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Synaptic enhancement induced dynamical change of functional connections between neurons in living neuronal networks.

Suguru N. Kudoh[†‡], Takahisa Taguchi[†] and Shunsuke Yuba [†]

†Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST)
1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan
‡ Interactions and intelligence Area, PRESTO, Japan Science and Technology Agency (JST)
Kawaguchi Center Building, 4-1-8, Honcho, Kawaguchi, Saitama, 332-0012, Japan
Email: author1@domain1, author2@domain2

Abstract–The neurons in dissociated culture system formed living neuronal networks autonomously, elongating neurites and establishing synaptic connections. The neuronal networks were re-organized depending spontaneous electrical activities. The spatio-temporal patterns of activity in these networks might reflect functional neuron assemblies. The functional connections between neurons were modified dynamically by synaptic potentiation and the process may be required for organization of the functional group of neurons. Such neuron assemblies are critical for information processing in brain. In order to visualize the functional connections between neurons, we have analyzed the autonomous activity of synaptically transmitted action potentials in living neuronal networks on a multi-electrode array, using "connection map analysis" that we developed for this purpose.

1. Introduction

One of the features of the brain is that it is a nonlinear dynamical system [8]. To elucidate the mechanism of information processing in brain system, we have to characterize dynamic feature of living neuronal networks. There have been many recent advances in theoretical research and physiological research [6, 13], but there is still a gap between these studies. To bridge this gap, it will be important to characterize the dynamics of neuronal networks. A dissociated culture system developed on multi-electrode array is particularly useful for this purpose [11]. Dissociated neurons begin to elongate neurites on the multi-electrode array and reconstruct a complicated living neuronal network. Spontaneous ensemble electrical activities are observed in developed networks, which seem to be able to perform certain types of information processing [4, 5]. It has been reported that cell assemblies in the brain are critical for information processing, such as that which occurs during perception or learning process. These functional assemblies of neurons are expressed by dynamic spatio-temporal patterns of correlation between neurons. Several physiological reports suggesting that cell assemblies in several regions of brain are related to memory [10, 12]. Our interest is in elucidating the relation-ship between such functional neuron assemblies and synaptic potentiation. We showed in previous papers that long lasting synaptic potentiation in the amplitudes of spontaneous synaptic currents (SSCs) is induced by a Mg^{2+}-free condition in dissociated neuronal networks [2-4]. Our results are consistent with the hypothesis that synaptic potentiation modifies the flow of information in part by reorganizing cell assemblies in living neuronal networks. To test this hypothesis, we have analyzed the autonomous electrical activity in living neuronal networks on a multi-electrode array, using "connection map analysis" that we developed for this purpose.

2. Methods

2.1. Primary culture of rat hippocampal neurons

The conduct of all experimental procedures was governed by the Guidelines for the Care and Use of Laboratory Animals of the AIST Kansai Center. The hippocampal neurons were prepared from Wistar rats on embryonic day 17 (E17) or E18 and cultured by a previously described method [2-4]. Briefly, the hippocampuses of rat embryos were dissociated by treatment with 0.175% trypsin (Invitrogen-Gibco, U.S.A.) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) supplemented with 10 mM glucose at 37^{o}C for 10 min. They were then plated on a poly ethylene-imine- (or poly-D-lysine) -coated MED probe (Alpha MED Science, Japan), which has 64 planar microelectrodes on dish. Half of the culture medium was renewed every two days. The medium consisted of 45% Ham's F12, 45% Dulbecco's modified minimum essential medium (Invitrogen-Gibco, U.S.A.), 5% horse serum (Invitrogen-Gibco, U.S.A.), and 5% fetal calf serum (Invitrogen-Gibco, U.S.A.), supplemented with 100 U/ml penicillin, 100μg/ml streptomycin (Invitrogen-Gibco, U.S.A.), and 5μg/ml insulin (Sigma-Aldrich, U.S.A.). The dissociated neurons were cultured for 12-60 days at 37^{o}C in 5% CO_2/95% air at saturating humidity.

