Spike Timing of Lacunosom-Moleculare Targeting Interneurons and CA3 Pyramidal Cells During High Frequency Network Oscillations in vitro

Jay Spampanato and Istvan Mody*

Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA

Running head: Spike timing during high frequency oscillations

* Corresponding Author
Istvan Mody, Ph.D.
UCLA
Neurology NRB1 Rm. 575D
635 Charles E. Young Drive South
Los Angeles, California 90095-7335
Ph: 310-206-4481
Fax: 310-825-0033
E-mail: Mody@ucla.edu

Number of figures: 9

Number of tables: 0

Number of pages: 34
ABSTRACT

Network activity in the 200-600 Hz range termed high-frequency oscillations (HFO) has been detected in epileptic tissue from both humans and rodents, and may underlie the mechanism of epileptogenesis in experimental rodent models. Slower network oscillations including theta and gamma oscillations as well as ripples are generated by the complex spike timing and interactions between interneurons and pyramidal cells of the hippocampus. We determined the activity of CA3 pyramidal cells, oriens lacunosum-moleculare (O-LM) and radiatum lacunosum-moleculare (R-LM) interneurons during HFO in the in vitro low Mg\(^{2+}\) model of epileptiform activity in GIN mice. In these animals, interneurons can be identified prior to cell-attached recordings by the expression of green-fluorescent protein (GFP). Simultaneous local field potential recordings from stratum pyramidale and on-cell recordings of individual interneurons and principal cells revealed three primary firing behaviors of the active cells: 36% of O-LM interneurons and 60% of pyramidal cells fired action potentials at high-frequencies during the HFO. R-LM interneurons were biphasic in that they fired at high-frequency at the beginning of the HFO but stopped firing before its end. When considering only the highest frequency component of the oscillations most pyramidal cells fired on the rising phase of the oscillation. These data provide evidence for functional distinction during HFOs within otherwise homogeneous groups of O-LM interneurons and pyramidal cells.

Keywords: spike timing, high frequency oscillation, interneuron, pyramidal cell, CA3 hippocampus, in vitro
INTRODUCTION

Hippocampal synchronized network activity is associated with well-defined behavioral states and with the development of seizures. Slow oscillations (5-10 Hz; theta) are associated with active exploration and rapid eye movement (REM) sleep (O'Keefe and Nadel, 1978; Buzsaki, 2002) while faster (30-100 Hz; gamma) activity is associated with awake, attentive behavior and REM sleep (Singer, 1993; Gray, 1994; Fries et al., 2001; Buzsaki et al., 2003). The very fast (100-200 Hz; ripples) activity superimposed on sharp waves lasting 50-100 ms is correlated with memory formation during slow-wave sleep and awake immobility (Ylinen et al., 1995). Ultrafast oscillations (200-600 Hz) termed high-frequency oscillations (HFO) have been detected in epileptic tissue of humans and rodents (Bragin et al., 1999a; Bragin et al., 1999b; Bragin et al., 2002) and may underlie epileptogenesis in rodent models (Bragin et al., 2004).

Theta, gamma and ripple oscillations are generated by the complex spike timing and interactions between interneurons and pyramidal cells of the hippocampus (Klausberger et al., 2003; Hajos et al., 2004; Mann et al., 2005; Gloveli et al., 2005). The O-LM interneurons provide theta rhythm inhibitory feedback (Klausberger et al., 2003; Gloveli et al., 2005) and fire phase locked to the gamma rhythm (Hajos et al., 2004). In contrast, R-LM interneurons fire with no specificity during gamma oscillations (Hajos et al., 2004). O-LM cells fire at high frequencies under artificial stimulation (Zhang and McBain, 1995; Aradi and Maccaferri, 2004; Fujiwara-Tsukamoto et al., 2004; Gloveli et al., 2005) and they respond specifically to high-frequency synaptic input (Pouille and Scanziani, 2004) but they are completely silent during ripple activity (Klausberger et al., 2003). Moreover, O-LM interneurons fire only at low frequencies while phase-locked to theta (Klausberger et al., 2003) and gamma oscillations (Hajos et al., 2004) possibly due to a lack of high-frequency input from pyramidal cell collaterals.
However, pyramidal cells fire action potentials at high-frequencies in some *in vitro* models of epileptiform activity (Mody et al., 1987; Aradi and Maccaferri, 2004; Dzhala and Staley, 2004; Lasztoczi et al., 2004) suggesting a possible role for O-LM cells in HFO.

