Repetitive Spreading Depression-Like Events Result in Cell Damage in Juvenile Hippocampal Slice Cultures Maintained in Normoxia

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Abstract

Prolonged seizures, e.g. induced by fever, experienced early in life are considered a precipitating injury for the subsequent development of temporal lobe epilepsy. During *in vitro* epileptiform activity spreading depressions (SDs) have often been observed. However, their contribution to changes in the properties of juvenile neuronal tissue is unknown. We therefore used the juvenile hippocampal slice culture preparation (JHSC) maintained in normoxia (20% O₂, 5% CO₂, 75% N₂) to assess the effect of repetitive SD-like events (SDLEs) on fast field potentials and cell damage. Repetitive SDLEs in the CA1 region could be induced in about two thirds of the investigated JHSCs (n = 61) by repetitive electrical stimulation with 2 to 200 pulses. SDLEs were characterized by a transient large negative field potential shift accompanied by intracellular depolarization, ionic redistribution, slow propagation (assessed by intrinsic optical signals) and glutamate receptor antagonist sensitivity. The term ‘SDLE’ was used because evoked fast field potentials were only incompletely suppressed and superimposed discharges occurred. With 20 ± 1 repetitive SDLEs (interval of 10-15 min, n = 7 JHSCs) the events got longer, their amplitude of the 1st peak declined, while threshold for induction became reduced. Evoked fast field potentials deteriorated and cell damage (assessed by propidium iodide fluorescence) occurred, predominantly in regions CA1 and CA3. As revealed by measurements of tissue pO₂ during SDLEs repetitive transient anoxia accompanying SDLE might be critical for the observed cell damage. These results, limited so far to the slice culture preparation, suggest SDs to be harmful events in juvenile neuronal tissue in contrast to what is known about their effect on adult neuronal tissue.
Introduction

Spreading depression (SD) is characterized by i) a transient large negative field potential shift accompanied by intracellular depolarization of neurons and glial cells and redistribution of ions, ii) a suppression of evoked and spontaneous neuronal activity and iii) a slow wave-like propagation (Nicholson and Kraig 1975; Kruger et al. 1996; Martins-Ferreira et al. 2000; Somjen 2001). It was first described as a propagating silencing of neuronal activity measured by cortical electrography in anaesthetized rabbits (Leão 1944). Since then, it has been observed in almost all the gray matter of different species, preparations and ages using several triggers, e.g. high potassium, repetitive electrical stimulation or hypoxia (Somjen 2001). With regard to the partly confusing terminology we follow the suggestion made by Somjen (2001) who distinguishes between normoxic SD and hypoxic SD-like depolarization. In addition, we use the term ‘SD-like event (SDLE)’ employed by Kovács et al. (1999) to describe events reminiscent of SDs but containing superimposed discharges, recorded during Mg\(^{2+}\)-free perfusion of juvenile hippocampal slice cultures (JHSCs) maintained in 95% oxygen tension.

From a clinical perspective, SD is worth elucidating because it is considered to play a pathophysiological role in several diseases, e.g. epilepsy, migraine, head trauma, subarachnoid hemorrhage and stroke (Gorji 2001; Petzold et al. 2003). The understanding of its effect on functional and structural properties of neuronal tissue, particularly with respect to pathological sequelae, is crucial in order to estimate the benefit of a potential therapy aimed at prevention of SD. In terms of neuronal survival, SDs per se do not lead to cell damage in healthy adult neuronal tissue (Nedergaard and Hansen 1988). In juvenile tissue, repetitive SDs potentially affect cellular and synaptic properties (Hablitz and Heinemann 1989; Kunkler and Kraig 1998) and were observed during epileptiform activity (Kreisman and Smith 1993; Psarropoulou and Avoli, 1993; Gloveli et al. 1995). Prolonged epileptiform activity has been shown to result in cell damage and other long-term effects dependent on age and epilepsy model used (Heinemann et al. 2002; Cilio et al. 2003; Dube et al. 2004). The effects of
prolonged epileptiform activity, e.g. prolonged febrile seizures, are intensively studied in the juvenile hippocampus because alterations there are considered a prerequisite for the subsequent development of mesial temporal lobe epilepsy (French et al. 1993). While repetitive SDs occur during epileptiform activity their contribution to alterations in juvenile neuronal tissue is unknown.

In order to approach this issue we present a reliable method to evoke SDLEs in normoxic juvenile hippocampal slice cultures (JHSCs) by repetitive electrical stimulation. We chose JHSCs for several reasons. In contrast to the juvenile intact brain the problem of access to the hippocampus is circumvented and SDLEs are reliably evocable. High-resolution techniques, e.g. fluorescence imaging, can be applied in slices, which is of advantage for elucidating mechanisms of cell damage (Kovács et al. 2002). In contrast to acute slices the culture preparation does not interfere with acute changes following the preparation itself, e.g. traumatic cell damage. The culture preparation offers opportunities for preincubation experiments in order to evaluate neuroprotective strategies (Pringle et al. 1997) and it allows the study of long-term effects by repeated measurements (Gutierrez et al. 1999a). The main disadvantage is the culture process itself, which could change the properties of the tissue (Gutierrez et al. 1999b), potentially also increasing its vulnerability.

We used that method to evoke repetitive SDLEs and focused on the alterations of electrophysiological characteristics of SDLE and evoked fast field potentials. Additionally, we measured tissue pO₂ during SDLE and assessed cell damage by the fluorescent dye propidium iodide (PI). PI is impermeable to cells with an intact plasma membrane, but if the plasma membrane is damaged it intercalates with DNA to form a fluorescent complex. PI fluorescence is well correlated with conventional markers of cell death (Vornov et al. 1991; Newell et al. 1995).
Materials and Methods

Slice culture preparation

All procedures were carried out in accordance with the recommendations of the Berlin Animal Ethics Committee. Combined entorhinal cortex-hippocampal slice cultures were prepared from postnatal 6-9 days old Wistar rats and cultured for 8-13 days in vitro corresponding immature rats of postnatal age 15-20 following Stoppini’s method (Stoppini et al. 1991) as described previously (Pomper et al. 2001). Briefly, after decapitation, the hippocampi with the entorhinal cortex attached were dissected and 400 µm slices were cut under sterile conditions in gassed (95% O₂ / 5% CO₂) ice-cold minimal essential medium (MEM; Gibco, Eggenstein, Germany) at pH 7.35. Two slices were seated on each culture membrane (0.4 µm Millicell culture plate inserts; Millipore, Eschborn, Germany). The membranes were placed in 6-chamber incubation wells (Falcon, New Jersey, USA) and incubated in a medium containing 50% MEM, 25% Hank’s balanced salt solution (HBSS; Sigma, Deisenhofen, Germany), 25% horse serum (Gibco), 2 mM L-glutamine, 10000 U/ml penicillin and 10000 µg/ml streptomycin, pH 7.4 in an incubator (Hereaus; Hanau, Germany) at 36.5°C containing room air enriched with 5% CO₂. The culture medium was completely replaced the first two days and thereafter twice a week.

