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# Electrical coupling underlies high-frequency oscillations in the hippocampus *in vitro*

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**Coherent oscillations, in which ensembles of neurons fire in a repeated and synchronous manner, are thought to be important in higher brain functions. In the hippocampus, these discharges are categorized according to their frequency as theta (4–10 Hz)[1], gamma (20–80 Hz)[2] and high-frequency (~200 Hz)[3–5] discharges, and they occur in relation to different behavioural states. The synaptic bases of theta and gamma rhythms have been extensively studied[6,7] but the cellular bases for high-frequency oscillations are not understood. Here we report that high-frequency network oscillations are present in rat brain slices *in vitro*, occurring as a brief series of repetitive population spikes at 150–200 Hz in all hippocampal principal cell layers. Moreover, this synchronous activity is not mediated through the more commonly studied modes of chemical synaptic transmission, but is in fact a result of direct electrotonic coupling of neurons, most likely through gap-junctional connections. Thus high-frequency oscillations synchronize the activity of electrically coupled subsets of principal neurons within the well-documented synaptic network of the hippocampus.**

The synaptic networks that generate theta and gamma rhythms depend critically on the time course of synaptic inhibition by interneurons that release γ-aminobutyric acid (GABA), which coordinate the discharge of principal cells[6,7]. Such mechanisms appear to be too slow[7] to control high-frequency oscillations of ~200 Hz recorded from freely moving rats *in vivo*[3–5]. High-frequency oscillations occur predominantly in the CA1 pyramidal layer, usually superimposed on negative 'sharp waves' lasting tens of milliseconds. High-frequency activity occurs at rest or during sleep and can be coherent over ~5 mm along hippocampal pyramidal layers[3,4].

We now report the occurrence of high-frequency oscillations in rat hippocampal slices *in vitro*. Low-pass filtering of extracellular field potentials at 400–500 Hz unmasked repetitive, short high-frequency discharges (Fig. 1). High-frequency oscillations occurred in 45 of 47 slices from 27 juvenile (postnatal day P18–26) rats, and in slices from two immature (P5) and four adult (>6 weeks old) rats. Autocorrelograms and power spectra revealed waveform repetition periods of 3–8 ms (125–333 Hz; Fig. 1A). Mean intraburst frequencies were as follows: for CA1, 180 ± 47 Hz (±s.d., $n = 33$ individual high-frequency events at independent sites in 20

