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Interneuron and pyramidal cell interplay during *in vitro* seizure-like events

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Abstract

Excitatory and inhibitory (EI) interactions shape network activity. However, little is known about the EI interactions in pathological conditions such as epilepsy. To investigate EI interactions during seizure-like events (SLE), we performed simultaneous dual and triple whole-cell and extracellular recordings in pyramidal cells and oriens interneurons in rat hippocampal CA1. We describe a novel pattern of interleaving EI activity during spontaneous *in vitro* SLEs generated by the potassium channel blocker 4-aminopyridine in the presence of decreased magnesium. Interneuron activity was increased during interictal periods. During ictal discharges interneurons entered into long-lasting depolarization block (DB) with suppression of spike generation; simultaneously, pyramidal cells produced spike trains with increased frequency (6–14Hz) and correlation. Following this period of runaway excitation, interneuron post-ictal spiking resumed and pyramidal cells became progressively quiescent. We performed correlation measures of cell-pair interactions using either the spikes alone or the subthreshold postsynaptic interspike signals. EE spike correlation was notably increased during interneuron DB, while subthreshold EE correlation decreased. EI spike correlations increased at the end of SLEs, while II subthreshold correlations increased during DB. Our findings underscore the importance of complex cell-type specific neuronal interactions in the formation of seizure patterns.

Keywords

hippocampus; epilepsy; inhibition; excitation; correlation

Introduction

Epileptic seizures are often described as “hypersynchronous” events arising from decreased inhibition with excessive excitation (Penfield and Jasper 1954; Wyler and Ojemann 1982; Westbrook 1991). However, inhibition is retained in samples from human epileptics (Babb et al. 1989; During and Spencer 1993; Menendez de la Prida et al. 2002), and interneuron activity is essential for the generation of *in vitro* epileptiform activity in human (Cohen et al. 2002) and animal (Higashima et al. 1996; Avoli et al. 1996a; Lopantsev and Avoli 1998; Velazquez and Carlen 1999; Fujiwara-Tsukamoto et al. 2004) tissue. Additionally, EEG

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(Schiff et al. 2005) and *in vitro* synaptic currents (Netoff and Schiff 2002) suggest that seizures may not be characterized by a straightforward synchrony increase.

Previous *in vitro* studies of intracellular excitatory-inhibitory (EI) interplay focused primarily on stimulation-induced epileptiform activity (Perez-Velazquez and Carlen 1999; Perez-Velazquez 2003; Fujiwara-Tsukamoto et al. 2004, 2005). In these paradigms, tetanic stimulation replaced pre-ictal and ictal onset dynamics and the resulting afterdischarges demonstrated synchronous activation of inhibitory and excitatory cells (Fujiwara-Tsukamoto et al. 2004). However, we know little about EI interactions and synchrony during spontaneous pre-ictal, ictal, and post-ictal events.

To investigate EI, excitatory-excitatory (EE), and inhibitory-inhibitory (II) interactions in robust seizure-like events (SLEs), we used a 4-aminopyridine (4-AP) and decreased magnesium seizure model. Simultaneous dual and triple whole-cell recordings of inhibitory oriens interneurons and excitatory pyramidal cells, along with extracellular potential measurements, were performed in the CA1 of rat hippocampal slices. We report a novel pattern of EI activity interplay during these spontaneous SLEs.

Methods

Animals

Experiments were performed on Sprague Dawley rats (P18–P35) with a George Mason University approved protocol.

Electrophysiology

Animals were anesthetized with diethyl-ether, decapitated, brains were removed, hippocampi isolated, and transverse 350 μ m sections cut in cold dissection buffer (in mM: 2.6 KCl, 1.23 NaH₂PO₄, 24 NaHCO₃, 0.1 CaCl₂, 2 MgCl₂, 205 sucrose, 20 glucose) using a vibratome.

