Circuit topology for synchronizing neurons in spontaneously active networks

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Spike synchronization underlies information processing and storage in the brain. But how can neurons synchronize in a noisy network? By exploiting a high-speed (500–2,000 fps) multineuron imaging technique and a large-scale synapse mapping method, we directly compared spontaneous activity patterns and anatomical connectivity in hippocampal CA3 networks ex vivo. As compared to unconnected pairs, synchronically coupled neurons shared more common presynaptic neurons, received more correlated excitatory synaptic inputs, and emitted synchronized spikes with approximately $10^2$ times higher probability. Importantly, common presynaptic parents per se synchronized more than unshared upstream neurons. Consistent with this, dynamic-clamp stimulation revealed that common inputs alone could not account for the realistic degree of synchronization unless presynaptic spikes synchronized among common parents. On a macroscopic scale, network activity was coordinated by a power-law scaling of synchronization, which engaged varying sets of densely interwoven (thus highly synchronized) neuron groups. Thus, locally coherent activity converges on specific cell assemblies, thereby yielding complex ensemble dynamics. These segmentally synchronized pulse packets may serve as information modules that flow in associatively parallel network channels.

Strong Spike Synchronization Between Synaptically Connected Neurons. In rat entorhino-hippocampal slice cultures, 104 pairs of adjacent CA3 pyramidal cells (PCs) were randomly selected for whole-cell recordings (Fig. L4). The connectivity density among CA3 PCs was 28.8%. This connection ratio is higher than the connectivity (2–8%) reported in acute slice preparations (35, 36). In acute hippocampal slices, 75% to 90% of the axons of CA3 pyramidal neurons are amputated even in 500-μm-thick slices (37). Therefore, organotypically cultured ex vivo networks are likely to self-recover their complexity to a realistic extent. In support of this, we found that neither levels nor patterns of spontaneous excitatory postsynaptic currents (sEPSCs) or inhibitory postsynaptic currents (sIPSCs) differed between ex vivo and in vivo hippocampal neurons (Fig. S1). As the ex vivo recovery of slice cultures occurs without external inputs (unlike normal development), this work will describe the “default” network dynamics that emerge under disturbance-free conditions.

Of 104 PC pairs, we encountered 16 bidirectionally connected pairs. Given the connection probability $p$ and the total number of pairs $N$, the statistically expected number of bidirectional pairs would be $Np^2$, i.e., 8.6 pairs. In our datasets, therefore, the number of bidirectionally connected pairs was 1.9 times higher than expected ($P = 0.012$) (30), suggesting that CA3 recurrent networks are topologically biased to enhance local connectivity.

When the neuron pairs were held in current-clamp mode, we sometimes found spontaneous spikes synchronized (Fig. 1B). To quantify the synchrony level, we introduce the scalar measure $S_n$ based on statistical salience of the observed number of synchronized spikes relative to chance. Specifically, we counted all synchronized spikes, i.e., any pairs of spikes that concurred in two neurons within a given time lag. If neuron, and neuron, are independent units that fire in a random manner, the probability $P(n)$ that they exhibit $n$ synchronized spikes during the observation period $t$ is given by the Poisson equation:

$$P_i(n) = \frac{m_i^n}{n!} e^{-m_i}$$

where $m_i$ is the expected number of synchronized spikes, i.e., $f_i \times j_i \times t_i$, and $f_i$ and $j_i$ denote the spike rates of neuron, and neuron, respectively. When spikes synchronize $n$ times, the probability $P_i(n)$ (rareness) is given as follows:

$$P_i = \sum_{k=0}^{\infty} P_i(k) = 1 - \sum_{k=0}^{n-1} P_i(k)$$

Then, we defined the surprise index $(S_n)$ as $-\log_2 P_i$ (38).

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For extremely synchronized pairs with $S > 100$ bits, $S$ is denoted as 100 bits to avoid arithmetic precision problems in computing floating-point numbers. Thus, $S$ ranges between 0 and 100 bits, with higher $S$ reporting stronger synchronization.

$S$ varies as a function of time windows for synchrony detection. It peaked at time windows of 10 to 50 ms (Fig. 1C). In accord with this, cross-correlation histogram of spike timings in neuron pairs (Fig. 1C Inset) showed a peak at 0 ms with an SD of 25 ms (fitted to the Gaussian curve). We therefore selected the 10-ms bin in the following analysis, unless otherwise specified.

