Supporting Online Material for

Locally Synchronized Synaptic Inputs

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Locally Synchronized Synaptic Inputs
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1. MATERIALS and METHODS

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

We report all averaged values as the means ± S.D. unless otherwise specified.

1.1 Slice culture preparation

Organotypic slice cultures were used for functional spine imaging because the transparency of this tissue allowed optical access to a large number of spines en masse. Entorhinal-hippocampal organotypic slices were prepared from 7-d-old Wistar/ST rats (S1). Rat pups were anesthetized by hypothermia and decapitated. The brains were removed and placed in aerated ice-cold Gey’s balanced salt solution supplemented with 25 mM glucose. Horizontal entorhinal-hippocampal slices were cut at a thickness of 300 µm using a vibratome. The slices were placed on Omnipore membrane filters and incubated in 5% CO₂ at 37°C. The culture medium, which was composed of 50% minimal essential medium, 25% Hanks’ balanced salt solution, 25% horse serum, and antibiotics, was changed every 3.5 d. In some experiments, slices were cultivated in the same medium with the addition of 100 µM L,d-2-amino-5-phosphonopentanoic acid (AP5) from day 0 in vitro. Experiments were performed at 12–19 d in vitro unless otherwise specified.

1.2 Electrophysiology

An entorhinal-hippocampal slice was placed in a recording chamber and perfused at 30–32°C at a rate of 3–4 ml/min with artificial cerebrospinal fluid (aCSF) consisting of 127 mM NaCl, 26 mM NaHCO₃, 3.5 mM KCl, 1.24 mM KH₂PO₄, 1.0 mM MgSO₄, 1.8–2.0 mM CaCl₂, 10 mM glucose,
and 200 µM Trolox. For dendrite patching, Alexa Fluor 488 was electroporated into the somata of CA3 pyramidal cells by applying one or two rectangular negative pulses (10 ms, 10–15 V) through a glass pipette (4–6 MΩ) loaded with 1 mM Alexa Fluor 488. The apical dendrites were visualized and targeted under Nipkow-disk confocal control using Alexa Fluor 488-conjugated albumin-coated glass pipettes (14–20 MΩ) (S2) filled with 130 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 10 mM phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, and 0.2% biocytin. Dendrites were current-clamped at approximately -70 mV (the reversal potential of GABA), and spontaneous excitatory postsynaptic potentials were recorded. For soma patching, whole-cell recordings were made from CA3 pyramidal cells and parvalbumin-positive fast-spiking cells using a MultiClamp 700B amplifier. For pyramidal cells, patch pipettes (4–6 MΩ) were filled with 130 mM CsMeSO₄, 10 mM CsCl, 10 mM HEPES, 10 mM phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, 0.2% biocytin, and 200 µM Fluo-5F. For parvalbumin-positive cells, the same pipettes were filled with 130 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 10 mM phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, 0.2% biocytin, and 200 µM Fluo-5F. Signals were low-pass filtered at 2 kHz and digitized at 20 kHz. Spontaneous and miniature excitatory postsynaptic currents (EPSCs) were recorded at -70 mV. Miniature EPSCs were recorded in the presence of 1 µM TTX and 50 µM picrotoxin. In some experiments, electrical field stimulation (50 µs, 3–6 V) was applied every 5 s using monopolar tungsten electrodes to generate synchronous inputs to a patched neuron. Electrodes were placed in the CA3 striatum radiatum at least 300 µm away from the imaged dendrites of the patched cells. During stimulation, a low concentration (2 nM) of TTX was perfused to reduce the spontaneous firing of surrounding neurons.
1.3 Ex vivo calcium imaging of synaptic inputs

