In vivo dual intra- and extracellular recordings suggest bidirectional coupling between CA1 pyramidal neurons

Edith Chorev and Michael Brecht
Bernstein Center for Computational Neuroscience, Humboldt University of Berlin, Berlin, Germany

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Chorev E, Brecht M. In vivo dual intra- and extracellular recordings suggest bidirectional coupling between CA1 pyramidal neurons. J Neurophysiol 108: 1584–1593, 2012. First published June 20, 2012; doi:10.1152/jn.01115.2011.—Spikelets, small spikelike membrane potential deflections, are prominent in the activity of hippocampal pyramidal neurons in vivo. The origin of spikelets is still a source of much controversy. Somaically recorded spikelets have been postulated to originate from dendritic spikes, ectopic spikes, or spikes in an electrically coupled neuron. To differentiate between the different proposed mechanisms we used a dual recording approach in which we simultaneously recorded the intracellular activity of one CA1 pyramidal neuron and the extracellular activity in its vicinity, thus monitoring extracellularly the activity of both the intracellularly recorded cell as well as other units in its surroundings. Spikelets were observed in a quarter of our recordings (n = 36). In eight of these nine recordings a second extracellular unit fired in correlation with spikelet occurrences. This observation is consistent with the idea that the spikelets reflect action potentials of electrically coupled nearby neurons. The extracellular spikes of these secondary units preceded the onset of spikelets. While the intracellular spikelet amplitude was voltage dependent, the simultaneously recorded extracellular unit remained unchanged. Spikelets often triggered action potentials in neurons, resulting in a characteristic 1- to 2-ms delay between spikelet onset and firing. Here we show that this relationship is bidirectional, with spikes being triggered by and also triggering spikelets. Secondary units, coupled to pyramidal neurons, showed discharge patterns similar to the recorded pyramidal neuron. These findings suggest that spikelets reflect spikes in an electrically coupled neighboring neuron, most likely of pyramidal cell type. Such coupling might contribute to the synchronization of pyramidal neurons with millisecond precision.

Spikelets in CA1 pyramidal neurons, also known as fast prepotentials, were first described in vivo over a half a century ago by Kandel and Spencer (1961). They were shown to correlate with the location of the animal, similar to firing in place cells (Epsztein et al. 2010). To date, the mechanisms underlying their occurrence remain controversial. The suggested mechanisms are 1) dendritic spikes (Kamondi et al. 1998; Kandel and Spencer 1961), 2) ectopic spikes (Avoli et al. 1998; Stasheff et al. 1993), and 3) spikes in an electrically coupled neuron (MacVicar and Dudek 1981; Mercer et al. 2006; Schmitz et al. 2001; Vignmond et al. 1997). These different mechanisms were explored in various experiments, mostly in vitro. In most cases the spikelets had to be evoked either by stimulation (Schmitz et al. 2001) or by inducing a pathological seizure-like state in the slices (Avoli et al. 1998; Stasheff et al. 1993). It is not entirely clear whether the spikelets observed under these different conditions are the same as the spontaneous spikelets recorded in CA1 pyramidal cells in vivo.

Dendritic spikes, the first suggested source for spikelets, in CA1 pyramidal neurons had variable effects on the soma: small-amplitude excitatory postsynaptic potentials (EPSPs), partial spikelike responses, and even somatic action potentials (Golding and Spruston 1998; Kamondi et al. 1998; Losonczy et al. 2008). However, after the backpropagation of a somatic action potential into the dendrites the probability for dendritic spikes is largely reduced because of the inactivation of voltage-dependent sodium channels (Remy et al. 2009). This is in contrast to the observed mixtures of spikes and spikelets during bursts in in vitro recordings (Epsztein et al. 2010). Ectopic spikes, the second suggested source for spikelets, have been shown to occur spontaneously in hippocampal pyramidal cells under pathological conditions. In those cases they appeared in somatic recordings as spikes of varying amplitudes (Avoli et al. 1998). Under nonpathological conditions, antidromic stimulations induced a full-blown spike, which exhibited a sharper rise than orthodromically triggered action potentials. When stimulating putative axons of nearby cells (Schmitz et al. 2001), spikelets could be evoked in the soma. The proposed mechanism was that the stimulation evoked spikes in adjacent axons and that these signals traveled into the recorded cell’s axon via axo-axonal coupling. The signal then backpropagated in the axon, generating a spikelet somatically. Spikelets evoked in this fashion were sensitive to intracellular blockage of voltage-dependent sodium channels as well as to gap junction blockers. Recently, spikes occurring during ripples in slice preparations were shown to be ectopic spikes; these spikes were riding on spikeletlike events (Bähr et al. 2011).

Theoretical and experimental work on cortical pyramidal cells suggests yet another variation of the ectopic origin of spikelets. This work proposed that spikelets are spikes initiated at the axon that fail to invade the soma and trigger a somatic action potential (Hu et al. 2009). In this study antidromic stimulation was used to trigger spikes in the soma. After hyperpolarizing the soma the same stimulation failed to trigger a full-blown spike, and instead spikelets were recorded somatically. The action potentials were riding on top of these spikelets, similar to what was observed for spikelets and “shouldered” spikes in spontaneous recordings in vivo (Epsztein et al. 2010).

