Synchronization of GABAergic Inputs to CA3 Pyramidal Cells Precedes Seizure-Like Event Onset in Juvenile Rat Hippocampal Slices

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Abstract

Here we address how dynamics of glutamatergic and GABAergic synaptic input to CA3 pyramidal cells contribute to spontaneous emergence and evolution of recurrent seizure-like events (SLEs) in juvenile (P10-13) rat hippocampal slices bathed in low-[Mg^{2+}] artificial cerebrospinal fluid. In field potential recordings from CA3 pyramidal layer a short epoch of high frequency oscillation (HFO; 400-800 Hz) was observed during the first 10 ms of SLE onset. GABAergic synaptic input currents to CA3 pyramidal cells were synchronized and coincided with HFO, while the glutamatergic input lagged by ~10 ms. If the intracellular [Cl\textsuperscript{-}] remained unperturbed (cell-attached recordings) or was set high with whole-cell electrode solution, CA3 pyramidal cell firing peaked with HFO and GABAergic input. By contrast, with low intracellular [Cl\textsuperscript{-}], spikes of CA3 pyramidal cells lagged behind HFO and GABAergic input. This temporal arrangement of HFO, synaptic input sequence, synchrony of GABAergic currents, and pyramidal cell firing emerged gradually with preictal discharges until the SLE onset. Blockade of GABA\textsubscript{A} receptor mediated currents by picrotoxin reduced the inter-SLE interval and the number of preictal discharges, and did not block recurrent SLEs. Our data suggest that dynamic changes of the functional properties of GABAergic input contribute to ictogenesis and GABAergic and glutamatergic inputs are both excitatory at the instant of SLE onset. At the SLE onset GABAergic input contributes to synchronisation and recruitment of pyramidal cells. We conjecture that this network state is reached by an activity-dependent shift in GABA reversal potential during the preictal phase.
Introduction

Epileptic seizures manifest as uncontrolled limb movements, loss of consciousness and posture, and high-amplitude electroencephalogram (EEG) signals with complex dynamics (Dikanev et al. 2005; Jirsch et al. 2006; Schiff et al. 2000; Worrell et al. 2004). How synchronised activation of large neuronal populations – reflected by the large-amplitude EEG signal of seizures – is brought about is a question of high theoretical and clinical importance. In vivo, high-frequency oscillations (HFOs) >200 Hz preferentially occur at the seizure onset (Bragin et al. 2005; Jirsch et al. 2006), a feature manifested in vitro (Dzhala and Staley 2003a; Khoshravani et al. 2005; Lasztóczi et al. 2004). This temporal alignment of HFOs and transition to seizures suggests a causal link between the two phenomena (Bragin et al. 2000, 2002; Jirsch et al. 2006; Lasztóczi et al. 2004). HFOs are linked to hyper-excitability and synchrony of neuronal networks, the two hallmarks of epileptiform activity (Le Van Quyen et al. 2006; McCormick and Contreras 2001; Perez Velazquez and Carlen 2000; Traub et al. 2001). Hyper-excitable state, that renders the network prone to spontaneous seizures, may result from disruption of balance between excitation and inhibition. This could be either a consequence of alterations in glutamatergic and/or GABAergic synaptic input per se (Cossart et al. 2001; Epsztein et al. 2006; Trotter et al. 2006), or a positive shift in the reversal potential of GABA_A receptor mediated currents (Cohen et al. 2002; Dzhala and Staley 2003b; Fujiwara-Tsukamoto et al. 2007; Khazipov et al. 2004; Köhling et al. 2000; Lopantsev and Avoli 1998; Perez Velazquez 2003).

Excitatory action of the inhibitory transmitter amino acid GABA has been suggested to be responsible for increased seizure-susceptibility of immature brain (Dzhala et al. 2003b, 2005; Khazipov et al. 2004; Tyzio et al. 2007). Although the direct excitatory action of GABA has been demonstrated in the neonatal brain (postnatal days<8) (Khazipov et al. 2004; Rivera et al. 1999; Tyzio et al., 2007) other, more complex ways of GABAergic excitation have been
established in adult animals and in human epileptic brain (Cohen et al. 2002; Epsztein et al. 2006; Fujiwara-Tsukamoto et al. 2003, 2007; Kaila et al. 1997; Lamsa and Kaila 1997; Marty and Llano 2005; Staley et al. 1995; Perez Velazquez 2003). Transitory between neonatal and adult, slices from juvenile (P10-13) rat hippocampus are frequently used in experimental epilepsy research, as they are more susceptible to develop seizures than slices from adults (Heinemann et al. 1991; Köhling et al. 2000; Lasztóczi et al. 2004). Interestingly, experimental data supported either excitatory (Dhzala et al. 2003b; Khazipov et al. 2004) or inhibitory/shunting (Rivera et al. 1999; Tyzio et al. 2007) actions of GABAergic transmission at this age. Although the susceptibility to seizures of the juvenile brain is well documented, information on the short-term dynamics of glutamatergic and/or GABAergic synaptic drive contributing to ictogenesis is much more scarce.

Juvenile rat hippocampal slices bathed in low-\([\text{Mg}^{2+}]\) artificial cerebrospinal fluid (ACSF) express preictal discharge trains with increasing similarity to SLE onset, confinement of field potential (fp) HFOs (400-800 Hz) to the start of discharges and gradual emergence of synchronized synaptic currents onto CA3 pyramidal cells (Lasztóczi et al. 2004). Although the contribution of these synchronised inputs to SLE initiation was documented (Lasztóczi et al. 2004), the relative weight of GABAergic versus glutamatergic components has not been established. Glutamatergic (Dzhala and Staley 2004; Palva et al. 2000) and GABAergic (Jones and Barth 2002; Khalilov et al. 2005; Palva et al. 2000) synaptic interactions were implicated in the generation of HFOs and seizure-related synchrony. Accomplished by a detailed analysis of temporal relationships between pyramidal cell firing, fp activity and synaptic input sequences during SLEs, the aim of the present study was to dissect GABAergic and glutamatergic contributions to SLE dynamics.
**Materials and Methods**

Animals were kept and used in accordance with the European Council Directive of 24 November 1986 (86/609/EEC) and the Hungarian Animal Act, 1998 and associated guidelines. All efforts were made to reduce animal suffering and the number of animals used. Transverse, 400 μm thick hippocampal-entorhinal slices were cut in horizontal plane from brains of juvenile (P10-13) male Wistar rats (Toxicoop, Budapest, Hungary), as described elsewhere (Lasztóczi et al. 2004). Slices were put in a submerge-type recording chamber continuously perfused by ACSF at 36°C (composition in mM: 124 NaCl, 10 glucose, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂ and 26 NaHCO₃; saturated with carbogen gas). To evoke spontaneous recurrent SLEs, the perfusing solution was switched to one with no added Mg²⁺ ions and [K⁺] raised to 5 mM (low-[Mg²⁺]-ACSF). In a subset of experiments the CA3 region was isolated by knife cuts after the SLE activity has been established.

Recordings were performed as described previously (Lasztóczi et al. 2004) with minor modifications. Signals obtained by Multiclamp700A amplifier (Axon Instruments, Foster City, CA) were low-pass filtered at 2 kHz and digitised at 10 kHz (Digidata1320A, Axon Instruments). For single cell recording CA3 pyramidal cells were identified visually. Extracellular (2-5 MΩ) and cell attached (5-9 MΩ) electrodes were filled with ACSF. For cell attached recordings holding potential was set to –70 mV. Experiments were discarded if signs of partial ”break-in” had occurred [e.g. spontaneous postsynaptic currents (sPSC) appeared].

