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

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INTRODUCTION

Spreading depression (SD) is characterized by a transient large negative field potential shift accompanied by intracellular depolarization of neurons and glial cells and redistribution of ions, a suppression of evoked and spontaneous neuronal activity, and a slow wave-like propagation (Kruger et al. 1996; Martins-Ferreira et al. 2000; Nicholson and Kraig 1975; Somjen 2001). It was first described as a propagating silencing of neuronal activity measured by cortical electrography in anesthetized 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, repetitve 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 Mg2+-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 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 (Nederkaard 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 (Gioveli et al. 1995; Kreisman and Smith 1993; Psarropoulou and Avoli 1993). Prolonged epileptiform activity has been shown to result in cell damage and other long-term effects dependent on age and epilepsy model used (Cilio et al. 2003; Dube et al. 2004; Heinemann et al. 2002). 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). Although repetitive SDs occur during epileptiform activity, their contribution to alterations in juvenile neuronal tissue is unknown.

To approach this issue, we present a reliable method to evoke SDLEs in normoxic 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, something of advantage for elucidating mechanisms of cell damage (Kovács et al. 2002). In contrast to acute

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slices, the culture preparation does not interfere with acute changes after the preparation itself, e.g., traumatic cell damage. The culture preparation offers opportunities for preincubation experiments to evaluate neuroprotective strategies (Pringle et al. 1997), and it allows the study of long-term effects by repeated measurements (Gutierrez et al. 1999). The main disadvantage is the culture process itself, which could change the properties of the tissue (Gutierrez and Heinemann 1999), 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 partial oxygen pressure (pO$_2$) 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 (PI). PI fluorescence is well correlated with conventional markers of cell death (Newell et al. 1995; Vornov et al. 1991).

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- to 9-day-old Wistar rats and cultured for 8–13 days in vitro corresponding immature rats of postnatal age 15–20 following Stoppi’s method (Stoppi et al. 1991) as described previously (Pomer 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% O$_2$-5% CO$_2$) 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 six-chamber incubation wells (Falcon) and incubated in a medium containing 50% MEM, 25% Hank’s balanced salt solution (HBSS; Sigma, Deisenhofen, Germany), 25% horse serum (Gibco), 2 mM t-glutamine, 10,000 U/ml penicillin, and 10,000 µg/ml streptomycin, pH 7.4 in an incubator (Hereaus; Hanau, Germany) at 36.5°C containing room air enriched with 5% CO$_2$. The culture medium was completely replaced the first 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% O$_2$-5% CO$_2$-75% N$_2$) artificial cerebrospinal fluid (ACSF) containing (in mM) 129 NaCl, 3 KCl, 1.25 NaHPO$_4$, 1.8 MgSO$_4$, 1.6 CaCl$_2$, 21 NaHCO$_3$, and 10 glucose; pH 7.4. In the first set of experiments, 61 JHSCs were studied electrophysiologically inclusive of the application of repetitive electrical stimulation with 2–200 pulses of maximal intensity aimed at SDLE induction. In 7 of 39 SDLE-positive JHSCs, each positioned on a 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 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 (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$^+$, Ca$^{2+}$) 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 (Fig. 4A). JHSCs, which presented with stimulus-evoked response amplitudes <0.5 mV, initial occurrence of epileptiform activity or morphological imperfection 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 BX 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), 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 first and second peaks and latency to first 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 N-methyl-D-aspartate (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

pO$_2$ on the surface and in different depths of JHSCs maintained in the interface chamber was measured using an oxygen sensitive electrode (Clark-Style Microelectrode, No. 737GC, Diamond General Development, Ann Arbor, MI). 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 <150 µm thick. In the second set of experiments, pO$_2$ 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, 100 × amplified), extracellular potassium concentration (1.6 Hz low-pass filtered, 50 × amplified) and pO$_2$ (1.6 Hz low-pass filtered, 10 × amplified) were simultaneously recorded and analyzed using Spike2 software (Cambridge Electronic Design).

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 (Petzold et al. 2005; Weissinger et al. 2000). 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 ($T_0$) and was subtracted from each subsequent image of the series ($T_t$). This series of subtracted images revealed changes in light transmittance (LT) over time

