Analysis for the mechanism of autonomous network reorganization in dissociated culture

Ai Kiyohara¹,², Takahisa Taguchi¹,² and Suguru N. Kudoh¹
¹. Cell Engineering Research Institute (RICE), National Institute of Advanced Industrial Science and Technology (AIST)
1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan
². Graduate School of Science, Osaka University
Machikaneyama, Toyonaka, Osaka 560-0043, Japan
email: ai-kiyohara@aist.go.jp

Abstract—The dissociated culture is a suitable system to observe behaviors of functional molecules and an intercellular interaction in detail. The cultured neuronal network was confirmed to have a mechanism to modify own functional structure automatically. We found characteristic transient high frequency burst activity (HFB) during culture days. The neuronal network generated such particular spontaneous activity, suggesting that the HFB autonomously constructed a heterogeneous network structure suitable for neurotransmission. After the HFB, some neurons became to have many inputs while others became to have little. We confirmed that the HFB terminated within 10 days, without a drastic change of the number of neurons. It suggests that the termination of the HFB activity was not caused by the decrease of the neurons, but was performed by the modification of the intercellular interaction.

I. INTRODUCTION

To understand the function of brain, we have to examine the interaction between neurons and its dynamics for adaptation to ambient environment. For this purpose, using dissociated neurons, we prepared a reconstructed neuronal network which keeps basic characters of neurons in a brain. With the system, we can observe and operate the network dynamics of neurons. To discuss the dynamics in dissociated neurons, we have to consider of the time scale of reconstruction of a neuronal network, stability of the network and constancy of electrical activity. Recent study reported that spontaneous action potentials became to more and more complex depending on culture days, and the activity pattern increased variability [1]. In the study using more number of dissociated neural networks, it was reported that dissociated cultures of the rat cerebral cortex increased the frequency of bursts after 3 weeks from cultured day, thereafter the synchronization of the spontaneous action potentials between neurons increased [2]. These results indicated that the frequency of spontaneous action potential increased depending on culture days even after the drastic change of the pattern of neuronal activity. It is possible that the process reconstructs the neuronal network connectivity between neurons.

II. METHOD

A. Primary culture of rat hippocampal neurons

We isolated a rat hippocampus and cultured hippocampal neurons on the multi-electrode-array dish. The hippocampal region was cut off from Wistar rats on embryonic day 18, and neurons were dissociated by 0.175% trypsin (Invitrogen-Gibco, Carlsbad, California, U.S.A.) in Ca²⁺- and Mg²⁺- free phosphate-buffered saline (PBS-minus) supplemented with 10 mM glucose at 37 °C for 10 min. The cells were suspended in Dulbecco’s modified Eagle’s medium (Invitrogen-Gibco, Carlsbad, California, U.S.A.) containing 5 % fetal calf serum (Invitrogen-Gibco, Carlsbad, California, U.S.A.), 5 % horse serum (Invitrogen-Gibco, Carlsbad, California, U.S.A.), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen-Gibco, Carlsbad, California, U.S.A.), and 5µg/ml insulin (Sigma-Aldrich, US). The MED prove was coated with 0.02 % poly-ethylene-imine (Sigma-Aldrich, US). Neurons were plated on a MED probe, having 64 planar microelectrodes in the center of the culture dish at 37 °C in 5 % CO₂ / 95 % air at saturating humidity. Half of the culture medium renewed every 2 days.

B. Developmental changes of neuronal activity

Neurons grew neuritis and constituted a network by the self-organization without innate connections at all. Spontaneous extracellular action potentials were recorded for 10 minutes in normal culture medium at 10-120 days in vitro (DIV). Extracellular potentials were collected through 64 electrodes simultaneously with the integrated MED64 system (Alpha MED Science, Japan) [3] at a sampling rate of 10 kHz. We observed changes of spontaneous action potentials during culture days. The recoded action potentials were analyzed using our developed program, MEDFAUST.
C. Immunohistochemistry

Neurons were fixed with 4 % paraformaldehyde (Wako, Japan) for 20 min at room temperature and permeabilizing them for 20 min in 0.3 % Triton-X100 (Sigma-Aldrich, US). Then they were exposed to primary antibodies in 1% BSA in PBS for 60min. Primary antibodies were mouse anti-neuronal nuclei (NeuN) monoclonal antibodies from mouse brain (2 µg / ml, MILLIPORE) and rabbit anti-S-100 protein monoclonal antibodies from bovine brains (17.2 µg / ml, Sigma-Aldrich, US). Primary antibodies were visualized with fluorochrome-conjugated second antibodies (10 µg / ml, Invitrogen-Gibco, Carlsbad, California, U.S.A.) in 1% BSA in PBS for 60min. Second antibodies are Alexa Fluor 568 goat anti-rabbit IgG for S-100 and Alexa Fluor 488 goat anti-mouse IgG for NeuN.