For voltage clamp, pipettes (4-6 MΩ) were filled with a solution containing (in mM) 130 CsMeSO₃, 10 NaCl, 0.05 CaCl₂, 2 ATP (magnesium salt), 1 EGTA and 10 HEPES (pH set to 7.3 with 1N CsOH). To suppress escape action currents 5 mM QX 314 (Tocris, Bristol, UK) was added. Cells were voltage-clamped around 0 mV (at -5, 0 or +5 mV), -45 mV (at -40, -45 or -50 mV) or –30 mV (-25, -30 or -35 mV) to record GABAergic (outward), glutamatergic (inward) or combined (outward-inward) currents, respectively. The holding potential was
adjusted in individual experiments to a level where outward or inward sPSCs (as appropriate) just disappeared. These holding potential values were in agreement with the reversal potentials experimentally determined for pharmacologically isolated spontaneous GABAergic and glutamatergic sPSCs (see the Results). If signs of seal deterioration (large sustained inward currents with increased noise) or cell closure (reduction of sPSC amplitude associated with slowing of their dynamics and a shift in the holding current towards zero) occurred, the recordings were discarded from analysis. For current clamp with low-[Cl⁻] intracellular solution pipettes (3-5 MΩ) contained (in mM) 135 KGlucnate, 10 NaCl, 0.05 CaCl₂, 2 ATP (magnesium salt), 1 EGTA and 10 HEPES (pH set to 7.3 with 1N KOH). In the case of high-[Cl⁻] condition intracellular solutions [KGlucnate]=85 mM and [KCl]=50 mM were applied with all other concentrations unchanged. In order to explore cellular responses we applied hyperpolarizing (30-100 pA) and depolarising (30-200 pA) current steps. In some cases ±50 or 100 pA current steps were also applied while recording SLEs under current clamp, but this did not affect spike timing, and therefore data were pooled. To determine the reversal potential of synaptic currents, pharmacologically isolated miniature excitatory postsynaptic currents (mEPSCs) or miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 1 μM tetrodotoxin and 100 μM picrotoxin or 1 μM tetrodotoxin, 10 μM CNQX and 50 μM D,L AP-5, respectively while the holding potential was changed between -80 mV and +80 mV in 20 mV steps. Linear function (Origin 6.1) has been used to fit the data: The value obtained for 0 pA current with the standard error of the fit was defined as the reversal potential. Voltages recorded or commanded in whole cell configuration were subject to a junction potential correction of 15 mV calculated with the built-in application of Clampfit, based on the composition of intracellular and extracellular solution.

Two to ten SLEs were analysed from each slice. Being not fully developed, first SLEs were discarded. SLE intensity was calculated as the standard deviation (SD) of high-pass filtered
(>1 Hz) fp trace containing the whole SLE, normalized to the SD of preceding period and when <200 %, SLEs were discarded due to the inability to unequivocally define event timing. Preictal discharges were numbered with negative numbers starting from the SLE onset backwards (e.g. p-1, p-2, p-3…etc.). Due to different numbers (4.3 on average) of preictal discharges N decreased for preictal discharges more distant from the SLE onset. For quantitative analyses of preictal trends, p-5 to p-1 and SLE onset were included.

Whenever possible, we identified the start of fp discharges by the peak of negative deflection (Lasztóczi et al., 2004). If this was missing, start points were identified visually with the experimenter blind to the contents of the other channel. Relationship between the fp discharges and spike timing of pyramidal cells was explored by constructing spike probability histograms within a 200 ms window centred at the start of fp discharges. Spike peak times were identified semi-automatically (MiniAnalysis, Synaptosoft, Decatur, GA) and inspected visually. Amplitude threshold for action potential and action current detection were set to 17-20 mV and 13-20 pA, respectively. Partial spikes (if 10-90 % slope >2.5 mV/ms) were not discriminated from full spikes. We constructed separate spike density histograms for ten different discharge categories (preictal discharges p–5, p-4, p-3, p-2 and p–1, secondary preictal discharges, SLE onsets, tonic discharges, clonic discharges and secondary clonic discharges) by pooling all the relative spike time data for all discharges of all SLEs within a category. To make sure that one discharge contributes to a particular bin with a maximum of one spike we chose a 3 ms bin size, allowing us to calculate the spike probability histograms by simply normalizing the spike density histograms to the number of discharges within a category. Histograms were fitted with built-in Origin 6.1 functions (OriginLab Co., Northampton, MA). Gaussian model was better for tonic and secondary discharges, while the skewed Extreme model was better for others. All histograms were fitted first with one and then with the sum of two functions. Multiple peaks were accepted if resulted in an increase in
R² of more than 0.01. The smaller peaks at the extremes of histogram windows in case of secondary and tonic discharges represent spikes associated with neighbouring discharges. Therefore – although two to three component fits accounted for their presence – values are reported for the relevant component only.

For discharge-associated outward and inward current transients (compound postsynaptic currents; comPSC) amplitudes and 10-90 % current rise times were measured using the Mini Analysis software. To analyse changes in current parameters within the SLE, we used a time normalization protocol separately for the tonic and the clonic periods. For this we divided both periods into six equal-length epochs (epochs 1 to 6 for both tonic and clonic periods in Figure 4), first averaged the parameters within an epoch, followed by averaging across SLEs. For temporal analysis of synaptic input sequences, 200 ms current traces centred at the start of fp discharge were extracted. These were scaled between their minima and maxima, and averaged across discharges. This analysis was performed on representative SLEs (one for outward and one for inward if available) from each slice. Due to the plateau-like nature of glutamatergic comPSCs under some conditions, for the purpose of display on Figure 8, time of occurrence of GABAergic and glutamatergic currents were defined by the time when currents reached 90% of the maximum in the analysis window.

In order to extract the intensity of high frequency fp activity with temporal resolution comparable to other analyses we transformed fp traces containing SLEs into 400-800 Hz intensity traces with a sliding window approach. To avoid phase shifts introduced by most filters, we band pass filtered the down-sampled (5 kHz) SLE-containing traces by calculating the difference between a low-pass filtered (<800 Hz) and a high-pass filtered (>400 Hz) trace. We used Gaussian type digital filters (pClamp9), as they do not introduce phase shifts. The resulting trace was transformed into an intensity trace by calculating the SD of the trace with a continuously sliding 3.2 ms window. 200 ms epochs centred at the fp discharge start were
finally extracted and averaged across different SLEs. This analysis was performed on one representative SLE for each slice.

Unless stated otherwise in the text, one-way ANOVA followed by Bonferroni’s multiple post hoc comparison was used for statistical testing with \( P<0.05 \) considered significant and data are reported as mean ± SD. Data processing, analysis and graphical representations were executed with pClamp9 (Axon Instruments), Origin 6.1 (OriginLab Co.), MiniAnalysis (Synaptosoft), Corel9 (Corel Inc. Dallas, TX) software, and self-devised scripts in MATLAB 6.5 (MathWorks Inc, Nattick, MA) environment.

**Results**

In 76 % of juvenile (P10-13) rat hippocampal slices perfusion with low-[Mg\(^{2+}\)] ACSF resulted in recurrent SLEs with 760±230 s latency, 82±28 s length and 335±133 s inter-SLE interval (Figure 1A; \( N=70 \) slices from 38 animals; Anderson et al. 1986; Walther et al. 1986). First signs of forthcoming SLEs were the appearance of large amplitude (>0.5 mV) discharges that consisted of 0.1-0.3 s positive shift with fast (>100 Hz) oscillation superimposed (Figure 1B; preictal discharges). Further discharges of the preictal period (there was 4.3 ± 2.8 on average) showed gradual build-up until the SLE onset (Figure 1B; Lasztóczi et al. 2004; Khoshravani et al. 2005). A decelerating train of tonic discharges followed the SLE onset, with the tonic-to-clonic transition marked by the reappearance of secondary discharges (Figure 1B).

