Supporting Information

Tajima et al. 10.1073/pnas.1705981114

SI Methods

Cell Culturing. All experimental protocol was approved by the Committee on the Ethics of Animal Experiments at the Research Center for Advanced Science and Technology, the University of Tokyo (Permit number: RAC130106).

A protocol for cell culturing had been reported previously (26). Briefly, E18 Wistar rat cortices were dissociated using trypsin and mechanical trituration. Next, 20–40 kJ/L neurons and glia were seeded over an area of ∼12 mm² on top of the CMOS chip. Layers of poly (ethyleneimine) followed by laminin were used to adhere cells. Plating media consisted of Neurobasal-B27 supplemented with 10% horse serum and 0.5 mM GlutaMAX during the first 24 h. Growth medium consisted of DMEM supplemented with 10% horse serum, 0.5 mM GlutaMAX, and 1 mM sodium pyruvate. Experiments were conducted inside an incubator to control environmental conditions (36 °C and 5% CO₂).

CMOS-Based Recording and Stimulation of Network Activity. Cultured neuron activities were simultaneously recorded with high-density MEA as described before (26, 27). Cortical networks were grown over 11,011-electrode CMOS-based MEAs (52, 53), which provide enough spatial and temporal resolution to detect action potentials from any neuron lying on the array: 1.8 × 2.0-μm² area containing 8.2 × 5.8-μm² electrodes with 17.8-μm pitch, sampled at 20 kHz. Subsets of 126 electrodes can be read out (and stimulated) at one time, and electrode selection can be reconfigured within a few milliseconds. To identify the locations of neurons growing over the array, a sequence of about 100 recording configurations were scanned across the whole array while recording spontaneous activity. Locations of active somata were identified because action potentials could usually be detected from multiple nearby electrodes. Electrode selection was then reconfigured such that a single electrode was assigned to each identified soma, and spontaneous activities were measured simultaneously from all of these cells. The putative neuron types (excitatory or inhibitory) were estimated based on spike shapes (Fig. S3). We could record from 93, 47, 98, 92, and 53 neurons in Chip 1437, 1440, 1444, 2427, and 2440, respectively.

Microstimulation-elicited network activities were then investigated to characterize a pairwise synaptic strength between neurons. An adequate stimulating electrode was explored such that a single target neuron was directly activated through axonal stimulation and that the directly evoked spikes were exclusively measured from the target cell. The directly evoked spikes could be easily distinguished from postsynaptic activations because they were very reliable (i.e., 100 spikes elicited out of 100 stimulation trials) and exhibited a small temporal jitter (Fig. S4). The microstimulation was a single, positive–negative, biphasic pulse with a charge-balanced amplitude of ±300–900 mV and a duration of 200 μs/phase, and was delivered 100 times every 3 s.

Burst Detection. Burst in spontaneous activity was detected modifying a protocol that was previously established by the authors’ group (27). The previous study proposes to determine the threshold for burst detection based on the distribution of ISI of consecutive spikes (27). In the bursting neurons, the ISI distribution typically has a bimodal structure whose valley can be used as an objective criterion for burst detection. In this study, we first computed sequences of population firing rate that were normalized such that it ranges from 0 to 1. We defined this as the sequence of global state bₜ, where t is the time. This was to use a common burst-detection threshold across different preparations of neuron cultures, which generally vary in terms of the absolute firing rate. We used 5-ms bin for the firing rate computation. We next derived the distributions of inverse firing rate over the bins. This yielded an ISI distributions for each preparation, in which we confirmed that all of them had bimodal shapes (Fig. 1D, Inset). For each preparation, we selected the smallest ISI providing the valley of distribution as the burst-detection threshold. The burst periods were determined by the at least three consecutive sequences of bins that have average ISIs under this threshold, identifying 26 bursts on average for each preparation. We defined the peak burst timing by selecting the center of the bin having the smallest average ISI (i.e., the largest global state) within each burst period.

Estimation of Synaptic Connectivity from Electrical Stimulation. Synaptic connectivity was estimated based on the evoked responses during electrical stimulation experiment. To reliably stimulate a single neuron, we searched a stimulation site around a target neuron in each MEA that could elicit an action potential exclusively at the target neuron. After the careful selection of stimulation sites, we could evoke the action potentials at almost 100% probability for each single stimulation, with very little jitters in the timings of action potentials in the stimulated neurons. We first aligned the spike raster to the timings of stimulation. We next computed the sequence of firing rate xᵢₜ in a way described above. The firing rates before the stimulation (−500 ms < t < −100 ms) were used to produce the reference probability distribution of each neuron’s firing rate, P(xᵢ). Next, we computed the probability, P(xᵢ > xᵢⱼ), for each time bin centered at t in a short poststimulation period (2.5 ms < t < 15 ms); the responses during 0 ms < t < 2.5 ms were omitted to eliminate the artifacts of electrical stimulation. We defined the smallest time t that satisfies P(xᵢ > xᵢⱼ) < 0.01 in the poststimulation period as the latency of neuron i. The neurons that had at least one time bin satisfying this condition were defined as “downstream” cells of the stimulated neuron; the neurons that did not have any such time bin were defined as “non-downstream” cells.