The third option, electrotonic coupling, implies that there is a second unit whose firing is the cause of spikelets. In vitro work directly demonstrated current transfer between CA1 pyramidal neurons (Mercer et al. 2006). Such connected couples, although rare, demonstrated strong coupling. Furthermore, evoked action potentials in one cell induced spikelets in the
connected cell. Those spikelets were similar in kinetics and amplitude to those described in vivo (Epsztein et al. 2010). However, the in vitro electrotonic coupling could result from membrane fusion due to slicing procedures (Gutnick et al. 1985). While such an artifact is likely to be resistant to pharmacological treatments, spikelet frequency was shown to be modulated with treatments known to affect the conductivity of gap junctions (Valiante et al. 1995). Moreover, spikelets, triggered by stimulation of putative axons of adjacent neurons, were abolished by the gap junction blocker carbenoxolone (Schmitz et al. 2001). However, in the absence of strong anatomical evidence for gap junctions between hippocampal pyramidal neurons in general (Hamzei-Sichaniet al. 2007) and between CA1 pyramidal cells in particular, membrane fusion is still a concern.

A fourth postulated mechanism for coupling is field effect coupling. In this scenario the spikelet is thought to be triggered because of the synchronized activity of the network that causes a redistribution of charge across the cell membrane, thereby polarizing it (Vigmond et al. 1997). This mechanism is thought to play a critical role during highly synchronized states of the network such as during epileptic discharge. Under nonpathological conditions the effect should be rather small and could not explain 10-mV spikelets.

We investigated the origin of spikelets in vivo, where they occur spontaneously. We asked whether spikelets in one cell correlate with spikes of another cell in its vicinity. To this end we performed simultaneous intra- and extracellular recording (Henze et al. 2000) to monitor the firing of one intracellularly recorded neuron (n = 36) and of additional extracellularly recorded units in its vicinity. We report that spikelets are often correlated with and preceded by firing of another unit. The timing was too fast for chemical transmission. Firing patterns of these secondary units are indistinguishable from those of CA1 pyramidal neurons. We further show that spikes and spikelets can be generated within very short time intervals and that these two signals seem to trigger each other with a characteristic delay of 1–2 ms, thus indicating bidirectional, a hallmark of electrotonic coupling.

MATERIALS AND METHODS

Experiments

Male Wistar rats (24–28 days old) were anesthetized with a ketamine-xylazine mixture (initial dose 8 mg ketamine and 1.2 mg xylazine per 100 g and then a third of the initial dose every 45 min). Rats were then placed in a stereotaxic frame. Animal temperature was monitored with a rectal probe and maintained at 36°C. A craniotomy was made to target the dorsal CA1 hippocampal area (3.5 mm posterior and 2.5 mm lateral in relation to bregma), and the dura was removed. The depth of the CA1 pyramidal layer was detected by ripple activity measured with a glass extracellular electrode (~2 MΩ). This was followed by dual intracellular and extracellular recordings, performed with custom-made constructs (Fig. 1A) consisting of a patch pipette (5–7 MΩ) glued to a tungsten electrode (1 MΩ). Distances between the patch pipette and tungsten electrode tips were between 18.8 and 140.5 μm (average 72.8 μm ± 46.4 μm, mean ± SD). Recordings were targeted to the pyramidal cell layer where blind in vivo whole cell recordings of CA1 pyramidal neurons were obtained (Margrie et al. 2002). In additional experiments (n = 11, of which 3 exhibited spikelets) we used only a patch pipette to record from CA1 pyramidal neurons; if the neuron exhibited spikelets, we injected brief current pulses of varying amplitudes and in a random order into the cells to evoke action potentials (pulse length 20 ms; pulse amplitudes 0, 300, 400, 500 pA). Patch pipettes were filled with an intracellular solution containing (in mM) 135 K-gluconate, 10 HEPES, 10 Na2-phosphocreatine, 4 KCl, and 4 MgATP, with Na2GTP (pH adjusted to 7.2) as well as biocytin (~0.05%). For intracellular recordings we used the WPC-100 amplifier (Abimex-Zech electronic, Friedland, Germany). Extracellular recordings were done with the EXT 10-2F amplifier (NPI electronic, Tamm, Germany).

After recording, animals were perfused with phosphate buffer followed by a 4% (wt/vol) paraformaldehyde (PFA) solution. After the perfusion, the brains were removed and stored overnight at 4°C in a 4% PFA solution. After 24 h, the PFA solution was replaced with phosphate buffer until sectioning of the brain (no more than 2 days).