Pyramidal cells and interneurons were voltage-clamped at −30 mV and −70 mV, respectively, to facilitate channel currents mediated by NMDA receptors and calcium-permeable AMPA receptors. Images were acquired at 10–20 frames/s for 120–300 s using a Nipkow-disk confocal unit, a back-illuminated EM-CCD camera (512×512 pixels), and a water-immersion objective lens (40×, 0.80 NA). Fluorophores were excited at 488 nm (about 0.8 mW) and visualized with a 507-nm long-pass emission filter. To avoid a space clamp problem, only spines located within 200 µm of the soma were monitored. For each spine, the fluorescence change ΔF/F was calculated as (Ft – F0)/F0 where Ft is the fluorescence intensity at frame time t and F0 is the baseline. Input timings were determined as the onset of individual Ca2+ transients with an automatic machine-learning algorithm (S3). To examine the developmental effect of assembllet formation, slice cultures were used at 3–4 d in vitro. Because these immature networks showed a low level of spontaneous activity, the K+ concentration was increased to 4.0 mM. Under these conditions, CA3 pyramidal cells occasionally exhibited calcium elevations in their dendritic shafts, which were irrelevant to synaptic inputs and not considered in the data analysis. In some experiments, 1 mM AP5 or 1 mM picrotoxin was locally pressure-applied together with 40 µM Alexa Fluor 594 through a patch pipette placed near the imaged dendrites. The spatial spread of the ejected solution was monitored based on Alexa Fluor 594 fluorescence and set to be restricted within 100 µm (S4). The head width (φ) of each spine was measured in the Z plane that gave the maximal spine area of the Z-series confocal planes (0.2 to 0.4-µm steps), and the spine head size was calculated as 4π(φ/2)³/3.
1.4 *Ex vivo* calcium imaging of neuronal outputs

For calcium imaging of CA3 neurons, a glass pipette (4–6 MΩ) for dye loading was filled with aCSF that consisted of 100 μM OGB1-AM, 15% Pluronic F-127, and 10% DMSO. The tip of the pipette was inserted into the stratum pyramidale, and the dye solution was injected by manually controlling a 1-ml syringe pressurizer (10–30 hPa for 1–3 min). Images were acquired at 100 frames/s with a Nipkow-disk confocal unit, a high-speed back-illuminated CCD camera, a water-immersion objective lens (16×, 0.80 NA, Nikon, Tokyo, Japan), and Solis software (Andor). Fluorophores were excited at 488 nm and visualized with a 507 nm long-pass emission filter. In each cell body, the fluorescence change $\Delta F/F$ was calculated as $(F_t - F_0)/F_0$ where $F_t$ is the fluorescence intensity at frame time $t$ and $F_0$ is the baseline. Spike timings were determined as the onset of individual Ca$^{2+}$ transients with an automatic machine-learning algorithm that could accurately detect the timings with zero- or one-frame-jitter errors (S3).

1.5 *In vivo* calcium imaging of synaptic inputs

C57BL/6N mice (P30−35) were anesthetized by 1.5 mg/kg urethane. Depth of anesthesia was monitored regularly by observing whisker movements and the pinch withdrawal reflex of the hindlimb; additional doses of anaesthetic were given as needed. Body temperature was monitored by a rectal temperature probe and maintained at 37 ± 1°C using a heating pad. After removing the skin, a small craniotomy (2–3 mm in diameter) was performed above the barrel cortex (3 mm lateral to the midline and 1.5 mm posterior to the bregma). The dura was removed and exposed brain surface was subsequently covered by 1.5–2% agarose and coverslip to suppress movement artifact. Whole-cell recordings were made from layer 2/3 pyramidal cells under direct visual
control by shadow-patching method (S5). Patch pipettes (5−7 MΩ) were filled with internal solution contained 135 mM Cs-gluconate, 10 mM HEPES, 3 mM MgATP, 0.3 mM Na₂GTP, 10 mM Na-phosphocreatine, 5 mM QX-314 chloride, 300 µM Fluo-5F, and 50 µM Alexa594. During two-photon calcium imaging, neurons were voltage-clamped at -30−0 mV. Calcium imaging was performed using a two-photon microscope with a mode-locked Ti:sapphire laser (wavelength: 810 nm, average power: 5−25 mW) in conjunction with a 25× water-immersion objective lens. Fluorescence was divided into green (Fluo-5F) and red (Alexa 594) channels through a dichroic mirror and detected by a pair of photomultiplier tubes. Images were acquired at 6−8 frames/s.