To study the activity of pyramidal cells, O-LM and R-LM interneurons during HFO we utilized the *in vitro* low Mg$^{2+}$ model of epileptiform activity (Walther et al., 1986; Mody et al., 1987; Jones and Heinemann, 1988) and the genetic expression of green-fluorescent protein (GFP) in *GIN* mice to identify interneurons prior to cell-attached recordings. About 96% of the GFP-positive cells in the CA3 of *GIN* mice express somatostatin and mGluR1α (Oliva, Jr. et al., 2000), both markers of O-LM cells (Somogyi and Klausberger, 2005). Further anatomical analysis of the GFP-positive cells showed them to be O-LM and R-LM interneurons based on their dendritic morphology and axonal targeting (Oliva, Jr. et al., 2000). Following simultaneous field recordings from stratum pyramidale and single unit recordings of individual interneurons and principal cells we found three primary firing behaviors: 36% of the O-LM interneurons and 60% of the pyramidal cells fired action potentials at high-frequencies during HFO, while the remainder were silent during the HFOs. R-LM interneurons were biphasic in that they fired at high-frequency at the beginning of the HFO but stopped firing before its end. When considering only the early (first 30 ms) and typically the highest frequency component of the oscillations most pyramidal cells fired with phase association correlated with the rising phase of the oscillation.

**MATERIALS and METHODS**

*Slice Preparation*
Slices were obtained from adult (2-4 month old) male GIN mice (Oliva, Jr. et al., 2000) originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the UCLA Division of Laboratory Animal Medicine. In accordance with the UCLA Chancellor’s Animal Research Committee approved protocol, the mice were anesthetized with halothane prior to decapitation. Brains were quickly removed and placed into ice-cold normal artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 1.25 NaHPO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 10 D-Glucose, 1.5 Glutamine, 1.5 Na-Pyruvate with pH 7.3-7.4 when bubbled with 95\% O\textsubscript{2}-5\% CO\textsubscript{2}. Normal aCSF was supplemented with 3 mM kynurenic acid (Spectrum Chemical Mfg. Corp., Gardena, CA) for the duration of the cutting procedure. Slices (350 \textmu m thick) were cut in the horizontal orientation using a Leica VT1000S Vibratome, (Leica Microsystems, Wetzlar, Germany). The slices were then transferred to an interface style holding chamber containing normal aCSF at 32\degree C and allowed to recover for at least one hour prior to experimentation.

Local Field Potential and Cell-Attached Recordings

Slices were placed in a submerged recording chamber modified to promote laminar flow and perfused at >8 ml/min with an extracellular recording solution containing (in mM): 126 NaCl, 5 KCl, 1.2 CaCl\textsubscript{2}, 0.05 MgCl\textsubscript{2}, 1.25 NaHPO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 10 D-Glucose, 1.5 Glutamine, 1.5 Na-Pyruvate. The recording solution was aerated with 95\% O\textsubscript{2}-5\% CO\textsubscript{2} and the bath temperature was maintained at 34\degree C. Under these conditions, recordings in the CA3 pyramidal cell layer showed epileptiform bursting to occur within minutes of submersion in the recording solution. To consistently obtain spontaneous oscillations it was necessary to fit the chamber with small plastic fibers fixed parallel to the direction of solution flow thereby elevating the slices off
the glass coverslip. Microelectrodes (1-5 MΩ when filled) were pulled from 7740 glass (Garner Glass Company, Claremont, CA) and filled for field recordings with the extracellular solution and for cell-attached recordings with 126 mM NaCl and 10 mM HEPES adjusted to pH 7.2. The field potential recordings were obtained through an electrode placed in the stratum pyramidale of CA3 while simultaneous single unit recordings were made with a second electrode positioned less than 400 μm away from the field electrode. The recordings presented here were taken primarily from the sub region CA3c and CA3b however similar activity was observed in CA3a. The firing patterns of single cells were recorded in the cell-attached loose patch configuration, from visually identified pyramidal cells and green fluorescent interneurons. All recordings were obtained DC in the current clamp configuration (I=0) using the Axon Instruments MultiClamp 700A (Molecular Devices Corp., Sunnyvale, CA) at 10 kHz sampling frequency through a 3 kHz low pass filter with EVAN data collection software (in house).