Synaptically connected pairs were more synchronized than uncoupled pairs (Fig. 1D); the mean $S$ for synaptic pairs was higher by 24 bits than that for uncoupled pairs, indicating that, on average, synaptic pairs exhibited synchronized spikes with a $1.7 \times 10^7$ (i.e., $2^{24}$) times higher probability. Consistent with this, the probability to find synaptic pairs increased with $S$ (Fig. S2). Therefore, the functional and anatomical connectivity is reciprocally, if not perfectly, linked at the single-cell level.

**Common Presynaptic Neurons Shared by Synaptically Connected Neurons.** To probe the synaptic wiring pattern among CA3 PCs, we employed reverse optical trawling (RO廷), a new optical mapping method (39). While EPSCs were recorded simultaneously from two randomly selected PCs, a small number of nearby neurons were sequentially activated by iontophoretic application of glutamate through a glass pipette that was slowly moved to survey the surrounding network (Fig. 1E). Spikes of glutamate-activated neurons were captured as somatic Ca$^{2+}$ transients with high-speed fMCI (Fig. S3). Neurons that exhibited calcium transients immediately before EPSCs were statistically screened. With this procedure, we can identify $96\%$ of presynaptic cells projecting to the patch-clamped neurons (39). Fig. 1E–G shows an example of RO廷-identified synaptic connections, where nine neurons were sequentiually whole-cell recorded in pair, and 66 neurons were imaged with fMCI.

In these synapse connection maps, we often found “common” presynaptic neurons that projected to both of the two patched neurons. The mean ratio of common presynaptic neurons to the total presynaptic neurons that projected to at least one of the patched neuron pair was $13.2 \pm 2.5\%$. This proportion increased to $43.9 \pm 20.2\%$, however, when the postsynaptic pairs were synaptically connected (Fig. 1H; $n = 11$ pairs). This suggests that synaptic pairs receive more correlated inputs than nonsynaptic pairs. To confirm this, we carried out targeted patch-clamp recordings from spontaneously synchronized PC pairs, which were identified online by high-speed fMCI. After monitoring spontaneous spikes in current-clamp mode, sEPSCs and sIPSCs were recorded in voltage-clamp mode at $-90$ mV and $0$ mV, respectively. As expected, these PC pairs received highly correlated sEPSCs and sIPSCs (Fig. 2A Left). The cross-correlations peaked sharply at time lags of less than 1 ms (Fig. 2A Right). The half peak width in the cross-correlogram was $67.0 \pm 56.1$ ms for sEPSCs and $54.7 \pm 50.4$ ms for sIPSCs.

As control experiments, we also targeted less synchronized neuron pairs. In the low-$S$ pairs, sEPSCs were only weakly correlated (Fig. 2B), whereas sIPSCs were still highly correlated (Fig. 2B). Data are summarized in Fig. 2 C and D. The correlation coefficients of sEPSCs correlated positively with $S$ ($r = 0.34$, $P < 0.01$), whereas those of sIPSCs were almost always high, independent of $S$. These data imply that excitatory neurons are specifically wired to ensure local synchronization and that inhibitory activity is globally coherent in CA3 networks.

**Synchronized Inputs from Common Presynaptic Neurons.** How efficiently do common excitatory synaptic inputs produce synchronized firing? With the dynamic-clamp technique, we injected artificially generated “correlated” synaptic conductance patterns into two CA3 PCs under pharmacological blockade of fast synaptic transmission. The stimulus sweeps were constructed in silico from spike trains of 200 Poisson-firing presynaptic neurons, in which each spike was convolved with a unitary waveform of a fast excitatory
atory postsynaptic conductance. Correlated conductances were generated by overlapping some of the 200 presynaptic spike trains so that their correlation coefficients ranged from 0 to 1 (Fig. 2E). When PC pairs were stimulated with these conductances, they emitted spikes, some of which were found to synchronize (Fig. 2F). $\delta$ was calculated from the spike series and plotted against the correlation coefficients between the injected conductances ($n = 55$ pairs).