1.6 Transgenic mouse and behavioral task
The GFP-GluR1/c-fos transgenic mice were described previously (S6). Briefly, c-fos-tTA and tetO-GFP-GluR1 transgenes were microinjected into BALB/cByJ and C57BL/6J F2 hybrid embryos and implanted into pseudo-pregnant females. All transgenic mice were maintained as heterozygotes and were backcrossed to C57BL/6J mice. Mice were fed on a Dox diet (40 mg/kg of mouse chow) from the time of weaning (3−4 w old) and were used for experiments at 11−14 w of age. Only males were used for our experiments. Mice were individually housed for 1 w and deprived of Dox for 4 d prior to the experiments. On the experimental day, mice were placed in a novel rectangular chamber and allowed to explore it for 500 s. The mice were subsequently returned to their home cages and treated with a high dose of Dox (6 g/kg) to rapidly suppress additional expression of GFP-GluR1. After 24 h, the brains were sagittally sectioned at a thickness of 100 µm using a vibratome and fixed with 4% paraformaldehyde (PFA) for 1 h. Without permeabilization, the slices were incubated with a rabbit anti-GFP antibody at 4°C overnight and
were incubated with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody at 4°C overnight. Micropipettes were coated with DiI (1,1´-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate) dissolved in ethanol at 10 mg/ml. The tip of the DiI-coated micropipette was inserted at several positions in the CA1 pyramidal cell layer of fixed slices using a micromanipulator. Next, slices were placed in phosphate-buffered saline (PBS) at 4°C for 3–4 d to allow the DiI to spread throughout the dendritic arbor. For each spine, the volume-normalized expression level of GFP was calculated as $F_{\text{GFP}}/F_{\text{DiI}}$ where $F_{\text{GFP}}$ and $F_{\text{DiI}}$ were the mean fluorescence of GFP and DiI in the spine. Spines with GFP levels that exceeded 1×SD of the mean GFP level across all spines were defined as GFP-positive spines.

### 1.7 Immunohistochemistry

After recording, the slices were fixed with 4% PFA in PBS at 4°C overnight. The slices were blocked with 5% normal goat serum and 0.1% Triton X-100 in PBS at room temperature for 15 min and were incubated in primary anti-parvalbumin rabbit IgG antibody at 4°C overnight. Following incubation in the primary antibody, the slices were rinsed and treated with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody at room temperature for 5 h.

### 1.8 Biocytin reconstruction

To enhance the probability of synaptic transmission, the extracellular solution of 2.5 mM K⁺, 2.4 mM Ca²⁺, and 4.0 mM Mg²⁺ was used for dual patch-clamp recordings to search synaptically connected neuron pairs. For visualization of these neurons, the slices were fixed in 4% PFA and
0.05% glutaraldehyde in PBS at 4°C overnight. After the PBS washes, the slices were incubated with 0.3% H$_2$O$_2$ in PBS for 30 min. After permeabilization in 0.2% Triton X-100 in PBS for 1 h, the slices were processed with an ABC reagent at 4°C overnight. The slices were washed in PBS, equilibrated with Tris-buffered saline (0.05 M; pH 7.5) and developed with 0.0003% H$_2$O$_2$, 0.02% diaminobenzidine and 10 mM (NH$_4$)$_2$Ni(SO$_4$)$_2$ in Tris-buffered saline.
2. Supporting Figures and Video

**Figure S1** Two working hypotheses for the spatial patterns of dendritic inputs from presynaptic cell assemblies. In the clustered input model (A), afferents from synchronous neuron group 1 (or 2) converge onto a small segment of a postsynaptic neuronal dendrite; in the dispersed input model (B), the afferent terminals diverge over the dendritic trees.

