Cholinergic induction of network oscillations at 40 Hz in the hippocampus *in vitro*

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Acetylcholine is vital for cognitive functions of the brain. Although its actions in the individual cell are known in some detail[1], its effects at the network level are poorly understood[2]. The hippocampus, which receives a major cholinergic input from the medial septum/diagonal band[3], is important in memory[4,5] and exhibits network activity at 40 Hz during relevant behaviours[6]. Here we show that cholinergic activation is sufficient to induce 40-Hz network oscillations[7] in the hippocampus in vitro. Oscillatory activity is generated spontaneously in the CA3 subfield and can persist for hours. During the oscillatory state, principal neurons fire action potentials that are phase-related to the extracellular oscillation, but each neuron fires in only a small proportion of the cycles. Both excitatory and inhibitory synaptic events participate during the network oscillation in a precise temporal pattern. These results indicate that subcortical cholinergic input can control hippocampal memory processing by inducing fast network oscillations.

To investigate the effect of cholinergic activation on network activity in the hippocampus, we used the cholinergic agonist carbachol (20 μM), which induced fast oscillatory field activity in hippocampal slices (39 ± 3 Hz (mean ± s.d.), range 35–55 Hz; $n = 70$; Fig. 1a, b). The amplitude of the oscillation increased with increasing concentrations of carbachol up to 20–50 μM, above which no further change was observed (0.1–200 μM; $n = 3$; Fig. 1c). This effect was mediated by muscarinic acetylcholine receptors, as the selective muscarinic agonist muscarine (10–20 μM; $n = 2$) mimicked the effect, and the oscillation induced by carbachol was blocked by the muscarinic antagonists atropine (10 μM; $n = 8$), and pirenzepine (M1-subtype-selective; 10 μM; $n = 10$; Fig. 1a). The 40-Hz oscillatory activity appeared spontaneously, was continuous over prolonged periods (up to 3 h), and was often nested in theta frequency oscillations (Fig. 1d); similar features are regularly observed during 40-Hz oscillations *in vivo*[6], and are consistent with previous studies of cholinergically induced theta frequency oscillations *in vitro*[8-11].

Synchronous oscillations at 40 Hz *in vivo* are a collective behaviour of a distributed network of neurons, which cross areal boundaries[12]. Cholinergically induced oscillations at 40 Hz in hippocampal slices were seen in both the CA3 and the CA1 areas (Fig. 1a). Cross-correlations between different recording sites revealed a high degree of synchrony within the CA3 area, and a phase lag of CA1 relative to CA3. These results indicate that cholinergically induced 40-Hz oscillations are generated within the CA3 area and propagate to CA1. To test this assumption, we studied hippocampal slices with cuts made to separate the hippocampal subfields. Oscillations in CA3 persisted, whereas activity was observed in neither CA1 nor dentate gyrus ($n = 4$). Therefore, the CA3 network generates intrinsically synchronized 40-Hz oscillations, and cholinergically induced 40-Hz oscillations in CA1 depend on intact connections with CA3.

Inhibitory synaptic events mediated by GABA$_A$ (γ-aminobutyric acid A) receptors may be involved in synchronization of population activity[7,13-15]. To test whether rhythmic inhibition is also important during cholinergically induced oscillations, we applied a GABA$_A$

receptor antagonist ((−)-bicuculline methochloride, 1–10 μM). All field oscillations were blocked (Fig. 2a; $n = 7$), indicating that rhythmic inhibitory postsynaptic potentials (IPSPs) may be pivotal in phase-locking the activity of principal neurons. Furthermore, during cholinergic activation, as well as during control conditions[15], minimal stimulation of GABAergic interneurons in the presence of excitatory-amino-acid blockers entrained the firing of action potentials in the pyramidal neurons (data not shown).

