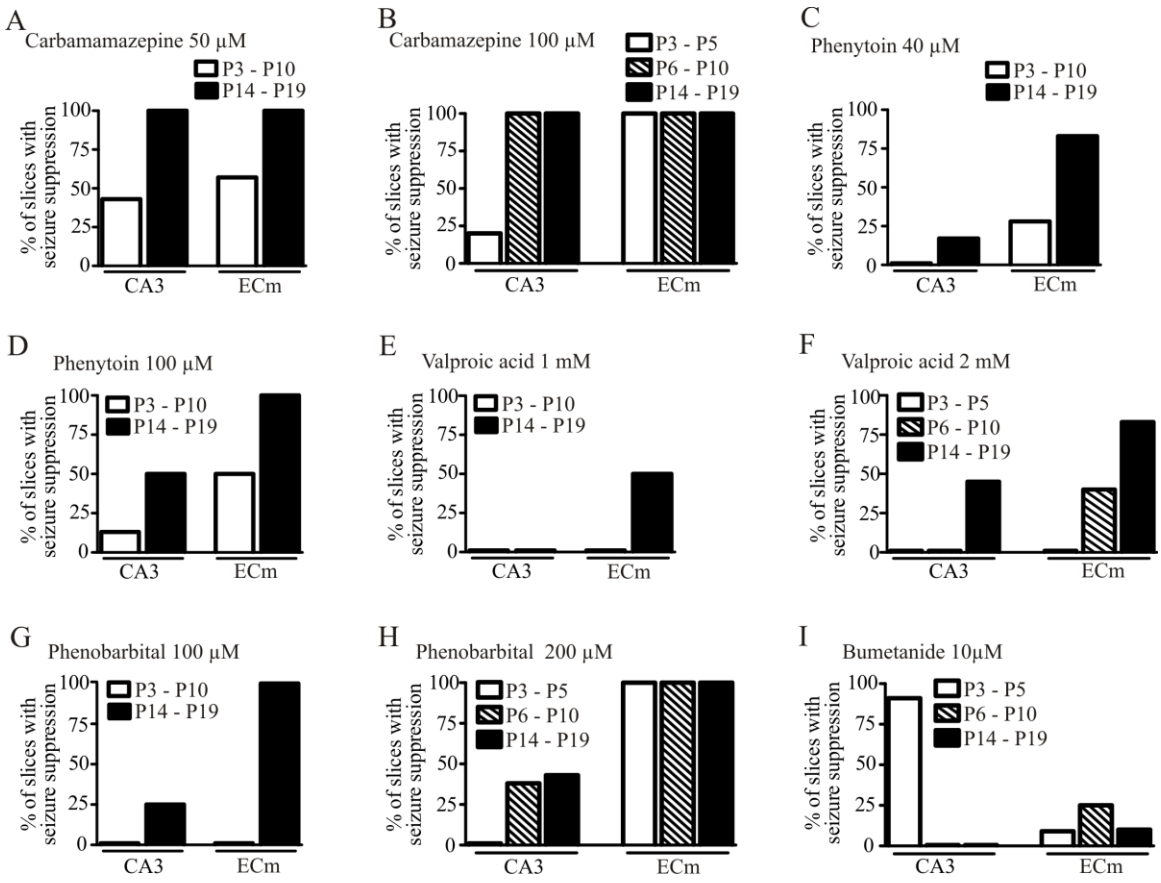

Figure 1. Percentages of slices in which AEDs and bumetanide completely blocked SLEs. As with almost every AED the P3 - P5 slices or P3 - P10 slices were more pharmacoresistant than P14 - P19 slices. In contrast to AEDs, bumetanide blocked the SLEs in CA3 in more than 90% of P3 - P5 slices but failed to block SLEs in CA3 at latter postnatal ages. SLEs in ECm were more sensitive to AEDs but less sensitive to bumetanide than the SLEs in CA3.