Slices were incubated for an hour in artificial cerebrospinal fluid (ACSF; pH 7.3, 30°C, in mM: 130 NaCl, 0.6 MgSO₄, 3.5 KCl, 1.2 CaCl₂, 10 glucose, 2.5 NaH₂PO₄, 24 NaHCO₃) aerated with 95% O₂–5% CO₂, transferred to a submersion recording chamber (Warner Instruments) and perfused (1 ml/min, 34°C). Borosilicate glass micropipettes (4–7 M Ω) for current clamp recordings contained (in mM): 116 K gluconate, 6 KCl, 0.5 EGTA, 20 HEPES, 10 phosphocreatine, 0.3 NaGTP, 2 NaCl, 4 MgATP, and 0.3% Neurobiotin (pH 7.25, 295 mOsm). Extracellular recordings were performed with micropipettes (1–3 M Ω , 0.9% NaCl).

Alignment of the seizure start times was based on an identifiable feature of the extracellular recording - the fast positive extracellular shift (FPES, 1–5 mV, Fig. 1D). Oriens interneurons (Supplementary Materials) were targeted because of their importance and vulnerability in epilepsy (Oliva et al. 2002; Santhakumar and Soltesz, 2004; Sanon et al., 2005), and their role in network synchronization (Pike et al. 2000; Gillies et al. 2002). Seven of 12 interneurons that underwent depolarization block (DB) displayed orienslacunosum moleculare (OLM) interneuron membrane characteristics and bipolar horizontal dendritic morphology (Maccaferri and McBain 1996; Maccaferri and Lacaille 2003, Supplementary Materials). Cells selected for patching were 30–200 μ m apart (average 122 μ m). An extracellular electrode was placed in the pyramidal near the patched cells. A subset of experiments was performed after isolating CA1 (n=13) by making cuts from alveus to moleculare lacunosum on each side of CA1. Recordings were performed using Axoclamp 2B and 2A, MCC 700 (Molecular Devices), and Grass P16 amplifiers, filtered (5 KHz whole-cell, 1 KHz extracellular), and digitized at 10 KHz (Digidata and Pclamp7, Molecular Devices).

SLEs appeared following 100 μ M bath-application of 4-AP (Sigma) and raising the temperature to 36°C. Increasing concentrations of 4-AP were used (up to 200 μ M) if SLE formation was not observed within 20 min. We fortuitously discovered that decreasing the Mg concentration to 0.6 mM, although insufficient by itself to promote SLEs, had a profound affect on prolonging 4-AP induced SLEs. 4-AP increases inhibitory and excitatory signaling (Avoli et al. 1988, 1996b; Somjen 2004), while decreased Mg partially removes n-methyl-d-aspartate (NMDA) receptor blockade and divalent charge screening. A similar strategy employs elevated potassium with decreased magnesium (Khosravani et al. 2003, 2005; Isaev et al 2005).

Histochemistry

Neurobiotin filled cells were reconstructed with *camera lucida* (Fig. 2A and Supplementary Material). For the cells that exhibited DB, the outline of the slices and stratum pyramidale were drawn using *camera lucida* and overlaid (to the best fit) in Photoshop (Adobe; Fig. 2E).

Correlation analysis

To track the temporal evolution of EE and EI synchrony, two unbiased crosscorrelations were calculated using subthreshold membrane potentials and spike times of pairs of neurons over discrete time windows. We use crosscorrelation to infer the presence of synchronization (Netoff and Schiff 2002). Dual correlation analyses allowed us to compare synchrony in subthreshold inputs and suprathreshold neuronal outputs. Alternate methods to achieve a similar analysis used median filters to remove spikes (Mooney et al. 2001; Buonomano 2003), which alter the spectra and are less suitable for correlation measures.

For spike correlation, a simple threshold was used to identify discrete spike times. To measure subthreshold correlations, data between spike initiation and termination were discarded and correlations were performed using interspike data (Fig. 1A and 1B). The spike data gaps were retained, leaving the time relationships among the residual subthreshold voltages unchanged. This subthreshold activity reflects synaptic network inputs to the cells, as well as intrinsic voltage-gated subthreshold responses. In contrast, spike cross-correlation reflects neuronal output (Supplementary Material). We applied an estimate of the expected crosscorrelation variance given each time series' autocorrelation and time length. Only statistically significant correlations, greater than two standard deviations of the expected value, were included in the analysis (full details in Supplementary Materials).