Fig. 1. Dual intra- and extracellular recordings from a CA1 pyramidal neuron reveal a correlated extracellular signal for intracellular spikelets (inSpikelets). A: custom-made construct consisting of a patch and a tungsten electrode glued together with their tips close to each other. B: magnified view of the tips. C: morphological reconstruction of a recovered hippocampal CA1 neuron, labeled with biocytin and visualized by the avidin-biotin-peroxidase method. Or, stratum oriens; Pyr, stratum pyramidale; Rad, stratum radiatum; Mol, stratum lacunosum-moleculare. D: snapshot of the recording from the cell in C. Black trace shows the intracellular membrane potential, filled and open arrows mark intracellular spikes (inSpikes) and inSpikelets, respectively. Gray trace shows the simultaneously acquired extracellular recording. Arrows point to primary extracellular signals (PESs) and secondary extracellular signals (SESs) (filled and open arrows, respectively). E: black traces are superpositions of all inSpikes. The corresponding traces from the extracellular recording are shown in gray. For every inSpike there was a corresponding PES. F: black traces are superpositions of all inSpikelets. The corresponding traces from the extracellular recording are shown in gray.
Brains were sectioned to 150-μm slices and processed with the avidin-biotin-peroxidase method to visualize the biocytin-filled cell morphology. Cell morphology was then reconstructed with NeuroLucida software (Fig. 1C; Microbrightfield, Williston, VT). We recovered 28 of 36 cells; in all cases only single cells were recovered. Chemicals were purchased from Sigma-Aldrich. All experiments complied with German regulations on animal welfare and were approved by an ethics committee (LAGeSO-Landesamt fuer gesundheit und Soziales).

We performed 41 such paired recordings, of which only 36 were used for analysis. The discarded recordings either were recordings from interneurons (as was evident from histology and by other criteria such as firing rate, firing pattern, and spike width) or had intracellular recording of poor quality. Cells were used for analysis only if their membrane potential was more hyperpolarized than −55 mV and they had overshooting spikes. Seal formation was between 1 and 10 GΩ. Average input resistance was 40 ± 4 MΩ, and average series resistance was 64 ± 6 MΩ.

Data were collected and digitized at 50 kHz (NI PCI-6251 National Instruments card with self-programmed software using LabVIEW, National Instruments) and stored on a PC for off-line analysis.

Analysis

Intracellular spike and spikelet detection. Intracellular spikes and spikelets were detected on the derivative of the membrane potential data. This was essential for eliminating slow-frequency modulations of the membrane potential. Since spikelets have slower rise time than spikes, a double-threshold strategy was sufficient to detect both spikes and spikelets and to differentiate between the two. The criterion for spikes was crossing of both thresholds, while for spikelets the criterion was crossing the lower but not the higher threshold. Thresholds were set manually. For those cases in which the spikelets triggered spikes during their rising phase, we followed by looking at all spikes and checking whether their derivative displayed a shoulder and if so whether this shoulder fit the kinetics of spikelets (Epsztein et al. 2010). Spike waveforms were aligned to each other by maximizing the cross correlation between all spikes to a randomly selected spike. Spikelet waveform alignment was done in the same fashion. The correlated extracellular traces were taken after high-pass filtering of the trace (cutoff frequency 600 Hz, using symmetrical filtering to avoid phase distortion of the signal).

Extracellular spike detection and sorting. Traces were high-pass filtered (cutoff frequency 600 Hz, using symmetrical filtering to avoid phase distortion of the signal). Spikes were detected by using a threshold on the filtered data. Spikes were aligned to each other by maximizing the cross correlation between all spikes to a randomly selected spike. Spike sorting was done by principal component analysis. Clustering was done by manually identifying cluster centers in the first three principal components space and then using the k-nearest neighbor approach, with k = 3, to form the clusters. For a cluster to be considered as a single unit it had to pass the refractoriness period test (i.e., no interspike intervals < 1 ms).

All analysis was done with MATLAB scientific programing language (MathWorks, Natick, MA).

RESULTS

To simultaneously record intra- and extracellularly from CA1 pyramidal neurons we manufactured constructs in which a patch pipette and tungsten electrode were closely glued together (Fig. 1, A and B). average tip-to-tip distance 72.8 ± 46.4 μm, mean ± SD). We obtained blind in vivo whole cell recordings (Margrie et al. 2002) in rats anesthetized with a ketamine-xylazine mixture. We labeled the cells with biocytin to enable the identification and three-dimensional reconstruction of the recorded cells (Fig. 1C). In none of the experiments in which we could recover the recorded neuron by biocytin fills (n = 28 cells) did we observe dye coupling. The intracellular spikes (inSpikes) are visible in the extracellular recording as well as other units (Fig. 1D).

Spikelets Are Correlated with Extracellular Signals Different from Those Associated with Spikes