This inconsistency between artificial and spontaneous synaptic inputs suggests that naturally occurring EPSCs involve more information than captured by simple cross-correlations, thereby efficiently synchronizing postsynaptic neurons (40, 41). To seek the underlying information, we examined the dynamics of spontaneous firing activity of CA3 neuron populations. Using high-speed fMCI, the spatiotemporal pattern of spontaneous network activity was reconstructed with the millisecond resolution from 85 ± 25 neurons ($n = 14$ slices), ranging from 53 to 137 neurons (Fig. 3A and Movie S1). Within a given 10-ms period, on average, only a small number of neurons (0.19 ± 0.61% of the total cells; $n = 14$ slices) were active, whereas the network occasionally exhibited large synchronous events that involved up to approximately 50% neurons (Fig. 3A Lower). The frequency of the synchrony sizes was approximated by a power-law distribution $P(n) \approx n^{-\alpha}$, where $n$ denotes the synchrony size and $P(n)$ the probability of observing size-$n$ synchrony (Fig. 3B; $\alpha = -2.6$). A peri-synchronization time histogram revealed that, in a synchronous event, the network barraged spikes during a period of approximately ±20 ms (Fig. S4). During synchronization, CA1 networks showed high-frequency field oscillations (Fig. S5), which closely resembled sharp-wave/ripples observed in the hippocampus of quiescent or sleeping animals (42).

We computed $\delta_S$ for all neuron pairs in a raster plot and drew the S-based connectivity map (Fig. 3C). Synchronization was sparse: $\delta_S$ was 0 bits in 80.3 ± 17.9% pairs, whereas nonzero $\delta_S$ conformed to a log-normal Gaussian distribution (Fig. 3D). $\delta_S$ did not correlate with the physical cell-to-cell distance as a whole ($r = 0.096, P > 0.1$), but pairs with $\delta_S > 50$ bits were always located within a distance of 200 μm (Fig. 3E). Note that closely spaced spikes (>50 Hz firing) were hardly separated in fMCI data (Fig. S6A) because Ca$^{2+}$ transients had a long decay constant of approximately 500 ms (43). Therefore, fMCI tends to underestimate $\delta_S$, whereas $\delta_S$ obtained by fMCI was almost linear with electrophysiologically obtained $\delta_S$ ($r = 0.77, P < 0.01$; Fig. S6B).

After constructing the $\delta$ maps, we conducted ROTing in the same network to search common presynaptic neurons. The common presynaptic pairs exhibited significantly higher $\delta_S$ than unshared pairs (Fig. 3F; P < 0.01, Kolmogorov-Smirnov test). This suggests that common parents are more prone to synchronize. In other words, inputs into common postsynaptic pairs will be more correlated than “Poisson-correlated” inputs, which we used in the dynamic-clamp experiments (Fig. 2E).

Given that network synchronization magnitudes were power-law tailed, we now generated the synaptic conductance patterns from power law-scaled presynaptic spike trains by keeping the overall presynaptic firing rate the same as in the Poisson simulation (Fig. 3G). Neuron pairs stimulated by the scaled pattern conductances exhibited strongly synchronized spikes, sometimes reaching an $\delta_S$ of 100 bits (Fig. 3 H and I).

**Ensemble Dynamics of Spontaneous Activity.** Based on the matrices of $\delta_S$ obtained by fMCI, we constructed dendrograms using the method of Ward, a hierarchical clustering algorithm (Fig. S7A). It disclosed the “cliqueness” of synchronous neurons. We then examined whether the $\delta_S$ matrices included small-world attributes (27). By setting various lower-limit thresholds on the $\delta_S$ matrices, we depicted the synchrony graphs as a function of the threshold (Fig. S7B). For any threshold, the extracted graph exhibited the “small-worldness” (Fig. S7 C–E).

Using the affinity propagation algorithm (44), the order of neurons was sorted so that higher $\delta_S$ pairs were more neighbor d in the matrix, and the enumerated neurons were clustered into subgroups. In the representative data shown in Fig. 4A, 96 neurons were separated into 15 groups. On average, each movie contained $16.1 \pm 7.6$ groups, and each group comprised $5.8 \pm 4.1$ neurons ($n = 14$ slices). This clustering was validated with ROTing: the connection probability among within-group neurons was $37.6 \pm 17.8\%$, significantly higher than the across-group connectivity ($27.0 \pm 8.6\%; P = 0.04$, Wilcoxon signed-rank test; $n = 14$ slices; Fig. 4B).