Electrophysiology

One Millicell culture plate carrying two JHSCs was transferred to an interface recording chamber and perfused at 1.6 ml/min with prewarmed (34°C), gassed (20% O₂, 5% CO₂, 75% N₂) artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl 129; KCl 3; NaHPO₄ 1.25; MgSO₄ 1.8; CaCl₂ 1.6; NaHCO₃ 21; glucose 10; pH 7.4. In the first set of experiments, 61 JHSCs were studied electrophysiologically inclusive the application of repetitive electrical
stimulation with 2-200 pulses of maximal intensity aimed at SDLE induction. In 7 out of 39 SDLE-positive JHSCs, each positioned on a separate culture plate, repetitive SDLEs were evoked every 10-15 min with the stimulus intensity and pulse number required to induce an SDLE in a given culture in order to study their effect on field potentials, ion exchange and cell damage. The remaining JHSC on each culture plate served as control (non-SDLE control group).

After an equilibration period of 15 min a bipolar platinum wire stimulating electrode was placed in alveus/stratum oriens of CA1 region to stimulate antidromically outgoing axons of pyramidal cells. Both layers could not be distinguished with certainty by the microscope used in the electrophysiological set up. Thus we refer to both regions as antidromic stimulation site (see Fig. 4A). In some slice cultures an additional stimulating electrode was placed at the hilar border of the CA3 region for orthodromic activation. Extracellular ion concentrations (K⁺, Ca²⁺) and field potentials related to ground (chamber fixed pellets washed by bath solution) were recorded simultaneously using double-barreled ion-sensitive/reference electrodes manufactured and calibrated as described elsewhere (Heinemann and Arens 1992). The recording electrode was positioned in the pyramidal layer of CA1 (see Fig. 4A). JHSC, which presented with stimulus-evoked response amplitudes lower than 0.5 mV, initial occurrence of epileptiform activity or morphological imperfectness were not investigated. Evoked field potentials and spontaneous activity were 3 kHz low-pass filtered and amplified. For intracellular recordings made from CA1 pyramidal cells, glass microelectrodes (70-100 MΩ) were pulled with a Brown Flaming horizontal puller (Sutter Instruments) and filled with 2 M potassium acetate. Recordings related to ground potential were performed with a SEC-05 LX npi amplifier (npi electronic GMBH, Tamm, Germany) operating in bridge mode. Extracellular and intracellular data were acquired, stored and analyzed using an ITC-16 interface (Data acquisition Interface, InstruTECH, USA), TIDA software (TIDA 4.1, Heka Elektronik, Lamprecht, Germany) and Signal software (Version 2.15, Cambridge Electronic
Design, Cambridge, UK). The population spikes of evoked fast field potentials were analyzed with respect to amplitude and latency. The population postsynaptic potential was determined by the area under the curve, thereby calculating the sum of the area under the curve of both, the early positive and late negative part of the postsynaptic field potential. SDLEs were electrophysiologically analyzed with respect to amplitudes of 1st and 2nd peaks and latency to 1st peak of the large negative field potential shift, related to baseline before stimulation, and the half maximal duration.

The role of glutamate receptors was studied by bath application of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV, 60 µmol/l) and the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 60 µmol/l). Both drugs were dissolved in ACSF.

**Measurement of partial oxygen pressure**

Partial oxygen pressure (pO2) on the surface and in different depths of JHSC maintained in the interface chamber was measured using an oxygen sensitive electrode (Clark Style Microelectrode, # 737GC, Diamond General Dev. Corp., Ann Arbor, USA). The maximal measured depth had to be restricted to 100 µm because deeper measurements would have increased the risk of electrode damage by the culture plate carrying the slice culture. JHSCs were about 150 µm thick. In the second set of experiments, pO2 was measured continuously during electrical stimulation trains with and without subsequent SDLEs. The oxygen sensitive electrode was positioned close to the ion-sensitive/reference electrode in the pyramidal layer of CA1. Field potential (1 kHz low-pass filtered, 100x amplified), extracellular potassium concentration (1.6 Hz low-pass filtered, 50x amplified) and pO2 (1.6 Hz low-pass filtered, 10x amplified) were simultaneously recorded and analyzed using Spike2 software (Cambridge Electronic Design, Cambridge, UK).
**Imaging intrinsic optical signals**

The imaging of intrinsic optical signals was performed employing an in-house software (Dr. H. Siegmund, Johannes-Müller-Institut für Physiologie, Charité Universitätsmedizin Berlin) as described previously (Weissinger et al. 2000, Petzold et al. 2005). In brief, JHSCs were transilluminated from below using a voltage-regulated halogen lamp and viewed from above with an upright binocular microscope (MZ6; Leica, Bensheim, Germany). Video frames were acquired with a CCD camera (VC-1910; Sanyo, Osaka, Japan) and digitized using a frame grabber board (pciGrabber 4plus; Phytec, Mainz, Germany). The first image in a series, captured before electrographic onset of SDLE, served as control (T0) and was subtracted from each subsequent image of the series (Tx). This series of subtracted images revealed changes in light transmittance (LT) over time:

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LT = \frac{T_x - T_0}{T_0} \times 100 = \frac{\Delta T}{T_0} \%
\]

LT changes were displayed using grayscale intensity scales. Regions of interest were selected over specific slice culture areas to quantify and compare LT changes in these areas. In order to analyze propagation of a single SDLE in time and space subsequently captured images were compared. Corresponding pixels in consecutive images exceeding a previously chosen threshold of light intensity change clearly above the optical noise were marked. By this approach a circumscribed area of intrinsic optical signal associated with SDLE could be displayed. Changes in this area exhibited specific spread patterns from one image to another such as region of onset, direction and extent of propagation and the velocity of spread. The latter could be determined by analysis of the temporal interval between the images.
**Measurement of cell damage**