2.2. Spike recording by the multi-electrode array

Spontaneous extracellular action potentials were recorded in the standard external bathing solution 10-60

days after the start of the culture. The standard external bathing solution contained (in mM) 120 NaCl, 3 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Na-Hepes (pH 7.3). The Mg^{2+}-free external bathing solution contained the same compounds except for the absence of $MgCl_2$. The osmolarity of each solution was adjusted to 300 mOsm with sucrose. The data were gathered with the MED64 system (Alpha MED Science, Japan) [7] at a sampling rate of 10 or 20 kHz. All experiments were carried out at room temperature (20-25oC). The recorded spikes were automatically analyzed using a program MEDFAUST developed by us. Spontaneous events were detected when the amplitudes of spikes exceeded a pre-specified threshold. Detected spikes were classified by their amplitude -versus- decay-time distributions using modified k-means cluster analysis and classified to each neuron units. All these process performed automatically by MEDFAUST.

2.3. Connection Map Analysis

All possible combinations of pairs of spike trains recorded by the multi-electrode array were subjected to cross-correlation analysis. Then connectivity indices were calculated for each pair. The connectivity index is defined as follows:

$$Connectivity = A_{peak} \times \left(w_1 \times CI + \frac{w_2}{\Delta t + 1} + \frac{w_3}{Kurtosis} \right)$$

$$w_1 = \frac{1}{100}, w_2 = 1, w_3 = 1 \qquad (1)$$

Where Apeak is an area within the 2msec range around the peak of cross-correlofunction of the pair, Atotal is the total area of cross-correlofunction, delta-t is the distance of the peak of the cross-correlofunction from 0. The connectivity index indicates the degree of robustness of the relationship between the pair. The mean and standard error of all connectivity indices were calculated. When the value of the connectivity index exceeded a criterion, the mean plus standard error, the pair was assumed to be functionally connected. Each neuron is denoted as a small point in maps, and lines between the points represent the connectivity between the neurons indicated by these points. The color of the lines indicates the relative value of the connectivity index. Functional connections between all combinations of recorded spike trains are depicted simultaneously in this 2-D map. In this study, we generated connection maps from data recorded for 3min to 10min, the bin width for calculation of the cross-correlation was 5msec, and the range of the calculation of cross-correlofunction was 50 msec. Connection maps of serial experiments were expressed as follows. First, connection maps were generated for sequential recordings. Then each point indicating a neural unit in the second recording was shifted to the lower right. The parameters of classified APs, such as mean amplitudes, were compared with the parameters of the initial reference recording, and each point was identified as being the same as an individual unit seen in the initial recording. These units were then depicted at the same position as in connection map of the initial recording, and other units (including newly detected units) were left behind at the shifted positions. Units with more than 20% fluctuation of the parameters in sequential records were identified as distinct neurons.

3. Results and Discussions

3.1. Spike activities in living neuronal networks on a multi-electrode array

The MED probe is a multi-electrode array with 64 planar microelectrodes that can be used to obtain multi-channel field action potentials in dissociated culture systems. Fig. 1 shows an example of a two-dimensional cultured neural network of rat hippocampal cells. Robust neurites formed complex networks from about E18 DIV7, and spontaneous action potentials (SAPs) began to be observed frequently from about the same period. Fig. 2 shows its spontaneous electrical activities. Each frame represents recorded signals from one electrode and the position of the frame corresponds to the relative position of the electrode. SAPs of presynaptic neurons evoked synaptic currents in postsynaptic neurons, and spontaneous synaptic currents (SSCs) often evoked action potentials. Synaptic transmission is the origin of the SAPs and SSCs observed by whole-cell patch clamp recordings performed in dissociated cultures (data not shown).

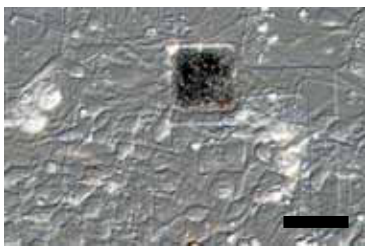

Fig. 1 An example of a cultured living neuronal network (E18D9). The black bar indicates 50μm.