$$LT = \frac{T_t - T_0}{T_0} \times 100 = \frac{\Delta T}{T_0} \%$$

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. 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 that 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 molar 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 two 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 GmbH, Hamburg, Germany) equipped with a water immersion objective (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. Pixel pictures (640 × 480) were taken from three regions: dentate gyrus (DG), CA3, and CA1. The following seven areas were analyzed separately: 1) molecular layer (ml), 2) granule cell layer (gcl), and 3) hilus (hil) of DG; 4 and 5) stratum pyramidale (sp) and 6 and 7) stratum lucidum-molecular membrane 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, MD). 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 means ± SE 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 nonparametric Mann-Whitney-U-Test for unpaired data were 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 $pO_2$ with Clark-type oxygen-sensing electrodes. At the surface of JHSCs, $pO_2$ was 120 mmHg, and it declined to 67 mmHg at a depth of 100 μm. The effect of 25-μm steps on $pO_2$ decline attenuated with increasing depths. This indicates that the nadir of $pO_2$ was nearly reached at 100 μm (Fig. 1). The measured values of $pO_2$ correspond to in vivo data obtained under normoxic conditions in the rat brain (4–80 mmHg) (Vovenko 1999) and lie above those measured in patients during the recovery period after 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 ± 5.5 (SD) mV and 2.4 ± 0.7 (SD) ms in 61 JHSC, respectively. As shown in Fig. 2, blockade of glutamatergic transmission via non-NMDA receptors by CNQX (60 μM) abolished the postsynaptic potential almost completely, whereas the population spike was preserved. The NMDA-receptor antagonist APV (60 μM) had no significant effect on either population spike or postsynaptic potential. The facts that the first population spike preceded the postsynaptic potential and that it was preserved while glutamatergic transmission was blocked indicated the first population spike was antidiromically, i.e., nonsynaptically evoked. The fact that the postsynaptic potential was abolished by CNQX but preserved by APV indicates it is 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 (evoked SDLE). Following this protocol, SDLEs in CA1 were significantly different between SDLE-positive and -negative JHSCs. The number of stimuli required for inducing SDLEs was superimposed among 50% (n = 7), 75% (n = 4), and 100% (n = 8) of maximal stimulation intensity. A nonsystematic 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 10–15 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 in the following text refer, if not stated otherwise, to 39 SDLEs (1 of the 1st 3 induced SDLEs per SDLE-positive JHSC). The large negative field potential shift typical of SD consisted of an early negative first peak followed either by a second peak divided by a brief decline (69%, Fig. 3A) or by a prolonged plateau before recovery to baseline (31%). The amplitude of the first peak was $-31.1 \pm 1.0 \text{ mV}$, and the latency to first peak was $6.1 \pm 0.7 \text{ s}$. Half-maximal duration of SDLEs was $21.9 \pm 1.6 \text{ s}$. The negative field potential was accompanied by intracellular depolarization and typical ion redistribution (Fig. 3). The extracellular potassium concentration rose from 3 to $49.3 \pm 4.9 \text{ mM}$ with a latency to peak of $11.0 \pm 0.8 \text{ s}$ (n = 24), and the extracellular calcium concentration fell from 1.2 to $0.3 \pm 0.1 \text{ mM}$ with a latency to peak of $20.9 \pm 3.1 \text{ s}$ (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, tonic-like high-frequency primary discharges with short latency and duration and the endpoint around the first negative peak and 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 after an SDLE (Fig. 3A). During SDLEs, neurons depolarize up to a membrane potential of $-40 \text{ mV}$. 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, the population spike was blocked during the plateau phase of an SDLE while the postsynaptic potential was only strongly reduced.

**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 seven JHSCs. Typical recordings are shown in Fig. 4. SDLEs spread always from the site of stimulation to other parts of the hippocampus.

**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) and hilar-CA3 (right) stimulation are illustrated.** The traces show the effect of different bath solutions [normal artificial cerebrospinal fluid (ACSF), 60 μM 2-amino-5-phosphonovaleric acid (APV) wash in, 60 μM 6-cyano-7-nitroquinoxalene-2,3-dione (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.
the subiculum and toward 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 because this sensitivity is another feature of SDs. APV (60 μM) reversibly blocked induction of SDLEs in 9 of 14 JHSCs even when stimulus intensity was increased to supramaximal intensities and when stimulus trains of 10 s
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): after 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⁺]. Bottom 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 time scale as the electrophysiological changes of SDLE.
(20 Hz) were applied. In the remaining JHSCs, the threshold for induction of SDLE was increased. The latency to the first 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 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**

To answer the question whether repetitive SDLEs result in neuronal dysfunction in JHSCs, we first 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 h. In total, 22 ± 1 SDLEs (inclusive 3 spontaneous SDLEs in 2 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 first 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 second 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 three 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**

To assess cell damage as potential cause of electrophysiological observed neuronal dysfunction, JHSCs that 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 ± 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 with 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 pO2 continuously during SDLE. Surprisingly, we realized that in a depth of 50 μm the tissue became anoxic (Fig. 7A), defined by a pO2 value of ≤5 mmHg. Anoxia lasted 18 ± 1.4 s in 13 of 15 SDLEs in four JHSCs investigated. In 100 μm, the largest depth measured, the duration of anoxia was almost doubled to 30.7 ± 9.5 s (3 SDLEs in 3 JHSC). The dependency of pO2 alterations during SDLE on depth is exemplified in Fig. 7A. No changes occurred in the gas phase directly above JHSC (pO2 = 140 mmHg) indicating constant oxygen supply at the gas-liquid interface. At 50 μm, the minimal pO2 of 2.7 ± 0.8 mmHg (n = 15 SDLEs) was on average reached after 16.7 ± 0.4 s. The half-maximal duration was 54.9 ± 1.9 s.