4. Discussion

In the present studies, the suitability of OHSCs for developing the models of epileptiform activities was tested. It was found that in OHSCs, SLEs induced by low Mg^{2+} or 4-AP are resistant to the standard AEDs that suggest this preparation may serve as a model of drug refractory epilepsy [1,37]. When the compounds, which enhance the GABA-mediated effects, were tested, they blocked the pharmacoresistant SLEs completely; hence the pharmacoresistant of SLEs in OHSCs cannot be due to the altered and/or impaired GABA-ergic mechanisms. Interestingly, AEDs completely blocked the HFS induced PADs in OHSCs. Thus, a single OHSC provides two different *in vitro* models of epileptiform activities with different pharmacological sensitivity. The age specific effects of AEDs were tested in acute slices prepared from 3 - 19 day old rats and the pharmacoresistance was found strongest in slices prepared from 3 - 5 day old rats. Bumetanide, a NKCC1 blocker, showed the anticonvulsant effect during first postnatal week in the area CA3 and in latter ages it was found ineffective.

Validity of our in vitro model of pharmacoresistant SLEs in OHSCs

It has been shown that SLEs induced either by 4-AP or low Mg^{2+} in OHSCs are pharmacoresistant. 4-AP blocks certain K^+ channels, in particular those from the Kv1 and Kv3 families. Changes in K^+ current properties have been found in human epileptic hippocampal tissue [3,41]. The reasons for seizure induction in the low Mg^{2+} model are the facilitated activation of NMDA receptors, and it is generally agreed that NMDA receptor activation promotes limbic epileptogenesis [23,25]. Decreased Mg^{2+} concentrations are a critical factor in eclampsia where normalization of Mg^{2+} levels blocks seizures and improves outcome [10].

The validity of our *in vitro* pharmacoresistant model in OHSCs is built on tissue properties. In few respect, morphological properties of OHSCs are comparable with properties of human epileptic tissue. Thus, projections from area CA1 to the dentate gyrus and back-projections from area CA1 to CA3 have been described [6,16,40]. Moreover, mossy fibers from granule cells may display axonal sprouting and recurrent connectivity [7,16,40] comparable to that observed in patients with epilepsy [13,14] and in animal models of temporal lobe epilepsy [24]. 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 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 [9]. Secondly, the clinical evidence for de novo pharmacoresistance in many patients with temporal lobe epilepsy 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 an epileptic tissue or the immaturity of one particular neuronal or transmitter system is solely responsible for the pharmacoresistance in OHSCs.

Effects of compounds that enhance GABA mediated action in OHSCs

As a part of my PhD project the effects of compounds that enhance GABA-mediated actions on SLEs in OHSCs were investigated [35]. We found that low Mg^{2+} induced SLEs were dose dependently suppressed by $GABA_A$ receptor agonists muscimol, isoguvacine and alfaxalone and by the GABA uptake inhibitor nipecotic acid, whereas, the $GABA_B$ receptor agonist baclofen attenuated but did not suppress SLE. These findings demonstrate that in OHSCs $GABA_A$ receptors are functional and mediate inhibitory effects on SLEs. The results do not support the hypothesis that in OHSC altered and/or impaired GABA-ergic mechanisms contribute to drug resistant seizure like activity. The failure of benzodiazepines and phenobarbital to block the SLEs at therapeutic doses [1], and the effectiveness of muscimol, isoguvacine, nipecotic acid, and alfaxalone to block the SLEs may be due to their differences in mechanisms to modulate the responses of GABA as they have different binding sites at $GABA_A$ receptor complex [30]. However, the ineffectiveness of benzodiazepines in blocking SLEs in OHSCs cannot be attributed to a lack of receptors. At P10, the $GABA_A$ receptor subunits α1 and α2 in the rat hippocampus mediating the anticonvulsive actions of diazepam [12,19] are present in concentrations only slightly higher (α1) or lower (α2) than in adults, and interneurons in the hippocampus strongly express the α1 subunit [39]. Their ineffectiveness in suppressing SLEs in OHSCs is also not due to the rundown of GABA-mediated functions. Studies have shown that during status epilepticus, GABA-ergic mechanisms fail and seizures become self-sustaining and pharmacoresistant. For example, benzodiazepines are effective early in the course of status epilepticus but significant pharmacoresistance develops within few minutes and benzodiazepines fail to arrest status epilepticus by 45 min [11,22,33]. It may be hypothesized that after prolonged epileptiform activity the releasable pool of GABA is diminished. We found however, that the ineffectiveness of 1,4-benzodiazipines and the other compounds tested in suppressing SLEs in OHSCs was independent on whether or not we applied them before or after inducing SLEs thus rundown of GABA may not be responsible for pharmacoresistance in OHSCs. Our study does not

exclude the possibility that susceptibility to seizure like activity and resistance of SLEs to clinically employed AEDs is caused by immaturity of receptors and channels other than subserving the GABA system.