In 9 of 36 CA1 pyramidal dual recordings we observed intracellular spikelets (inSpikelets). The fraction of cells showing spikelets was lower than previously reported (Epsztein et al. 2010), probably because in this study the animals were anesthetized while in the previous study animals were initially anesthetized before the administration of an antide and the waking up of the animals. Figure 1C shows the reconstruction of one cell that had inSpikelets. The inSpikelets were associated with secondary extracellular signals (SESs), different from the primary extracellular signals (PESs) associated with inSpikes (Fig. 1D). In this example, we observed not only a perfect correlation between inSpikes and PESs (Fig. 1E) but also a striking correlation between inSpikelets and SESs (Fig. 1F). In eight of nine recordings in which we detected inSpikelets, the inSpikelet-triggered averages of the extracellular signals revealed spikelike signals. In seven of these, inSpikelets co-occurred with well-separated large-amplitude SESs, different from the PESs. In those cases in which a secondary extracellular unit was identified, an inSpikelet accompanied every SES (100% of the detected SESs were accompanied by an inSpikelet; SES number was 8,665, n = 7). Conversely, almost every inSpikelet was correlated with SES (~96% of inSpikelets were accompanied by detected SESs; number of inSpikelets was 9,024, n = 7); between inSpikes and detected PESs this figure was ~99%, number of inSpikes = 42,978, n = 36). The lower correlation observed between SESs and inSpikelets than for PESs and inSpikes was probably due to the fact that successive inSpikes in bursts have decreased amplitudes and attenuated kinetics (Buzsáki et al. 1996). As a result, the extracellular signals of consecutive spikes in bursts became smaller and hence harder to detect. Similar attenuation was also observed for bursts of inSpikelets and their correlated extracellular signals; since SES amplitudes were often smaller than PESs to begin with, the detection of late events in those bursts was even more prone to failure. However, even though we were sometimes unable to detect the extracellular correlates of late inSpikes, we did see an extracellular correlate of inSpikelets, events that are of much smaller amplitude (amplitude: inSpikelets 6.3 ± 3.1 mV, 5th inSpikes in burst 44.6 ± 14.2 mV; mean ± SD) and slower kinetics (initial rise slope: inSpikelets 5.35 ± 3 V/s, 5th inSpikes in bursts 37.6 ± 18 V/s; mean ± SD). This suggested to us that the SESs we recorded were not the extracellular manifestation of the inSpikelets.

Membrane Potential Affects Intracellular inSpikelet but Not SES Waveforms

To further explore the independent sources of inSpikelets and SESs we tested the voltage dependence of the inSpikelet shapes and their correlated SESs. Figure 2A shows the averaged inSpikelet traces at different membrane potentials from one such recording. The amplitudes of inSpikelets became smaller in hyperpolarized membrane potentials (Fig. 2A, left,
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and Fig. 2, B and D), and the general shape was altered. In contrast, the correlated SESs remained unchanged (Fig. 2A, right). Thus, unlike for inSpikes, where the PES is the extracellular signal, the SESs are records of different, yet highly correlated, events. We further observed that the frequency of both inSpikelets and inSpikes increases when depolarizing the membrane potential (Fig. 2C), with inSpikes showing greater sensitivity.

Spikelets Are Preceded by Firing of a Secondary Unit

Since inSpikelets and SES are correlated events, it was interesting to assess whether there was a temporal relation between them. PESs and inSpikes, by definition, occur simultaneously. Therefore the temporal relation between defined points on the two waveforms can be compared with the interval between the same defined points of inSpikelets and SESs to evaluate their temporal order. To this end, we aligned the inSpikes and inSpikelets to one another (Fig. 3A) at the points of maximal change of the derivative (i.e., the inflection point; Fig. 3A); we then plotted the correlated normalized extracellular signals on top of each other (Fig. 3B), and we repeated this process for all eight recordings that had SESs. We then measured the time delay between the first peak of the extracellular signal and the point of alignment. Figure 3C summarizes the results, showing that SES waveforms tended to precede PES waveforms ($P < 0.05$, Wilcoxon rank sum test), indicating a temporal order and therefore a likely causal relation between SES and inSpikelets. The delay between the two events cannot be explained by chemical transmission, and therefore we conclude that inSpikelets are triggered by action potentials in an electrically coupled secondary unit or compartment (i.e., axon or dendrites).

Figure 3, E and F, show the amplitude relationships of the PESs and SESs. In all but one of our recordings, PESs had bigger amplitudes than SESs (Fig. 3E). The amplitudes of SESs were within the range of PESs recorded with the same type of electrodes. Furthermore, the shapes of SESs did not statistically differ from the shapes of PESs, as revealed by a principal component analysis of the average waveforms of both populations (Fig. 4). This suggests that SESs are usually of smaller amplitude than PESs because the electrode is further away from their source. This bias is probably due to the fact that we tried to manufacture the constructs such that the patch and tungsten electrodes were close by. It implies that the source of SESs should be capable of generating currents comparable to the currents generated by somatic spikes. The dendrites seem unlikely candidates because only small-amplitude dendritic spikes seem to occur in bursts while fast, large spikes do not (Kamondi et al. 1998). Additionally, dendritic spikes are unlikely to follow somatic action potentials because of the inactivation of voltage-dependent sodium channels induced by the backpropagation of the spike (Remy et al. 2009). This is in contrast to the observed mixtures of spikes and spikelets during bursts (Epstein et al. 2010). Furthermore, currents generated by such spikes are expected to be small and to attenuate rapidly with distance (Buzsáki et al. 1996). Moreover, dendritic spikes can be initiated in multiple locations on the dendritic tree, and if they were to generate somatic spikelets then a heterogeneous population of spikelets would have been expected, in contrast to the single or rarely two populations of spikelets observed in our recordings.