Interestingly, there was no correlation between the latency to first peak of the large negative field potential shift and the latency to minimal pO2. However, we found a strong negative correlation between the latency to the half-maximal negative field potential shift and the pO2 at this time, i.e., the later the field potential shift was detected by the recording electrode the lower was the pO2 value there (Fig. 7B). This correlation is not attributable to stimulation induced neuronal activation because there was no correlation between pulse number and pO2 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 toward 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 \( \leq 20 \text{ mmHg} \) at 50 \( \mu \text{m} \) when 200 pulses were applied. The maximal decrement of pO2 correlated very strongly to the maximal increment of extracellular potassium concentration \([K^+]\), 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 SDLEs (B). In C and D, error bars indicate SE \((n = 7)\).

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
DISCUSSION

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

SDLE versus SD

As already mentioned in results, 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 toward 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 after electrical stimulation trains have been demonstrated before by Shin et al. (1992) in 95%-oxygen tension gassed JHSC.

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.

**FIG. 6.** Cell damage measurement after repetitive SDLEs. A: example of a JHSC that 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 with an only slight staining in the pyramidal layer of CA1 in a JHSC without repetitive SDLEs (B). In addition, the stratum lacunosum-molecular 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 nonSDLE group) and the matched JHSCs belonging to the incubator control group. Error bars indicate SE. *, significant differences (P < 0.05) between SDLE-group (●) and non-SDLE group (○).
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$^{2+}$/H$^{+}$ and high K$^{+}$/H$^{+}$ (Gloveli et al. 1995; Kreisman and Smith 1993). 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; Psarropoulos 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 (Kovács et al. 1999; Kunkler and Kraig 1998; 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 reorganiza-

**FIG. 7.** Changes of pO$_2$ induced by electrical stimulation and SDLE. A: changes of [K$^+$]$_o$ (top trace), pO$_2$ (middle trace), and field potential (fp, bottom 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. B: pO$_2$ at the time of half-maximal large negative field potential shift of SDLE is plotted vs. 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: maximal decrement of pO$_2$ (Δ pO$_2$) induced by electrical stimulation without subsequent SDLE depends on the number of stimulation pulses. Error bars denote SDs. D: maximal decrement of pO$_2$ (Δ pO$_2$) induced by electrical stimulation without subsequent SDLE correlates strongly with the maximal increment of [K$^+$]$_o$ (Δ [K$^+$]$_o$, r = 0.9, P < 0.001).
tion in JHSC (Gutierrez and Heinemann 1999). 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**

To take advantage of the JHSC preparation, some effort was made to induce SD in JHSC. Kunkler and Kraig (1998) introduced a method of SD induction using hilar electrical stimulation in JHSCs gassed with 95% oxygen tension and perfused with modified ACSF. 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-thirds 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 <20 mmHg at a depth of 50 μm 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 SD. This indicates that SDLEs were initiated in normoxia.

Another argument for SDLEs evoked in normoxia is the sensitivity toward the NMDA receptor antagonist APV, a feature untypical for hypoxic SD-like depolarizations (Lauring 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 (Buchheim et al. 2002; Gorji et al. 2000) as well as slice cultures under hypoxic conditions (Kunkler and Kraig 1998, 2004). However, other studies suggested a relative resistance of CA3 neurons toward SDs (Aitken et al. 1998; Schiff and Somjen 1987). 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 (Gido et al. 1994; Nedergaard and Hansen 1988). 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 toward 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 one to five 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 (Gido et al. 1994; Nedergaard and Hansen 1988). 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 PO2, 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 Ca2+ 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 toward 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-wk-old rats than in 6- and 9-wk-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 after 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., ~100 μm in JHSCs and ~200–300 μm in 400-μm-thick acute slices. Those distances are considerable larger than the typical intercapillary distance of ~50 μm in the intact brain. Otherwise, decreased PO2 values were also found during SDs in the intact brain (Lukyanova and Bureš 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 with 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 PO2 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 SD associated anoxia applies to the juvenile hippocampus of the intact brain remains to be clarified by the use of an in vivo approach. If the observed effect holds true, in vivo then slice cultures are an appropriate tool to study the underlying mechanism of cell damage and neuroprotective strategies.

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