Neurons within a group were sparsely distributed over the imaged field (Fig. 4C). The distribution of the cell-to-cell distance between within-group neurons did not differ from that of across-group neurons (Fig. 4D; $P > 0.1$, Kolmogorov-Smirnov test; $n = 14$ slices), indicating no spatial bias of synchronous spike patterns. Raster plots sorted along the groups demonstrated that within-group neurons frequently exhibited synchronized spikes (Fig. 4E Upper). Interestingly, the internal structure of synchronization was dynamic, that is, different synchrony events recruited different sets of neuron groups (Fig. 4E Lower).
Information Flow from CA3 to CA1. Finally, we addressed how these CA3 cell assembly activities are transferred to downstream CA1 networks. After sorting CA3 neurons based on $S$ matrices, we applied ROTing to probe the CA3-to-CA1 synaptic connectivity. An example map is presented in Fig. 5A, which shows the projection pattern from 90 CA3 neurons to seven patch-clamped.
CA1 PCs. CA3 PC pairs that projected convergently to the same CA1 PCs had statistically higher S than the other CA3 PC pairs (Fig. 5B; $P < 0.01$, Kolmogorov-Smirnov test). Thus, synchronous CA3 activity tends to converge on the same CA1 neurons.

Discussion
In this study, we used high-speed fMCI to capture the fine-scale structure of spontaneous CA3 network activity and analyzed the organization and generation of spike synchronization with the aid of large-scale optical mapping and dynamic-clamp techniques. We found that complex recruitments of highly synchronized cell assemblies constitute large-scale network synchronization and that the assemblies emerge through presynaptic synchronization.

Origin of Synchronization. More synchronized neuron pairs were more likely to be synchronically linked. We do not think, however, that this synaptic link is enough to synchronize these neurons, because a single synapse is too weak to depolarize beyond the spike threshold. Rather, correlated synaptic inputs from multiple common presynaptic neurons (30–33, 45) are more plausibly causal of spike synchronization. Two lines of evidence are in favor of this hypothesis. First, ROTing revealed that synchronically coupled CA3 PC pairs shared numerically more presynaptic CA3 PCs. Second, double whole-cell recordings of neuron pairs revealed that more synchronized pairs received more correlated sIPSCs.

We also found that common parent PCs were strongly synchronized. This indicates that these common parents are also under the further-upstream innervation by common “grandparent” PCs. This suggests synchronous activity flow, that is, synchronous pulse packets flow across densely connected neuron groups in recurrent networks. This idea is supported by our dynamic clamp data showing that common presynaptic neurons need to be synchronized to evoke a realistic level of postsynaptic synchronization, although common inputs from randomly spiking neurons can do neurons to some extent.

In the dynamic clamp experiments, however, we did not consider the dendritic properties. Dendrites exhibit nonlinear excitation through spatiotemporal input summation and dendritic spikes. It is also possible that synchronized spikes result from nonlinear dendritic computation with convergent inputs of synchronous activity into specific dendritic branches. Research into this possibility is under way in our laboratory with multiple patch-clamp recordings targeting dendrites (46).

Cell Assemblies in Small-World Networks. Consistent with previous reports showing that the functional and anatomical connectivity among individual neurons exhibits small-world architectures (26, 27, 47, 48), the CA3 networks also included small-world topology. The small-world network is theoretically believed to allow fast information transfer with low wiring costs (49), the co-existence of information segregation and integration (50), and synchronization (51). Neurons within a small-world cluster, classified by affinity propagation, were sparsely distributed in space. In hippocampal place cell activity recorded by multiple unit electrodes in vivo, it is also reported that synchronous cliques are dispersed across the electrodes (17). Despite this apparent randomness of neuron locations, within-group neurons were preferentially interconnected. This may emerge from target-selection mechanisms, such as activity-dependent synaptic plasticity. Moreover, synchronous CA3 neuron groups converged onto the same CA1 neurons. Thus, the CA3-to-CA1 connectivity forms relatively independent routes that carry CA3 ensemble activities to specific CA1 neuron subsets (Fig. S8). Synchronous modules may serve as endogenous building blocks that embody the diversity and complexity of information processing in associative and parallel networks.

Coherent Inhibitory Networks. In the rat neocortex, the axons of inhibitory neurons highly arborize, twisting and turning, seemingly rummaging among their postsynaptic targets (52). Therefore, single interneurons may promiscuously provide nearby PCs with correlated inhibitory inputs. Moreover, interneurons are reciprocally connected through synaptic contacts and gap junction (53–55). Thus, their interplay may give rise to globally coherent inhibition of PC networks (56). Indeed we found that sIPSCs were correlated between virtually all PC pairs. Inhibitory inputs limit the window available for temporal summation and increase the temporal precision of PC firing (57). They also entrain network activity by interacting with intrinsic oscillatory mechanisms of PCs (58). We thus speculate that interneurons couple relatively independent PC subgroups and orchestrate complex network synchronization.