In order to study temporal relationship between cellular events and fp, these should be recorded sufficiently close. Dual extracellular recordings in CA3 addressed this issue, with the distance between electrodes ranging from 26 μm to 895 μm (\( N=19 \) slices from 15 animals). Delay histograms of tonic, clonic and secondary clonic discharges peaked at 0 ms for distances <100 μm (\( N=7 \) slices), whereas non-zero peaks were frequently found for
distances >100 μm. Therefore, in parallel single-cell and fp recordings we placed the two electrodes within 100 μm.

Spikes coincide with the start of fp discharges: cell attached recordings

We first addressed the spike timing of CA3 pyramidal cells in relation to epileptiform fp discharges. To keep the intracellular milieu as intact as possible and yet to record spike timing accurately we recorded action currents of CA3 pyramidal cells in cell attached voltage-clamp configuration (8 cells from 7 animals; Perkins 2006). Before switching the perfusion to low-[Mg²⁺] ACSF cells emitted action currents at a low overall rate (0.59±0.84 Hz) typically in short bursts of 2-6 spikes (intraburst frequency: 153±58 Hz), identifying the recorded cells as pyramidal cells. In low-[Mg²⁺] ACSF action current rate increased to 3.50±2.54 Hz indicative of network activation. During SLEs we found action currents associated with preictal discharges, SLE onsets and tonic and clonic discharges (Figure 2A and 2B; N=30 SLEs from 8 slices of 7 animals). Maximum instantaneous action current frequency during SLEs (including the preictal period) was 230±39.1 Hz with inter-spike intervals no shorter than 3.1 ms observed. Accordingly, binning histograms at 3 ms allowed us to accurately estimate spike probability without including adjacent spikes in any bin. The fp discharge start times were identified either by the minimum of negative deflection at their start or manually as appropriate (Lasztóczi et al., 2004; see Methods for details). This method has a high (ms range) accuracy for all the discharges starting with a negative fp deflection (late preictal discharges, SLE onset, tonic and clonic discharges; see Lasztóczi et al., 2004), and thus allows the temporal alignment of measures reflecting different aspects of network behaviour [reported as distributions for spike timing and mean current or intensity (± SE) for synaptic input and HFO].

Action currents were associated with the rising and sustained phases of early preictal discharges (p-5 in Figure 2C). This was reflected by the spike probability histogram (plot ‘p-
5’ in Figure 2D; pooled for 6 SLEs from 5 slices) well described with a single component curve with a peak at +42 ms (black line on Fig 2D; see Methods for the details on fitting procedures). SLE onsets displayed markedly different pyramidal cell firing probability distributions (Figure 2C and 2D, plot for SLE onset from 30 SLEs from 8 slices) with an earlier and sharper peak in addition to the more delayed one. Indeed this histogram was better fit with the sum of two components (see Methods for details) with peaks at +2.8 ms and +51 ms (black lines on Fig 2D). In parallel to the evolution of fp waveform (Figure 1B) spike probability histograms showed gradual transformation between p-5 and SLE onset (Figure 2C and 2D, pooled for 13, 17, 25 and 30 SLEs from 7, 7, 8 and 8 slices, from p-4 to p-1, respectively). Contrasting the relatively constant peak times (around 40 ms) and amplitudes of the slower component throughout the preictal period, the sharper and earlier component appeared at p-2 only, and had increasing amplitude and accelerating peak towards the SLE onset (Fig 2D; black lines). We frequently observed action currents aligned to the negative fp deflections marking the start of fp events. In agreement with this observation, the spike probabilities of the histogram zero bins were large throughout the preictal period (Figure 2D; Table I). This tendency was most obvious for the SLE onset where the firing probability was eventually at its maximum level within the zero bin (Figure 2D; see Figure 2C also).

Pyramidal cell firing was associated with the start of tonic (Figure 2C, middle plots), primary clonic (Figure 2C, right hand plots), secondary preictal and secondary clonic discharges as well. For these discharge types spike probability histograms (pooled for 30 SLEs from 8 slices) peaked with high values at 0 ms, and accordingly were fitted with curves peaking close to zero. Histogram for primary clonic discharges and the curve fitted to it were much like the histogram and the curve of SLE onset (Fig 2D; slow and fast components peaked at +41 ms and +0.9 ms, respectively).
The above data indicate that during the preictal period CA3 network gradually enters a state characterized by synchronous and accelerated action potential firing timed to the start of discharges. The question arises if this can be explained by the temporal organization of synaptic inputs to individual CA3 pyramidal cells. To address this we performed whole-cell voltage-clamp recordings from CA3 pyramidal cells with a CsMeSO\textsubscript{3} pipette solution during recurrent SLEs. In a set of pilot experiments we recorded pharmacologically isolated mEPSCs and mIPSCs (in the presence of 1 µM tetrodotoxin and 100 µM picrotoxin or 1 µM tetrodotoxin, 10 µM CNQX and 50 µM DL-AP-5, respectively) and determined their reversal potentials under control conditions. Recording currents at EPSC reversal potential would reflect dynamics of GABA\textsubscript{ergic} currents (and vice-versa) even in the absence of pharmacological receptor blockade (Trevelyan et al. 2006). The reversal potential of mIPSCs was -48±1.6 mV and that of mEPSCs was -5±2.8 mV (N=6 cells from 3 animals and N=4 cells from 3 animals for IPSCs and EPSCs, respectively). During low-[Mg\textsuperscript{2+}] induced recurrent SLEs cells were held either close to 0 mV (N=23 SLEs from 10 cells of 5 animals) or to -45 mV (N=17 SLEs from 8 cells of 5 animals) to record GABA\textsubscript{ergic} (outward) and glutamatergic (inward) currents, respectively (Figure 3A and 4A). In order to record the two components as separate as possible the exact holding potential for each experiment was determined by adjusting it in 5 mV steps to a level where outward or inward sPSCs (as appropriate) just disappeared.

Fp discharge-associated inward and outward current transients resulted from temporal summation of individual postsynaptic currents (Figure 3C, open arrow on clonic trace), as confirmed by their high amplitude, slow rise-time and rugged, uneven onset (Figure 3A, 3B, 3C and 4A). These are hereby referred to as compound postsynaptic currents (comPSC). Their 10-90 % rise time therefore estimates the synchrony of underlying individual PSCs (Lasztóczi
et al. 2004; faster rise indicates higher synchrony). During the preictal period (from p-5 to SLE onset) inward comPSC rising did not change (Figure 3B). The 10-90 % current rise times for discharges p-5 to SLE onset were 48±20 ms, 55±14 ms, 60±17 ms, 49±15 ms, 53±15 ms and 38±22 ms (N=6, 9, 10, 14, 17 and 17 SLEs from 3, 5, 6, 8, 8 and 8 slices, respectively; P=0.04 by ANOVA, but no significant differences within the preictal period with Bonferroni’s post-hoc test; Figure 3D, grey circles). By contrast, when cells held close to 0 mV, marked changes were observed in outward (GABAergic) current waveforms. The rising phase of comPSCs associated with early preictal discharges was slow (rise times frequently >100 ms), while comPSCs associated with late preictal discharges and SLE onsets rose fast with sharp upstroke (rise times frequently <20 ms; Figure 3C). Mean rise times from p-5 to SLE onset showed a decreasing trend and were 208±167 ms, 142±119 ms, 184±193 ms, 136±129 ms, 50±61 ms and 29±24 ms, respectively (Figure 3D, black circles; N=5, 7, 14, 22, 23 and 23 SLEs from 4, 5, 7, 10, 10 and 10 slices, respectively). The differences within outward comPSC rise times were highly significant (P<<0.05 for differences p-5 to p-1 and p-5, p-4, p-3 and p-2 to SLE onset; ANOVA followed by Bonferroni’s post-hoc test). Amplitudes were normalized to average preictal period values (including SLE onset) that amounted to 1116±446 pA and 1108±458 pA (outward and inward, respectively). Amplitude of glutamatergic currents showed mild increasing trend during the preictal period (Figure 3E, gray circles) but this reached significant levels only between p-5 and p-2, while outward currents showed no change but a significant drop to 76±20 % at SLE onset (Figure 3E, black circles; P<<0.05). This drop may in part result from SLE onsets frequently starting from an elevated baseline (residual current from the last comPSC; see Figure 4B and Discussion).