Data Analysis

Analysis was completed using both EVAN and Igor software (WaveMetrics, Lake Oswego, Oregon). Bursts were detected on their negative field spike. This was used to define the start of the oscillations. In all cases the detected points that began the negative field spike were subsequently labeled as time zero for alignment and quantification purposes. An inter-burst interval histogram was generated for each recording (binwidth = 0.1 s). A Gaussian was fit to the histogram to determine the average inter-burst interval and its standard deviation within the recording. Individually detected action potentials were then assigned to a burst if they occurred within 1 to 3 standard deviations before to 0.4 s after the start of the burst (4-fold longer than the average burst duration). A raster plot was generated by plotting the action potential time relative
to the start of each detected burst. To determine the action potential frequency the time leading up to a burst was binned at 100 ms and the time during and after a burst was binned at 20 ms. The number of action potentials in each bin was counted and the values and standard deviations were then scaled to 1 s to generate the average firing frequency (s\(^{-1}\)). Wigner transforms of field oscillations band pass filtered between 100 and 1000 Hz were calculated using the built-in function of Igor. In all cases the filtered data were phase adjusted to eliminate errors introduced during the filtering. For cells that fired during the HFO, phase association was determined using the Hilbert transform. Action potentials that occurred during the first 30 ms of the oscillations, the time period during which the strongest and highest frequency activity was observed, were then assigned an angle based on the corresponding phase using the built-in phase-corrected functions of IGOR. Circular statistics were used to calculate the mean angle and vector length for each cell and Rayleigh’s test was applied to determine the significance of the directionality of firing. More specifically the mean angle is determined by first calculating the mean sine (\(\bar{s}\)) and the mean cosine (\(\bar{c}\)) for each cell as follows:

\[
\begin{align*}
\bar{s} &= \frac{\sum \sin(a)}{n} \\
\bar{c} &= \frac{\sum \cos(a)}{n}
\end{align*}
\]

Where \(a\) is the angle for each action potential and \(n\) is the total number of angles. Then the mean angle (\(\bar{\theta}\)) is:

\[\bar{\theta} = \arctan\left(\frac{\bar{s}}{\bar{c}}\right)\]

The mean vector length (\(r\)) is then:

\[r = \sqrt{(\bar{s})^2 + (\bar{c})^2}\]
The polar plots displayed in Figures 8B and 9B are circular histograms of the probability of action potential firing at each phase of the oscillation.

**RESULTS**

*High-frequency oscillations in the submerged chamber.*

Perfusion of a Mg\(^{2+}\)-free or low Mg\(^{2+}\) recording solution has been shown previously to result in regular epileptiform activity in the CA1 and CA3 regions of the hippocampus *in vitro* (Walther et al., 1986; Mody et al., 1987; Jones and Heinemann, 1988). Though the activity was described as robust and reproducible, all of these studies used an interface style recording chamber which cannot be combined with the visualization and fluorescent identification of individual interneurons for single-cell recordings. By modifications of the recording chamber (see methods) and increasing the rate of perfusion to greater than 8 ml/min similar to what was reported recently to elicit stable gamma oscillations under similar conditions (Hajos et al., 2004; Mann et al., 2005) we were able to reliably reproduce regular epileptiform activity in the submerged chamber with no requirement for additional tetanization or drug application (Fig. 1). Figure 1A illustrates 60 s of a typical field recording from the CA3 pyramidal cell layer of a regularly bursting slice. The expanded burst demonstrates a standard field recorded waveform: each burst begins with a large fast negative field deflection followed by a slower, larger positive potential that upon return to baseline, overshoots to go negative again before settling more slowly back to baseline (Fig 1A, middle panel). The high frequency oscillations lasted on average for 108 ms (Fig 1C), as can be seen in the expanded trace band-pass filtered between 100-1000 Hz (Fig 1A, bottom panel). Individual epileptiform bursts consist of a complex and variable assortment of high frequency activity in the fast ripple band of 200-600 Hz demonstrated by the fast Fourier transform (Fig 1B) of the oscillation shown in Figure 1A. This
Spike timing during high frequency oscillations

Slice displayed continuous high frequency oscillations with an interburst interval of approximately 1.3 s (Fig 1D). For all experiments the average interburst interval was 2.3 ± 0.2 s (n=22, ± SEM) with a range between 1.1 and 5.2 s (Fig 1E). Within a given slice the bursts occurred regularly for up to several hours with little change.

**Spike timing of lacunosum-moleculare targeting interneurons during HFO.**

To determine the single unit firing patterns of principal cells and interneurons that generate the high-frequency field oscillations we performed simultaneous non-invasive loose-patch cell-attached recordings from visually identified pyramidal cells and GFP-expressing somatostatin O-LM and R-LM interneurons in the CA3 region of the hippocampus of GIN mice. O-LM and R-LM interneurons were scattered throughout each slice and were easily identifiable due to the high expression of GFP (Fig 2). These cells were previously characterized and concluded to be exclusively somatostatin expressing, mGluR1α expressing, lacunosum-moleculare targeting interneurons with dendritic arbors consistent with O-LM or R-LM interneurons (Oliva, Jr. et al., 2000). We observed a sparse and separated cellular distribution through out the hippocampus similar to that described by Oliva et.al. (Oliva, Jr. et al., 2000).