Statistical analysis

Averaged data representing spiking rates and crosscorrelation values are reported as mean $\pm$ standard deviation (s.d.). Analysis of variance (ANOVA) was used to determine whether the means of correlation measures from distinct SLE epochs were significantly different. Post-hoc Tukey multiple comparison tests (confidence limit $p < 0.001$) were performed following ANOVA. Because the number of SLEs measured per cell pair was variable (5–18), we also recalculated averages by randomly resampling to equalize the number of SLEs compared from each pair. Both pooled and resampled averages are reported.

Results

Recurrent SLEs (>15 sec ictal-like network events with extracellular potential shifts; Fig. 1A and 1D) were recorded in 30 neuronal pairs (18 dual and 4 triple) located in strata oriens and pyramidale, yielding over 250 SLEs for analysis. Qualitatively analogous SLEs were observed in experiments in which the CA1 region was surgically isolated ($n=13$ cells), suggesting that the excitatory Schaeffer collateral pathway is not necessary for local network SLE generation in the CA1 (Barbarosie and Avoli 1997; Netoff and Schiff 2002).

Pyramidal cells (n=35) fired at lower rates than interneurons prior to SLEs. Consistent early features of SLEs were a burst of pyramidal cell spikes (~1–3 seconds), coinciding with a FPES in the extracellular and intracellular potentials followed by a sustained negative extracellular shift (1–5mV, Fig. 1D). When multiple FPESs were seen, the final one just before the slow negative extracellular potential shift was selected as the temporal anchor point for subsequent analysis.

Sub- and suprathreshold crosscorrelation analyses of excitatory cell pairs (n=15) revealed qualitatively different results (Fig. 1E, 1F). Pyramidal cell spike output increased markedly during the SLEs (n = 15 pairs, 148 SLEs) and showed a considerable increase in spike crosscorrelation (Fig. 1E). In contrast, averaged subthreshold EE correlations (n=15 pairs; 148 SLEs) showed significantly decreased correlations at the start of the ictal-like event (Fig. 1F). This finding is consistent with previous results in which voltage-activated conductances during similar recordings were blocked (Netoff and Schiff 2002).

In the presence of 4-AP, oriens interneurons were more active and fired at higher frequencies than concurrently recorded pyramidal cells (Figs. 2–3). During SLEs, the interneurons and pyramidal cells exhibited a robust spiking interplay (Fig. 2), which took place over substantially longer time scales compared to the interplay seen in interictal bursts (Supplementary Fig. 1).

Interneuron firing rates peaked at 62 Hz just prior to entering into the long-lasting (5–40 sec) DB within the ictal discharge. DB was characterized by transmembrane potential depolarization (to –40 to –10 mV) and the inactivation of action potential generating mechanisms (Bragin et al. 1997; Bikson et al. 2003; Somjen 2004). DB was observed in 12 horizontal interneurons located in stratum oriens and the pyramidal-oriens layer border (Fig. 2E). Depolarization block was rarely pronounced in pyramidal cells, and when observed was significantly shorter (< 2 sec) than in interneurons (Figs. 2D). As seen in Figs. 2–3, pyramidal cells produced sustained barages of spikes during interneuron DB, typically firing 6–15 spikes per second (average 8.28 spikes for the first 5 seconds of the ictal event). Note the fortuitous extracellular single unit recording from the axon of the interneuron in Figure 2(C and D), confirming cessation of inhibitory spiking during DB. SLE termination was accompanied by the return of interneuron activity as pyramidal cells became progressively quiescent.

Figure 3 shows an example of simultaneous EE and EI interactions during a triple whole-cell and extracellular recording of a spontaneous SLE and the respective average (n=8 SLEs) firing rates. This example illustrates that the firing rates of the two recorded pyramidal cells changed together, with peak firing rates at the onset and sustained high firing rates during the interneuron DB.

Analysis of 102 SLEs from EI pairs are shown in Figures 3C–E (n=12 pairs). There was an increase in EI subthreshold correlations at the beginning and towards the end of the interneuron DB. With resampling, the increase in subthreshold correlations towards the end of the SLEs was the most robust feature (Fig. 3C).