If IPSPs are responsible for phase-locking the oscillatory activity of pyramidal neurons, a prolongation of their duration should decrease the oscillation frequency[7,16,17]. We tested this prediction
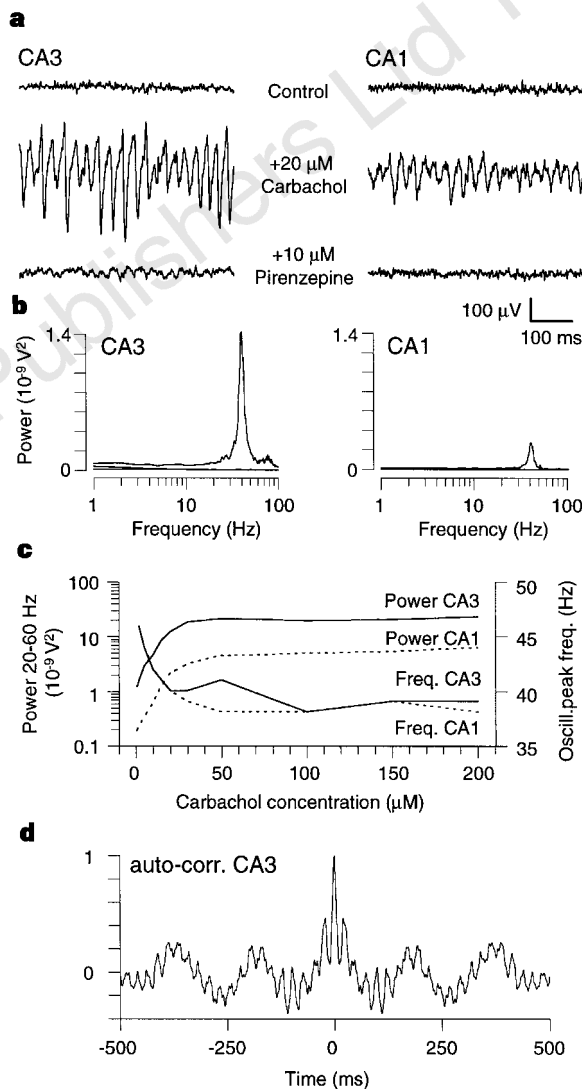