A pharmacosensitive model based on OHSCs

Phenytoin and carbamazepine completely blocked the HFS induced PADs in OHSCs in contrast to SLEs induced by lowering Mg^{2+} or by applying 4-AP, which demonstrate that the same preparation provides for a pharmacosensitive model when HFS is used [36]. There is one marked difference between local stimulation and whole preparation dependent epileptiform activity induced by convulsants. The latter may be more severe and severity of seizure activity might then account for pharmacoresistance.

Age and region specific effects of AEDs in acute hippocampal-entorhinal cortex slices

We have demonstrated that in acute hippocampal-entorhinal cortex slices shortly after birth 4-AP induced SLEs in CA3 and the medial entorhinal cortex (ECm) showed more resistance to AEDs when they were compared with the SLEs in the 3rd postnatal week. The entorhinal cortex was generally more sensitive to AEDs than the hippocampus. In early age group carbamazepine and phenytoin prolonged the duration of SLEs in CA3 by increasing clonic like activity but they blocked tonic like activity, whereas, valproic acid and phenobarbital prolonged duration of SLEs by increasing both tonic and clonic like activities [38].

We hypothesized that strong pharmacoresistant observed in slices prepared from first postnatal week might be due to the differential Cl^- transmembrane gradients. Depolarizing actions of GABA [4] in the first postnatal week were attributed to a strong expression of the cation-chloride cotransporter NKCC1 which causes accumulation of Cl^- inside the cells setting the reversal potential for inhibitory postsynaptic potentials to depolarized levels with respect to resting membrane potential. Up-regulation of the KCC2, chloride-extruding potassium - chloride cotransporter, and the subsequent enhancement in the efficacy of neuronal Cl^- extrusion is thought to be responsible for converting depolarizing and excitatory GABA responses of immature neurons to classical hyperpolarizing inhibition by the end of the second postnatal week [28]. As a part of my PhD project, a possible contribution of depolarizing GABAergic mechanisms to seizure like activity was analyzed by applying the inhibitor of NKCC1, bumetanide. It was surprising that bumetanide completely blocked SLEs in area CA3 only at P3 - P5. At latter postnatal ages bumetanide failed to suppress SLEs in the area CA3. In the ECm bumetanide showed blocking effects weaker than the area CA3. These findings support other evidence that the switch of $GABA_A$ receptors from being depolarizing early in life to its classical

hyperpolarizing effects latter on, occurs at different time points, in different regions [15]. We here propose that depolarizing GABA contributes in addition to enhanced susceptibility to seizure like activity in the neonatal rat hippocampus [34] to the pharmacoresistance of this activity. Our data indicate that in the area CA3 this contribution is most significant until postnatal day 5, and it is stronger in the area CA3 than in the ECm [38].

The direction of the effects of GABA (depolarizing/excitatory versus hyperpolarizing/inhibitory) is also influenced by the transmembrane gradient for bicarbonate as most GABA operated channels are to some extent also permeable for bicarbonate [28]. Hence, expression of the cytosolic carbonic anhydrase may influence efficacy of GABAergic drugs. A possible contribution of bicarbonate mediated GABA dependent depolarization to 4-AP induced SLEs was investigated by applying azetazolamide. The carbonic anhydrase blocker azetazolamide induced suppressive effects on 4-AP provoked SLEs which were dependent on postnatal age, drug concentration and region in the temporal cortex. The effects of acetazolamide on SLEs were similar to other AEDs but different from those of bumetanide [38]. The effects of azetazolamide can be explained by a blockade of carbonic anhydrase V causing intracellular acidosis and opposing thereby seizure like activity [5,20]. As significant seizure suppressing effects were seen already in P6 - P10 slices [38] a major contribution of a carbonic anhydrase VII blockade to these effects appears unlikely as carbonic anhydrase VII is expressed in CA3 in larger amounts only from P13 on [29].