The majority of R-LM interneurons (19/23) and approximately half of the pyramidal cells (14/29) and O-LM interneurons (12/26) were found to be inactive in the low Mg²⁺ recording solution. Inactivity was defined as failure to fire a single action potential over a period of several minutes while the CA3 region was observed to be actively bursting. The mechanism of inactivity cannot be defined through means of loose-patch recordings. It remains possible that these cells were disconnected from the network through the processing of the tissue for *in vitro* slice recordings. Another possibility stems from the use of a low Mg²⁺ and 5 mM K⁺ aCSF
solution which could have shifted some pyramidal cells and O-LM or R-LM interneurons into depolarization block which could have occurred through activation of NMDA or mGluR1α receptors on O-LM-interneurons (Baude et al., 1993). Depolarization block has been shown to be an important component of O-LM interneuron activity in much longer seizure-like events in the CA1 region (Ziburkus et al., 2006). Finally, it is likely that this inactivity represents one aspect of cellular assembly into functional units during HFOs. It is generally considered to be the case that higher frequency oscillations are the result of smaller numbers of participating cells in a given assembly (Buzsaki and Draguhn, 2004). The focus of this study was to determine the activity of cells that might contribute to the generation of hippocampal high-frequency oscillations and therefore from this point on we consider only the active cells in our analysis. In all experiments the recorded cells never fired action potentials at the 200-600 Hz frequency of the field oscillations.

Based on their firing properties during the HFO the active O-LM interneurons fell into two groups. The first group, representing 36% of active O-LM interneurons, fired rhythmically at an average basal rate of approximately 16 Hz between bursts and increased to an average instantaneous frequency of approximately 183 Hz simultaneous with the start of the oscillation (Fig 3). These cells exhibited some spike-frequency adaptation but were able to maintain very high firing rates during the HFO and on average, higher than basal firing rates for up to 200 ms from the end of the HFO. This activity is clearly illustrated by both the cell-attached recording (Fig 3B) and the raster plot of 100 sequential bursts (Fig 3C). The simultaneous field recording and Wigner transform are included for comparison of the HFO duration, frequency and timing with the cell activity (Fig 3A).
The second group, representing 63% of active O-LM interneurons, fired at a significantly higher average basal rate of 30 Hz in-between bursts (Student’s t-test, p<0.01) but abruptly stopped firing at the beginning of the epileptiform burst and remained silent for the duration of the HFO (Fig 4). These cells then slowly returned to their basal firing rate requiring more than 300 ms from the end of the HFO to fully recover (Fig 4B, C). The simultaneously recorded field oscillation, band pass filtered insert and Wigner transforms are included for comparison of the timing of the single cell activity with the network activity (Fig 4A). This group of O-LM cells was never observed to fire at the extreme high frequencies reported for the previous group.

R-LM interneurons were far less likely to be active in the low Mg\(^2+\) recording solution than both O-LM interneurons and pyramidal cells. All of the R-LM interneurons that were active (4/4) maintained the highest average basal firing rate at 39 Hz (significantly faster than both groups of O-LM interneurons, Student’s t-test, p<0.05). Each active R-LM interneuron increased firing to an average spontaneous rate of 135 Hz for a brief period simultaneous with the beginning of the field oscillations but rapidly slowed before the end of the epileptiform burst (Fig 5). In contrast to the O-LM interneurons that stopped firing during the HFO, R-LM interneurons quickly returned to their basal firing rate of 39 Hz within 50 ms following the end of the HFO. The raw trace shown depicts the irregular basal firing of the R-LM interneurons with no discernable change in behavior immediately prior to the field oscillation (Fig 5A, B). The raster plot demonstrates the consistency of this activity for 100 sequential bursts (Fig 5C).

**Spike timing of CA3 pyramidal cells during HFO.**

The basal firing rate of all active pyramidal cells was typically only a few Hz. Similar to the O-LM interneurons, active pyramidal cells could be classified into two groups based only on
their activity during the HFO. The first group, representing 60% of the active pyramidal cells, fired at very high frequencies simultaneous with the HFO (Fig 6). In step-wise fashion these pyramidal cells increased firing to an average instantaneous rate of 154 Hz and exhibited significant spike-frequency adaptation throughout the duration of the field oscillation (Fig 5A, B). In contrast to the high frequency firing observed in O-LM interneurons, pyramidal cells returned to their basal firing rate of only a few Hz coincident with the completion of the HFO; that is, they did not continue to fire at a higher than basal rate in the absence of a detectable field oscillation. The raster plot demonstrates the sporadic firing of pyramidal cells leading up to each burst and the decline in frequency during the HFOs (Fig 5C).