Ex Vivo Data. Our data were obtained exclusively from organotypic slice cultures, an ex vivo experimental network model, and hence must be carefully extrapolated to other neuronal systems, such mature brain networks in vivo. Nonetheless, it is still intriguing to find that such spontaneously reorganized ex vivo networks show highly nonrandom patterns of connectivity and activity. Our findings thus describe a primary regime of how neuronal networks intrinsically develop and operate in the “free-run” mode.

Materials and Methods
Experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number 19-43, A21-6) according to the University of Tokyo guidelines for the care and use of laboratory animals.

Entorhino-hippocampal organotypic slices were prepared from 7-d-old WistarST rats (SLC). Experiments were performed on days 7 to 11 in vitro. Patch-clamp recordings were carried out simultaneously from two to four PCs with two Axopatch 700B dual amplifiers (Molecular Devices). Whole-cell patch pipettes (4–6 MΩ) were filled with 135 mM K-glucurate, 4 mM KCl, 10 mM Hapes, 10 mM phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, and 0.2% biocytin. Dynamic-clamp stimulation was performed with a PCI-6024E data acquisition board (National Instruments) under a real-time Linux-environment.

For fMCI, slices were incubated with Oregon Green 488 BAPTA-1 AM at 37 °C for 1 h and imaged at 500 to 2,000 frames per s with a Nikon-Ts confocal unit (CSUX-1; Yokogawa Electric), a high-speed back-illuminated CCD camera (iXon DU860; Andor), and a water-immersion objective lens (magnification ×16, 0.80 NA; Nikon). Spike timings were determined with an automatic machine-learning algorithm. Spike train data used here are available online at http://hippocampus.jp/data. Further details are provided in SI Materials and Methods.
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Supporting Information

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SI Materials and Methods

Animal Experiment Ethics. Experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number 19-43, A21-6) according to the University of Tokyo guidelines for the care and use of laboratory animals.

Slice Culture Preparations. Entorhino-hippocampal organotypic slices were prepared from 7-day-old Wistar/ST rats (SLC) as previously described (1). Briefly, rat pups were anesthetized by hypothermia and decapitated. The brains were removed and placed in aerated, ice-cold Gey balanced salt solution supplemented with 25 mM glucose. Horizontal entorhino-hippocampal slices were made at a thickness of 300 μm by a vibratome (DTK-1500; Dosaka). They were placed on Omnipore membrane filters (JHWP02500; Millipore) and incubated in 5% CO₂ at 37 °C. The culture medium, composed of 50% MEM (Invitrogen), 25% Hanks balanced salt solution, 25% horse serum (Cell Culture Laboratory), and antibiotics, was changed every 3.5 days. Experiments were performed on days 7 to 11 in vitro. Although slice cultures are known to self-rewire and form abnormal connections that very rarely exist in normal conditions, such as CA1-to-CA1, CA1-to-CA3, and CA3-to-dentate gyrus connections (2, 3), these aberrant connections are not dominant in our slice culture preparations. ROTing, a synapse mapping technique described later, demonstrates that these abnormal connections are less than 0.5% of the total connections and that an overwhelming number of the connections project to their normal targets. This is probably because in our preparations, the entorhinal cortex is not dissected from slices of the hippocampal formation. Lesions of the entorhinal cortex are known to result in abnormal sprouting and reorganization of hippocampal networks in vivo and ex vivo (4, 5).

Ex Vivo Patch-Clamp Recordings. A slice was placed in a recording chamber perfused at 3 to 4 mL/min with artificial cerebrospinal fluid (aCSF), consisting of 127 mM NaCl, 26 mM NaHCO₃, 3.3 mM KCl, 1.24 mM KH₂PO₄, 1.0 mM MgSO₄, 1.0 to 1.2 mM CaCl₂, and 10 mM glucose at 30 to 32 °C. Whole-cell recordings were carried out simultaneously from two to four pyramidal cells. Patch pipettes (4–6 MΩ) were filled with 135 mM K-gluconate, 4 mM KCl, 10 mM Hepes, 10 mM phosphate, 4 mM MgATP, 0.3 mM NaGTP, and 0.2% biocytin. Single units were extracellularly recorded in the loose-cell–attached mode with aCSF-filled pipettes. To examine whether the recorded neurons were synaptically connected, aCSF was modified to 2.2 mM K⁺, 3.0 mM Mg²⁺, and 3.6 mM Ca²⁺ to reduce spontaneous activity and enhance the reliability of synaptic transmission (6).