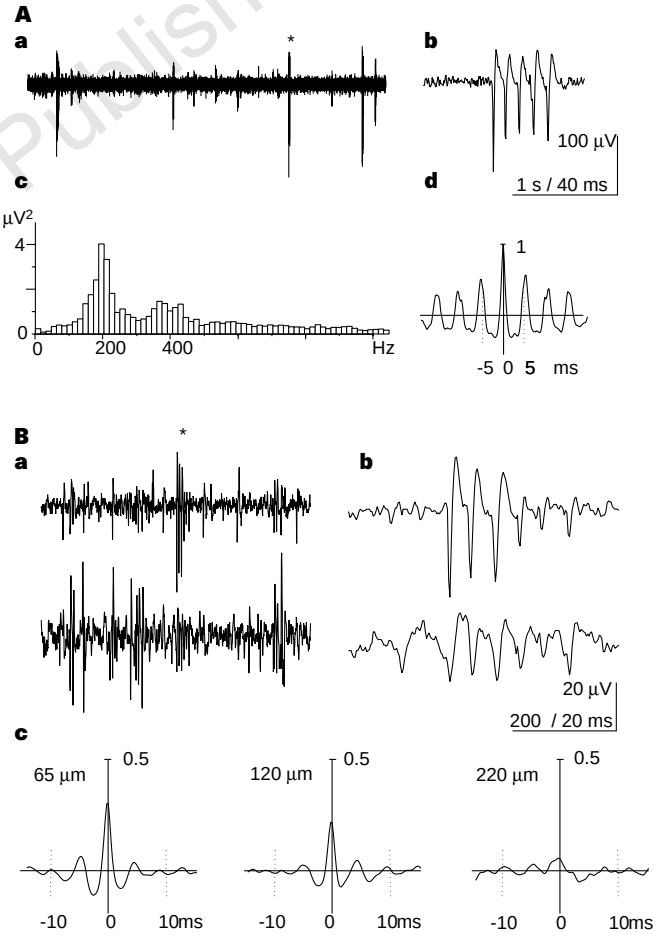


**Figure 1** High-frequency oscillations in CA3. **A**, Filtered (5–400 Hz bandpass) CA3 pyramidal layer field potential with spontaneous high-frequency oscillations. **a**, **b**, The 1-s time calibration applies to the left trace (**a**); the 40-ms scale applies to the right trace (**b**, from the section marked with an asterisk in **a**). The power spectrum **c** of the trace in **a** reveals a major component at ~180 Hz. The autocorrelation function (**d**) of the marked event in **b** shows peaks at +5.4 and –5.4 ms (185 Hz). Autocorrelations from 15 high-frequency oscillations at this site yielded a mean frequency of 207 ± 18 Hz. **B**, Paired field recording from CA3 pyramidal layer at varying distances. Traces in **a** show coherent events (the section marked with an asterisk is enlarged in **b**) as well as independent events. Cross-correlograms (**c**) of 5-s periods show coherent high-frequency oscillations when the spacing between electrodes is 65 μm or 120 μm (phase lags 0.1 and 0.2 ms, respectively), but not when the spacing is 220 μm.

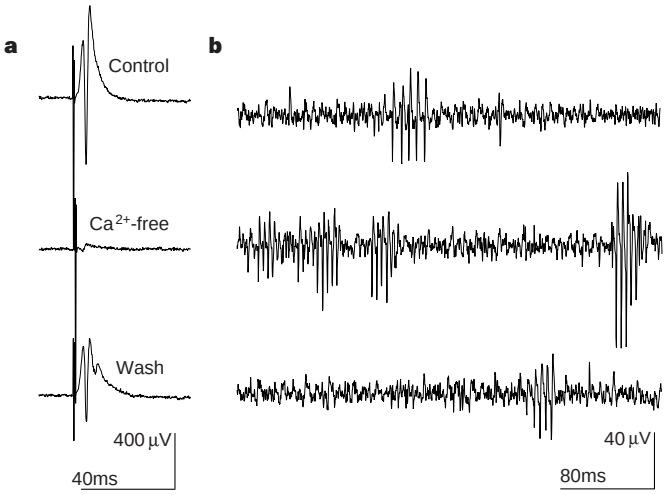


**Figure 2** High-frequency oscillations do not depend on synaptic transmission. **a**, Washout of $Ca^{2+}$ reversibly blocks field excitatory postsynaptic potentials and population spike responses recorded in the dentate granule cell layer following electrical stimulation of the perforant path. **b**, Spontaneous high-frequency activity recorded in CA3 of the same slice. When the postsynaptic response in (**a**) vanished because of washout of $Ca^{2+}$, high-frequency activity in CA3 increased (middle trace).