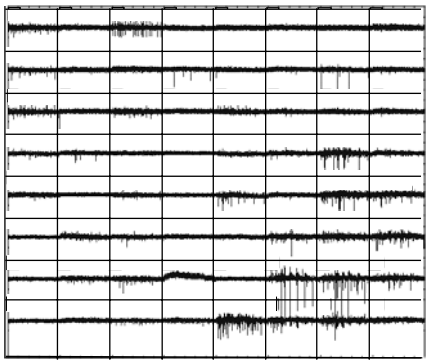

Fig. 2 Spontaneous spike activities recorded at E18D9. Vertical bar represents 250μV and horizontal bar represents 1 sec.

3.2. Hub-like structure of connection map

We used a "connection map analysis" to analyze recorded spike trains of spontaneous action potentials. Fig. 3 shows one example of a connection map of a living neuronal network (E17DIV19). Each point in the map indicates an identified neuron and lines represent the functional connections between the neurons indicated by the points. The number of inputs and outputs of each neuron varied even in same cultured network.

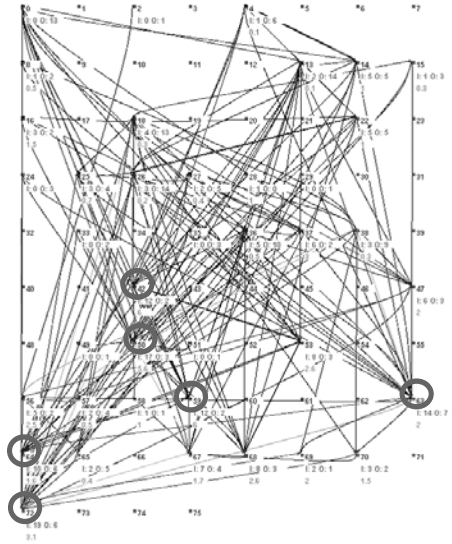

Fig. 3 An example of a connection map of a living neuronal network. Each point in the map represents an identified neuron and the lines represent functional connections between these neurons. Gray bold circles indicate the “hub”-like structure of the living neuronal network; each hub has >10 inputs from other neurons.

The distribution of connections of each neuron followed a power law distribution rather than Gaussian distribution (Fig.4). Several neurons had many inputs and outputs and others had only one or 2 links to another neuron. The hub-like neurons with many connections were observed in cultured network. These results suggest that the living neuronal network is not a random network but a scale-free network [1, 9]. The connection map contains all dynamic links over the entire recording time, and neurons included in several different neuron assemblies are expressed in the maps as nodes with many links. Thus, hub-like nodes in the connection map correspond to neurons associated with several overlapping assemblies of neurons. These features of a scale-free network may result in self-organization without any external inputs. In this study, the living neuronal networks received no external inputs during the incubation period but nevertheless organized into clusters with distinct spatio-temporal characteristics. This implies that spontaneous network activity is sufficient to organize such functional assemblies of neurons even in a dissociated culture system. Self-assembled structures may thus be formed by functional connections between neurons, and it is likely that modification of synaptic strengths would evoke the dynamic re-organization of these connections in the living neuronal network. To test this hypothesis, we transiently exposed living neuronal networks on multi-electrode arrays to a Mg^{2+}-free condition, which induces synaptic potentiation in dissociated neurons.