In Vivo Patch-Clamp Recordings. Male ICR mice (18–20 day old) were anesthetized with urethane (1.2–1.8 g/kg, i.p.). Animals were implanted with a metal head-holder and mounted on a custom-made stereotaxic fixture. A small craniotomy (approximately 1 mm²) was made at 2.5 mm caudal to the bregma and 2.2 mm ventrolateral to the sagittal suture along the surface of the skull, and the dura was removed. Whole-cell recordings were obtained with the blind patch-clamp approach (7).

Data Acquisition. All electrophysiological recordings were carried out using MultiClamp 700B amplifiers (Molecular Devices). Signals were low-pass filtered at 2 kHz and digitized at 20 kHz. Data were analyzed with custom-written scripts in IgorPro 6 (Wavemetrics). Excitatory and inhibitory postsynaptic current (EPSC and IPSC, respectively) were recorded at clamped voltages of −90 mV and 0 mV, respectively. EPSCs and IPSCs were computed on the assumption that EPSCs were recorded at the reversal potential for inhibition and that IPSCs were recorded at the reversal potential for excitation.

Dynamic-Clamp Stimulation. CA3 PCs were stimulated with the dynamic-clamp conductance injection technique. Synaptic events were modeled based on conductance g(t), and the command current signal I(t) was computed as a function of I(t) = g(t) × [V(t) − E_rev] (V(t), membrane potential; E_rev, reversal potential) under a real-time Linux environment and delivered into patch-clamped neurons at 20 kHz by a PCI-6024E data acquisition board (National Instruments). Conductance stimuli (30 s) consisted of a series of excitatory synaptic inputs, which were constructed from 200 Poisson spike trains convolved with a unitary conductance transient representing a fast excitatory synaptic response fitted by the dual exponential function (g_s × [exp(t/τ_a) − exp(−t/τ_d)]), where τ_a represents an activation time constant, τ_d a decay time constant, and g_s a scaling factor (τ_a = 0.5 ms, τ_d = 2 ms, and g_s = 1,000 pS). The average rate of the total input firing was 800 Hz. Two partially correlated conductance sweeps were simultaneously generated so that their correlation coefficient Ccorr ranged from 0 to 1 at roughly every 0.2 s; for an expected correlation level (c), two sweeps, g_s(t) and g_s(t + c), were constructed by overlapping (1 − c) × 200 Poisson trains. To make the scaled synaptic input sweep, presynaptic spikes were distributed in a power law across trains. During stimulation, intrinsic fast synaptic transmission was blocked in the presence of an inhibitor mixture of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 50 μM D,L-2-amino-5-phosphonopentanoic acid, and 50 μM picrotoxin.

FMI. Slices were incubated with 2 mM dye solution at 37 °C for 1 h (8). The dye solution was aCSF containing 0.0005% Oregon Green 488 BAPTA-1 (OGB-1) AM, 0.01% Pluronic F-127, and 0.005% Cremophor EL. After 1 h recovery, a slice was transferred to a recording chamber. Images were acquired at 500 to 2,000 frames per s with a Nipkow-disk confocal unit (CSUX-1; Yokogawa Electric), a high-speed back-illuminated CCD camera (IXON DU860; Andor), and Solis software (Andor). Fluorophores were excited at 488 nm with an argon laser (10–15 mW, 532-BS-AO4; Omnichrome) and visualized with a 507-nm filter. In each cell body, the fluorescence change ΔF/F was calculated as (F_H − F_D) / F_D, where F_H is the fluorescence intensity at frame time t, and F_D is baseline. Spike timings were determined as the onsets of individual Ca²⁺ transients with an automatic machine-learning algorithm that can accurately detect the timings within one-frame-jitter errors (9).