The second group, representing 40% of active pyramidal cells, similarly displayed a low basal firing rate between bursts; however they stopped firing during the field oscillations (Fig 7). In contrast to the O-LM interneurons that stopped firing during HFOs the pyramidal cells exhibited a unique firing pattern in that they specifically fired one or a few action potentials tightly synchronous with the negative field deflection signifying the start of the epileptiform burst (Fig 7A, B). The raster plot again demonstrates the sporadic firing nature of these pyramidal cells during the inter-event interval, the precise firing of a few action potentials at the start of the epileptiform burst and the consistent lack of firing during the HFO (Fig 7C). It is important to note that all of the active pyramidal cells demonstrated some level of sporadic firing between oscillations regardless of their behavior during the HFOs.

Figure 8A and 9A depict the summarized activity of each individual group described above. The light grey area signifies the start and average HFO duration. It is easy to see the contrast in behavior between the two groups of O-LM interneurons and the two groups of pyramidal cells as well as the biphasic activity of the R-LM interneurons. This figure also
depicts the rapid cessation of firing in pyramidal cells compared to the extended activity in the 
O-LM interneurons that fired at high frequency.

Because each individual HFO typically consisted of multiple bands of high-frequency 
activity that changed over time, we concentrated only on the first 30 ms to determine if the firing 
of any of the cells active during the HFO was associated with a specific phase of the oscillation. 
This duration was long enough to include the highest frequency and highest power activity which 
typically occurred at the start of the HFO. During this time period the majority of O-LM and R-
LM interneurons did not fire with any specific phase association. This can be seen in the polar 
histograms depicting their firing behavior (Fig 8B). In contrast, during the same period a 
majority of pyramidal cells did fire with a phase association corresponding to the rising slope of 
the oscillation (for 6/9 cells, Rayleigh’s test p<0.05 with a mean angle of 125° and a mean vector 
of 0.08). The polar histogram displays the average phase associated action potential probability 
of all of the active pyramidal cells (Fig 9B). The raw data demonstrates the phase association of 
action potential firing (Fig 9C) during the early high frequency, high power oscillation. In this 
example, the first action potential occurred immediately before the start of the HFO. The second 
through fifth action potentials fired on the rising phase of the 215-344 Hz oscillation and the last 
four action potentials fired out of phase.

**DISCUSSION**

We have presented here for the first time in adult mice, low Mg^{2+} induced high-frequency 
oscillations in a submerged hippocampal slice without the requirement of additional 
pharmacological agents or tetanus to further enhance neuronal excitability. The CA3 region
generated spontaneous epileptiform field oscillations in the HFO band of 200-600 Hz repeatedly and reliably at a rate of approximately 0.5 Hz. Once the HFOs were established cell-attached recordings were used to determine the activity of CA3 pyramidal cells and two types of interneurons: somatostatin expressing O-LM and R-LM interneurons. During epileptiform bursts active pyramidal cells were found to fire either at high frequencies on average 150 Hz or just 1 to 3 action potentials simultaneous with the start of the field oscillation. Similarly active O-LM interneurons fired either at high frequencies on average 180 Hz or not at all during the field oscillation. All active R-LM interneurons fired in a biphasic nature; that is they began to fire at high frequencies on average 140 Hz at the start of the epileptiform bursts but stopped firing halfway through. The field oscillations generated by CA3 in low Mg\(^{2+}\) varied at frequencies between 200 Hz and 600 Hz; however neither the principal cells nor the O-LM/R-LM interneurons fired action potentials at those frequencies. Finally, the firing of the majority of the pyramidal cells could be associated with the rising phase of the early and typically highest frequency portion of the field oscillation.

At all times basal activity between bursts was an order of magnitude higher (16-39 Hz) in the two anatomically defined groups of interneurons compared to the pyramidal cells (1-4 Hz). It is therefore likely that these dendritic targeting interneurons play some role in determining the inter-event-interval of HFOs. These two unique populations of interneurons are positioned to provide specific inhibition to the excitatory input arriving at the distal dendrites of CA3 pyramidal cells via entorhinal perforant path afferents. R-LM interneurons are likely to provide feed-forward inhibition due to a dendritic arbor primarily confined to strata radiatum and lacunosum-moleculare (Hajos and Mody, 1997; Oliva, Jr. et al., 2000). On the other hand, O-LM interneurons are specifically tuned to provide feed-back inhibition in response to high-
frequency input originating from pyramidal cell collaterals (Pouille and Scanziani, 2004). Our data further support the likelihood that O-LM interneurons are a critical component in the regulation of CA3 pyramidal cell high-frequency activity. Though there were two functionally different groups of O-LM interneurons and the majority was observed to be inactive during the periods of high-frequency pyramidal cell activity, the inactive group was also already firing action potentials rhythmically at a basal rate of approximately 30 Hz, twice the rate of action potential generation in the group that fired in concert with the high-frequency pyramidal activity. It is possible that the higher basal rate of firing could signify that these O-LM interneurons were already more depolarized and therefore incapable of responding to high-frequency input via pyramidal cell synapses. O-LM interneurons have previously been reported to be completely silent during hippocampal sharp wave ripple (100-200 Hz) oscillations (Klausberger et al., 2003). Likewise, the majority of O-LM interneurons in our study were also silent during the faster low Mg$^{2+}$ induced HFOs. In contrast however, we observed tonic firing between oscillations and very high-frequency firing in a number of O-LM interneurons during HFOs consistent with the hypothesis that low Mg$^{2+}$ induced HFOs are a better model of pathological high-frequency oscillations than sharp-wave ripples (Bragin et al., 1999a). It is possible this activity signifies a more urgent necessity to inhibit entorhinal input to CA3 during HFOs whereas doing so during sharp-wave ripples would disrupt the normal operation of the hippocampal-entorhinal circuitry (Chrobak et al., 2000).