For cell damage measurement in JHSCs which experienced repetitive SDLEs, the culture plate carrying both JHSCs, the one exposed to and the other one not exposed to repetitive SDLEs (SDLE and non-SDLE group), was transferred to the incubator after electrophysiological recordings and stained with 10 µmolar Propidium Iodide (PI, Molecular Probes, Leiden, Netherlands) in culture medium for 30 min and then washed with fresh culture medium for 60 min. In parallel, another culture plate carrying 2 JHSCs was taken out of the incubator and stained accordingly (incubator control group). After staining, a patch of culture plate carrying one JHSC was excised and mounted in a chamber on an inverted Olympus IX 50 microscope (Olympus Optical Co. GmbH, Hamburg, Germany) equipped with a water immersion objective 40x Uapo/340 (Olympus). The fluorescence imaging system consisted of a monochromator, a 16-bit CCD camera and the Windows NT-based image processing software (Till Photonics, Munich, Germany). PI was excited at 550 nm and fluorescence pictures were obtained at 605 nm. 640 x 480 pixel pictures were taken from three regions: Dentate gyrus (DG), CA3 and CA1. The following 7 areas were analyzed separately: (1) molecular layer (ml), (2) granule cell layer (gcl) and (3) hilus (hil) of DG; (4+5) stratum pyramidale (sp) and (6+7) stratum lacunosum-moleculare together with stratum radiatum (slm-sr) of CA3 and CA1 (Fig. 4A). The fluorescence signal was expressed in arbitrary units on a gray scale and the mean fluorescence signal of each area was determined using v1.29 ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). As previously applied (Pomper et al. 2004), for statistical evaluation imaging results were presented as ratio between the means of corresponding areas of both, JHSCs maintained in the interface recording chamber (SDLE and non-SDLE group) and the matched JHSCs belonging to the incubator control group.
**Statistics**

Values are expressed as mean ± standard error of the mean if not otherwise noted. Correlations were determined by the Pearson’s correlation coefficient (r). For comparison of cell damage between SDLE and non-SDLE group the non-parametric Mann-Whitney-U-Test for unpaired data was used. A value of p < 0.05 was regarded as significant.

**Results**

*Without electrical stimulation, JHSCs were normoxic in the interface recording chamber using 20% oxygen tension*

JHSCs were gassed with a 20% oxygen containing gas mixture and perfused with 20% oxygen gassed ACSF during electrophysiological recordings in the interface chamber. We previously reported this oxygen tension as the optimal one to prevent hyperoxic cell damage in JHSCs (Pomper et al. 2001). For comparison with the *in vivo* situation we measured pO₂ with Clark type oxygen sensing electrodes. At the surface of JHSCs pO₂ was 120 mmHg and it declined to 67 mmHg at a depth of 100 µm. The effect of 25-µm-steps on pO₂ decline attenuated with increasing depths. This indicates that the nadir of pO₂ was nearly reached at 100 µm (Fig. 1). The measured values of pO₂ correspond to *in vivo* data obtained under normoxic conditions in the rat brain (4-80 mmHg, see Vovenko 1999) and lie above those measured in patients during the recovery period following head injury (van den Brink et al. 2000). Thus, without electrical stimulation a hypoxic situation, which is known to induce SD-like depolarization, can be ruled out.
SDLEs induced by electrical stimulation

For electrical stimulation site we chose the outgoing axons of CA1 pyramidal cells located in alveus/stratum oriens. Electrical stimulation of alveus/stratum oriens evoked a field potential in the pyramidal layer of CA1 region composed of a population spike preceding a population postsynaptic potential (Fig. 2). Occasionally, one or two additional population spikes were superimposed on the postsynaptic potential. The mean maximal amplitude and latency of the first population spike before any treatment or occurrence of SDLE were 9.7 mV (± 5.5 mV S.D.) and 2.4 ms (± 0.7 ms S.D.) in 61 JHSC, respectively. As shown in Fig. 2, blockade of glutamatergic transmission via non-NMDA receptors by CNQX (60 µmolar) abolished the postsynaptic potential almost completely, while the population spike was preserved. The NMDA-receptor antagonist APV (60 µmolar) had no significant effect, neither on population spike nor on postsynaptic potential. The facts that the first population spike preceded the postsynaptic potential and that it was preserved while glutamatergic transmission was blocked indicates the first population spike as antidromically, i.e. non-synaptically evoked. The fact that the postsynaptic potential was abolished by CNQX but preserved by APV indicates it as a non-NMDA-receptor dependent component of evoked fast field potential. Thus, a stimulation of neurons that release glutamate occurred during alveus/stratum oriens stimulation.

While using the alveus/stratum oriens stimulation an input-output measurement was performed to determine the stimulation intensities necessary to evoke an antidromic population spike at threshold, 25, 50, 75 and 100% of maximal amplitude. Subsequently, stimulation trains (2, 3, 5, 10, 50, 200 pulses) of 20 Hz with the maximal stimulation intensity were applied to evoke SDLE. Following this protocol, SDLEs in CA1 were electrophysiologically observed in 64% of JHSCs (n = 61, Fig. 3A). The amplitude of the population spike could have been one parameter predicting the occurrence of SDLE. However, this amplitude whether evoked anti- or orthodromically did not significantly differ
between SDLE-positive and SDLE-negative JHSCs. The number of stimuli required for inducing SDLEs showed peaks at 2 and 200 pulses (n = 11 and n = 13 of 39 JHSC, respectively). The stimulation intensity threshold was evaluated in 19 out of 39 SDLE-positive JHSC using stimulus trains successful for induction of SDLE and using the stimulation intensities according to the input-output measurement. The stimulation intensity thresholds were almost equally distributed among 50% (n = 7), 75% (n = 4) and 100% (n = 8) of maximal stimulation intensity. A non-systematic comparison of the effect of the frequencies 10, 20 and 50 Hz indicated a best stimulation frequency at 20 Hz. Thus we chose this frequency for further experiments. SDLEs could repeatedly be evoked in JHSCs provided an interval of 10 to 15 min was left between the stimulus trains. Application of stimulus trains to the hilus could also elicit SDLEs, but these were not systematically studied. Occasionally, seizure-like events were elicited by stimulus trains below the threshold for SDLE induction. They were characterized by smaller field potentials shifts and superimposed discharges with an early tonic-like and a late clonic-like appearance.

**Electrophysiological characterization of SDLEs**

The characteristics of SDLEs described below refer, if not stated otherwise, to 39 SDLEs (one of the first three induced SDLEs per SDLE-positive JHSC). The large negative field potential shift typical of SD consisted of an early negative 1st peak followed either by a 2nd peak divided by a brief decline (69%, Fig. 3A) or by a prolonged plateau before recovery to baseline (31%). The amplitude of the 1st peak was -31.1 ± 1.0 mV and the latency to 1st peak was 6.1 ± 0.7 s. Half maximal duration of SDLEs was 21.9 ± 1.6 s. The negative field potential was accompanied by intracellular depolarization and typical ion redistribution (Fig. 3). The extracellular potassium concentration rose from 3 mM to 49.3 ± 4.9 mM with a
latency to peak of 11.0 ± 0.8 s (n = 24) and the extracellular calcium concentration fell from 1.2 mM to 0.3 ± 0.1 mM with a latency to peak of 20.9 ± 3.1 s (n = 8).