Figure 1 Generation and propagation of 40-Hz oscillations in hippocampal slices. **a**, Example traces of extracellular local field recordings in the middle to distal third of the stratum radiatum of the CA3 and CA1 areas. In control solution, no apparent rhythmic activity is seen. Bath application of the cholinergic agonist carbachol induces prominent oscillatory activity which is abolished by the addition of the muscarinic M1 receptor antagonist pirenzepine. **b**, Power spectra of control, carbachol-induced extracellular oscillations and after addition of pirenzepine obtained over 50-s periods in the CA3 and CA1 areas using a fast Fourier transform algorithm. Note the distinct peak at 40 Hz in both spectra only in the presence of carbachol, whereas the spectra obtained during the control period and after pirenzepine application appear as nearly overlapping flat lines. The oscillations were consistently more prominent in the CA3 region. **c**, Concentration dependence of the carbachol-induced field oscillation. Power and frequency are plotted as a function of carbachol concentration in CA3 (continuous line) and CA1 (dotted line). **d**, Auto-correlogram of extracellular oscillations in CA3, showing periodicity at gamma and theta frequencies.
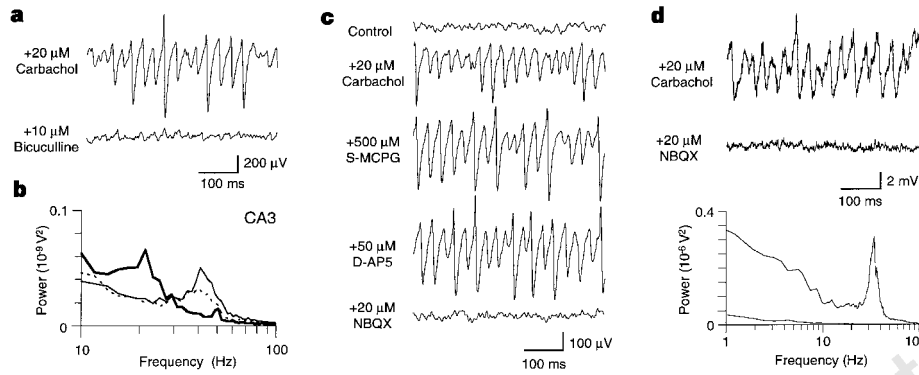
Figure 2 Pharmacological analysis of 40-Hz oscillations in the CA3 area. **a**, Extracellular field oscillations were blocked by bicuculline methochloride. **b**, Power spectra showing a reversible shift of the peak frequency of carbachol-induced oscillations following bath application of pentobarbitone. Thin line, carbachol; thick line, plus pentobarbitone; dotted line, wash. **c**, Persistence of extracellular field oscillations in the presence of the metabotropic glutamate receptor antagonist (S)-MCPG and the NMDA receptor antagonist D-AP5. In contrast, the non-NMDA glutamate receptor antagonist NBQX blocked the oscillation. **d**, Blocking by NBQX of intracellular oscillatory activity in a pyramidal cell held at –20 mV. Power spectra in the absence (top trace) and presence (bottom trace) of NBQX are shown in the lower panel.
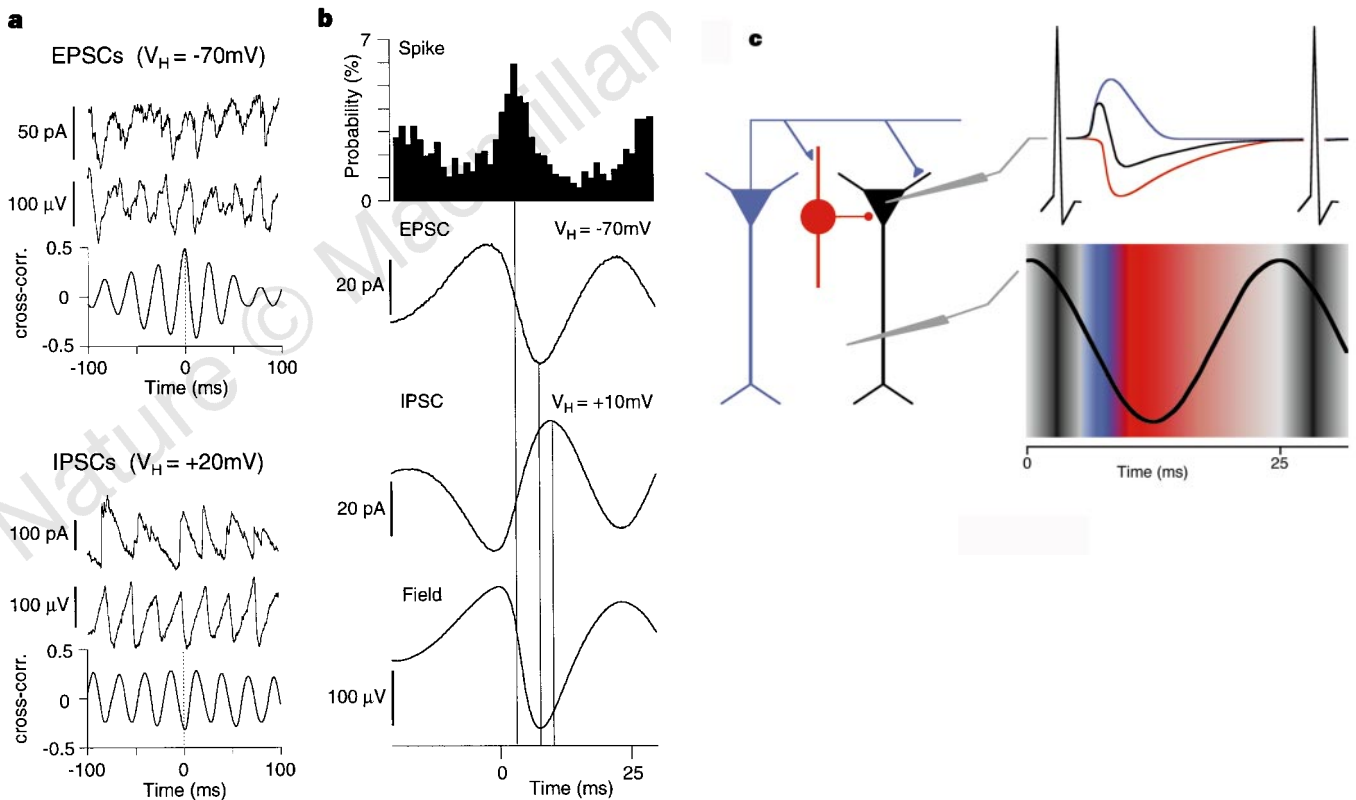