The percentage of active pyramidal cells that routinely participate in other fast network oscillations of the hippocampus has been reported to be quite low, ranging from 10% to 22% for sharp-wave ripples and high K$^+$ induced epileptiform activity respectively (Ylinen et al., 1995; Dzhala and Staley, 2004). In low Mg$^{2+}$ we found 48% of CA3 pyramidal cells fired spontaneous
Spike timing during high frequency oscillations

action potentials. The active pyramidal cells always fired action potentials associated with each HFO. As mentioned above, some CA3 pyramidal cells were observed to fire action potentials at very high frequencies during HFOs. This finding is consistent with previous reports of spontaneous epileptiform activity in CA3 (Mody et al., 1987; Aradi and Maccaferri, 2004; Dzhala and Staley, 2004; Lasztoczi et al., 2004). Of the active pyramidal cells, approximately 60% fired at high frequency during epileptiform bursts and maintained this firing pattern for many minutes i.e., the duration of the recordings. The remaining 40% fired only one or a few action potentials suggesting that despite their very similar morphology (Bilkey and Schwartzkroin, 1990) there may be at least two functionally different groups of pyramidal cells in CA3. Similar ratios of bursting vs non-bursting CA3 pyramidal cells have been reported following current injection under normal ionic conditions (Masukawa et al., 1982; Bilkey and Schwartzkroin, 1990). Though our data suggesting two functional groups of pyramidal cells in CA3 are consistent with these previous findings, we cannot rule out the possibility that the slice technique itself has altered the synaptic inputs to some pyramidal cells causing them to fire fewer action potentials during HFOs. It will be important for future studies to determine whether an individual CA3 pyramidal cell is predetermined to function in one of these two active states (or to be inactive) during spontaneous high frequency network oscillations in vitro and in vivo or is capable of switching between states in response to changes in incoming synaptic activity or changes in intrinsic properties.

When the full length HFO was considered for analysis of directional firing there was no apparent phase association for any of the cells studied here. However, when the analysis was restricted to the early highest power and typically highest frequency portion of the oscillation a phase association was apparent for the majority of the pyramidal cells that fired during the HFO.
During this time pyramidal cells fired on the rising phase of the oscillation, but then continued to fire with no phase association through the end of the oscillation. The loss of phase association could be attributed to their firing at a slower frequency than the field oscillation. Our data also suggest that while the LM targeting interneurons fire during the HFO, they are not the primary interneuronal components of the oscillation.

The focus of this study was to identify the firing activity of cells that participate in the generation of HFOs. Though aware of the limitations of the technique, we utilized cell-attached recordings to faithfully determine the firing properties of these cells without disruption of their intracellular milieu. In doing so we cannot ignore that some cells did not fire at all in our low Mg\(^2\+) slice preparation. The lack of firing of certain cells during and between an active action potential dependent network oscillation means that these cells are not contributing to the field oscillation. Thus, the oscillation is not dependent on their firing and it is not a novel finding that not all cells participate in larger network activities (Buzsaki and Draguhn, 2004). HFOs clearly and robustly occur with still many cells silent. Elucidating the mechanism of inactivity will not be a straightforward task. Without prior knowledge of the specific intracellular ionic concentrations of K\(^+\) and Cl\(^-\) one cannot utilize the more powerful technique of whole-cell recordings to address this question. Patching in the whole-cell configuration with these concentrations unknown will make the interpretation of the experiments very difficult. Furthermore, whether these cells fire after they are whole-cell patched or whether they are in a state of depolarization after they are whole-cell patched does not contribute to our understanding of the cellular activity required for the generation of network oscillations.

In conclusion, though high-frequency network oscillations can be recorded throughout CA3 stratum pyramidale our data suggest that this \textit{in vitro} epileptiform behavior is the result of a
smaller subset of synchronized neurons. Our data are also consistent with some functional
diversity within otherwise anatomically homogeneous populations of O-LM interneurons and
pyramidal cells in the CA3 area.