D. Inhibition of N-methyl-D-aspartireceptors (NMDA-Rs)

Amino-phosphonopentanoic acid (APV) (25 µM, SIGMA-Aldrich, US) which was a specific inhibitor of NMDA-Rs was dropped into the culture. Just after, spontaneous action potentials were recorded during 10 minutes by each culture.

III. RESULTS

We analyzed developmental changes of the pattern of spontaneous action potentials in cultured neuronal networks. The spontaneous action potential increased day by day. Spontaneous action potentials were observed from DIV 20, and a particular transient period with HFB lasting for about a week was observed from about 20-50 days.

HFB activity was observed at over a wide area of neuronal networks. After this high frequency of the spontaneous action potentials terminated, a steady state of an intermittent pattern was observed at about DIV 44.

After the termination of a high frequency burst period, the spatial patterns of the spontaneous activity also changed drastically. After the HFB, the remarkable change of a distribution of frequency of spontaneous activity over the electrodes was observed. There were a lot of low-frequency electrodes and a little of high frequency electrodes in a neuronal network (Fig.1). It is interesting that such a transient burst existed, and a different temporal pattern of spontaneous action potentials was observed after such a characteristic activity.

Fig.1 Appearance of the high frequency burst period. Initial cell density is 7800 cells / mm² A: Patterns of spontaneous action potential changed during culture days. B: Number of events in a 10 minutes recording on the each electrode. Vertical axis represents number of events from each electrode. Horizontal axis represents an electrode number.
We examined the number of neurons and the quantitative change by immunohistochemistry. Neurons decreased in DIV 7-14, and afterwards it stabilized without remarkable change (Fig.2 A). Neurons didn’t decrease during the high frequency burst period. On the other hand, glial cells increased gradually, ever since it kept increasing after HFB.

In addition, we also found that cultures resisting to the inhibition of NMDA-Rs appears after the high frequency burst period (fig.3). The result suggested that NMDA-Rs contributed to modifications of dynamics of neuronal network in early stages, but not after the high frequency burst period.

Fig.2 Changes of number of neurons during culture days. Vertical axis represents number of cells (cells / mm²) and horizontal axis represents culture age (day). Error bars indicates SE (N=5). A: Developmental change of number of neurons. B: Developmental change of number of glial cells.

Fig.3 Changes of the pattern of the action potential under the inhibition of the NMDA-Rs.
In previous report [4], composition pattern of NMDAR subunits changed to mature type during development. Accompanying to that, NMDA-mediated excitatory post synaptic currents became more susceptible to Mg\textsuperscript{2+} block, suggesting that NMDAR components of synaptic transmission decreased at resting membrane potential. In other words, dominant of synaptic transmission became to moreover non-NMDAR component, which is consistent with our results. In our result, the interesting point was that change of response against APV occurred after this maturation of NMDARs.

Even though expression of mature type of NMDAR subunits increased and increase of NMDARs under functional synaptic sites, contributions of NMDAR to normal synaptic transmissions decreased after the HFB. The result suggested that the change of network response against APV was caused by changes of functional connectivity between neurons.

We have following hypotheses: Before HFB, most of the synaptic connections was weak and temporal summations of over two inputs were required to evoke an action potential. In other words, there were a lot of “AND” elements. Then synaptic potentiation occurred during HFB, then after the HFB, strong synaptic connections increased and many neurons became to “OR” elements, suggesting mean temporal summation of inputs were no more been required for eliciting spontaneous action potentials. And hub-like neurons with many strong inputs increased after HFB, as a result of that, APV resistant networks increased.

IV. CONCLUSION

We found a characteristic transient high-frequency burst activity and that the activity plays an important role in the autonomous modification of the neuronal connections in the network. A heterogeneous distribution of connections in the network after the high frequency burst period seems to be suitable for information processing. Simultaneously, the number of neurons was stable during and after HFB. Consequently, the change after HFB was not controlled by the change of number of neurons, by interaction between neurons. Appearance of APV resistant network suggested that NMDA-Rs dependent network structure was changed after the high frequency burst period.

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