The axon is another candidate for generating the SES. In such a scenario the inSpikelet would result from an axonal spike to invade and trigger a somatic spike. It follows that the extracellular signal generated when the spike invades the soma (inSpike) should be a combination of the SES and the somatic spike currents; however, for inSpikes we do not get extracellular signals that are combinations of the PES and SES, unless the inSpike is a shouldered spike and then we see a SES followed by a PES. Alternatively, the PES could result from a spike initiated at the soma rather than the axon.

Fig. 2. SES shape is independent of changes in inSpikelet shape. A, left: averages of inSpikelet waveforms at different holding potentials. Right: corresponding averages from the extracellular recording showing the SES. B: average inSpikelet amplitude as a function of membrane potential ($V_m$) ($n = 1,444$). Error bars are standard deviations. C: frequencies of inSpikes and inSpikelets (black and gray, respectively) as a function of membrane potential. D: population analysis of the dependence of inSpikelet and SES amplitudes (black and gray, respectively) on membrane potential of the inSpikelets. For each cell ($n = 7$) the inSpikelets and SES amplitudes were normalized to the maximal amplitude. The normalized signals were then averaged depending on the membrane potential at which the inSpikelets were elicited and the standard deviation calculated. We then averaged the normalized amplitudes for each condition across all cells and we calculated the standard deviation of the standard deviations (bars).
Phase Plots Suggest That inSpikes and inSpikelets Are Not Initiated in the Somatic Compartment

To assess which of the all-or-none signals found in neurons exhibiting spikelets (Fig. 5A) are initiated somatically and which remotely, we analyzed the dependence of the thresholds of inSpikes, inSpikelets, and shouldered inSpikes (Fig. 5A) on the somatic membrane potential. Threshold was defined as the voltage where the voltage derivative crosses 20 V/s for inSpikes and 3 V/s for inSpikelets. If the threshold changes with membrane potential, it points to a remote location of the initiation, given that at the initiation site the threshold should be more or less constant (Hu et al. 2009). The phase plots for these three signals are shown in Fig. 5B. Note that the inSpikelet and the shouldered inSpike overlap at the beginning of their rising phase, suggesting that an inSpikelet is triggering an inSpike, resulting in a shouldered inSpike. This can be due to the inSpikelet triggering a spike at the soma or at the axon initial segment. In a study by Hu et al. (2009) it was shown that an antidromic stimulation led to shouldered action potentials at the soma. They demonstrated that the shoulder is due to an action potential at the axon initial segment, while the later full-blown action potential is due to the active initiation of a spike in the somato-dendritic compartments. The later part was shown to be blocked by hyperpolarization of the soma. In this scenario, the shoulder part of the action potential (i.e., inSpikelet) was shown to have a voltage-dependent threshold at the soma while the later somatic spike had a weak dependence on the somatic membrane potential. In our data, the thresholds of inSpikelets showed a dependence on the membrane potential similar to that in the Hu et al. study (Fig. 5C, middle), indicating that they are initiated at a different compartment. Similarly, inSpike thresholds were also voltage dependent at the soma (Fig. 5C, top), suggesting that they too are initiated at a different compartment, presumably the axon initial segment. The shoulders of shouldered inSpikes look indistinguishable from inSpikelets (Fig. 5C, bottom and middle). The later part of these shouldered inSpikes show a few interesting features differentiating them from the shouldered spikes observed by Hu et al. 1) The inSpike appears to begin at different phases of the inSpikelet, at times after the inSpikelet has begun its decay (Fig. 5C, bottom, inset). 2) The thresholds for this later phase seem as voltage dependent as inSpikes and inSpikelets (threshold was defined as crossing +20 V/s from beginning of rising slope of inSpike; Fig. 5C, bottom, inset). This suggests that both the shoulder part of shouldered inSpikes and the action potential part are initiated remotely. Such linear dependencies of the threshold on membrane potential for both inSpikes and inSpikelets were also apparent at the population level. Hence we have two signals, inSpikes and inSpikelets, that are initiated remotely, and at

Fig. 3. Comparison of the temporal and amplitude relations between the extracellular events corresponding to spikes and spikelets revealed that the units correlating with spikelets fire before spikelet onset. A: the averaged inSpike (black) and inSpikelet (gray) were aligned to one another at the point of maximal change in the derivative (dashed vertical line). B: averaged corresponding PES (dark green) and SES (light green) from 8 different cells (each row is for 1 cell). The traces were normalized to the same amplitude. Note that the light green traces slightly precede the dark green traces. C: quantification of the temporal relations. We measured the average delay between the alignment point (dashed vertical line) and the 1st peak for both the PES (dark green; n=36) and the SES (light green; n=8); error bars indicate the standard errors of the measurements. The mean intervals were significantly different (P < 0.05, Wilcoxon rank sum test) across the 2 groups. D: same as in A but with a different timescale. E: same traces as in B but not normalized. F: amplitudes of the SESs are plotted against the simultaneously acquired PESs; note that most SESs are smaller than their matched PESs.
times these two events co-occur, resulting in a superposition of these two events thus yielding shouldered inSpikes. The fact that the SES can be bigger than the PES rules out the hypothesis that the sources of SESs are spikes restricted to the axon while PESs are spikes initiated at the axon and invading the soma. This leaves spikes in coupled cells as the only plausible source for SESs.