Entering the tonic period there was a further substantial drop in GABAergic comPSC amplitude (to ~10 %), followed by a gradual increase, regaining the original (preictal period) amplitude at SLE end (Figure 3A and 3E, black circles; P<<0.05). In spite of increasing...
tendency, rise time of the outward comPSCs remained reduced from the SLE onset onwards (no further significant differences; Figure 3C and 3D, black circles). The glutamatergic inward comPSCs showed decreased amplitude and rise-time from the start of the tonic phase (P<<0.05; Figure 3E). The amplitude remained low for the rest of SLE (no further significant change), but the rise time gradually regained its original value during the clonic phase (Figure 3B and 3D, gray circles; P<<0.05). The temporal sequence of synaptic inputs was explored by time aligning current recordings from CA3 pyramidal cells to the start of preictal, tonic and clonic fp discharges. At holding potentials close to 0 mV (Figure 4A-C; N=10 SLEs) substantial outward currents were present already before and at the start of preictal discharges and SLE onsets (Figure 4C). We quantified the synaptic input at discharge start by the mean current found in the zero 3 ms bin (e.g. from -1.5 ms to +1.5 ms). This was 715±422 pA, 596±235 pA, 683±270 pA, 729±396 pA, 855±318 pA and 620±336 pA for GABAergic currents of discharges p-5 to SLE onset, respectively (N=3, 4, 6, 10, 10 and 10 SLEs, respectively; Figure 4E, upper plot). Inward comPSCs of preictal discharges and SLE onsets (cells clamped close to -45 mV) started at or after the start of the fp discharge (Figure 4A, 4B and 4C), and thus displayed small zero bin currents (55.9±16.3 pA, 14.4±2.15 pA, 64.5±37.4 pA, 53.5±44.5 pA, 82.6±106 pA and 135±83.5 pA from p-5 to SLE onset, respectively; N=3, 4, 8, 8 and 8 SLEs, respectively; Figure 4E, upper plot). Although the driving forces for the two types of comPSCs should be similar when recorded at the reversal potential of the other (they are at exactly the same distance from their reversal potential) other sources of variability could also affect these measurements. In order to assess the temporal organization of synaptic inputs with the exclusion of confounding amplitude changes, the current transients were scaled between the minimum and maximum found in the ±100 ms window, and the scaled currents were averaged across SLEs, giving fractional currents for the period sampled (Figure 4D). A progressive increase in the fractional zero bin (-1.5 ms to 1.5 ms) outward currents
was observed from p-5 to SLE onset (Figure 4E, bottom plot), while the peaks of the average
currents shifted closer to discharge start (+44.9 ms, +66.2 ms, +31.8 ms, +14.5 ms, +7.6 ms
and +4.4 ms from p-5 to SLE onset, respectively; exemplified with p-2 and SLE onset traces
displayed in Figure 4C and D). Contrasting the outward currents, fractional inward currents
were small within the zero bin throughout the preictal period (Figure 4E, bottom plot).
Moreover, instead of displaying clear peaks, average glutamatergic currents often did not start
to decay within the analysis window (Figure 4C and D). Statistical analysis disclosed
significant differences between zero bin fractional inward and outward currents for all
discharge types. Within inward and outward current group, comparisons showed significant
differences between outward fractional currents of p-4 and SLE onset only (P<<0.05).
Similarly to the SLE onset the scaled and averaged GABAergic outward current of the tonic
and clonic discharges peaked close to the discharge start (at +0.4 ms and +2.6 ms,
respectively), and displayed large fractional currents within the zero bin, not significantly
different from the SLE onset. The peak of the average inward current associated with tonic
discharges (Figure 4D) at +13 ms indicated that the glutamatergic component lagged behind
the start of discharge and the GABAergic current. Indeed fractional zero bin inward currents
were significantly smaller than the respective fractional outward currents (P<<0.05).
These data brought to surface a clear temporal sequence of synaptic events associated with
fp discharges: a transient and highly synchronized GABAergic input followed by a sustained
glutamatergic input. To further test the validity of this scenario, we recorded a combination
(−sum) of GABAergic and glutamatergic currents during SLEs by voltage clamping CA3
pyramidal cells to holding potential halfway between the reversal potentials for GABAergic
and glutamatergic currents (around −30 mV; N=9 SLEs from 5 cells; Figure 4A, 4B and 4C).
These recordings showed that the preictal discharges, the SLE onsets (except one with no
discernible GABAergic transient) and the clonic discharges started with an outward current
coincident with the start of fp discharges, and only later the inward component prevailed (Figure 4B and 4C). This is exactly what one would expect if the scenario outlined above was valid. The outward component associated with the start of tonic discharges was much smaller, if discernible at all (Figure 4C).

**Pyramidal cell spike-timing depends on intracellular [Cl⁻]: current clamp recordings**

The cell attached recordings disclosed pronounced synchronization and intense firing of CA3 pyramidal cells in a narrow time-window that is dominated by GABAergic input. One possible explanation for this paradox finding is that at the start of some discharges the inhibition provided by GABAergic input is diminished or GABAergic currents are functionally excitatory due to a shift in Cl⁻/HCO₃⁻ distribution. To explore how changes in GABAergic function would affect the spike timing we recorded and compared the timing of fp discharge-associated spikes from CA3 pyramidal cells in whole cell current clamp mode, with low-[Cl⁻] (10 mM) and high-[Cl⁻] (60 mM) intracellular solutions. Determined for pharmacologically isolated mIPSC under control conditions, reversal potential of GABAergic currents changed from -65±1.7 mV (low-[Cl⁻]; N=4 cells from 3 animals) to -42±1.8 mV (high-[Cl⁻]; N=5 cells from 3 animals) between the two intracellular solutions. Depending on the validity of our hypothesis forcing the GABAergic input hyperpolarizing or depolarising with setting the intracellular [Cl⁻] low or high should result in differential spike timing at the start of discharges.

With the low-[Cl⁻] solution in pipettes (N=12 cells from 8 animals) cells had resting membrane potential and input resistance of −79±8.6 mV and 189±66.0 MΩ, respectively. Depolarising current injections evoked a train of overshooting action potentials, starting with intrinsic burst in nine cells. Ten of the recorded cells were filled with AlexaFluor 488 (100-200 μM) and observed under confocal microscope. They showed morphology characteristic of pyramidal cells (data not shown). Perfusion of slices with low-[Mg²⁺] ACSF resulted in the
depolarization of cells to \(-72\pm 3.0\) mV (P<0.05; paired \(t\)-test; measured before SLEs). SLEs (N=37, from 12 cells of 8 animals) were associated with slowly decaying plateau-like depolarisation to \(-50\pm 4.0\) mV (Figure 5A), and were followed by membrane potentials significantly more negative than before SLEs \((-77\pm 4.8\) mV; P<0.05, paired \(t\)-test).