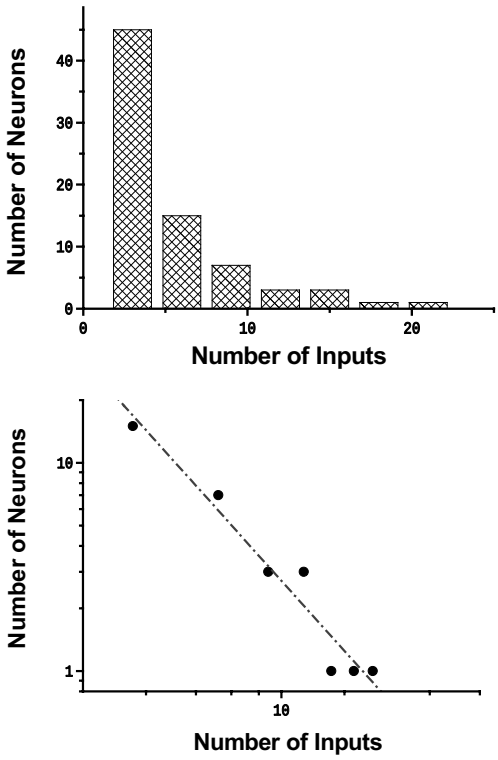

Fig.4 Upper Panel; An example of the distribution of input connection in neurons in a cultured living neuronal network. Lower panel; Log plot of histogram shown in A. Fitted line and 95% confidence interval (dotted lines) are also indicated.

3.3. Modification of neuron assemblies by the Mg^{2+}-free condition

We noticed that SAPs became more synchronized after the transient Mg^{2+}-free condition. We performed a connectivity map analysis of all combinations of spike trains recorded from a multi-electrode array before and after the induction of synaptic potentiation by transient Mg^{2+}-free condition. An example of sequential connectivity maps of a living neuronal network is shown in Fig. 5. The connection maps suggest that the functional connectivity between neurons changed drastically after the induction of synaptic potentiation in living neuronal networks cultured on a multi-electrode array. Several links between neurons were conserved after the induction of

synaptic potentiation, but additional connections emerged. Interestingly, there were newly emerged hub-like neurons (Fig. 5 arrow). This result suggests that additional neuron assemblies were formed or that existing neuron assemblies formed new association with groups of coupled neuron assemblies. This modification of functional structure may be the basic cellular mechanism of associative learning or the formation of perceptions. Our main conclusion is that spontaneous activity is enough to construct dynamic functional assemblies of neurons through self-organization even in small-scale living neuronal networks. Furthermore, synaptic potentiation can induce the re-organization of such assemblies of neurons.

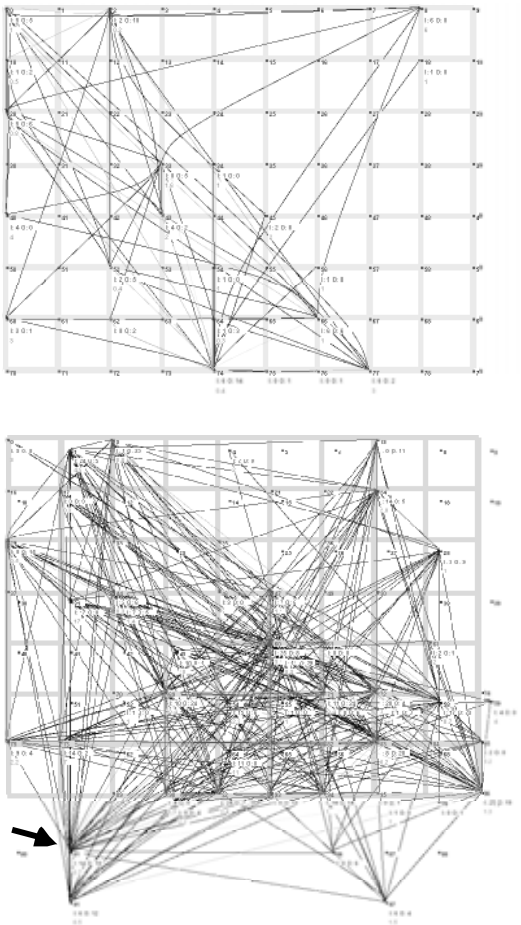

Fig.5 Effect of synaptic potentiation on a connection map. Connection map before (upper) and after (lower) induction of synaptic potentiation. Neuron units located on crossover points of the gray grids are have been identified as the same individual neuron units in initial and subsequent recordings; others are newly detected units. These maps were obtained from the same cultured neuronal network (E17D19).

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