So far, the electrophysiological properties of SDLEs in JHSCs matched those of SDs very well. However, our observations were less typical concerning another critical feature of SDs, namely the complete suppression of spontaneous activity and evoked field potentials. Thus we prefer the term ‘Spreading depression-like event’ instead of ‘Spreading depression’. In terms of spontaneous activity, the negative field potential shift was superimposed by discharges resembling epileptiform activity in 82% of 39 JHSCs. Two types of discharges could be distinguished, i) tonic-like high frequency primary discharges with short latency and duration and the endpoint around the 1st negative peak and ii) clonic-like low frequency secondary discharges with long latency and duration, typically superimposed around the second peak or plateau and persisting until recovery back to baseline. Both discharges occurred alone (tonic-like: 25%, clonic-like: 22%) or together with or without a silent period in between (25% and 28%, see the 1st SDLE in Fig. 5A for an example of continuous spontaneous activity during SDLE). Occasionally secondary discharges persisted for 1-2 min following an SDLE (Fig. 3A). During SDLEs neurons depolarize up to a membrane potential of -40 mV. In terms of evoked field potentials, alveus/stratum oriens or hilar evoked field potentials applied at various times after the induction of SDLEs were only incompletely suppressed. As shown in Fig. 3C for alveus/stratum oriens stimulation population spike was blocked during the plateau phase of an SDLE while the postsynaptic potential was only strongly reduced.
The propagation velocity of SDLE equaled that of SDs as revealed by Intrinsic Optical Signals

As some of the electrophysiological properties of SDLEs did not correspond to those of SDs we were interested to see whether the events were localized or would spread from the site of stimulation to other parts of the hippocampus. To this end we performed measurements of intrinsic optical signals during SDLEs in 7 JHSCs. Typical recordings are shown in Fig. 4. SDLEs spread always from an initiation site within the pyramidal layer of CA1 region in both directions, towards the subiculum and towards the CA3 region. CA3 was invaded in 43% and the subiculum in 29% of tested JHSCs. Spread into the hilus or DG was never noted. Starting from the pyramidal layer SDLEs invaded always the stratum radiatum and mostly also the stratum oriens of the CA1 region. SDLEs were optically characterized by a decreased light transmittance of maximal 0.65 ± 0.05% which lasted 43 ± 2 s. The mean propagation velocity was 3.26 ± 0.14 mm/min. This spread velocity is very similar to that recently measured for SDs in the CA1 region of acute slices from adult rats (4.1 mm/min) and falls into the range of propagation velocities observed for SDs in other regions, species or preparations (1.7 – 9.7 mm/min, see Buchheim et al. 2002, Petzold et al. 2005).

NMDA and non-NMDA glutamate receptor antagonists influence SDLE

Finally, we evaluated the sensitivity of SDLEs to glutamate receptor antagonists since this sensitivity is another feature of SDs. APV (60 µM) reversibly blocked induction of SDLEs in 9 out of 14 JHSCs even when stimulus intensity was increased to supramaximal intensities and when stimulus trains of 10 s (20 Hz) were applied. In the remaining JHSCs the threshold for induction of SDLE was increased. The latency to the 1st peak of the negative field potential shift was increased whereas the half maximal duration was decreased. Likewise, CNQX (60 µM) blocked induction of SDLEs in 6 out of 11 JHSCs over the full range of train
durations and stimulus intensity variations. In the remaining JHSCs an increase in threshold intensity for induction of SDLEs was noted.

**Changes in SDLE properties with repetitive induction**

In order to answer the question whether repetitive SDLEs result in neuronal dysfunction in JHSCs we firstly focused on changes in the properties of SDLE during repetitive induction. Therefore SDLEs were evoked every 10-15 min with the stimulus intensity and pulse number required to induce an SDLE in a given culture. Interval, stimulus intensity, and pulse number were determined at the beginning of an experiment and then kept constant. The maximal chamber time was limited to 6 hours. In total, 22 ± 1 SDLEs (inclusive 3 spontaneous SDLEs in 2 out of 7 JHSCs) were observed in this time period. SDLEs were analyzed with respect to their electrophysiological characteristics in relation to the running number of SDLEs, inclusive spontaneous SDLEs, starting to count with the first SDLE evoked at threshold. The following results refer to 20 ± 1 SDLEs (n = 7 JHSCs) evoked at threshold or occurring spontaneously.

The overall duration of SDLE increased (Fig. 5A). The half maximal duration of negative field potential shift increased by 95.8 ± 24.6 % with respect to an initial value of 16.6 ± 2.2 s. The half maximal duration was significantly correlated to the number of SDLEs (r = 0.4, p < 0.001, Fig. 5C). The amplitude of the 1st peak of negative field potential shift decreased by 17.9 ± 5.1 % with respect to an initial value of -34.8 ± 1.7 mV. The continuous decrement of the amplitude was also significantly correlated to the number of SDLEs (r = 0.4, p < 0.001, Fig. 5). In contrast, the amplitude of the 2nd peak (-33.1 ± 1.4 mV, n = 6) did not change. Repetitive SDLEs facilitated the induction of SDLE as indicated by a decreased pulse threshold of SDLE induction in all 3 JHSCs tested after having experienced on average 18 SDLEs.
Potassium and calcium redistributions associated with SDLEs were unaltered with respect to amplitude whereas the duration increased continuously in parallel to that of the negative field potential.

Repetitive SDLEs lead to deterioration of evoked field potentials

We then assessed neuronal dysfunction by analyzing the first alveus/stratum oriens-evoked field potential per SDLE with respect to first population spike amplitude, postsynaptic potential and number of additional population spikes over the time course of the experiment. In control experiments the amplitude of the evoked responses was largely unaltered (Pomper et al., 2001). In contrast, repetitive SDLEs deteriorated the population spike amplitude (Fig. 5B) as indicated by a significant positive correlation between the decrement of the population spike amplitude and the number of SDLEs (r = 0.3, p < 0.001). The population spike amplitude evoked before the 20th ± 1 SDLE declined by 34.9 ± 14.2 % with respect to an initial value of 10.8 ± 1.7 mV (n = 7, Fig. 5D). Synaptic transmission was reduced as revealed by a negative correlation between the area under the curve of the postsynaptic potential and the number of SDLEs (r = -0.2, p < 0.05). The population spikes of field potentials evoked by hilar-CA3-stimulation declined to a similar extent as those after alveus/stratum oriens stimulation and were strongly correlated to them (r = 0.9, p < 0.001). The number of additional population spikes increased with the number of SDLE (r = 0.4, p < 0.001) suggesting a loss of inhibitory control.