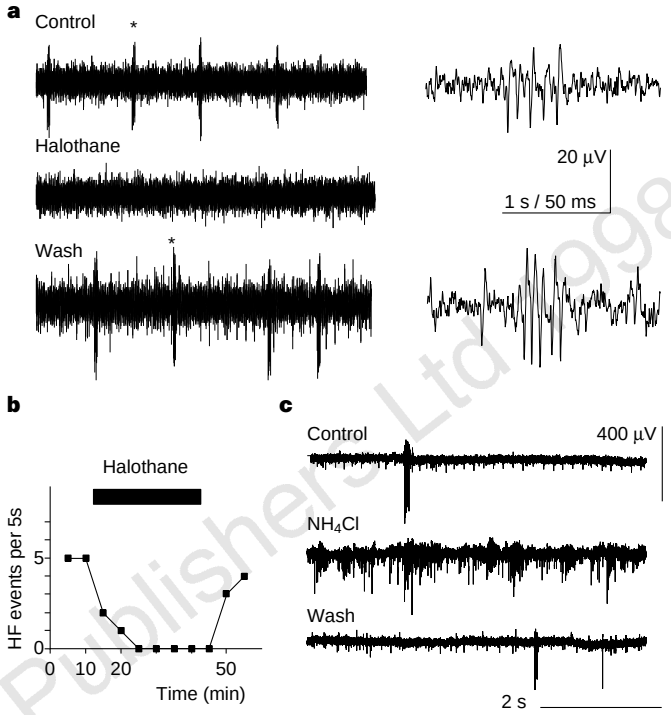


**Figure 3** Gap-junctional coupling and high-frequency oscillations. **a**, High-frequency oscillations in CA3 in normal ACSF (top) and in the presence of 5 mM halothane (middle) are shown. Halothane reversibly blocks the network activity. The 1-s time calibration applies to the left traces; the 50-ms scale applies to the expanded high-frequency events at the right. **b**, Time course of the blocking of high-frequency events by 5 mM halothane. The symbols indicate the number of high-frequency events per 5-s period recorded every 5 min. **c**, Intracellular alkalinization with 10 mM $NH_4Cl$ in the ACSF increased the number and duration of high-frequency oscillations in CA3.

slices); for CA3, $195 \pm 44$ Hz ($n = 26$); and for dentate, $161 \pm 31$ Hz ($n = 6$). High-frequency oscillations occurred more often when neuronal excitability was enhanced by elevated $K^+$ concentrations (5 mM), 20 μM carbachol, 70 μM 4-aminopyridine, or 0 mM $Ca^{2+}$ in the medium.

Laminar profiles in CA1 revealed pronounced high-frequency oscillations in the pyramidal cell layer but almost no detectable field potentials in the dendritic layers on either side ($n = 4$ of 4 slices tested). Moving the recording site along the cell layer revealed local patches of greater or less high-frequency activity.

To determine whether the high-frequency waveform reflects unit activity (that is, extracellularly recorded action potentials of single neurons) or coherent discharges in a neuronal ensemble, we inserted a second electrode at varying distances along the pyramidal layer. In four of five slices, we found clear correlations (but not identity), and consistently short phase lags, between high-frequency oscillations if the interelectrode distance was ⩽120 μm (which corresponds to the diameters of 6–8 cells, or several hundred pyramidal cells in the slice; Fig. 1B). The presence of coherent as well as independent high-frequency oscillations at two different recording sites makes it unlikely that high-frequency field potentials reflect action potentials of a single cell.

Synchronization of high-frequency oscillations *in vivo* has been attributed to GABAergic synaptic inhibition[4]. In our experiments *in vitro*, high-frequency activity persisted after addition of the $GABA_A$ receptor antagonist bicuculline (50 μM, $n = 13$). Further addition of ionotropic glutamate receptor antagonists (20 μM NBQX, and 40 μM D-AP5; $n = 8$) also failed to block high-frequency oscillations. To block chemical synaptic transmission completely, we used a nominally calcium-free solution. Postsynaptic field potentials ceased within 5–10 min of switching to this solution (Fig. 2a), but coherent high-frequency oscillations always became more, rather than less, prominent (9 of 9 slices, Fig. 2b). This was also the case when calcium-free artificial cerebrospinal fluid (ACSF) with 1 mM EGTA was used or when the $Mg^{2+}$ concentration in the calcium-free ACSF was increased from 4 to 7 mM. Thus, spontaneous high-frequency oscillations persist in the absence of calcium-dependent synaptic transmission.