Figure 3 Temporal relationship between intracellular events and local field oscillations in CA3. **a**, Synaptic currents recorded at holding potentials of –70 mV (EPSCs) and +20 mV (IPSCs), respectively, plotted above the concomitantly recorded local field oscillation and the corresponding cross-correlogram. **b**, Occurrence of action potentials and synaptic events relative to the extracellular field oscillation. Time zero was arbitrarily set to the positive peak of the extracellular field oscillation in the stratum radiatum. Action potentials (top) are plotted as a probability histogram, showing the occurrence of all action potentials recorded over 10,000 cycles. Synaptic events (EPSC, IPSC) and field are shown as the average over 3,000 cycles. **c**, Proposed mechanism underlying the generation of 40-Hz field oscillations, based on information about the circuitry of the CA3 region (left). Pyramidal neurons (blue) form excitatory synapses with other pyramidal neurons (black) through local axon collaterals, which also activate GABAergic interneurons (red) involved in a local inhibitory feedback circuit. Action potentials in a few pyramidal neurons therefore produce a monosynaptic EPSP (blue, top right) in neighbouring pyramidal neurons that is rapidly curtailed by a disynaptic IPSP (red, top right). The resulting summated potential is shown as a black trace (top right). Bottom right, approximate time relations between action potentials and synaptic events, relative to the field oscillation. The respective time windows, illustrating the peak probability of intracellular events, are shown in black for action potentials, blue for EPSPs and red for IPSPs.

by adding a barbiturate, which prolongs the decay time of IPSPs. Indeed, increasing the concentrations of pentobarbitone (5–20 μM) gradually decreased the frequency of the carbachol-induced oscillation, emphasizing the role of rhythmically occurring IPSPs in pacing the activity of the hippocampal network[7,13,15] (Fig. 2b; $n = 3$).

We next determined whether glutamatergic excitatory mechanisms are necessary for the emergent population oscillation. Metabotropic glutamate receptors are not necessary, because the receptor antagonist (S)-α-methyl-4-carboxyphenylglycine (MCPG, 200–500 μM; $n = 6$; Fig. 2c) failed to block carbachol-induced oscillation, yet did prevent 40-Hz oscillations induced by the metabotropic glutamate receptor agonist (RS)-3,5-dihydroxyphenylglycine (DHPG, 10 μM; $n = 2$). Likewise, NMDA (N-methyl-D-aspartate) receptors are not necessary, because the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (D-AP5, 50 μM; $n = 6$) failed to reduce the extracellular oscillation (Fig. 2c). However, in contrast to 40-Hz oscillations induced by metabotropic glutamate receptors in the CA1 area[7], cholinergically induced oscillations require ionotropic non-NMDA glutamate receptors. In both CA3 and CA1 the oscillatory activity was completely abolished by the non-NMDA glutamate receptor antagonist 6-nitro-7-sulphamoylbenzo(f)-quinoxaline-2,3-dione (NBQX, 20 μM; $n = 5$; Fig. 2c, d) as well as by the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonist GYKI52466 (30–50 μM; $n = 4$)[18]. Thus, tonic and/or phasic excitatory activity seems to be important for these oscillations. Recurrent excitatory feedback in the CA3 region could be involved in the generation of the oscillations, and propagation to CA1 may depend on the excitatory Schaffer collateral input onto CA1 neurons.

During 40-Hz oscillations in the hippocampus in vivo, individual pyramidal neurons do not fire action potentials at every cycle[6]. To analyse the frequency of action potentials and their temporal relation to synaptic events, we recorded action potentials during cell-attached patch-clamp recordings from CA3 pyramidal neurons followed by whole-cell recordings of synaptic events from the same neurons (Fig. 3a). We then temporally related both action potentials and synaptic events to extracellular field oscillations ($n = 3$; Fig. 3b). Each pyramidal neuron fired in fewer than 5% of the cycles. Most action potentials occurred 3–4 ms after the positive peak of the field oscillation and 56% of the action potentials fell within <20% of the cycle. The average maximum excitatory postsynaptic current (EPSC) occurred 3–4 ms after the action potential, whereas peak inhibitory currents reached their maximum 1–3 ms after the maximum EPSC (Fig. 3b).