Instantaneous action potential frequencies were always <170 Hz.

Preictal discharges and SLE onsets were accompanied with depolarisations and action potential firing (Figure 5A and 5C). The spike probability histogram of early preictal discharges showed that pyramidal cells did not fire before or at the start of the event, but firing probability smoothly increased during the first 40-50 ms of the discharge (plot ‘p-4’ in Figure 5C; pooled for 27 SLEs from 7 animals). The firing pattern associated with SLE onset was markedly different (Figure 5A and plot for the SLE onset in Figure 5C; pooled for 37 SLE onsets from 8 slices). Firing probability remained low (0.08) at the start of fp discharge, but increased sharply shortly afterwards, and reached peak (0.41) at the +6 ms bin. This pattern was adequately described by the sum of two components (see Methods): a sharper and earlier, peaking at +4.6 ms and a wider and later peaking at +19 ms. Spike probability histograms of preictal discharges (plots ‘p-4’ to ‘p-1’ in Figure 5C; pooled for 27, 34, 36 and 37 discharges of 7, 7, 7 and 8 animals, respectively), disclosed the evolution of this pattern with increasing and accelerating fast fit component (peak times of +15 ms, +16 ms, +10 ms and +4.9 ms for p-4 to p-1 discharges, respectively; Figure 5C). Spike probability in zero bins was relatively low throughout the preictal period and the SLE onset indicating that not much firing was associated with the start of discharges (Figure 5A and 5C). Clonic discharges were also associated with depolarising potentials and action potential firing (Figure 5A and 5C). The overall firing pattern associated with clonic discharges was similar to that of the SLE onset (Figure 5A and 5C, pooled for 37 SLEs from 12 cells of 8 animals). As indicated by the low value of firing probability at the zero bin, spikes rarely timed to the start of discharge, but
were synchronized to the +9 ms bin (Figure 5A and 5C). Indeed, in several cases clonic
discharges and SLE onsets started with a short hyperpolarisation under whole cell conditions
(data not shown), thus displaying clear inhibition during a time period when cells recorded in
cell attached mode showed peak firing. Tonic discharges of the first few seconds, as well as
secondary preictal and secondary clonic discharges often lacked action potentials probably as
a consequence of depolarisation induced inactivation (Ziburkus et al. 2006). The histograms
of tonic (Figure 5C; pooled for 37 SLEs from 12 cells of 8 animals), secondary preictal and
secondary clonic (not shown) discharges had peaks lagging behind start of fp discharge
(Figure 5C). Noticeably, the spike probabilities in zero bin were much lower than peak values.

We also recorded CA3 pyramidal cells filled with high-[Cl\textsuperscript{−}] intracellular solution that made
GABA\textsubscript{ergic} inputs depolarising (see above; N=11 cells from 6 animals, Figure 5B and 5D).
Resting potential of the cells was -73±7 mV which increased to -60±6.6 mV after changing
the perfusion solution to low-[Mg\textsuperscript{2+}] ACSF. In all cases rhythmic membrane depolarisations
(of 24±12 mV amplitude) associated with action potential bursts appeared after a few minutes
in low-[Mg\textsuperscript{2+}] ACSF. Membrane potential changes associated with SLEs were similar to
those recorded from cells filled with low-[Cl\textsuperscript{−}] intracellular solution (plateau like
depolarization to -44±4 mV, and hyperpolarisation to -79±5 mV after SLEs).

Preictal discharges (p-4, p-3, p-2, p-1) and SLE onsets (pooled for 6, 9, 11, 13 SLEs from
4, 5, 5, 5 animals, respectively) were accompanied with depolarisations and action potential
firing (Figure 5B and 5D). However, in contrast to the results obtained with low-[Cl\textsuperscript{−}]
intracellular solution the spike probability histogram of preictal discharges p-4 to p-1 showed
that pyramidal cells often fire before or at the start of the event, but less after that (Figure 5C
and 5D). Cell firing associated with SLE onset showed a different pattern. Spike probability
peaked in the -3 ms bin and was smaller but still high in the 0 ms bin. Spiking activity – just
like in case of preictal discharges – decreased following the discharge start (Figure 5B and
Clonic discharges were also associated with depolarising potentials and action potential firing (Figure 5B and 5D pooled for 16 SLEs from 11 cells of 6 animals). Firing probability started to increase and actually reached its peak (at -3 ms) before the discharge start, remained high in the 0 ms bin and dropped sharply in the forthcoming bins. This pattern was described by the sum of two components: a sharper, peaking at -1.8 ms and a wider and smaller peaking at -5.8 ms (due to the small number of action potentials we did not use fits to analyse preictal discharges and SLE onsets for the high-[Cl⁻] intracellular solution). The high firing probability preceding the preictal, SLE onset and clonic discharges (Figure 5B and 5D) can be explained by the intense (and in this case excitatory) GABAergic input preceding these discharges (data not shown, but see Figure 3C and Fig 4C), while the silencing after the discharge start is most likely the consequence of inactivation caused by the joint action of glutamatergic and depolarising GABAergic inputs. Under high-[Cl⁻] conditions GABAergic excitation may contribute to depolarization induced block of action potentials during the tonic phase as well, as firing probability associated with tonic discharges (Figure 5B and 5D pooled for 16 SLEs from 11 cells of 6 animals) was low but precisely timed to the start of the discharge when compared to the low-[Cl⁻] situation (compare Figure 5D to Figure 5C).

These data show that the spike timing of CA3 pyramidal cells at the start of SLE onset and clonic discharges is dependent on intracellular [Cl⁻], and is thus critically regulated by GABAergic inputs. Since under undisturbed intracellular conditions (cell-attached measurements) the spike timing at these SLE stages resembled the spike timing observed with high-[Cl⁻] rather than low-[Cl⁻] intracellular solution, our data also imply a relatively high GABA reversal potential at the SLE onset and throughout the SLE.

*HFOs at SLE onset are confined to the start of discharge*

Although 400-800 Hz fp activity was present during interictal periods as well, large increases in this band were associated with preictal discharges, SLE onsets and clonic
discharges. (N=70 SLEs from 70 slices of 38 animals, Figure 6A; see Methods). At SLE
onsets, clonic discharges and some late preictal discharges HFOs appeared as short (5-20 ms)
epochs of fp activity confined to the start of these discharges (Figure 6B; Lasztóczi et al.,
2004). Sometimes – especially at SLE onsets – these HFOs were composed of only a few
cycles. Temporal patterns of HFOs were investigated by averaging the HFO intensity traces
for distinct discharge categories across different SLEs with the start of fp discharge assigned
as the zero time point (Figure 6C). For early preictal discharges (see the plot ‘p-5’ on Figure
6C; pooled for 23 SLEs) large HFO intensities were distributed across long periods (~100 ms)
mainly after the start of the discharge. Only minor changes in temporal HFO intensity
distribution were observed for the next preictal discharges (p-4 to p-1; N=32, 47, 60 and 69
SLEs, respectively; Figure 6C), although at p-1 a peak located very close to the start of the
discharge (at +0.8 ms) emerged. The HFO intensity distributions for SLE onset (N=70 SLEs)
and clonic discharges (N=70 SLEs), displayed much clearer peaks again very close to the start
of discharge (both at +0.4 ms). HFO intensity had peaks associated with the start of tonic and
secondary discharges (0 ms; tonic; pooled for 70 SLEs), or very close to those (at +0.6 ms and
+0.4 ms, for secondary preictal and secondary clonic discharges, respectively). These data
identify a short epoch of HFO in the 400-800 Hz range confined to the start of fp discharges
at the SLE onset and during subsequent SLE periods (Lasztóczi et al., 2004).