**Secondary Units Are Indistinguishable from CA1 Pyramidal Units**

To assess the identities of these coupled units, we analyzed their firing patterns (Fig. 6). We used the inSpikelets rather than the SESs since spike sorting is less reliable than the intracellular detection of inSpikelets. The inter-inSpike interval histograms (Fig. 6A) were compared with the inter-inSpikelet interval histograms (Fig. 6B). In cells that fired bursts (8 of the 9 cells showing spikelets) both inSpikes and inSpikelets showed the characteristic ∼5-ms refractory period corresponding to the minimal value of inter-inSpike/inSpikelets intervals during bursts. In five cells (n = 9) the distributions of intervals of inSpikes and inSpikelets were not significantly different by Kolmogorov-Smirnov test (P > 0.05). We also checked how similar the inter-inSpike distributions of these 9 different cells were; we found that 5 of 36 possible combinations were found to be similar (P > 0.05, Kolmogorov-Smirnov test). For the inter-inSpikelet interval distributions, 6 of 36 possible combinations were found to be similar (P > 0.05, Kolmogorov-Smirnov test). This implies that connected units exhibit more similar firing patterns than nonconnected units. This suggests that both the primary cell and the coupled neuron are from the same class, in this case CA1 pyramidal neurons.

**Connections Between Primary and Secondary Units Are Bidirectional**

Electric coupling often, but not always (Csicsvari et al. 1999), leads to bidirectional coupling between neurons. Such connectivity can lead to mutual excitation. In our case, mutual excitation would mean that spikes in one cell excite spikes in the coupled neurons and vice versa. This is the same as showing that the probability for inSpikes increases directly after inSpikelets and that the probability of inSpikelets increases directly after inSpikes (Fig. 7A). When we analyzed the distribution of inSpikelet to inSpike intervals (Fig. 7, B–D, top) and of inSpike to inSpikelet intervals (Fig. 7, B–D, bottom), clear peaks in intervals of 1–2 ms were apparent in both conditions. This indicates that the connections are bidirectional, as expected from electrical synapses. It appears that the interval for inSpikes to follow inSpikelets is slightly shorter than the interval for inSpikelets to follow inSpikes (Fig. 7, B–D). In the case in which the inSpike triggers an inSpikelet, a longer delay is expected as the inSpike needs to travel to the other cell, generating there an inSpikelet, which will in some

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**Fig. 4.** PES and SES waveforms are not statistically different. We collected all PESs (n = 36) and SESs (n = 9) and normalized them to the same amplitude. We then did a principal component analysis of these waveforms. We plotted the 2nd and 3rd principal components vs. the 1st principal components (left and right, respectively). Black and gray dots mark PES and SES, respectively. Note that both occupied the same space.

**Fig. 5.** inSpikelets and inSpikes are not initiated within the soma. A: examples of the 3 all-or-none events found in CA1 pyramidal neurons exhibiting inSpikelets: inSpikelet (blue), inSpike (gray), and a shouldered inSpike (black). B: the corresponding phase plots (membrane potential derivative vs. membrane potential) of the signals in A. C: phase plots of inSpikes (top), inSpikelets (middle), and shouldered inSpikes (bottom). Inset, bottom: zooming in on 4 shouldered inSpikes. Green and purple, events where the inSpike started on the rising phase of the inSpikelet; blue and black, events where the inSpike was initiated after inSpikelets begun to decay. Dots mark the thresholds.
cases trigger an inSpike, which will travel back and form an inSpikelet. This also explains the lower counts observed for inSpike-to-inSpikelet intervals than for inSpikelet-to-inSpike intervals. This bidirectional excitation with characteristic intervals of 1–2 ms (but shorter intervals are also observed) is a hallmark feature of electrical coupling. The 8-ms peaks observed for inSpikelets following inSpikes are most likely due to the bursty nature of these events. Synchronized spikes in both cells will not result in inSpikelets, as no potential gradient is present; later unsynchronized spikes in a burst will result with inSpikelets following inSpikes with ~8-ms intervals.

This latter analysis was done on spontaneous firing. One might argue that the observed timing of events is due to network activity rather than a functional connection between the units. To overcome the network contribution, we did additional experiments in which we repeated inSpikelet-inSpike correlation analysis but for evoked inSpikes and inSpikelets. We recorded from 11 cells, of which 3 had inSpikelets. In those three cells we injected brief current pulses (20 ms) of varying amplitudes (0, 300, 400, 500 pA) in a randomized order. Figure 8A shows examples of responses to such pulses. We then detected all inSpikelets and inSpikes, which occurred during pulses. Figure 8B shows examples of inSpikelets superimposed on top of each other during rest (Fig. 8B, top) and during depolarizing pulses (Fig. 8B, bottom). We calculated the time intervals between inSpikes and inSpikelets and plotted histograms of these time intervals (Fig. 8C) during depolarized pulses and during rest; 1,500 interleaved pulses were used for rest and depolarized conditions. The histograms were normalized to the number of inSpikelets. This particular cell did not show bursting activity of either inSpikes or inSpikelets under resting conditions, as can be seen by the lack of peaks in the histogram for the rest condition. During the depolarizing pulses more inSpikes were triggered by inSpikelets, as is evident from the ~1–2 ms peak in the correlogram (Fig. 8C). Also, more inSpikelets were triggered by inSpikes, as is evident from the

Fig. 6. Comparing firing patterns of spikes and spikelets. A: interspike interval histograms for 5 CA1 pyramidal neurons. B: interspikelet interval histograms for the same 5 CA1 pyramidal neurons. One-millisecond bins were used. The spike/spikelet counts were normalized to their total number.