**Acknowledgements:** We thank E. Marton, M. Rafizadeh and R. M. Lazaro for technical
assistance and Dr. G. Faas for valuable discussion.

**Grants:** This work was supported by NIH/NINDS grant P50 NS002808 to I. Mody.

**Disclosures:** The authors have no disclosures to make.

2. **Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA and Somogyi P.** The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* 11: 771-787, 1993.


Figure 1: Low Mg$^{2+}$ induced spontaneous high-frequency activity in CA3 stratum pyramidale recorded in a submerged chamber. A. Top: Field recording from stratum pyramidale of CA3 after perfusion of aCSF containing 0.05 mM Mg$^{2+}$. Field activity could be observed within minutes of submersion in the recording media. Middle: One burst is expanded to illustrate the typical field waveform. Bottom: The expanded high-frequency oscillation is shown band-pass filtered between 100 to 1000 Hz. B. Fast Fourier transform of the expanded HFO showing high power in the 200-600 Hz range. C & D. Histograms of the duration (C) and inter-event intervals (D) of the first 100 high-frequency oscillations of the recording shown in A. The event duration histogram is fit with a Gaussian centered at 108.1 s with a standard deviation of 12.9 s. The inter-event interval histogram is fit with a Gaussian centered at 1.2 s with a standard deviation of 0.2 s. E. The mean inter-burst interval histogram of 22 slices shows that the time between the bursts was variable, ranging between 1-4 s for 91% of the slices (20/22).

Figure 2: GFP-expressing interneurons in the CA3 region of the hippocampus. Left Panel: 10x magnification of the CA3 region of a GIN mouse. GFP expressing cells can be seen throughout the three layers: stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR). Middle Panel: Higher magnification (40x) of two interneurons in oriens demonstrating easily identifiable O-LM cell bodies and proximal dendrites. Right Panel: Higher magnification (40x) of two interneurons in the stratum radiatum. R-LM cell somata were typically small.
Figure 3: Increased firing of O-LM interneurons during high-frequency oscillations. A. Top: Field recording from stratum pyramidale of CA3 after perfusion of 0.05 mM Mg$^{2+}$ aCSF. Inset: Expanded trace of the HFO band-pass filtered between 100-1000 Hz. Scale bars are 50 $\mu$V and 25 ms. Bottom: Wigner transform of the band pass filtered field recording demonstrating the frequency spectra of the oscillations present during the HFO. B. Loose-patch cell-attached recording during the field burst illustrated in A, from a visually identified GFP expressing O-LM interneuron located less than 300 $\mu$m away from the field electrode. Some basal firing activity prior to the HFO is followed by an immediate increase in firing simultaneous with the start of the HFO. C. Raster plot of action potential firing during 100 sequential bursts illustrates the consistency of this pattern of activity over time.

Figure 4: Decreased firing of O-LM interneurons during high-frequency oscillations. A. Top: Field recording from stratum pyramidale of CA3 after perfusion of 0.05 mM Mg$^{2+}$ aCSF. The inset shows the burst in A band-pass filtered between 100-1000 Hz. Scale bars are 200 $\mu$V and 25 ms. Bottom: Wigner transform of the band pass filtered field recording demonstrating the frequency spectra of the oscillations present during the HFO. B. Loose-patch cell-attached recording during the field burst illustrated in A, from a visually identified GFP expressing O-LM interneuron located less than 300 $\mu$m away from the field electrode. High basal firing activity can be seen prior to the HFO followed by an immediate cessation of firing simultaneous with the start of the HFO. C. Raster plot of action potential firing during 100 sequential bursts illustrates the consistency of this pattern of activity over time. This cell sometimes fired one or two action
potentials in rapid succession just after the negative field spike which can be seen in the dark band of the raster plot.

**Figure 5: Biphasic firing of R-LM interneurons during high-frequency oscillations.** A. Top: Field recording from stratum pyramidale of CA3 after perfusion of 0.05 mM Mg\(^{2+}\) aCSF. The inset shows the burst in A band-pass filtered between 100-1000 Hz. Scale bars are 200 μV and 25 ms. Bottom: Wigner transform of the band pass filtered field recording demonstrating the frequency spectra of the oscillations present during the HFO. B. Loose-patch cell-attached recording from a visually identified GFP expressing R-LM interneuron during the filed burst illustrated in A located less than 300 μm away from the field electrode. The irregular basal firing activity (~39 Hz) prior to the HFO is increased dramatically coincident with the start of the HFO. R-LM interneurons fired a few action potentials in rapid succession before likely entering into depolarization block, from which they recovered immediately following the HFO. C. Raster plot of action potential firing during 80 sequential bursts illustrates the consistency of this pattern of activity over time.