*SLEs are generated within the network of the CA3 region*

Trevelyan et al. (2006) described synaptic input sequences resulting from discharge spread
in the neocortex. In order to interpret our data, it is essential to know if SLEs (and individual
discharges within) are generated locally or the CA3 network is driven by synchronized input.
When the CA3 region was isolated from other hippocampal regions by a knife cut (N=4 slices
from 4 animals) after the third SLE had occurred, subsequent to a 10-15 minute-long silent
period SLEs reappeared in the CA3 region in 3 out of 4 slices. This proves that the isolated
CA3 is able to generate and support epileptiform activity, but does it generate SLEs in the intact slice preparation as well? To answer this question we mapped potential sources of synchronized input to the CA3 with double extracellular recordings. Concurrently with large amplitude (>1 mV) SLEs in the CA3, only tiny (usually <0.2 mV) fp fluctuations (4 slices) or no activity (2 slices) were detected in the granule cell layer of the dentate gyrus. Recordings from layer II/III of the entorhinal cortex (N=8 slices), disclosed epileptiform activity that was temporally uncoupled from the activity in the CA3 in 6 slices and SLEs delayed in the entorhinal cortex by 16-47 s in two slices. Even if the SLE activity was overlapping in the two regions, individual discharges showed no temporal correlation. SLEs in the CA3 region had always their counterparts in the CA1 region (N=8 slices). The delay (spread time from CA3 to CA1) was positive for the preictal, the secondary preictal, the tonic, the clonic and the secondary clonic discharges (in 92±8.9 %, 99±2.6 %, 95±6.3 %, 98±2.7 % and 93±7.1 %, respectively). These data strongly argue that SLEs and their individual discharges were generated within the CA3 network.

The role of GABA in regulating SLE genesis of juvenile slices

The above results clearly indicate that an intense GABA_A receptor dependent activity is present at the SLE onset in a region where the discharges are originating from, coincident with high firing probability of pyramidal cells. To explore how these currents are involved in the regulation of SLE recurrence patterns we applied a selective GABA_A receptor antagonist, picrotoxin (PTX).

In voltage clamp experiments with the cells held at 0 mV, PTX blocked outward currents associated with SLE onsets, thus providing pharmacological evidence on the GABAergic nature of these currents (Figure 7A and 7E). The effect was dependent on PTX concentration (1µM PTX: 74±6%, P<0.01, N=3, 10 µM PTX: 7±3%, P<0.001, N=4, 100µM PTX: 0.2±1%, p<0.001, N=3; outward current amplitude in percent of control outward currents at the SLE
onset; Figure 7A). PTX significantly decreased inter-SLEs intervals, but only higher concentrations were effective (10 µM PTX: 62±7%, N=5; 100 µM PTX: 45±11%, p<0.01, N=5; mean ± SE, in percent of control; Figure 7B and 7E). 10 µM PTX decreased the number of preictal discharges before SLEs (25±16%, P<0.01, N=5; mean ± SE, in percent of control) but did not change the duration of SLEs (Figure 7C and 7D). Effects of 100 µM PTX on the number of preictal discharges were not determined because due to the fast recurrence (small inter-SLE interval) the last clonic discharges of an SLE were hard to discriminate from the preictal discharges of the next). After 20 minutes of washout of either 1 µM or 10 µM PTX, the amplitude of outward currents were not significantly different from control values, and inter-SLE intervals were not different from values measured in control slices. Interestingly, the number of preictal discharges remained significantly lower than control (1µM picrotoxin: 30±20%, 10 µM picrotoxin: 44±25%, p<0.05, N=5, mean ± SE, in the percent of control).

Discussion

By examining the functional dynamics underlying the genesis of spontaneous, recurrent SLEs in the CA3 region of the hippocampal slices from juvenile rats (P10-13) we have found parallel changes in fp discharge waveform, HFO, synaptic input dynamics and CA3 pyramidal cell spike timing characteristics during the preictal period. All these changes reflect the gradual build-up of a network state that can be referred to as ictogenesis culminating in the SLE onset. Figure 8 summarizes the most important properties (Figure 8A) and the development (Figure 8B) of this network state. Once this network state has been reached, it is maintained throughout the SLE (Figure 8B) as to most parameters showed similar dynamics during the SLE onset and primary clonic discharges. The most striking feature of the temporal organization of synaptic currents impinging onto single CA3 pyramidal cells at the instant of SLE onset is the advanced arrival of GABAergic input followed by glutamatergic excitation (Fig 8A, see also Fig 4). Similar temporal sequence of synaptic inputs has been shown to
represent inhibitory mechanism temporarily blocking the spread of epileptiform discharges in the neocortex (Trevelyan et al., 2006). The situation in the hippocampus is somewhat different, as this pattern is not associated with SLE spread but ictogenesis instead, most probably because the discharges are generated within the network of hippocampal CA3 under our conditions. The question arises how the CA3 pyramidal cells are recruited into the SLE onset and subsequent discharges, once they receive such a strong GABAergic inhibition first? When their intracellular milieu is intact, e.g. recorded with cell-attached pipettes, pyramidal cells show maximal firing precisely at the start of the SLE onset (and clonic discharges) at a time when they receive mostly GABAergic input (Fig 8A). Intense and synchronous firing of pyramidal cells is also reflected by the strong glutamatergic input that follows the synchronized GABAergic input with a delay consistent with monosynaptic activation. A substrate to such a monosynaptic fast excitation is most likely the abundant recurrent collateral system of the CA3 region (Li et al., 1994; Debanne et al. 1995, Wittner et al. 2007).