A significant increase in EI spike correlations was observed at the beginning (unweighted) and towards the end of the SLEs (weighted averages; about 45 seconds, Fig. 3D). These EI subthreshold and spike findings are consistent with the fact that pyramidal cells and interneurons were active together at the onset and offset of the depolarization block.

The spike rate averages (n=12 pairs, 102 SLEs) in Figure 3E show a consistent robust sequence starting with intense interneuron firing, followed by a peak in pyramidal cell firing during interneuron DB, and concluding with the return of intense inhibitory firing as pyramidal activity decays. The terminal decay in pyramidal cell firing frequency is associated with the second

peak and sustained firing in interneurons recovering from the block (about 35 seconds, Fig. 3E and inset).

Figure 4 shows an example of two interneurons participating in a SLE. Although spiking activity ceased as both interneurons entered DB, subthreshold correlation values nevertheless increased ($n=13$ SLEs, 3 pairs). The finding that pyramidal cell spike correlations are increased during interneuron DB (Fig. 3C) is consistent with synchronous excitation contributing to the subthreshold II correlations during their DB. In contrast, the loss of inhibitory spike outputs are consistent with the decrease in EE subthreshold correlations during DB (Fig. 1F).

Discussion

Our fundamental finding is the novel interleaved spiking pattern between pyramidal cells and interneurons during spontaneous *in vitro* hippocampal seizure-like events. Our analysis of spike output between EI pairs revealed that pyramidal cells and interneurons became activated at different times during SLEs. Oriens interneurons were more active at SLE onset, followed by a period of DB during which pyramidal cells exhibited runaway excitation. SLE termination was characterized by the oriens interneurons emerging from DB into progressively more active spiking and the simultaneous gradual reduction of pyramidal cell spiking.

Neuronal interplay and depolarization block may have been first observed in pioneering *in vivo* studies of single intracellular neocortical and hippocampal seizure recordings from untyped cells (Kandel and Spencer 1961; Matsumoto and Marsan 1964). More recently, a strong inhibitory component of seizures with increased inhibitory spiking before and after paroxysmal discharges was observed in single intracellular *in vivo* recordings from fast spiking putative interneurons (Timofeev et al. 2002). Additional recent *in vivo* observations have clearly shown that precisely interleaved activity of subpopulations of inhibitory cells coordinate normal hippocampal theta rhythms and sharp waves (Klausberger et al. 2003).

Examples of *in vitro* EI interplay have been observed in spontaneous bursts with elevated K^+ (Aradi and Maccaferri 2004). We have also observed similar activity in our preparation (Supplementary Fig. 1), and have observed that the spontaneous SLEs in our solution retained features of the burst EI interplay, but at the much longer time scale of seizures. This is in contrast to tetanically stimulated seizure-like afterdischarges that show an apparent EI synchronization (Perez-Velazquez and Carlen 1999; Fujiwara-Tsukamoto et al. 2004). We are aware of no other observations of such EI interactions during spontaneous SLEs.

In our work we explicitly calculated the correlation between distinct neuronal subtypes. We found that correlations during SLEs depended qualitatively upon whether subthreshold or spike information was considered. During the SLE onset, we observed a decrease in the subthreshold input correlation among EE pairs. This finding is consistent with our previous work in which we pharmacologically suppressed active conductances (Netoff et al. 2002). EE spike output correlation, on the other hand, increased during SLEs. Similar increases in spike correlation were observed *in vivo* between nearby putative neocortical pyramidal cells (Timofeev et al. 2004).

It remains to be explained how hippocampal pyramidal cells, with sparse associational connections, exhibit prominent spike synchronization in the setting of decreased subthreshold correlations. It is possible that such spikes synchronize through their ephaptic electric fields (Traub 1985a,b) or through gap junction connectivity between the axons of pyramidal cells (Draguhn et al. 1998). These effects are likely to be more effective when interneurons coordinate their DB activity via their own gap junctions (Zhang et al. 2004), thus releasing the pyramidal cells from inhibition concurrently. Indeed, our preliminary evidence suggests that

the gap junction blocker carbenoxolone in our preparation blocks SLEs, and at low concentrations, shortens DB in interneurons (Supplementary Figure 2).