**Note:** The present work supports the clustered input model.
Figure S2 Dendrites receive spatially biased synchronous inputs. (A) A stacked confocal image of a dual-patched CA3 pyramidal cell. Alexa Fluor 594 was electrophoretically injected from the soma, and the visualized dendritic trees were targeted for current-clamp recording using fluorophore-coated glass pipettes (S2). CA3 pyramidal cells are suitable for dual dendritic patching because their thick apical dendrites are usually bifurcated as shown in this photograph. (B) Typical traces of spontaneous fluctuations in membrane potentials simultaneously recorded from two dendrites (black traces). Dynamics of membrane potentials were largely similar between the dendrites, but large postsynaptic potentials occasionally occurred in only one dendrite (arrowhead), which suggests spatially biased synchronous synaptic inputs. The bottom blue trace indicates the time change in the Euclidean distance between two membrane potentials (10-ms bins). (C) The Euclidean distance showed a heavily tailed distribution with a small fraction of time spent for large distances (real, for 3 min). As a control, a Poisson distribution was obtained by assuming that the Euclidean distance fluctuated randomly due to background noise (chance). The noise level was obtained in the same dataset. The real distribution was more heavily tailed than the Poisson distribution ($p < 0.01$, Kolmogorov-Smirnov test), indicating that spatially biased synchronous inputs occurred more frequently than would be expected by chance. Similar results were obtained in all three of the recorded neurons.
Figure S3 Calcium elevations in spines work as reliable reporters of synaptic inputs. (A) The timing of spine calcium events (top) is associated with the EPSC timing (bottom). Twenty raw calcium traces (gray) and their mean trace (black) recorded from a spine are shown with the patch-clamp traces corresponding to time. The red-colored period indicates the video frame (100 ms) that showed the onset of the calcium events. (B-G) Blocking NMDA receptor activity abolished spontaneous calcium spine activity. (B) AP5 (1 mM) was locally applied to the observed dendritic trees through a puffing micropipette. The AP5 gradient was defined by the fluorescence of Alexa 594 from the same puffing micropipette. (C) Spontaneous synaptic activity before and after the local application of AP5. (D,E) The amplitude and frequency of the sEPSCs were not changed by AP5 application ($p > 0.1$, paired $t$-test, $n = 4$ neurons for each). (F) A representative calcium trace (top) and raster plot (bottom) show that AP5 application decreased the frequency of the spine calcium transients. The period of AP5 application is shown by a black line (top) and a gray area (bottom). (G) The frequency of calcium events in the spines was reduced by AP5 application ($p < 0.01$, paired $t$-test, $n = 4$ videos from 4 neurons).
Figure S4 Firing rates of neurons are approximately two times greater than spine activity rates. (A) Representative data of multi-neuron calcium imaging from the CA3 stratum pyramidale of a slice culture under the same experimental conditions, including temperature and extracellular ionic concentrations, as those used for the spine calcium imaging. (A) A raw confocal image; Left bottom: locations of 97 neurons. (B) A raster plot of calcium events of the 97 neurons. (C) Distribution of the frequency of spontaneous calcium activity of individual CA3 neurons. 