This tight temporal association between GABAergic input and CA3 pyramidal cell firing, together with limited coincident glutamatergic drive is paradoxical given that at the developmental stage of the animals used in the present study (P10-13) GABA provides hyperpolarizing/shunting inhibition to CA3 pyramidal cells (Rivera et al. 1999; Tyzio et al. 2007). Our observation that when recorded in whole cell mode timing of action potentials is critically dependent on the Cl⁻ concentration of intracellular solution suggests that, indeed functional state of GABA_A receptor mediated inhibition is critical for the spike timing of pyramidal cells at the start of discharges. As the unperturbed pyramidal cells showed spiketime similar to high-[Cl⁻] loaded cells we conjecture that GABAergic currents are excitatory and (at least partially) drive the cells to threshold at the start of SLE onset and clonic discharges. However, the fact that GABA_A receptor blockade by PTX decreases inter-SLE interval indicates that during the time between two SLEs, GABA might provide inhibition,
just like under control conditions (Rivera et al. 1999; Tyzio et al. 2007). Together these data point to a possible activity-dependent depolarising shift in the GABA<sub>A</sub> reversal potential during the preictal period. For the early postnatal days GABA action is depolarising and remains vulnerable even after P10 (Dhzala et al. 2003b; Khazipov et al. 2004; Le Van Quyen et al. 2006; Tyzio et al. 2007). Increasing extracellular [K<sup>+</sup>] (to 5 mM in our case) further challenges the systems responsible for the Cl<sup>-</sup> homeostasis maintaining hyperpolarizing GABA<sub>A</sub> receptor mediated currents (Dhzala et al. 2003b; Korn et al. 1987; Traynelis and Dingledine 1988). Under such circumstances excessive GABAergic activation may induce activity-dependent rearrangement of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> distribution (Staley et al. 1995; Kaila et al. 1997; Lamsa and Taira 2003; Fujiwara-Tsukamoto et al. 2007), a critical determinant of the functional effect of GABA<sub>A</sub> receptor mediated conductance (Staley et al. 1995; Lamsa and Kaila 1997; Rivera et al. 1999). Our data imply intense GABAergic input to CA3 pyramidal cells during all preictal discharges that may well result in activity-dependent weakening of GABAergic inhibition or eventually emergence of GABAergic excitation during the preictal period. The extent of anion gradient rearrangements is also frequency- and synchrony dependent (Staley et al. 1995; Lamsa and Taira 2003). Thus the synchronisation of GABAergic inputs during the preictal period may actively support such a process and may represent a vicious circle of ictogenesis. In support of the requirement of functional alterations in GABA<sub>A</sub> receptor mediated effects for SLEs to occur we demonstrated that PTX shortens inter-SLE interval and reduces the number of preictal discharges. The tight temporal association between GABAergic inputs and CA3 pyramidal cell firing developed gradually during the preictal period. In cell attached mode, firing only partially overlapped with GABAergic input during preictal discharges but was coincident at SLE onset, tonic and clonic discharges (Figure 8B). However, when the intracellular [Cl<sup>-</sup>] was high even the small GABAergic input resulted in strong firing (e.g. before the start of early preictal discharges).
By contrast, under low intracellular [Cl\(^-\)] condition the GABAergic input coincided with suppressed firing even for late SLE stages, such as SLE onset or clonic discharges, and CA3 pyramidal cell spike distribution came closer to the glutamatergic input dynamics (Figure 8B: tonic and clonic discharges). Role for depolarising GABA in ictogenesis was suggested by several studies conducted using different seizure models (Lopantsev and Avoli 1998; Köhling et al. 2000; Cohen et al. 2002; Dzhala and Staley 2003b; Perez Velazquez, 2003; Fujiwara-Tsukamoto et al. 2003, 2007; Khazipov et al. 2004; Dzhala et al. 2003b, 2005), which might be further modulated by differential regulation of spatially segregated GABAergic responses on dendritic and somatic receptors (Cossart et al. 2001, Gulledge and Stuart 2003; Marty and Llano 2005, Romo-Parra et al. 2008). The activity-dependent changes in anion distribution and consequent mutual excitation of interneurons (Lamsa and Taira 2003; Fujiwara-Tsukamoto et al. 2007), together with gap junction coupling (Beierlein at al., 2000; Nyikos et al. 2003; Tamás et al. 2000) might also contribute to synchronization of GABAergic cells.

Hippocampal interneurons, the most probable players of timing are heterogeneous (Freund and Buzsáki 1996; Parra et al. 1998; Somogyi and Klausberger 2005), have differential effects on principal cells (Miles et al. 1996) and diverge in their relation to physiological oscillations (Klausberger et al. 2003; Somogyi and Klausberger 2005) and SLE patterns (Fujiwara-Tsukamoto et al. 2004; Ziburkus et al. 2006; Spampanato and Mody 2007). Although further research on firing patterns of identified interneurons is required to disclose the source of GABAergic inputs described here, one possible candidate is axo-axonic cells, showing a firing pattern consistent with such a role during tetanically induced after-discharges (Fujiwara-Tsukamoto et al. 2004), and evoking excitatory responses under control conditions in vitro (Szabados et al. 2006; but see Glickfeld et al., 2008). The function of GABAergic input is also outlined by its pronounced reduction during the tonic period, most probably due to action potential inactivation in interneurons (Ziburkus et al. 2006) or reduction of driving
force. This may result in a runaway excitation through tonic period (Ziburkus et al. 2006), but as soon as GABAergic input recovers to amplitude comparable to glutamatergic, the pattern of clonic activity with all its characteristic similarities to the SLE onset returns.

Our results show that at the start of SLE onset and clonic discharges intense firing of CA3 pyramidal cells is not only coincident with a strong GABAergic synaptic input but also with a negative fp deflection-associated short epoch of 400-800 Hz fp HFO (Lasztóczi et al., 2004). The idea of a causal relationship between HFOs (>200 Hz) and seizure genesis is based on spatial and temporal correlation of the two. In a series of studies Bragin et al. (1999, 2000, 2003) have shown that in vivo oscillatory activity >200 Hz is spatially restricted to the epileptogenic focus, and formulated a hypothesis that the emergence of a hyper-synchronous neuronal sub-network drives seizures (Bragin et al. 2002). Pathological HFOs are not only spatially restricted to the site of ictogenesis but they are also enhanced immediately at the seizure onset in both animal models (Bragin et al. 2005) and human temporal lobe epilepsy (Jirsch et al. 2006) suggesting an even more direct link between the two phenomena. In vitro studies of HFO have shown that epileptogenesis (Khalilov et al. 2005; Mochovos et al. 2008) and ictogenesis (Dzhala and Staley 2003a; Khoshrvani et al. 2005; Lasztóczi et al. 2004) are dependent on HFOs. In the low-[Mg²⁺] model oscillatory activities >400 Hz behave distinctly from those <400 Hz (Lasztóczi et al. 2004), but whether >200 Hz activities are heterogeneous is at present unknown (Buzsáki and Draguhn 2004). At the SLE onset and at the start of clonic discharges transient GABAergic input coexists with the 400-800 Hz HFO, while glutamatergic excitation is largely absent. This may indicate GABAergic mechanisms as the source of fp HFO. Indeed, GABAergic mechanisms were implicated in the generation of fast rhythms, including ripples (Ylinen et al. 1995), sensory-evoked HFOs ( ~600 Hz; Jones and Barth 2002), neonatal network oscillations (Palva et al. 2000), and oscillations associated with epileptiform activity (Khalilov et al. 2005). However, pyramidal cell firing associated with
HFO was also observed in the present study, and some HFOs are resistant to GABA<sub>A</sub> receptor blockade (Behrens et al. 2007). As an alternative mechanism, Dzhala and Staley (2004) suggested that fast ripples are initiated and synchronized by excitatory interactions between pyramidal cells. In case, the maximum inter-spike interval recorded in the cell-attached mode (~3 ms in the present study; Dzhala and Staley, 2004) would give ~300 Hz oscillations, much like those superimposed on sustained phase of preictal discharges (Dzhala and Staley, 2004; Lasztóczi et al. 2004; Khoshravani et al. 2005) but slower than HFOs found at start of discharges (Lasztóczi et al. 2004; this study). This hypothesis could however still account for short 400-800 Hz epochs assuming two or more misaligned pyramidal cell subpopulations (Foffani et al. 2007).

In conclusion our data point out that at the SLE onset (and further discharges of SLE) there is a close temporal association between network phenomena such as a strong GABAergic input to, and intense firing of CA3 pyramidal cells, and HFOs in the CA3 pyramidal layer. The paradox co-existence of GABAergic input and intense firing together with a delayed glutamatergic excitation to these same cells, suggests that at the SLE onset GABA is excitatory rather than inhibitory, and might actively contribute to synchronization of pyramidal cells. Underlying the gradual development of network synchrony the genesis of epileptiform activity may possibly involve an activity-dependent depolarizing shift of GABA inputs during the preictal phase.

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References


Legends to figures

Figure 1. Temporal structure of SLEs

(A) Representative fp recording of an SLE from the CA3 pyramidal layer of a hippocampal slice from a P12 rat. The bar above marks different SLE periods. (B) Discharges of different periods on a faster time scale. Traces are enlarged from SLE in (A). The four preictal discharges on the left are numbered from early to late as p-4, p-3, p-2 and p-1. Note that from p-1 onwards, all discharges start with negative fp deflections. In subsequent analyses the peaks of these negative fp deflections are used for the temporal alignment of discharges. Arrowheads mark secondary discharges.