Repetitive SDLEs result in cell damage

In order to assess cell damage as potential cause of electrophysiological observed neuronal dysfunction, JHSCs which underwent repetitive SDLEs (SDLE group) were compared with
respect to their PI fluorescence intensity to those maintained in the interface chamber without SDLE (non-SDLE group) for the same time period of $316 \pm 9$ min total chamber time. As depicted in Fig. 6, PI staining was significantly increased in CA1 and CA3 region of JHSC belonging to the SDLE group compared to the non-SDLE group. No differences were observed among the areas sp and sr-slm of both regions. There was only a small albeit significant increase in PI staining in the granule cell layer of dentate gyrus.

**SDLE is accompanied by transient anoxia**

In view of hypoxia as a potential mechanism of cell damage due to repetitive SDLEs we recorded $pO_2$ continuously during SDLE. Surprisingly, we realized that in a depth of $50 \, \mu m$ the tissue became anoxic (Fig. 7A), defined by a $pO_2$ value of less than or equal to 5 mmHg. Anoxia lasted $18 \pm 1.4$ seconds in 13 out of 15 SDLEs in 4 JHSCs investigated. In 100 $\mu m$, the largest depth measured, the duration of anoxia was almost doubled to $30.7 \pm 9.5$ s (3 SDLEs in 3 JHSC). The dependency of $pO_2$ alterations during SDLE on depth is exemplified in Fig. 7A. No changes occurred in the gas phase directly above JHSC ($pO_2 = 140$ mmHg) indicating constant oxygen supply at the gas-liquid interface. At $50 \, \mu m$, the minimal $pO_2$ of $2.7 \pm 0.8$ mmHg ($n = 15$ SDLEs) was on average reached after $16.7 \pm 0.4$ s. The half maximal duration was $54.9 \pm 1.9$ s.

Interestingly, there was no correlation between the latency to 1st peak of the large negative field potential shift and the latency to minimal $pO_2$. However, we found a strong negative correlation between the latency to the half maximal negative field potential shift and the $pO_2$ at this time, i.e. the later the field potential shift was detected by the recording electrode the lower was the $pO_2$ value there (Fig. 7B). This correlation is not attributable to stimulation induced neuronal activation because there was no correlation between pulse number and $pO_2$ at the time of half maximal negative field potential shift. Rather, it appeared that a spread of
hypoxia preceded the spread of the negative field potential shift possibly due to propagated synaptic activity or by diffusion of oxygen towards the initiation site.

**Electrical stimulation decreases pO₂ considerably**

The large decline of pO₂ during SDLE raises the question to what extent the electrical stimulation by itself causes changes of pO₂. As shown in Fig. 7C, stimulation trains without subsequent SDLEs led to considerable decline of pO₂ reaching values below 20 mmHg at 50 µm when 200 pulses were applied. The maximal decrement of pO₂ correlated very strongly to the maximal increment of extracellular potassium (Fig. 7D), which is in line with previous in vivo as well as culture studies (Lewis and Schuette 1975; Kann et al. 2003) and indicates the Na-K-ATPase as the main oxygen consumer. Concerning the contribution of stimulation induced decline of pO₂ to the SDLE associated anoxia we did not find a significant correlation between the pulse number used for SDLE induction and the duration of anoxia. Furthermore, the pulse number did not positively correlate to the extent of cell damage determined after repetitive SDLEs in the experiment described before. These two results indicate that SDLEs by themselves are accompanied by transient anoxia and induce cell damage if evoked repetitively.

**SDLEs are initiated in normoxia**

It is well known that hypoxia facilitates SD-like depolarizations. As shown before pO₂ lies in the normoxic range in JHSCs without electrical stimulation. Nevertheless, the stimulation associated pO₂ decline, which in part reaches hypoxic values, could be the main factor for SDLE initiation in JHSC. Following this hypothesis one would expect low pO₂ values during the early phase of SDLE. We determined pO₂ at the time when the negative field potential shift reached half of its maximum, which is readily recognizable and with certainty after SDLE initiation. At this point, the mean pO₂ was 46.3 ± 5.1 mmHg at 50 µm (n = 15 SDLEs,
the whole range of pO₂ values is plotted in Fig. 7B). Taken the other measurements of pO₂ into account it is very unlikely that pO₂ reaches hypoxic values deeper within the tissue. In conclusion, JHSCs are normoxic during SDLE initiation.

Discussion

The main findings of this study are: a) SDLEs, initiated in normoxia, were reliably evoked by electrical stimulation trains in JHSCs when a 20% oxygen tension was used, b) the characteristics of SDLEs in JHSCs equaled those of SDs with the exception of an incomplete suppression of evoked and spontaneous activity during SDLE, c) repetitive induction of SDLEs decreased the amplitude of the 1st peak of negative field potential shift, increased the duration of SDLE and lowered the threshold for induction of SDLE, d) it deteriorated evoked fast field potentials, and e) it led to cell damage, which f) might be the effect of repetitive anoxia associated with SDLE.

SDLE versus SD

As already mentioned in the result section we favor the term ‘Spreading depression like event’ because not all of the criteria for ‘Spreading depression’ are fulfilled. Arguments for SD are the transient large negative field potential shift paralleled by intracellular neuronal depolarization, the typical ion redistribution, the spread characteristics inclusive propagation velocity and the sensitivity towards glutamate receptor antagonists. Arguments against SD are the occurrence of spontaneous activity and the only incomplete suppression of evoked fast field potentials. In terms of evoked fast field potentials a complete suppression of the alveus/stratum oriens evoked population spike was observed while part of the postsynaptic potential remained indicating a loss of the ability to generate action potentials in neurons at the recording site in the pyramidal layer of CA1 region. That might be due to a depolarization
block of voltage-gated Na\(^+\)-channels in pyramidal cells and fits the criteria of SD. In terms of spontaneous activity we report primary and secondary discharges. Obviously, there were enough cells excited synchronously in the CA1 region despite the parallel occurrence of SD. Those discharges consisted of extra- and intracellular measured postsynaptic potentials whereas population spikes or action potentials only occurred at the beginning or end of a SDLE. That is in line with the incomplete suppression of evoked postsynaptic potentials but complete suppression of evoked population spikes during SDLE. The fact that even SDLEs without superimposed discharges showed an incomplete suppression of evoked postsynaptic potentials implies that it is not the actual occurrence of superimposed discharges but the potential to their occurrence, which makes the distinction between SDLE and SD.

Occasionally, we observed seizure-like events in the pyramidal layer of CA3 while SDLEs with synchronized discharges occurred in CA1 and vice versa (data not shown). Accordingly, bath application of APV abolished SDLEs whereas seizure-like events with a similar discharge pattern occurred in some JHSCs. These observations suggest SDLEs to be SDs with superimposed seizure-like events. Seizure-like events following electrical stimulation trains have been demonstrated before by Shin et al. in 95%-oxygen tension gassed JHSC (Shin et al. 1992).