Note: The mean ± S.D. of the event frequency from 699 neurons was 3.6 ± 10.3 min⁻¹. Because the activity rate of 1,084 spines was 1.5 ± 3.8 min⁻¹ (Fig. 1E,G), the frequency of spine events was 58% lower than that of the soma events. Conversely, the rate of transmitter release or action potential propagation failure (S7) during the patch-clamp recordings from synaptically connected pairs of CA3 pyramidal neurons in the slice cultures is approximately 50% (S8). Therefore, the activity frequency of the spines was consistent with the firing rates of the neurons, which indicates that our spine imaging captured the majority of the synaptic inputs (S9).
Figure S5 Spine activation is spatially clustered on dendrites: reshuffling simulation. The distribution of the path length between two coactivated spines was compared to that in surrogates, which was obtained by a random shuffling of the spine identity in each dataset. In this shuffling method, only the temporal patterns of calcium activity were exchanged between spines. Thus, the location of individual spines and the temporal and population modulations in the overall spine activity were completely preserved. This tightly restricted data shuffling was designed to avoid a false-positive overestimation of the significance level, which may occur in a simple Monte-Carlo simulation. For each dataset, 100 surrogates were generated. The cumulative probability of the path distances between all possible pairs of synchronized spines was compared between the original datasets (thick lines) and their surrogates (thin gray lines). (A) Spontaneous spine activity of CA3 pyramidal cells in control slices after 12–19 d of culture in vitro. (B) Spontaneous dendrite activity of CA3 fast-spiking parvalbumin-positive interneurons in control slices after 12–19 d of culture in vitro. (C) Spontaneous spine activity of layer 2/3 pyramidal cells in the somatosensory cortex of anesthetized mice. (D) Stimulation-evoked spine activity of CA3 pyramidal cells in control slices after 12–19 d of culture in vitro. (E) Spontaneous spine activity following application of picrotoxin to the dendrites of CA3 pyramidal cells in the control slices after 12–19 d of culture in vitro. (F) Spontaneous spine activity of CA3 pyramidal cells in slices cultured in the present of AP5 for 12–19 d. (G) Spontaneous spine activity of CA3 pyramidal cells in control slices after 3–4 d of culture in vitro. (H) GFP-positive spines of CA1 pyramidal cells in vivo.
Figure S6 Synaptic inputs are not clustered in fast-spiking parvalbumin-positive interneurons. (A) Biocytin reconstruction of a recorded interneuron. The cell type was identified by its non-adaptive spiking pattern (inset) and immunohistochemical staining for intra-pipette biocytin (green) and parvalbumin (PV, magenta; bottom). (B) Three dendritic branches were confocally time-lapse imaged, and the video was line-scanned post hoc. (C) Detection of synaptic input loci from spontaneous calcium activity. Each red dot represents the site (spot) that gave the strongest fluorescent signal in a calcium event, and its subsidiary line shows the region of a significant fluorescence increase. (D) Representative raster plot of calcium events. (E) Probability of observing co-activated calcium spots as a function of the path distance between the coactivated spots. Spatial clustering of calcium activity was not observed (|Z| ≥ 0.63, p ≤ 0.53; n = 11 videos from 8 neurons). The same conclusion was drawn from the reshuffling simulation (Fig. S5B).

Note: Inhibitory interneurons exhibited highly localized calcium activity in their aspiny dendrites, as reported previously (S10, 11). Because they are highly excitable and can fire in response to a single excitatory input (S12), they may not require dendritic integration via synaptic clustering. For another type of interneuron, see ref. S10.
**Figure S7** Four possible mechanisms that can explain spatially clustered synaptic activation. (A) A population of spontaneously synchronized presynaptic neurons (cell assembly) project convergently to a narrow segment of a postsynaptic dendrite and simultaneously activates the adjacent spines. (B) A single presynaptic axon makes multiple “en passant” synapses with a narrow dendritic segment of a single neuron (S13) and simultaneously activates adjacent spines. (C) Diffusible molecules, such as glutamate, released from a single synapse spread to neighboring spines (S14) and synchronize the activity of adjacent spines. (D) Broad excitatory synaptic inputs are spatially segregated by local inhibitory inputs.