Figure 2. Spike timing of CA3 pyramidal cells during SLEs.

(A) A representative SLE recorded simultaneously from CA3 pyramidal layer and from a CA3 pyramidal cell in cell attached mode (38 μm apart). Open and filled arrowheads point to preictal discharges and SLE onset, respectively. The arrow marks the position of the tonic-to-clonic transition. (B) The preictal period of the SLE in (A) on a faster time-scale. Open and closed arrowheads point to discharges as in (A). (C) fp (grey traces) and cell attached (black traces) recordings of individual discharges time-aligned to the start of fp discharges (vertical dashed lines). On left hand plots preictal discharges and the SLE onset are displayed. Seven representative tonic and seven representative clonic discharges are overlaid in middle and right hand plots, respectively. (D) Spike probability histograms deduced from cell attached recordings for different discharge categories (as indicated). Vertical dashed lines indicate the time of discharge starts in the fp trace. Black traces are fits and their sums.

Figure 3. Changes in glutamatergic and GABAergic currents during SLEs.
Representative SLEs recorded from CA3 pyramidal cells under voltage clamp at −40 mV (upper trace) and 0 mV (lower trace). Arrows mark the tonic-to-clonic transition. (B and C) glutamatergic inward (B) and GABAergic outward (C) current transients associated with preictal discharges and SLE onset (upper traces), five representative tonic discharges (middle traces) and five representative clonic discharges (bottom traces). Open arrow in C marks an individual postsynaptic current with different (faster) kinetics. Traces are scaled vertically and aligned to peaks. For clarity the decay of the preceding p-1 was omitted for the SLE onset trace of the GABAergic input. Traces are enlarged from SLEs in (A). (D and E) Mean rise-time (D) and amplitude (E) of GABAergic (black) and glutamatergic (grey) comPSCs associated with discharges of different SLE periods. Amplitudes were normalized to the mean of preictal period. O: SLE onset.

Figure 4. Temporal patterns of synaptic inputs during SLEs.

(A) Representative SLEs recorded simultaneously from CA3 pyramidal layer (fp; upper traces) and from a voltage clamped CA3 pyramidal cell located 78 μm apart (lower traces). The cell was voltage clamped at −45 mV (upper plot), 0 mV (middle plot) or −25 mV (bottom plot) to record pure glutamatergic, pure GABAergic, or combined currents, respectively. (B) Preictal periods of SLEs in (A) displayed on a faster time scale. Open arrowheads mark the preictal discharges while the filled arrowhead the SLE onsets. (C) fp discharges (grey) and current transients (black) from SLEs in (A). Discharges were scaled vertically and aligned to the start of the fp discharge (vertical red lines). For tonic and clonic periods 5-5 representative discharges are displayed. (D) Mean fractional GABAergic (red line) and glutamatergic (green line) currents in an analysis window of ±100 ms. Red and green shaded areas represent the ± SE range. The zero bin (from −1.5 to +1.5 ms) is indicated by the grey vertical bar. For the ease of kinetic comparison both outward and inward currents are presented in upward
direction. (E) Mean GABAergic (red) and glutamatergic (green) currents (upper plot) and fractional currents (lower plot) at the discharge start (within the zero bins) for discharge categories as indicated on the abscissa.

**Figure 5. Spike timing of current-clamped pyramidal cells during SLEs**

(A and B) Representative fp (grey traces) and current-clamp (black traces) recordings with low 10 mM [Cl⁻] (A) and high 60 mM [Cl⁻] (B) pipette solution of individual discharges time-aligned to the start of the fp discharges (vertical dashed lines). On left hand plots preictal discharges and the SLE onset (bottom traces) are displayed. Five representative tonic and five representative clonic discharges are overlaid in middle and right hand plots, respectively. (C and D) Spike probability histograms for different discharge categories as indicated above the plots. The starts of individual fp discharges were assigned as the zero time-point (vertical dashed lines). Spike outputs were pooled into 3 ms bins from all discharges available. Black traces represent fit components and their sum.

**Figure 6. Temporal patterns of HFOs during SLEs**

(A) fp recording (upper trace) of an SLE from the CA3 pyramidal layer, the trace band-pass filtered between 400 and 800 Hz (middle trace), and fp intensity in 400 to 800 Hz band integrated with a 3.2 ms sliding window (bottom trace). Open and filled arrowheads point to preictal discharges and SLE onset, respectively. The arrow marks the position of the tonic-to-clonic transition. (B) The SLE onset and a clonic discharge enlarged from the SLE shown in (A). The traces are as in (A). The vertical dashed lines mark the discharge start. (C) Average intensities of activity in 400-800 Hz band during different discharge categories (as indicated above the plots). The grey shaded areas represent the ± SE ranges. The vertical dashed lines mark the point of alignment (discharge start).
Figure 7. The effect of picrotoxin on the amplitude of the outward currents associated with SLE onsets and different SLE parameters

(A) Effect of different concentrations (1 µM, 10 µM and 100 µM) of picrotoxin on the amplitude of outward currents associated with the SLE onset. (B) Effect of different concentrations (1 µM, 10 µM and 100 µM) of picrotoxin on inter-SLE interval. (C) Effect of different concentrations (1 µM, 10 µM and 100 µM) of picrotoxin on duration of SLEs. (D) Effect of different concentrations (1 µM, 10 µM and 100 µM) of picrotoxin on the number of preictal discharges observed before the SLEs. (A, B, C and D) Hatched bars indicate control slices, open bars 1 µM picrotoxin, light grey bars 10 µM picrotoxin and dark grey bars 100 µM picrotoxin. All values (mean ± SE) are expressed as the percent of control values, measured before drug application. *p<0.05, **p<0.01, ***p<0.001. (E) Representative recording from CA3 pyramidal layer demonstrating the effect of 10 µM picrotoxin on SLE activity (field potential trace) and on outward currents associated with the SLE onset (voltage-clamp trace).

Figure 8. Contribution of postsynaptic currents to the synchronization of CA3 pyramidal cells during the development of epileptiform activity.

(A) (Upper plot) Representative fp discharge during the clonic phase of the SLEs. (Middle plot) Mean fractional GABAergic (red line) and glutamatergic (green line) currents centred on the fp discharge start in an analysis window of ±100 ms (replotted from Figure 4D). Red and green shaded areas represent the ± SE range. For the ease of kinetic comparison both outward and inward currents are presented in upward direction. (Bottom plot) Spike probability histograms for CA3 pyramidal cell discharges relative to the fp discharge start in whole-cell current clamp using intracellular solutions with high (60 mM, purple trace) and
low (10 mM, blue trace) [Cl–] and in cell-attached mode (black trace). Spike outputs were pooled into 3 ms bins from all discharges available. The grey vertical bar indicates the zero bin (-1.5 ms to +1.5 ms).

(B) Delay of postsynaptic currents and individual CA3 pyramidal cell discharges to the start of fp discharges in the different phases of the SLEs. Red rectangle: GABAergic postsynaptic currents (see Methods); green circle: glutamatergic postsynaptic currents (see Methods); purple triangle: cell discharge under whole-cell current clamp configuration with high (60 mM) [Cl–] intracellular solution; blue upside-down triangle: cell discharge under whole-cell current clamp configuration with low (10 mM) [Cl–] intracellular solution; black cross: cell discharge under cell-attached configuration.