The occurrence of superimposed discharges interpretable as seizure-like events raises the question to what extent we succeeded in isolating SDs from seizure-like events, which was one main goal of the study. Using the described method of induction by stimulation we succeeded in isolating SDLEs from other types of epileptiform activity. The question remains whether SDLEs in JHSCs are equivalent to juvenile SDs.

**SDLE might be the juvenile subtype of SD**

Except for a brief initial period of discharges there is no spontaneous or evoked activity during the classical SD in adult neuronal tissue (Grafstein 1956). This type of SD was also
observed in juvenile tissue after bath application of low Mg\(^{2+}\) and high K\(^+\) (Kreisman and Smith 1993; Gloveli et al. 1995). In contrast, others reported superimposed discharges during the falling phase of the negative field potential shift and most notably during the rising phase back to baseline in SDs induced by hyperthermia, bath applied 4-aminopyridine as well as pressure ejection of 2 M KCl in the acute juvenile hippocampal slice preparation (Haglund and Schwartzkroin 1990; Psarropoulou and Avoli 1993; Wu and Fisher 2000). In juvenile slice culture, superimposed discharges were also shown in both, low Mg\(^{2+}\) associated SDLEs and SDs evoked in sodium acetate containing ACSF (Kunkler and Kraig 1998; Kovács et al. 1999; Pomper et al. 2004). About 20% of SDLEs in the present study were even continuously superimposed with discharges, which might be the extreme of SD-associated spontaneous activity and potentially facilitated by the synaptic reorganization in JHSC (Gutierrez et al. 1999b). Because our data are consistent with the cited previous studies on juvenile tissue it is conceivable to consider SDLE, characterized by the potential to superimposed postsynaptic activity, the juvenile subtype of SD.

**SDLE induction in JHSC**

In order to take advantage of the JHSC preparation some effort was made to induce SD in JHSC. Kunkler and Kraig introduced a method of SD induction using hilar electrical stimulation in JHSCs gassed with 95% oxygen tension and perfused with modified ACSF (Kunkler and Kraig 1998). In this study, ACSF contained sodium acetate instead of sodium chloride to facilitate SD induction as well as a β-adrenergic agonist, isoproterenol, and raised Ca\(^{2+}\) to prevent seizure-like events, which are also facilitated by sodium acetate. We observed SDLEs in JHSC as one type of epileptiform activity in the low Mg\(^{2+}\) model of epilepsy, initially in the commonly applied 95% (Kovács et al. 1999) and recently also in 20% oxygen tension (Pomper et al. 2004). In this model the occurrence of SDLEs is not predictable, which makes it impossible to investigate their effect on neuronal tissue separately from those of
other types of epileptiform activity, i.e. seizure-like events and repetitive tonic discharges. Apart from the low Mg\(^{2+}\) model, we frequently observed paired pulse induced or even spontaneously occurring SDLEs in JHSC perfused with normal ACSF and gassed with 20% oxygen tension (Pomper et al. 2001). That led us to study systematically the electrical induction of SDLEs in JHSC maintained in 20% oxygen. We found that electrical stimulation trains from 2 to 200 pulses induced SDLEs in about two third of JHSCs, which we consider a satisfactory reliability. Particularly, when SDLEs occurred once in a JHSC, they could be induced repetitively provided that an interval of 10-15 min was maintained between stimulus trains.

When considering the cause of facilitated SDLE induction in 20% oxygen tension hypoxia in the depth of JHSC could be responsible. This would implicate SDLEs to be actually hypoxic SD-like depolarizations. The hypoxic threshold, defined by the pO\(_2\) at which SD-like depolarizations occur, was recently determined with 6.7-7.9 mmHg at the nadir in acute adult hippocampal slices (Foster et al. 2005). Without electrical stimulation, we measured 67 ± 1.4 mmHg close to the nadir in JHSCs. This value is far above the hypoxic threshold. During electrical stimulation without subsequent SDLEs we obtained minimal pO\(_2\) values below 20 mmHg at a depth of 50 \(\mu\)m when 200 pulses were applied. Thus, it is likely that at the nadir of JHSCs the hypoxic threshold was at least sometimes reached when high pulse numbers were used. However, the hypoxic threshold was reached too late to be responsible for SDLE initiation as demonstrated by a pO\(_2\) value of 46.3 ± 5.1 mmHg at the time of half maximal negative field potential shift of SDLE. This indicates that SDLEs were initiated in normoxia.

Another argument for SDLEs evoked in normoxia is the sensitivity towards the NMDA receptor antagonist APV, a feature untypical for hypoxic SD-like depolarizations (Lauritzen and Hansen 1992; Obeidat et al. 2000). Notably, in some JHSCs, APV did not block SDLEs but only increased their threshold of induction. This decreased sensitivity might result from a
stimulation pulse-induced increase of extracellular potassium concentration that has been shown to lower the efficacy of various NMDA receptor antagonists (Petzold et al. 2005).

As revealed by IOS-measurements SDLEs spread into CA3 in almost half of the experiments. This is in line with a number of recent studies demonstrating induction and/or spread of SDs in CA3 using acute slices (Gorji et al. 2000; Buchheim et al. 2002) as well as slice cultures under hyperoxic conditions (Kunkler and Kraig 1998, 2004). However, other studies suggested a relative resistance of CA3 neurons towards SDs (Schiff and Somjen 1987; Aitken et al. 1998). It awaits further investigations to elucidate whether differences in experimental condition, age or species contribute to this discrepancy.