Local Field Potential Recordings and Ripple Detection. In some experiments, CA1 local field potentials were recorded during fMCI monitoring of the calcium activity of CA3 neurons. Glass pipettes were filled with 2 M NaCl and placed in CA1 stratum pyramidale. To extract the ripple wave activity, the recorded data were band-pass filtered at 150 to 300 Hz. Ripple-like events were automatically detected based on their oscillatory powers and durations; the root mean square (3-ms window) of the band-passed signal was used to detect the ripple wave with a power threshold of 5 SDs with 10 ms in duration.
ROTing. The ROTing technique was used to find synthetically coupled neurons located in an fMCI-imaged region (6). Immediately after monitoring spontaneous activity with fMCI, aCSF was modified to 2.2 mM K⁺, 3.0 mM Mg²⁺, and 3.6 mM Ca²⁺ to reduce spontaneous activity and the resultant plasticity of synaptic wiring that may occur. In slices loaded with OGB-1 AM, two CA3 or CA1 PCs were voltage-clamped at -70 mV, and 10 μM glutamate was locally puffed through ionophoretic pipettes (approximately 1 μL, 3–10 μA for each 1–5 s) to evoke spikes in a few CA3 neurons located in an fMCI-targeted region. The pipette was slowly moved over CA3 networks, and the evoked spikes were monitored by fMCI at 50 frames per s with an Andor DV897 CCD camera. These spike timings were statistically compared with EPSCs recorded in the patch-clamped neurons to determine the responsible presynaptic pyramidal neurons.

Nissl and Biocytin Staining. After each experiment, the slice was fixed in 4% paraformaldehyde overnight at 4 °C and 0.2% Triton X-100 overnight and then incubated overnight at 4 °C with NeuroTrace (N-21482, 1:100; Molecular Probes) or streptavidin–Alexa Fluor 594 conjugate (1:1,000; Invitrogen-Molecular Probes) for Nissl or biocytin staining, respectively. Based on biocytin staining, data obtained from PCs were selected to analyze patch-clamp data.

Surprise Index as Pairwise Correlation. We focused on pairwise spike correlations (10, 11), rather than higher-order correlations, because this study aimed to attribute spike synchronization to synaptic connection, i.e., a structural relationship between two neurons. Our high-speed scanning did not allow imaging for a period of more than 3 min, and the numbers of spontaneous spikes were often insufficient to precisely calculate the correlation coefficient (12). To estimate the pairwise similarity in our point-process dataset, therefore, we considered the probability that spikes can synchronize by chance. Synchronized spike pairs (SSPs), defined as any pairs of spikes that concurred in two neurons, were detected with a time window of 10 ms. If neuron, and neuron, are independent units that fire in a random manner, the probability P(n) that they exhibit n SSPs during the observation period t is given by the Poisson equation:

\[ P_{i,j}(n) = \frac{m_{i,j}^n}{n!} e^{-m_{i,j}} \]

where \( m_{i,j} \) is the expected number of SSPs, i.e., \( f_i \times f_j \times t \), and \( f_i \) and \( f_j \) denote the spike rates of neuron, and neuron, respectively. When SSPs occur n times, the probability (i.e., rareness) is:

\[ \bar{P}_{i,j} = \sum_{k=n}^{\infty} P_{i,j}(k) = 1 - \sum_{k=0}^{n-1} P_{i,j}(k). \]

The surprise index (\( S_{i,j} \)) was defined as follows (13):

\[ -\log_2 \bar{P}_{i,j}. \]

For extremely synchronized pairs with an S of more than 100 bits, S is denoted as 100 bits to avoid arithmetic precision problems in computing floating-point numbers.

Data Representation. We reported all averaged values as means ± SDs.

Fig. S1. Spontaneous synaptic activity in hippocampal slice cultures is similar to that in in vivo hippocampus. (A) Representative whole-cell patch-clamp traces of spontaneous excitatory postsynaptic current (Upper: sEPSC at −90 mV) and inhibitory postsynaptic current (Lower: sIPSC at 0 mV) recorded from hippocampal neurons in a slice culture preparation (ex vivo) and the hippocampus of a urethane-anesthetized mouse (in vivo). (B) We calculated the mean and SD as well as the third and fourth standardized moments, i.e., skewness, and kurtosis, of spontaneous excitatory postsynaptic conductance (Upper: sEPSC) and inhibitory postsynaptic conductance (Lower: sIPSC; n = 3 cells for each). No parameters did show statistical differences. Data are means ± SEM.