In contrast to EE, EI correlation was elevated at the beginning and end of SLEs. These findings are consistent with the fact that EI spike synchrony can only occur when both populations of cells are firing – at the beginning and towards the end of inhibitory DB. The loss of inhibitory spikes during DB is coincident with the simultaneous decrease in pyramidal cell subthreshold EE synchronization. Lastly, subthreshold II correlation increased while the interneurons in DB stopped firing and pyramidal cell spike correlation increased. We suggest that correlated EE spiking during interneuron DB delivers synchronous synaptic currents to interneurons at a time during which they are unable to respond by spiking.

Nonetheless, we are cautious in evaluating data following spike extraction. The residual subthreshold time series likely undersampled synaptic currents that are coincident with spike generation, potentially leading to a spurious decrease in subthreshold EE correlation. However, our results are consistent with earlier findings during active conductance blockade (Netoff et al. 2002). It may be possible that overestimation of correlation due to the presence of active membrane conductances, and underestimation through time series undersampling following spike extraction, may have had partially offset each other.

Although, synchrony has often been implicated in seizure generation (Westbrook 1991), we are cautious in our interpretation of the observed correlation patterns in our experiments. Despite consistent patterns of correlation changes in the SLEs, we cannot infer a causal role for synchrony in orchestrating the neuronal interplay observed. Furthermore, it remains unknown what synchronization and neuronal interplay patterns will be observed in other *in vitro* and endogenous *in vivo* seizures when similarly examined.

Previous work has shown that following stimulation, chloride loading inside pyramidal cells causes inhibition to become excitatory (Fujiwara-Tsukamoto et al. 2004,2005). It is possible that excessive inhibitory activity at the start of our SLEs became excitatory in its effects on pyramidal cells. Preliminary voltage clamp observations (Ziburkus et al. 2005) suggest that not only pyramidal cells but interneurons as well may have elevated intracellular chloride in this model.

Most of our recorded interneurons resembled O-LM cells in their membrane properties (Fig. 2A) and horizontally oriented dendritic projections (Fig. 2B). O-LM cells also constitute the overwhelming majority of the oriens interneurons (Freund and Buzsaki 1996; Somogyi and Klausberger 2005). Nevertheless, we recognize that we likely included interneurons other than O-LM cells in our sampling.

Although seizures have traditionally been treated as monolithic entities – ictal versus non-ictal – there is little to support this notion. Our findings suggest that the interplay of cellular activity supports a natural partitioning of these *in vitro* SLEs into stages. The motif of excessive inhibition, subsequent inhibitory breakdown through DB, and the concurrent increase in excitatory output could underlie some of the structured patterns seen in EEGs during seizures. In recent work, discrimination of human scalp and intracranially recorded seizures into at least 3 dynamical stages was shown (Schiff et al. 2005). The experimental findings in this present report illustrate a set of cellular interactions that underlie the evolution of stages in these *in vitro* SLEs. It will remain a challenge to identify the relevant cellular mechanisms that underlie the evolution of endogenous seizure stages *in vivo*. Finally, if inhibitory and excitatory cell types fire at different times during seizures, then our findings suggest several novel therapeutic targets for seizure control, including blocking excessive inhibitory network activity and preventing depolarization block.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Analysis of the SLEs in pyramidal cell pairs

A. Left trace shows the complete voltage signal recorded from a pyramidal cell during SLE; right contains an expanded excerpt from the left. **B.** Examples of the same signal as in A with the spikes removed (regions in gray boxes). The residual interspike voltage signal was used for subthreshold correlation analysis. **C.** Photomicrograph of two neurobiotin filled pyramidal cells in the CA1. *s.* - stratum; *l.m.* - lacunosum moleculare; *r.* - radiatum; *p.* - pyramidale; *o.* - oriens. **D.** Whole-cell recordings from two pyramidal cells (triangles) and an extracellular electrode (EC). Following some pre-ictal spikes, pyramidal cells fire a prolonged burst of spikes riding on a wave of intracellular depolarization (ictal event), followed by post-ictal spikes of decaying frequency. The inverted triangle (30sec., D and E) is aligned with the FPESs used to denote the start of the ictal events. **E.** The crosscorrelation between each pair was measured within one-second nonoverlapping windows beginning 30 seconds before until 70 seconds after the FPES. Graphs of crosscorrelation values for spikes (top) and subthreshold voltages (bottom). Black diamonds represent unweighted pooled and gray squares weighted resampled averages. ANOVA results for one-second windows showed that crosscorrelation values for subthreshold voltages ($df=7199$, $F=1.38$, $p=0.0083$ for unweighted) and for the