5. Conclusion

We conclude that a single OHSC can express both pharmacoresistant and pharmacosensitive epileptiform activities. Low Mg^{2+} or 4-AP models based on OHSCs can be used as an *in vitro* model of pharmacoresistant mesial temporal lobe epilepsy. In addition OHSCs could be used to evaluate the effects of chronic application of drugs as they remain viable for several weeks in the incubator and drugs may be applied during prolonged incubation periods. Based on the results in acute hippocampal entorhinal cortex slices we conclude that pharmacoresistance is strongest around first postnatal week in rodents. Expressions of the NKCC1 co-transporter in immature brain contribute to pharmacoresistance during first postnatal week and bumetanide has a potential to be used as an antiepileptic drug in neonates. Thus, our data might explain the severe pharmacoresistance noted frequently in babies particularly when they are born preterm. Pharmacoresistance in OHSCs is at least in part due to the developmental characteristics of immature temporal cortex, however, the effects of reorganization during incubation time on pharmacoresistance and other mechanisms may not be ruled out since we have observed pharmacoresistance in OHSCs stronger than in acute slices.

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Statement of own contribution for submitted publications

- *First publication*

Albus K, **Wahab A**, Heinemann U (2008). Standard antiepileptic drugs fail to block epileptiform activity in rat organotypic hippocampal slice cultures. *British Journal of Pharmacology* 154:709-724.

Contribution: 40%

Detailed contribution: Participation in protocol designing and performing experiments including preparation and maintenance of organotypic hippocampal slice cultures, recordings of field potentials and extracellular potassium changes, data analysis and preparation of manuscript.

- *Second publication*

Wahab A, Heinemann U, Albus K (2009). Effects of gamma-aminobutyric acid (GABA) agonists and a GABA uptake inhibitor on pharmacoresistant seizure like events in organotypic hippocampal slice cultures. *Epilepsy Research* 86:113-123.

Contribution: 70%

Detailed contribution: Protocol designing and performing of experiments including recordings of field potentials and extracellular potassium changes, preparation and maintenance of organotypic hippocampal slice cultures, data analysis and its interpretation, writing of manuscript.

- *Third publication*

Wahab A, Albus K, Heinemann U (2010). Drug refractoriness of epileptiform activity in organotypic hippocampal slice cultures depends on the mode of provocation. *Epilepsy Res* 90(3):304-308.

Contribution: 45%

Participation in performing experiments including preparation and maintenance of organotypic hippocampal slice cultures, recordings of field potentials and extracellular potassium changes, and preparation of manuscript.

- *Fourth publication*

Wahab A, Albus K, Heinemann U (2010). Age and region specific effects of anticonvulsants and bumetanide on 4-aminopyridine induced seizure like events in hippocampal-entorhinal cortex slices. *Epilepsia* (in press).

Contribution: 80%

Detailed contribution: Protocol designing and performing of experiments including recordings of field potentials and extracellular potassium changes, preparation of acute hippocampal-entorhinal cortex slices, data analysis and its interpretation, writing of manuscript.

- *Fifth publication*

Wahab A, Albus K, Gabriel S, Heinemann U (2010). In search for models of pharmacoresistant epilepsy. *Epilepsia* 51 Suppl 3:154-159.

Contribution: 30%

Detailed contribution: Participation in writing this review. Processing the peer review.

Prof. Dr. Uwe Heinemann Abdul Wahab

Curriculum vitae

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

Publications oral and poster presentations

List of own publications which are included in the thesis with impact factors

(i). Albus K, **Wahab A**, Heinemann U. Standard antiepileptic drugs fail to block epileptiform activity in rat organotypic hippocampal slice cultures. *British Journal of Pharmacology* 2008; 154:709-724.

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(ii). **Wahab A,** Heinemann U, Albus K. Effects of gamma-aminobutyric acid (GABA) agonists and a GABA uptake inhibitor on pharmacoresistant seizure like events in organotypic hippocampal slice cultures. *Epilepsy Research* 2009; 86:113-123.

(2008 Impact factor: 2.4)

(iii). **Wahab A,** Albus K, Heinemann U. Drug refractoriness of epileptiform activity in organotypic hippocampal slice cultures depends on the mode of provocation. *Epilepsy Res* 2010; 90:304-308.

(2008 Impact factor: 2.4)

(iv). **Wahab A,** Albus K, Heinemann U. Age and region specific effects of anticonvulsants and bumetanide on 4-aminopyridine induced seizure like events in hippocampal-entorhinal cortex slices. *Epilepsia* 2010; (in press).