Fig. S2. The ratio of synaptic pairs increases as a function of S. CA3 PCs were randomly selected, and their spontaneous spike activities were recorded in current-clamp mode. The probability to find synaptically connected pairs were plotted against the spike synchronicity S.
Fig. S3. High-speed fMCI. (A) Confocal images of the CA3 PC layer in an OGB-1 AM–loaded (Left) and post hoc Nissl–stained (Center) slice. Neurons are Nissl-positive and thus distinguishable from nonneuronal cells. (B) Simultaneous loose-patch recording and calcium imaging reveals that action potentials evoke somatic calcium transients. (C) Twenty neurons were monitored at 2,000 frames per s. Spontaneous \( \Delta F/F \) traces of individual neurons (Right), the locations of which are also shown (Left).

Fig. S4. Temporally sparse synchronization in spontaneously spiking CA3 networks. (A) (Upper) Rastergram of spontaneous spikes in 96 neurons monitored by fMCI (same as Fig. 3A in the main text). (Lower) Time histogram of the percentage of coactivated cells to the total imaged neurons (2-ms bin). (B) Time expansion of a single synchrony event marked by an asterisk in A. (C) Peri-synchronization time histograms in which the peak time of each synchrony event (≥15 × SDs from the mean activity) was aligned at time 0 ms (bin, 2 ms; \( n = 985 \) events of 14 slices). (D) Power-law distributions in synchrony size for various bin sizes (2, 10, 50, and 100 ms) with the scaling exponents of –3.4, –2.6, –2.3, –2.0, and –1.9, respectively (\( n = 14 \) slices). The power law was robust for bin sizes of 2 to 100 ms.
Fig. S5. Synchronization is accompanied by ripple-like high-frequency oscillations. Local field potentials (band-filtered at 150–300 Hz) were recorded from CA1 stratum radiatum (Upper), whereas CA3 network activity was monitored with fMCI (Lower). The event marked with an asterisk was magnified in time (Right). Ripple-like high-frequency oscillations occurred with CA3 network synchronization.

Fig. S6. Comparison of optically and electrophysiologically determined $S$ values. (A) Distribution of interspike intervals in spontaneous activity of neurons recorded by whole-cell current-clamp technique ($n = 154$ cells; black) and high-speed fMCI ($n = 1,193$ cells; green). The latter often missed “bursty” spikes with intervals of less than 20 ms. (B) Relationship between $S$ of intracellularly recorded neuron pairs and its modified value ($S'$) in which spikes in greater than 50-Hz burst firings, except for each first spike, were removed from the spike series. Each dot represents a single neuron pair. The gray line indicates the linear diagonal $S = S'$, and the green the linear least-square fit.
Fig. S7. Small-world architectures in synchrony-based connectivity. (A) The $S$ matrix shown in Fig. 3A in the main text is hierarchically shown in a dendrogram format. (B) In the data for Fig. 3A in the main text, synchronized pairs were extracted at the $S$ thresholds of 25, 15, and 5 (bits). Circles and lines in each graph indicate the extracted neurons and suprathreshold links, respectively. (C and D) Two metrics, i.e., the mean clustering coefficient $C$ (a measure of how frequently neighbors of each cell are also neighbors of each other) and the mean shortest path length $L$ (the average length of the shortest path between two cells), were compared with those of 200 equivalent random graphs. The random graphs maintained the total numbers of neurons and links, but all links were randomly rewired. Chance is represented as the mean (line) ± SD (shade) of 200 randomly rewired graphs. At any given $S$ threshold, $C$ was consistently larger than chance ($C_{\text{rand}}$), whereas $L$ was close to chance ($L_{\text{rand}}$). (E) For a small-world network, the ratios $\lambda = L/L_{\text{rand}}$ and $\gamma = C/C_{\text{rand}}$ are expected to be approximately 1 and greater than 1, respectively. Therefore, we calculated the scalar measure of small-worldness, defined as $\gamma/\lambda$ (14). The small-worldness was consistently higher than the chance level, i.e., 1, over the entire $S$ threshold range.
Fig. S8. Network organization and cell assemblies. The connectivity of CA3 PCs is densely clustered among specific neuron subsets (different colors). These interwired neurons thus receive correlated excitatory inputs and thereby generate highly synchronized spikes. CA3 inhibitory interneurons provide strongly correlated background inhibition, which may integrate CA3 PC groups. The synchronized CA3 spikes are specifically transmitted to subsets of CA1 PCs through relatively independent channels (arrows).

Movie S1. Spontaneous activity of CA3 neurons in a slice loaded with OGB-1 AM. Time is compressed by a factor of 2.