spikes ($df=7199$, $F=14.72$, $p<0.0001$) significantly changed across the evolution of SLEs. Tukey multiple comparison tests ($p<0.001$ for unweighted and $p<0.05$ for weighted averages) confirmed that correlation values at the start of the ictal event (inverted triangle mark) significantly decreased for the subthreshold voltages and increased for the spikes.

Figure 2. EI interplay during SLEs

A. Examples of pyramidal (triangle) and oriens interneuron (ellipse) membrane properties following 500 msec (scale bar) negative and positive current injections. **B.** Camera lucida microscope drawings of the dendrites of the cells in A. s. - stratum; o. - oriens; p. - pyramidale; r. - radiatum. Scale bar - 50μm. **C.** Repetitive SLEs and EI interplay. The interneuron (ellipse) is more active in 4-AP than the pyramidal (triangle) cell. EC denotes a fortuitous extracellular single unit recording of action potentials from the axon of this interneuron. The EC electrode was placed in oriens, about 75μm from the interneuron soma. **D.** Expanded time base illustrating robust EI interplay during the SLEs. Note the cessation of axonal spikes in EC during interneuron DB. **E.** Locations of interneurons exhibiting DB. Horizontal and rounded

interneuron somata were mapped following neurobiotin histochemistry. Gray stars indicate interneurons that underwent DB in isolated CA1.

Figure 3. EI interplay during SLEs

A. Traces from simultaneous whole-cell measurements from one interneuron (blue, ellipse) and two pyramidal cells (red, triangles), and an extracellular DC mode recording (EC). Inverted black triangles denote the FPESs. **B.** Average spike rates of the three cells (interneuron - blue circles, pyramidal 1 - red diamonds, pyramidal 2 - red triangles). Note that the plot in B is an average (n=8 SLEs in the experiment), and hence is not precisely time locked to the single SLE example shown in A. **C.** EI crosscorrelation averages. The means of EI subthreshold crosscorrelation values were significantly different (ANOVA: $df=10199$, $F=4.27$, $p<0.0001$) at distinct SLE stages (Tukey multicomparison, green asterisks). Unweighted pooled subthreshold EI crosscorrelation values (represented as black diamonds) peaked at the onset of SLEs, followed by a decrease, and then a gradual increase towards the end of the SLEs (Tukey multiple comparison test, $p<0.001$). With weighted resampled averages (gray squares) the peak at the onset of SLEs was less prominent, but the significance of the increase in subthreshold EI correlations was retained ($p<0.05$). Inverted black triangles denote the FPESs in C, D, inset in E. **D.** A significant increase in EI spike correlations was observed at the beginning (unweighted, blue asterisk) and following the DB in interneurons (weighted averages, green asterisk). (ANOVA, $df=10199$, $F=3.27$, $p<0.0001$; Tukey multiple comparison test, $p<0.001$ for unweighted (black diamonds) and $p<0.005$ for weighted resampled (gray squares) averages). **E.** Cumulative average spike rates (n=15 oriens interneurons and n=40 pyramidal cells from pairs and single whole cell recordings). Note the peak of inhibitory spiking preceding the FPES (dark blue - I unweighted pooled; light blue - I weighted resampled). Excitatory firing rates increase as interneurons enter into the DB and decreased as interneurons exit the DB (red - E unweighted pooled; orange - unweighted pooled). Inset shows average spike rates at expanded time scale.

Figure 4. II activity during SLEs

A. Whole cell measurements from two oriens interneurons accompanied by the average cross correlation (*CC*) measures of suprathreshold (**B**) and sub-threshold (**C**) signals ($n=13$ SLEs, 3 pairs). Inverted triangle indicates the FPES. Note the clear loss of spiking and increase in subthreshold correlations during DB in II pairs (unweighted averages).