The precise temporal sequence of action potentials preceding EPSCs, which, in turn, precede IPSCs, leads us to suggest the following model (Fig. 3c). Action potentials in a few pyramidal neurons produce a monosynaptic EPSP in neighbouring pyramidal neurons. This EPSP remains subthreshold in most neurons because it is rapidly curtailed by a disynaptic IPSP. This model is consistent with established effects of acetylcholine-receptor activation in the hippocampus, including excitation of pyramidal neurons[19,20], suppression of synaptic transmission at intrinsically originating excitatory synapses[21] and an increase in the excitability of interneurons[22]. The delay between EPSPs and IPSPs is consistent with reported spike–spike latencies for unitary pyramidal/interneuron connections in the CA3 area[23]. However, alternative mechanisms may also contribute: individual neurons may exhibit pacemaker-like oscillatory properties[24,25] and cholinergic tonic excitation may foster the emergence of oscillatory activity in a network of mutually connected interneurons[7], which, by virtue of their divergent output[26], phase the activity of both principal neurons and interneurons.

Although we have not studied alternative mechanisms that may be involved in the generation of 40-Hz oscillations[27], our data are consistent with the involvement of mutual excitatory connections between pyramidal neurons and with the existence of a fast-feed-back inhibitory loop, suggested earlier as mechanisms involved in cortical network oscillations[16,28]. The importance of excitatory connections[29] in the network oscillation reported here indicates that the synaptic strengths of specific excitatory connections in the network are instrumental in controlling which neuronal elements are active. We thus propose that cholinergic septal activation can induce an oscillatory state in the hippocampal network, in which the temporal and spatial structure of cortical (for example, entorhinal) inputs may govern information processing by selecting which subset of principal neurons leads the oscillation. Furthermore, a Hebbian type of plasticity[5] may be important in modulating the strength of phase-related intrinsic and/or extrinsic inputs, thereby providing an effective mechanism in which information is encoded. □

Methods

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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 intra-burst frequencies were as follows: for CA1, 180 ± 47 Hz ($\pm$s.d., $n = 33$ individual high-frequency events at independent sites in 20
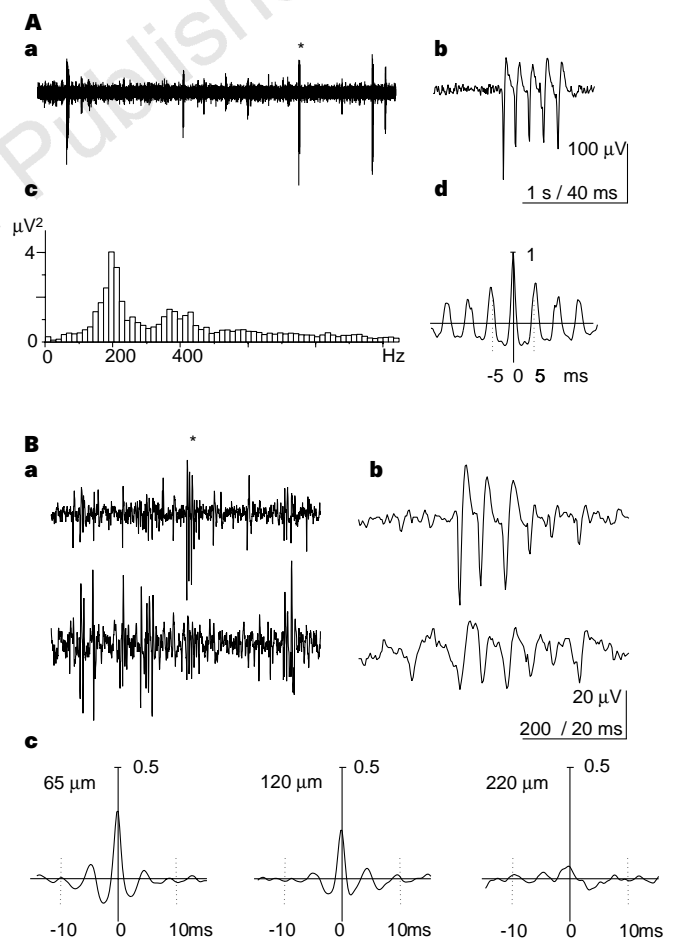

Figure 1 High-frequency oscillations in CA3. **A**, Filtered (5–400 Hz bandpass) CA3 pyramidal layer field potential with spontaneous high-frequency oscillations. **a**, **b**, The 1-s time calibration applies to the left trace (**a**); the 40-ms scale applies to the right trace (**b**, from the section 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.