**Effect of repetitive SDLE on JHSC**

There are several changes in JHSCs that develop continuously with the running number of repetitive SDLEs, suggesting a cumulative effect of repetitive SDLEs on JHSCs. First of all, SDLEs were prolonged in terms of large negative field potential shift and ion redistribution. This suggests a consecutive failure of ionic clearance mechanisms attributable to neurons and glial cells. Increased duration of SDLE might cause increased Ca\(^{2+}\) influx, known to activate signal transductions pathways potentially leading to cell damage. This seems to contradict prior studies. While the duration of the first four to eight SDs became also successively prolonged in the adult intact brain (Herreras and Somjen 1993) there was no neuronal damage observed after repetitive SDs *in vivo* (Nedergaard and Hansen 1988; Gido et al. 1994). However, in both studies focusing on neuronal damage there was no lengthening of SDs reported or recognizable in the provided figures. This discrepancy could be related to the fact that in the latter studies SDs were elicited in the parietal cortex whereas Herreras and Somjen (1993) studied hippocampal SDs. Thus, the increasing duration could reflect a characteristic of the hippocampus and could even be the essential prerequisite for subsequent cell damage (Kawasaki 1988). The amplitude of the large negative field potential shift also declined,
which could be explained by a decreased cell population participating in SDLE-associated intracellular depolarization. This interpretation is supported by the decline of population spike and postsynaptic potential, pointing towards neuronal dysfunction. Because decreased population spike is considered indicative of underlying neuronal cell damage in hypoxia as well as in hyperoxia we assessed cell damage by PI staining. This measurement confirmed the “electrophysiological” assumption. Cell damage occurred predominantly in CA1 and CA3 regions. Cells of both regions participated in SDLE as revealed by IOS-measurements. Cells in DG were also stained with PI, although IOS-measurement did not show involvement of this region in SDLE. This might be explained by the fact that IOS-measurement was only performed in the first 1-5 SDLEs of JHSCs other than those used for repetitive SDLE induction. Therefore, it cannot be ruled out that SDLEs spread into DG if evoked more often. This suggestion is indirectly supported by the fact, that SDLE induction in the CA1 region was facilitated by repetitive SDLE as revealed by decreased pulse threshold.

The PI staining used in this study is a well-suited method to assess cell damage. However, it does not distinguish among cell types affected. Arguments for the involvement of principle neurons in CA1, CA3 and DG are the decline of anti- and orthodromic evoked fast field potentials and the PI staining in the principal cell layers of CA1, CA3 and DG (upper blade). Arguments for additional cell damage of inhibitory interneurons are the increase of the number of additional population spikes with repetitive SDLEs and the PI staining in sr-slm, a layer containing dendrites of pyramidal cells as well as interneurons. However, this layer is also rich of glial cells. The exact contribution of the different cell types awaits further clarification.

Previous in vivo studies showed that repetitive SDs do not result in cell damage in healthy adult neuronal tissue assessed by histopathology 4-7 days thereafter (Nedergaard and Hansen 1988; Gido et al. 1994). In contrast, repetitive SDs that occur in the perifocal zone of a focal infarct region extend the infarction (Busch et al.1996; Hartings et al. 2003). The perifocal
zone is considered a compromised region that converts SD into a harmful event. Contributing factors might be low values of pO$_2$, pH, energy substrates, altered ionic concentrations and glial dysfunction (Largo et al. 1996, Lian and Stringer 2004). In particular, the combination of hypoxia and long-lasting Ca$^{2+}$ influx during hypoxic SD-like depolarizations are thought to be critical for subsequent cell damage (Somjen 2001). In the present study, the repetitive anoxic periods accompanying SDLEs and their potential prolongation with increasing duration of SDLEs might have been the main compromising factor leading to the observed cell damage.

Whether or not the repetitive anoxic periods are causative two interpretations for the observed cell damage in JHSCs remain apart from the aforementioned possibility of a hippocampal-specific vulnerability towards SDs. First, the juvenile tissue by itself corresponds to a compromised situation by immaturity of glial cells and neurons. One feature of immature tissue is the frequent physiological occurrence of apoptosis, necessary for tissue reorganization, which points to an increased cell damage potential in juveniles. Increased susceptibility to SDs combined with the potential to superimposed discharges and other types of epileptiform activity in addition to increased cell damage potential appears a harmful combination eventually evolving to hippocampal sclerosis. With regard to the anoxic periods during SDLEs one could argue that either the occurrence of anoxic periods or the vulnerability to anoxia is increased in juvenile tissue. The former argument is supported to some extent by Mayevsky et al. (1982), who reported NADH to be in the reduction cycle indicating an unmet oxygen demand in response to SD in the young but not adult rat brain during anesthesia. With respect to the latter argument it has been shown that the hippocampus is less vulnerable to hypoxia during the first postnatal days. This initially higher resistance to hypoxia disappears during the first postnatal week in CA1 region (Nabetani and Okada 1994). During the third week of postnatal development, which corresponds to the age of JHSCs, the hypoxic vulnerability is considered to have reached the adult level (Towfighi et al. 1997).
Interestingly, Yager and Thornhill (1997) reported a higher hypoxic vulnerability in 3-week old rats than in 6- and 9-week old rats, which supports the raised argument.

Second, the slice culture preparation in contrast to the intact brain constitutes a compromised situation. The slice preparation associated deprivation of afferent input and traumatic cell injury as well as the culturing process itself could have influenced the vulnerability to SDLEs. Additionally, the anoxic periods accompanying SDLEs could be an exclusive feature of slice cultures. At comparable baseline pO₂ values the amount of pO₂ decline following electrical stimulation trains in acute hippocampal slices of adult animals (Foster et al. 2005) was similar to that we measured in JHSCs. This suggests that oxygen consumption and diffusion barrier do not fundamentally differ between acute and cultured slices as well as between juvenile and adult slices. Accordingly, anoxic periods during SDLEs could be characteristic of slice preparations in general due to large diffusion distances, e.g. about 100 µm in JHSCs and about 200-300 µm in 400-µm-thick acute slices. Those distances are considerable larger than the typical intercapillary distance of about 50 µm in the intact brain. Otherwise, decreased pO₂ values were also found during SDs in the intact brain (Lukyanova LD and Bureš J 1967; Mayevsky et al. 1980). Using the near-infrared spectroscopy Wolf et al. (1996) demonstrated a shift of cytochrome aa₃ toward a more reduced state, which was interpreted as an increased unmet oxygen demand of mitochondria despite excessive blood oxygenation, possibly by a diffusion barrier. Finally, it remains to be clarified whether the amount of pO₂ decline during SD in the juvenile hippocampus in vivo is similar to that observed during SDLEs in JHSCs.

In conclusion, we established a model of SDLE initiation in JHSC by using stimulus trains in a 20% oxygen tension. This oxygen supply could be demonstrated as being normoxic compared to the in vivo situation ruling out the hypoxic cause of SD initiation. Further, we showed a high accordance of these SDLEs with regard to well known features of SD in other systems, i.e. field potential shift, intracellular depolarization, ionic redistribution, spread
properties. One exception was observed, namely the incomplete depression of spontaneous and evoked neuronal activity. We applied the method to evoke SDLEs repetitively and provide evidence that repetitive SDLEs result in neuronal cell damage in JHSCs, assessed by field potential deterioration, ionic disturbances and PI staining. Based on measurements of pO$_2$ repetitive anoxia associated with SDLEs might be responsible for the observed cell damage. Whether the effect of SDLEs on cell damage as well as the occurrence of SDLE associated anoxia applies to the juvenile hippocampus of the intact brain remains to be clarified by the use of an \textit{in vivo} approach. If the observed effect holds true \textit{in vivo} then slice cultures are an appropriate tool to study the underlying mechanism of cell damage and neuroprotective strategies.
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References


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Figure Legends

Fig. 1:

**Measurement of pO2 in JHSC without electrical stimulation.**

The mean pO2 is plotted versus the position of the oxygen recording electrode beginning at the gas phase above JHSC, followed by the liquid phase at the surface and different depths in 25-μm-steps. Within each of 6 JHSCs measurements at two different sites within the pyramidal layer of CA1 region were performed. At every site measurements consist of two trials, one while paling in, the other one while taking out the electrode. Error bars indicate standard deviations of in total 24 measurements.