Synchronized 'field bursts' can occur in CA1 in the absence of synaptic transmission[8,9] and appear to be mediated—in part—by gap junctions[9,10]. We therefore tested three gap-junction blockers. Octanol (1 mM, $n = 7$), halothane (5 mM, $n = 3$, Fig. 3a, b) and carbenoxolone[11] (100 μM, $n = 6$) all reversibly suppressed spontaneous high-frequency activity. The common action of these three agents is to block gap junctions. Halothane and carbenoxolone did not alter input resistance, time constants and discharge behaviour of CA1 neurons, but octanol reduced evoked repetitive-spike firing in 2 of 5 cells ($n \geqslant 5$ for each substance). Conversely, high-frequency oscillations occurred more frequently and were prolonged on adding $NH_4Cl$ (10 mM, $n = 5$, Fig. 3c), an agent that causes intracellular alkalinization[12], which enhances gap-junction coupling[13].

To identify the cell types involved in the network oscillations, we made whole-cell current clamp recordings from 23 regular-spiking neurons in the pyramidal layer, and from 15 putative interneurons, in the vicinity of the field potential electrode. One putative CA1 pyramidal cell recorded in calcium-free solution participated unambiguously in high-frequency discharges (Fig. 4). Most negative-going population spikes within the high-frequency oscillations were accompanied by an all-or-nothing depolarization resembling 'spikelets' or 'fast prepotentials'[10,14]. Field potential spikes remained similar whether the recorded cell exhibited subthreshold spikelets or full action potentials, indicating that the high-frequency field potential oscillations were not produced by this one cell but were generated by a multicellular network of neurons.

Computer simulations based on a detailed compartmental model of CA3 pyramidal cells[15] showed that fast coupling mediated by gap junctions is possible (Fig. 4D). Realistically fast coupling potentials (or spikelets) were best reproduced when gap junctions were located