According to the evoked response latencies, we identified the short- (<10 ms) and long-latency (≥10 ms) downstream cells for each stimulated neuron. The multisynaptic downstream cells for a stimulated neuron comprised relatively small fraction n (17% on average) of the entire population. This indicates that they are subsets of neurons receiving effectively strong input from the stimulated cell via relatively a small number of path length, although the interaction with longer path length, which was not detected here, could include the larger fraction of the cell population.

Causal Network Analysis. Pairwise causal interaction among neurons was estimated based on a variant of CCM. CCM was developed by Sugihara et al. (31) as an extension of nonlinear forecasting method of time sequence based on nearest-neighbor models (20, 54). The method is capable of detecting relatively weak causal interactions in deterministic dynamical systems (which can include some stochastic components) if the system and observations are deterministic, the embedding dimension is sufficiently large, and data size is sufficiently large for the given embedding dimension. A variant of CCM that can be used for systems in which the variables have heterogeneous time scales (such as in neural system) was developed by the authors (32).

Suppose that we want to know the interaction from neuron x to another neuron y. We first reconstruct the state-space trajectories of each neuron x in the delay coordinates,
$\mathbf{x}^\text{max} = (x_t, x_{t-\tau}, \ldots, x_{t-(d_{\text{max}}-1)\tau})$, where $d_{\text{max}}$ represents the maximum number of dimensions (number of delay coordinates) to be considered, $t$ is the time point, and $\tau$ is the unit delay length. We used $\tau = 5$ ms $d_{\text{max}} = 8$ in this study. In the embedding-based analyses, we convolved the firing-rate sequence of each neuron with a Gaussian kernel that has half-width-of-half-height of 25 ms, and normalized such that each neuron trace ranges from 0 to 1. To avoid a known vulnerability of the state-space reconstruction to the time-scale heterogeneity (55), we projected delay-coordinate space. We set $k=4$, weight $w(|\mathbf{x}_0^d - \mathbf{x}_0'|) = |\exp(-|\mathbf{x}_0^d - \mathbf{x}_0'|)|/\sum_{s.t. \mathbf{x}_s^d \in \mathbf{B}^d(\mathbf{x}_0')} |\exp(-|\mathbf{x}_0^d - \mathbf{x}_s'|)|$, and $|\mathbf{x}_0^d - \mathbf{x}_s'|$ as the square distance between $\mathbf{x}_0^d$ and $\mathbf{x}_s^d$ in the data analysis. Note that $\rho_{\text{true}}(\mathbf{x}_0^d) = 1$ means perfect prediction. We selected the optimal dimension $d = d^*$ so as to maximize the prediction performance, $\rho_{\text{true}}(\mathbf{x}_0^d)$. Typical value of $d^*$ was distributed from 2 to 4 in the current data.

\[ y(t) = \frac{1}{C_0} \sum_{s.t. \mathbf{x}_s^d \in \mathbf{B}^d(\mathbf{x}_0')} w(|\mathbf{x}_0^d - \mathbf{x}_s'|) y_s \]

with the $k$-nearest-neighbor set $\mathbf{B}(\mathbf{x}_0^d)$ of $\mathbf{x}_0^d$ in the delay-coordinate space. We used $\mathbf{B}(\mathbf{x}_0^d)$ to construct the transformed vector: $\mathbf{x}_0^d = R \mathbf{x}_0^\text{max}$. A $d$-dimensional delay vector $\mathbf{x}_0^d$ is constructed by selecting the first $d(\leq d_{\text{max}})$ components of $\mathbf{x}_0^\text{max}$. The causal interaction form neuron $x$ to another neuron $y$ is quantified based on the correlation coefficient, $\rho_{x,y}(\mathbf{x}_0^d)$, between the true ($y(t)$) and forecast ($\hat{y}_t(\mathbf{x}_0^d)$) signals, where

\[ \hat{y}_t(\mathbf{x}_0^d) = \sum_{s.t. \mathbf{x}_s^d \in \mathbf{B}^d(\mathbf{x}_0')} w(|\mathbf{x}_0^d - \mathbf{x}_s'|) y_s \]

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Fig. S3. Microstimulation-based estimation of synaptic connectivity in a pairwise manner. (A) Axonal stimulation on an arbitrary neuron elicited bidirectional action potential propagation. (B) Raw data of neural responses at a putative presynaptic neuron and postsynaptic neuron. Data from 100 trials are superimposed. (C) Raster plot of B. Antidromic, direct action potentials exhibited precise temporal responses, while orthodromic, synaptic action potentials were elicited stochastically with significant temporal jitters. (D) Poststimulus spike histograms of C.
Fig. S4. Identification of excitatory and inhibitory neurons. (A) Representative neurons in immunostaining with MAP2 and GABA. Action potentials below the insets were obtained at white rectangles, putatively from a neighboring neuron in a circle. The peak-to-peak time, $T_{pp}$, was defined as time duration from negative peak to positive peak of action potential. (B) Histogram of $T_{pp}$. Excitatory neurons (green) had larger $T_{pp}$ than inhibitory neurons (magenta). K-means method to $T_{pp}$ was used to separate excitatory and inhibitory neurons.