**Note:** To discriminate these four mechanisms, we carried out three series of analyses (i-iii) as follows. (i) Electrical stimulation. Field stimulation of CA3 stratum radiatum evoked massive activation of fibers passing near the stimulating electrode tip and mimicked network synchronization. This synchronization consisted of artificially selected neurons and did not reflect intrinsically occurring cell assembly dynamics. If mechanism B or C is true, even stimulation-evoked synchronization would produce spatially clustered spine activity. In fact, stimulation did not evoke clustered activity (Fig. 2A bottom, S5D). Thus, mechanisms B and C are unlikely. (ii) Anatomical reconstruction of synaptic contacts. The site where an axon fiber approaches a dendritic spine (an axo-dendritic proximity) is a candidate location for where the axon makes synaptic contacts. Biocytin was injected into synaptically connected neurons and *post hoc* labeled with avidin and diaminobenzidine, and the neuronal morphology was inspected by optical microscopy. We found that only 7.7% of the analyzed synapses had multiple contact sites (Fig. S8). Given that 31.5% of the spines participated in assemtles, mechanism B cannot fully explain the assemblet dynamics. (iii) Local blocking of GABAergic inhibitory inputs. Picrotoxin was locally applied to the imaged dendrites to block inhibitory transmission. Even under disinhibited conditions, clustered spine activation was intact (Fig. S5E, S9). This result excludes mechanism D. Note that mechanism D is also inconsistent with the sparseness of assemblies; assemtles usually appeared alone (Fig. S10A) and were unlikely to result from segregation by inhibition.
Figure S8 Single axo-dendritic proximities have single putative synaptic contacts. A pair of synaptically connected CA3 pyramidal cells were filled with biocytin and reconstructed using a camera lucida. Circles represent axo-dendritic proximities, i.e., candidates of synaptic contacts (S15), and these regions are shown in high-magnification photographs. Traces are unitary EPSCs recorded by dual patch-clamping from neurons 1 (pre) and 2 (post). The reconstruction was repeated for axons from 12 neurons, and 51 of 55 (92.7%) proximities had single contact sites, whereas other proximities (7.3%) had two or three contact sites. Given that 31.5% of the spines participated in assemblents, multiple synapses of single axons onto small dendritic segments, if there are any, are unlikely to contribute significantly to the functional synaptic clustering observed in spine calcium imaging.
**Figure S9** Spine activation is clustered in locally disinhibited dendrites. (A) Dendrites of CA3 pyramidal cells were treated with a local puffing of 1 mM picrotoxin, and the spines were imaged in the area where the Alexa-estimated concentration of picrotoxin was greater than 100 µM. (B) The probability of observing co-activated spines ($|Z| \geq 3.97, p \leq 7.2 \times 10^{-5}; n = 8$ videos from 8 neurons). The control is the same as in Fig. 2A. The Kolmogorov-Smirnov test indicated no significant difference between control and picrotoxin-treated dendrites ($p > 0.1$). The same conclusion was obtained from the reshuffling simulation (Fig. S5E).
**Figure S10** Assemblets are sparse over dendritic trees. (A) Distribution of the number of assemblies coactivated within a time window of 100 ms. (B) Distribution of the path distance between any pair of synchronized assemblets. The chance level and its 95% confidence interval (purple) were estimated from the distribution at distances of more than 100 µm. Coactivation seemed to be rare when assemblets were within 70 µm of one another.
Figure S11 Dendritic branches are heterogeneous in assembles. (A) Distribution of the path length of single hot zones. Mean ± S.D. = 7.7 ± 6.7 µm, CV = 0.88, n = 79 hot zones in 11 neurons. (B) Distribution of the number of assemblies emitted by single hot zones per minute. Mean ± S.D. = 2.7 ± 4.8 min⁻¹, CV = 1.76. (C) Distribution of the density of hot zones per 50 µm of dendritic length. Mean ± S.D. = 1.8 ± 1.2, CV = 0.68.