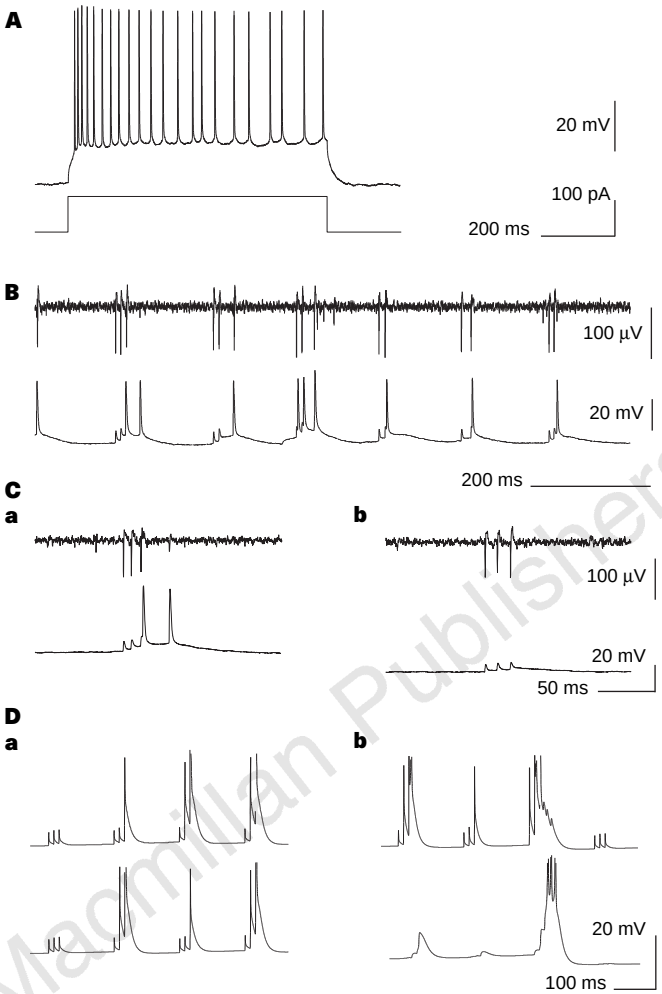


**Figure 4** Cellular activity during high-frequency oscillations. **A**, Voltage response of a CA1 neuron in the principal cell layer to current injection (120 pA, whole-cell recording). The appearance of the neuron under DIC, the spike frequency adaptation, and the presence of small after-hyperpolarizations indicate that the CA1 neuron is a pyramidal cell. **B**, Parallel recording of cell potential (bottom trace) and nearby field potential (top trace; sites were about 1-cell-diameter apart) in calcium-free solution. Each field oscillation is accompanied by a depolarization and each population spike within the oscillation is accompanied by a 'spikelet' in the cell. **Ca**, This expanded trace shows that high-frequency activity is much larger than the field associated with isolated full action potentials. **b**, Hyperpolarization abolishes action potentials but not spikelets during high-frequency oscillations. **D**, Simulation of a pair of pyramidal cells (as described in ref. 15), coupled either by **a**, a gap junction between axons in an unmyelinated section within 75 μm of the initial segment, or by **b**, a gap junction between somata. The simulated gap junctions were non-rectifying and the resistance was adjusted so that some of the coupling potentials were ~5 mV (191 MΩ in **a**; 20.5 MΩ in **b**). The distal axon of cell 1 (upper traces) was stimulated with triplets of small current pulses (period 12 ms, sufficient to induce antidromic spikes) every 150 ms. When the gap junctions are between axons (**a**), the potentials in cell 2 reflect axonal spikes that either block distal to the initial segment (~5 mV) or in the initial segment (~15 mV), or invade the soma (action potentials truncated). The smaller potentials resemble experimental records (**B**). In contrast, somatic gap junctions lead to coupling potentials in cell 2 that are smoother and slower than in experimental records.

between axons rather than between either somatic or dendritic compartments. Gap-junctional coupling between axons differs physically from coupling between somata or dendrites, as long as the density of sodium channels in the axonal membranes is high enough, because the postjunctional membrane can fire and can lead, by antidromic propagation, to the partial or full somatic action potentials observed. If the axonal sodium-channel density were low, then coupling would be largely passive and electrotonically filtered. Simulations of networks of 3,072 pyramidal cells show that an average of as few as 1.6 gap junctions per neuron can generate high-frequency oscillations without the need for chemical synapses (data not shown). The presence of small numbers of gap junctions on axons electrotonically distant from the soma may explain the lack of change in input resistance on addition of gap-junction blockers.

We conclude that spontaneous high-frequency oscillations at ~200 Hz occur in all principal cell layers in hippocampal slices *in vitro*, are generated in small networks of pyramidal cells, and are synchronized by electrotonic coupling, probably through gap junctions. Ephaptic or electric field effect interactions are unlikely to contribute greatly to synchronizing high-frequency oscillations, because the fields are smaller than the experimental estimate of the gradients (>5 mV per mm) needed to affect neuronal excitability[16].

These *in vitro* high-frequency oscillations closely resemble the high-frequency 'ripples' observed in the rat hippocampus *in vivo*[3–5]. We found a similar morphology of the events, laminar confinement to the pyramidal cell layer, participation of a small proportion of principal neurons in each 'ripple', and suppression of the oscillations by halothane. However, *in vivo*, high-frequency oscillations are coherent over ~5 mm of CA1 pyramidal layer and are superimposed on negative waves that start in CA3 and are transmitted synaptically through CA1 to the entorhinal cortex (sharp waves)[3]. *In vitro*, the coherent high-frequency activity was contained within small (≤120 μm) areas in physiological ACSF. 'Sharp waves' with long-range propagation of high-frequency oscillations only occurred in epileptogenic solutions, for example in the presence of bicuculline (not shown).