(2008 Impact factor: 3.73)

Additional Publications

(i). Wahab A, Albus K, Gabriel S, Heinemann U. In search for models of pharmacoresistant epilepsy. A review. *Epilepsia* 2010; 51 Suppl 3:154-159.

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(ii). Wahab A, Haq RU, Ahmed A, Khan RA, Raza M. Anticonvulsant activities of nutmeg oil of *Myristica fragrans*. *Phytotherapy Research* 2009; 23(2):153-158.

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(iii). Wahab A, Ahmed E, Nawaz SA, Sharif A, Haq RU, Malik A, Choudhary MI, Raza M. A pharmacological and toxicological evaluation of *Haloxylon recurvum*. *Natural Product Research* 2008; 22(15):1317-1326.

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Oral presentations

(i). **Wahab A,** Albus K, Heinemann U. Selective drug resistance in immature rat temporal cortex structures. *Fourth Annual Epicure Meeting*. In Marseille, France, January 29-30, 2010.

(ii). **Wahab A**, Albus K, Heinemann U. Pharmacoresistance in immature rat temporal cortex in INMED-INSERM UniteÂ 29, 163, route de Luminy, Marseille, France, 31 March 2009.

(iii). **Wahab A**, Albus K, Heinemann U. Investigation into mechanism of pharmacoresistance of seizure like events in organotypic hippocampal slice cultures. "*Berlin Brain Days*". in Charité-Universitätsmedizin, Berlin, Germany, 29th to 30th November 2007.

(iv). **Wahab A**, Albus K, Heinemann U. Effects of GABA agonists, GABA uptake inhibitor and glycine agonist on pharmacoresistant seizure like events in hippocampal slice cultures. "*18th European Student Conference*", in Charité-Universitätsmedizin Berlin, Germany, October 7-11, 2007.

(v). **Wahab A**, Ahmed A, Khan RA, Raza M. Anticonvulsant Efficacy and Safety Profiles of Some Medicinal Plants *"3rd Biennial Conference on Pharmacology & Therapeutics",* January 14-17, 2005, Karachi, Pakistan.

Poster presentations

(i). **Wahab A**, Albus K, Heinemann U. Selective drug resistance in immature rat temporal cortex. *"8th Göttingen Meeting of the German Neuroscience Society 2009"*. March 25-29, 2009, Göttingen, Germany.

(ii). **Wahab A,** Albus K, Heinemann U. Selective drug resistance in immature hippocampal-entorhinal cortex slices. *"Berlin Brain Days"*, organized by Charité – Universitätsmedizin, Berlin, Germany, 10th to 12th December 2008.

(iii). **Wahab A**, Albus K, Heinemann U. Effects of GABA agonists and a GABA uptake inhibitor on pharmacoresistant epileptiform activity in hippocampal slice cultures. *"The 37th Annual Meeting of the Society for Neuroscience"*, Nov. 3-7, 2007, San Diego, California, USA.

(iv). Albus K, **Wahab A**, Heinemann U. Standard antiepileptic drugs fail to block epileptiform activity induced by low magnesium or 4-aminopyridine in rat hippocampal slice cultures. "7th Göttingen Meeting of the German Neuroscience Society 2007". March 29-April 01, 2007, Göttingen, Germany

(v). Albus K, **Wahab A**, Heinemann U. In vitro alternatives to animal models for screening and evaluation of anticonvulsive compounds. *"14th Congress on Alternatives to Animal Testing - Linz 2007"*. September 28th – 30th 2007. University of Linz, Austria.

(vi). **Wahab A**, Ahmed A, Khan RA, Raza M. Anticonvulsant efficacy and safety profile of nutmeg oil of Myristica fragrans. *"Proceeding of the 58th Annual Meeting of the American Epilepsy Society",* December 3-8, 2004, New Orleans, USA. Epilepsia 2004, 45 (Supl. 7): Abstract p. 217.

(vii). **Wahab A**, Raza M. Fifty years of anticonvulsant medicinal plants research. A review. *"7th Eurasia Conference on Chemical Sciences"* March 09-12, 2002, Karachi, Pakistan. Abstract p. 175.

Erklärung

Ich, Abdul Wahab, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

A new model of pharmacoresistant seizure like events and age specific effects of antiepileptic drugs

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

Berlin, den 19 January 2010 Abdul Wahab

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