Note: All of these parameters varied greatly from dataset to dataset, which suggests that hot zones are non-uniform over the dendritic trees.
**Figure S12** Spines that participate in assembles have large heads. The cumulative distribution of the head sizes of spines that participated in at least one assembl (assembl, n = 341 spines) was significantly rightward compared to spines that were not involved in an assembl (other, n = 743 spines; p < 0.01, Kolmogorov-Smirnov test).
**Figure S13** Chronic blockade of NMDA receptors does not affect synaptic activity. Slices were cultivated in the chronic presence of 100 µM AP5 for 12–19 d *in vitro*. (A) Biocytin visualization of spines in the basal dendrites of control or AP5-treated CA3 pyramidal cells. (B) Spine density was not different between control and AP5-treated pyramidal cells (control, $n = 18$ branches from 9 cells; AP5, $n = 21$ branches from 10 cells). (C, D) Neither the amplitude nor the frequency of miniature EPSCs (C; mEPSC, $n = 11$ and 12 cells, respectively) or spontaneous EPSCs (D; sEPSC, $n = 25$ cells each) was different between the control and AP5-treated pyramidal cells. Data are the means ± S.E.M.
Figure S14 Clustered synaptic plasticity in behaving adult mice. (A) Schematic representation of the transgenic mouse system. GFP-fused GluR1 AMPA receptor subunit (GFP-GluR1) was expressed under control of the c-fos promoter (S6). The c-fos promoter drives activity-dependent expression of the tetracycline-regulated transactivator (tTA), which, in turn, activates transcription of the tetO promoter-linked GFP-GluR1 in a doxycycline (Dox)-regulated manner. (B) Experimental design. Mice were deprived of Dox for 4 d and were exposed to a novel environment for 500 s. After 24 h, approximately 25% of CA1 pyramidal neurons expressed GFP-GluR1. GFP-positive spines were examined in Dil-labeled CA1 pyramidal cells. (C) Left, representative confocal image of GFP and Dil fluorescence. Right, detection of GFP-positive spines (red) in a Dil-labeled dendrite (black). (D) Representative spatial distribution of GFP-positive spines (red, arrowheads) in the dendrites of three neurons. (E) The probability of observing GFP-positive spines around a given GFP-positive spine in the three cells shown in D. (F) The averaged probability of observing GFP-positive spines in 19 neurons. The chance level and its 95% confidence intervals were obtained from the distribution of distances of more than 10 µm. GFP-positive spines were clustered within 8 µm of one another (|Z| ≥ 4.02, p ≤ 5.7×10^{-5}; Fig. S5H; n = 19 neurons).

Note: Newly synthesized AMPA receptors are preferentially inserted into previously activated spines and contribute to long-term potentiation. Therefore, we used GFP-GluR1 to probe a part of spines that underwent long-term potentiation.
Figure S15 A possible mechanism underlying the emergence of assemlels. Step 1: Individual neurons in groups of synchronized neurons (cell assemblies 1 and 2) make synapses with postsynaptic dendrites in a spatially non-selective manner (non-selective innervation). Step 2: NMDA-dependent long-term potentiation occurs simultaneously in spines that receive synchronized inputs in spatially narrow dendritic regions (clustered plasticity), inducing spine enlargement (S16). Step 3: Synapses that did not exhibit long-term potentiation are weakened or eliminated (pruning). During this network-wide reorganization of synaptic competition, convergently synchronized synapses become predominant.

Note: This model was inspired by the concepts proposed by Govindarajan et al. (S17), DeBello (S18), and Larkum and Nevian (S19). Clustered plasticity may be induced by a synchronous input-induced massive depolarization of a small dendritic segment and/or by intracellular diffusion of plasticity-associated molecules to nearby spines (S20-24).


**Movie S1** Time-lapse confocal imaging of calcium activity from spines. A neuron was loaded with Fluo-5F. The changes in the fluorescent signal are shown in a pseudo-color scale and superimposed onto a time-averaged Fluo-5F image.
3. References