Fig. 7. Mutual excitation between coupled units during spontaneous activity. A, top: black, superposition of traces where inSpikes followed inSpikelets with short delay; gray, corresponding extracellular traces showing traces where PES followed SES. Bottom: black, same as top but traces where inSpikelets followed inSpikes with short delay; gray, cases where SES followed PES with short delay. B, top: histogram of the intervals between inSpikelets and later inSpikes from same cell as in A. Bottom: histogram of the intervals between inSpikes and later inSpikelets from same cell as in A. C and D: same as in B but from 2 additional cells.
increased frequency of events between −1 and −2 ms. Peaks were also observed at ±5 ms corresponding to the typical firing frequencies observed during depolarizing pulses. While both inSpikes and inSpikelets frequencies increase during depolarization, the effect was more prominent for inSpikes (Fig. 8, D and E). Figure 9A shows the averaged inSpikelet-inSpike cross correlograms from two additional cells. These cells were bursting during the resting condition, as is evident from the ±8 ms peaks in the cross correlogram (Fig. 9A). During depolarizing pulses more inSpikelets were triggered by inSpikes (compare −1-ms peaks between rest and depolarized condition, Fig. 9A). The fraction of spikelets that triggered inSpikelets increased as well (compare +1 ms peaks, Fig. 9A).

It should be emphasized that the increase in inSpikelet frequency is only partially due to inSpikes triggering inSpikelets. InSpikelets, which were not preceded by inSpikes, are more prominent after 4 ms from pulse onset (Fig. 9B). This increase can be explained by the indirect depolarization of the coupled neuron by the current injections.

Therefore inSpikelets both are triggered by and trigger inSpikes, and this occurs both in network-driven activity as well as during evoked activity. Thus we conclude that this bidirectional relation is independent of the network state and reflects a direct connection between inSpikes and inSpikelets.

**DISCUSSION**

Spikelets are a major part of CA1 pyramidal neurons’ electrical repertoire; however, to date the mechanisms underlying their occurrences are still debated. In this report we show, in vivo, that inSpikelets are time locked to SESs, and while these two signals are highly correlated, they are not manifestations of the same process. We demonstrate that the SES waveforms precede those of PESs, suggesting that SESs trigger inSpikelets. We show that SESs and PESs are statistically indistinguishable and that the sources of SESs generated large currents, arguing for the coupled cell origin for SESs. We further show that these two coupled cells mutually excite each other with a short time delay. Our interpretation of the data, in light of what has been published about spikes in the different compartments of CA1 pyramidal neurons and following Occam’s razor principle, supports the coupled cell hypothesis for inSpikelet origin. However, it is important to point out that much of the knowledge about dendritic spikes and ectopic spikes in CA1...
pyramidal neurons comes from in vitro work and might not hold true under in vivo conditions.

Origin of the Secondary Extracellular Signal

The possible sources of the SES are an extracellular recording of the inSpikelet, a dendritic spike, an axonal spike, or a spike in a coupled cell. As we showed, the inSpikelets but not the SESs are influenced by membrane potential manipulations, indicating that the SESs are not extracellular records of somatic inSpikelets.

The hypothesis that the SES is the extracellular manifestation of a dendritic spike is unlikely, as the expected signals of such events are very small (Buzsáki et al. 1996). The amplitudes of SES signals we record are well within those reported for units. In one recording, the SES amplitudes were even bigger than the PES amplitudes (Fig. 3). Another indication against a dendritic source is the fact that SESs often follow somatic spikes, which because of backpropagation renders the voltage-gated sodium channels of the dendrites largely inactivated (Remy et al. 2009).

The extracellular signal expected for a spike restricted to the axon is less clear. While the axon is a very thin structure, it is a very specialized one, with very high densities of channels. The SESs were recorded extracellularly with tungsten electrodes inserted blindly, implying that if the source is axonal it should generate very high current densities much bigger than those generated by the larger soma. Further evidence against the idea of an axonal source of SESs is the indication that both inSikes and inSpikelets are generated remotely to the soma (Fig. 5). The inSikes are most likely initiated at the axon initial segment and then invade the somatic compartment; thus the corresponding extracellular signal (i.e., PES) should be a combination of the axonal and somatic currents. If an inSpikelet is a spike restricted to the axon, then the PES waveform should be a combination of the SES waveform and a somatic component. However, such combinations are only observed for shouldered inSikes or for inSpike-triggered inSpikelets.