**Figure 6: Increased firing of a CA3 pyramidal cell during high-frequency oscillations observed in 9 out of 15 pyramidal cells.** A. Top: Field recording from stratum pyramidale of CA3 bathed in 0.05 mM Mg\(^{2+}\) aCSF. The inset shows the illustrated burst band-pass filtered between 100-1000 Hz. Scale bars are 200 μV and 25 ms. Bottom: Wigner transform of the band pass filtered field recording demonstrating the frequency spectra of the oscillations present
during the HFO. B. Loose-patch cell-attached recording from a visually identified CA3 pyramidal cell less than 100 μm from the field electrode. This trace demonstrates no basal firing activity prior to the HFO followed by an immediate increase in firing simultaneous with the start of the HFO. C. Raster plot of action potential firing during 100 sequential HFOs illustrates the consistency of this pattern of activity over time as well as the intermittent low-frequency inter-event firing during some sweeps.

Figure 7: Decreased firing of a CA3 pyramidal cell during high-frequency oscillations observed in 6 out of 15 pyramidal cells. A. Top: Field recording from the CA3 stratum pyramidale in 0.05 mM Mg^{2+} aCSF. Inset: The burst in A is shown band-pass filtered between 100-1000 Hz. Scale bars: 200 μV and 25 ms. Bottom: Wigner transform of the band pass filtered field recording demonstrating the frequency spectra of the oscillations present during the HFO. B. Loose-patch cell-attached recording from a visually identified CA3 pyramidal cell less than 100 μm from the field electrode. Note the low basal firing activity prior to the HFO followed by the firing of 1 to 3 precisely timed action potentials simultaneous with the start of the HFO. C. Raster plot of action potential firing during 100 sequential HFOs illustrates the consistency of this pattern of activity over time. Though this particular pyramidal cell can be seen to increase firing immediately prior to the start of the oscillation this was not seen in the averaged data.
**Figure 8: Average firing rates of O-LM interneurons and R-LM interneurons during HFO.**

A. The average firing rates of the three groups of interneurons categorized by their activity during the HFO. To calculate the specific rate of firing of each cell the time prior to each HFO was binned into periods of 100 ms while the time during and after each HFO was binned into 20 ms periods. Action potentials were then summed per bin and averaged across the cells within each group. These data were then scaled to obtain the number of action potentials per second (Hz). The area shaded in grey represents the start and duration of an average HFO burst. Error bars are standard deviations. (n for each group: O-LM ON=4, O-LM OFF=7, R-LM=4). B. Spike probability polar histograms were calculated according to the phase of firing for the O-LM ON and R-LM interneurons during the first 30 ms of the corresponding HFOs. Solid lines represent the average data and dotted lines are ± the standard deviation. In each plot the circle maximum probability is 0.06 and the x-axis and y-axis ticks represent probabilities toward the origin of 0.04 and 0.02, the origin is zero.

**Figure 9: Average firing rates and phase association of CA3 pyramidal cells during HFO.**

A. The average firing rates of the two groups of CA3 pyramidal cells categorized by their activity during the HFO were calculated as described in the legend to Figure 8. (n for each group: PYR ON=9, PYR OFF=6). B. The spike probability polar histogram of PYR ON cells calculated according to the phase of firing during the first 30 ms of the HFO demonstrates a phase association corresponding to the rising phase of the oscillation with a mean angle of 125°. Six out of nine PYR ON cells demonstrated significant directionality in firing (Rayleigh’s test, p<0.05). The solid line represents the average data and dotted lines are ± the standard deviation. In the plot the circle maximum probability is 0.06 and the x-axis and y-axis ticks represent
probabilities toward the origin of 0.04 and 0.02, the origin is zero. C. A CA3 HFO band pass filtered between 100 and 1000 Hz (top) and the corresponding single unit recording of one phase associated CA3 pyramidal cell (bottom). The grey box indicates the first 30 ms of the oscillation with a peak frequency of 215 Hz, which was used for analysis of phase association. During this time, 4 out of 6 action potentials were associated with the rising phase of the oscillation.
FIGURE 1

A

B

C

D

E
FIGURE 2
Figure 3

A

B

C

Spike timing during high frequency oscillations
Figure 4

A

B

C

Spike timing during high frequency oscillations
Figure 5

A

B

C

Spike timing during high frequency oscillations
Figure 6

A

B

C
Figure 7

A

B

C

Spike timing during high frequency oscillations
Figure 8
Figure 9

A

Pyramidal Cells

Time Relative to the Start of the Burst (s)

B

PYRON

C

100μV
10ms

300μV
10ms