Gap junctions between hippocampal neurons have been shown to exist[17]. CA1 pyramidal cells show calcium- and pH-dependent coupling[10,18,19] and contain connexin-43 messenger RNA[20], indicating that they can form gap junctions. Our results are consistent with the facts that gap-junction coupling is enhanced during intracellular alkalosis[13] (and decreased during acidosis[21]) and that gap-junction coupling is important in synchronous bursts in calcium-free medium[9,22]. Extracellular and intracellular potentials in CA1 pyramidal cells in calcium-free media[10,23] resemble the phenomena observed in our experiments (Fig. 4). Our simulations of pyramidal cells, and previous simulations of interneurons[24], show that as few as 1.6–2.0 gap junctions per cell can lead to synchronous neuronal activity (compare with Fig. 4D). In agreement with our findings, electrotonic coupling can synchronize activity in the developing hippocampus[25] and generate network oscillations in sympathetic preganglionic neurons[26] and the inferior olive[27]. Gap junctions can exist between axons in mammalian retina[28] and ectopic action potentials generated in axons need not invade the soma[29]. Our experiments and simulations indicate that gap junctions may occur between axons in the mammalian forebrain, and may be able to synchronize neuronal activity on a fast time scale. □

### Methods

We prepared transverse 400 μm hippocampal slices from Wistar rats of both sexes after anaesthesia, cervical dislocation, brain dissection and cooling to 4 °C in gassed (5% $CO_2$, 95% $O_2$) ACSF containing (in mM): NaCl, 135; KCl, 3; $NaH_2PO_4$, 1.25; $CaCl_2$, 2; $MgCl_2$, 4; glucose, 10; and $NaHCO_3$, 32. Slices were maintained for 1–10 h at room temperature in a gassed, ACSF-filled storage chamber. For recording, we transferred a slice to a chamber mounted on an upright microscope with a ×40 water-immersion objective and differential interference contrast (DIC) optics. The slice was superfused with ACSF at 34–35 °C containing 1 mM $Mg^{2+}$; in some experiments we used 5 mM KCl to enhance excitability. For field potential measurements we used a microelectrode filled with HEPES-buffered solution (pH 7.4), tip broken to diameter 15–30 μm, a chlorided silver wire reference electrode less than 2 mm from the microelectrode, and differential recordings by a Neurolog 104 amplifier (Digitimer). The signal was filtered at the indicated frequencies with an active filter (Digitimer NL 125). Data were stored and analysed on a PC using SIGNAL software (CED). For whole-cell patch-clamp recordings[30] we used a SEC 5L (npi electronics) in the switched current clamp or bridge mode. Intracellular solution contained (in mM): KCl, 135; $MgCl_2$, 2; $CaCl_2$, 1; HEPES, 10; EGTA, 10; pH, 7.2 (KOH). NBQX (6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione) and D-AP5(D-2-amino-5-phosphonopentanoic acid) were from RBI; other drugs were from Sigma.

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# Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins

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**In neural systems, information is often carried by ensembles of cells rather than by individual units. Optical indicators[1] provide a powerful means to reveal such distributed activity, particularly when protein-based and encodable in DNA[2–4]: encodable probes can be introduced into cells, tissues, or transgenic organisms by genetic manipulation, selectively expressed in anatomically or functionally defined groups of cells, and, ideally, recorded *in situ*, without a requirement for exogenous cofactors. Here we describe sensors for secretion and neurotransmission that fulfil these criteria. We have developed pH-sensitive mutants of green fluorescent protein ('pHluorins') by structure-directed combinatorial mutagenesis, with the aim of exploiting the acidic pH inside secretory vesicles[5,6] to monitor vesicle exocytosis and recycling. When linked to a vesicle membrane protein, pHluorins were sorted to secretory and synaptic vesicles and reported transmission at individual synaptic boutons, as well as secretion and fusion pore 'flicker' of single secretory granules.**

Wild-type green fluorescent protein (GFP) has a bimodal excitation spectrum[7,8] with peaks at 395 and 475 nm (Fig. 1a). Underlying the two maxima are protonated and deprotonated states of Tyr 66, which forms part of the chromophore[9–13]. Although critical residues exchange protons with the environment[10], the excitation spectrum of GFP is essentially unaltered between pH 5.5 and 10 (ref. 7 and