Another remaining hypothesis for the source of the SES is a somatic spike from a different unit. The waveforms of SESs are indistinguishable from those of PESs. While the SESs were usually smaller in size than the simultaneously recorded PESs, their amplitudes were in the same range and they were usually detectable and sortable. Thus we conclude that inSpikelets are time locked to spikes in a secondary unit.

Nature of the Primary and Secondary Unit Connection

The correlation between the SES and inSpikelets and their temporal order suggest that inSpikelets are triggered by firing in a second pyramidal neuron. The short delay between the SES and the inSpikelet is incompatible with chemical transmission, pointing to electrical transmission as the only plausible mechanism. The mutual excitation between inSikes and inSpikelets (i.e., inSikes in a secondary unit) further supports a bidirectional transmission mode, a signature of electrotonic connections rather than chemical connections. The mutual excitation is further supported by the dependence of inSpikelet frequency on membrane potential. Increasing spike frequency in one cell increases spiking frequency in the connected cell. Additional support comes from previous findings that treatments that affect the coupling conductance also affect inSpikelet occurrences (Church and Baimbridge 1991; Perez-Velazquez et al. 1994; Schmitz et al. 2001; Velazquez et al. 1997). Moreover, direct coupling between pyramidal neurons has been previously reported in a slice preparation (Mercer et al. 2006). Interestingly, the finding that the amplitudes of inSpikelets decrease in hyperpolarized and depolarized membrane potentials is in line with the dependence of both connexin 36- and pannexin-mediated coupling conductance on the voltage difference between the two coupled units (Bruzzone et al. 2003), but other mechanisms might also contribute to this phenomenon, such as active process or activity-induced plasticity of coupling (Haas et al. 2011).

Three forms of coupling are explored in the literature: coupling through gap junctions, coupling due to field effects, and coupling due to membrane fusion following damage to the tissue. The first two are physiological, while the latter is an artifact of the experimental intervention. Field effects were postulated to play a crucial role during states of synchronized firing, such as during epileptic discharge. There are no indications of such firing patterns during normal explorative or ketamine-xylazine anesthesia states, nor do we observe such firing in our recordings. Furthermore, the predicted shape of spikelets due to field effects is biphasic, a shape observed only in hyperpolarized membrane potentials in our experiments. Thus we conclude that field effects are not the main source for the spikelets we observe.

Gap junction- and membrane fusion-mediated coupling will look rather identical. The latter, however, will not be affected by pharmacological manipulations affecting gap junctions. Spikelets were shown to be blocked by such treatment (Schmitz et al. 2001). Furthermore, if membrane fusion as result of damage was such a prominent outcome, one would expect tetrad in vivo studies to detect such synchronized activity, but this is not the case. The damage inflicted on the tissue by our methods is rather minimal compared with those inflicted by other methodologies. In a less invasive methodology using fluorescent calcium imaging, Dombeck and colleagues found that neighboring units are highly synchronized (Dombeck et al. 2010). While most studies describe electrotonic connections as being weak, there is evidence that between principal neurons electrotonic connections are rather sparse and strong (Wang et al. 2010); this might be due to active amplification of the coupling currents by voltage-sensitive conductances. The exact means of coupling remains to be elucidated, but we think that the accumulating evidence argues against an artifact source for this phenomenon.

How Far Is This Secondary Unit from the Primary Unit?

The radius in which units are detectable and sortable depends on the exact means of recording. In a previous study using tetrodes and silicon probes, this distance was estimated to be ~50 μm in the lateral axis and ~100 μm in the vertical axis (Henne et al. 2000). In our data set, we could record clear units up to 140 μm away from the intracellular electrode (maximal lateral distance 80 μm, maximal vertical distance 138 μm). Thus the secondary unit should be within a 150-μm radius of our extracellular electrode, and with maximal radius of ~300 μm from the primary neuron. If we assume that the secondary unit is a pyramidal cell, then this distance becomes smaller because the pyramidal cells are restricted to a layer.
The firing patterns of the secondary extracellular units suggest that they are, indeed, pyramidal cells.

An open question is the exact location of the coupling between pyramidal cells. Theoretical and experimental work suggested axo-axonal coupling (Schmitz et al. 2001; Traub et al. 2002), yet other studies predict the coupling to reside within the proximal dendrites (Mercer et al. 2006). Our results cannot differentiate between the two possibilities.

**Functional Clusters of Hippocampal CA1 Pyramidal Neurons**

Our results show that, while there is no topographical organization in the hippocampus, small functional clusters are present. In this context it is noteworthy that a recent imaging study detected a higher than expected similarity of spatial modulation in directly adjacent hippocampal cells (Dombecker et al. 2010). In light of the present findings, we postulate that this imaging result reflects electrically coupled cells exhibiting similar place fields (Epshtein et al. 2010). Coupled close-by neurons coding for identical locations can mutually excite each other and increase the probability of firing at a given location, thus making the location code more reliable on trial-by-trial bases than if such connectivity was not present.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: E.C. and M.B. conception and design of research; E.C. performed experiments; E.C. analyzed data; E.C. and M.B. interpreted manuscript.