Fig. 2:

**Field potentials in CA1 and their responsiveness to glutamate receptor antagonists dependent on the stimulation site.**

Fast field potentials in the pyramidal layer of CA1 evoked by alveus/stratum oriens (left side) and hilar-CA3 (right side) stimulation are illustrated. The traces show the effect of different bath solutions (normal ACSF, 60 μmolar APV wash in, 60 μmolar CNQX wash in, wash out in normal ACSF) on population spikes and postsynaptic potentials. Note the suppression of postsynaptic potentials by CNQX at both stimulation sites while the population spike is preserved for alveus/stratum oriens stimulation indicating an antidromic component.

Fig. 3:

**Electrophysiological characterization of SDLE.**

(A) Simultaneous intracellular (upper trace, $V_{ic}$) and extracellular recordings (lower trace, field potential, fp) typical of SDLE are shown, recorded from the pyramidal layer of CA1 region related to ground potential and evoked by a stimulus train applied at alveus/stratum oriens. The large negative field potential shift consisted of a 1st and 2nd peak in this example.
It was superimposed by tonic-like high frequency discharges occurring before the 1st peak and clonic-like low frequency discharges after the 2nd peak lasting until recovery to baseline and followed by afterdischarges. The large negative field potential shift was paralleled by intracellular depolarization and the discharges by action potentials. In (B) the ion redistributions during SDLE are demonstrated by the alterations of extracellular concentrations of potassium \([K^+]_o\) and calcium \([Ca^{2+}]_o\) in comparison to the large negative field potential shift (fp). Recordings are from two different JHSCs. (C) Alveus/stratum oriens-evoked field potentials recorded in CA1 region at different times (C2) during SDLE (C1) show that the population spike was completely and reversible suppressed whereas the population postsynaptic potential was only strongly reduced with a longer lasting recovery period.

Fig. 4:

**SDLE spread measured by intrinsic optical signals.**

SDLE propagation was determined by measuring relative changes in light transmittance (LT) over time. The electrophysiological changes were recorded by a K+-sensitive microelectrode in CA1. (A) The anatomy of the slice culture is illustrated (DG, dentate gyrus; alv, alveus; so, stratum oriens; sp, stratum pyramidale; sr-slm, stratum radiatum and stratum lacunosum-moleculare; gcl, granule cell layer; ml, molecular layer; hil, hilus; stim, stimulation electrode; rec, recording electrode). (B) Following electrical stimulation of the outgoing axons of CA1 pyramidal cells, SDLE occurred in CA1, characterized by a transient decrease in LT that slowly propagated into CA3. (C) The electrophysiological changes of SDLE consisted of a negative shift of the DC-potential that was paralleled by a transient rise in \([K^+]_o\). Lower trace: a region of interest was placed close to the K+-sensitive microelectrode in CA1, demonstrating that the transient LT decrease occurred simultaneously with and at a comparable timescale as the electrophysiological changes of SDLE.
Fig. 5:

Effect of repetitive SDLEs on electrophysiological characteristics and evoked field potentials.

As exemplified in (A) for the 1st, 10th and 20th SDLE and quantified in (C) for the half maximal duration (half max dura) repetitive SDLE prolonged the large negative field potential shift and the increase of extracellular potassium concentration [K⁺]o continuously. Note also the decrease of the amplitude of large negative field potential shift recognizable in (A). In (B) and (D) the deterioration of evoked field potential population spike amplitude (Δ PS amp) is demonstrated. Additionally, the population postsynaptic potential decreased with repetitive SDLE (B). In (C) and (D) error bars indicate standard error of the mean (n = 7).

Fig. 6:

Cell damage measurement following repetitive SDLEs.

(A) Example of a JHSC experienced repetitive SDLEs and stained with PI for cell damage determination. Note the increased fluorescence signal in pyramidal layer of CA3 and CA1 forming a C-shaped band compared to an only slight staining in the pyramidal layer of CA1 in a JHSC without repetitive SDLEs (B). In addition, the stratum lacunosum-moleculare and stratum radiatum, both not clearly distinguishable, showed increased fluorescence signal indicating cell damage not confined to the pyramidal layer but also affecting interneurons or glial cells. The DG was almost spared from PI staining (bar = 400 µm). A quantification separated by areas is given in (C). Fluorescence signal is presented as ratio between the means of corresponding areas of both, JHSCs maintained in the interface recording chamber (SDLE and non-SDLE group) and the matched JHSCs belonging to the incubator control group. Error bars indicate standard error of the mean and asterisks indicate significant differences (p < 0.05) between SDLE-group (black) and non-SDLE group (white).
Fig. 7:

**Changes of pO$_2$ induced by electrical stimulation and SDLE.**

(A) Changes of [K$^+$]$_o$ (upper trace), pO$_2$ (middle trace) and field potential (fp, lower trace) dependent on different depths of electrode position (surface = 0 µm) during SDLEs evoked by 10 pulses within the same JHSC. Note the occurrence of anoxia at 50 µm and below. (B) The pO$_2$ at the time of half maximal large negative field potential shift of SDLE is plotted versus the latency between the electrical stimulation and this time. The linear curve fit illustrates the strong negative correlation ($r = -0.9$, $p < 0.001$). (C) The maximal decrement of pO$_2$ ($\Delta$ pO$_2$) induced by electrical stimulation without subsequent SDLE depends on the number of stimulation pulses. Error bars denote standard deviations. (D) The maximal decrement of pO$_2$ ($\Delta$ pO$_2$) induced by electrical stimulation without subsequent SDLE correlates strongly with the maximal increment of [K$^+$]$_o$ ($\Delta$ [K$^+$]$_o$, $r = 0.9$, $p < 0.001$).
Fig. 1

[Graph showing pO₂ (mmHg) vs depth (μm) with data points at 140, 120, 100, 83, 73, 67 for various depths (gas, 0, 25, 50, 75, 100).]

Fig. 2

[Graphs showing responses to ACSF, APV, CNQX treatments in alveus/stratum oriens and hilar-CA3 regions.]
Fig. 7

A

B

C
electrical stimulation trains without SDLE

D
electrical stimulation trains without SDLE