Archives Italiennes de Biologie, 145: 289-297, 2007.

MEA-BASED RECORDING OF NEURONAL ACTIVITY *IN VITRO*

Y. JIMBO

Graduate School of Frontier Sciences, University of Tokyo, 5-1-5, Kashiwanoha, Kashiwa-si, Chiba, 277-8561, Japan.

INTRODUCTION

In biological systems, information is stored and processed simultaneously in the same neuronal networks. The networks consist of a large number of neurons and their mutual connections. The weights of distributed synapses are adjusted in an activity-dependent manner during development as well as in matured neuronal networks. The activity-dependent synaptic plasticity has been extensively studied particularly in hippocampus, since the proposal of long-term potentiation (LTP) and depression (LTD) (1, 2, 6). Recently, spike timing dependent plasticity (STDP) has been reported and considered to be a strong candidate governing modification of the synaptic strength (10, 18). STDP suggested that the relative timings between neuronal firing and activation of synaptic inputs play a key role in synaptic modification. Probably, these synaptic changes are integrated and reflected in activity of neuronal ensembles. Little is known, however, about how this integration process is regulated and expressed in the network-wide activity.

To see activity of neuronal ensembles, one of the promising methods is microelectrode array (MEA) based recording. Since the first proposal of MEA by Gross (3) and Pine (14), this method was applied to various tissues such as dissociated cultures of spinal cord (4), retinal explants (11), hippocampal slices (17). The advantages of MEA are noninvasiveness and the capability of multi-site stimulation. The former allowed us of long-term monitoring of spontaneous activity during development (12, 16). The latter gave us a tool to study various aspects of evoked-activity characteristics including plasticity (7, 15). Noninvasiveness implies compatibility with other measurements, such as micropipette-recording and optical methods. Combination of these methods will be a useful tool to study neuronal network physiology.

In this paper, based on the advantages described above, we report two aspects of MEA-based recording. First, developmental changes of spontaneous activity in cortical neurons are shown. Rat cortical neurons are cultured on MEAs and the spontaneous activity is continuously monitored for two months. Because of the noninvasiveness of this method, we can keep the cell cultures for a long time under activity-recording conditions. The second one is application of multi-site stimulation to visualize tetanus-induced modification of evoked activity. A focal tetanic stimulation is

Corrisponding Author: dr. Y. Jimbo - E-mail: jimbo@miki.pe.u-tokyo.ac.jp

applied to cultured cortical neurons and how this tetanus affects 64 kinds of evoked activity is studied.

METHODS

Cell culture

Cortical tissue was obtained from E18 Wistar rat embryos and dissociated by trituration after digestion with 0.02% papain (Boehringer). Dissociated cells were plated on substrates coated with poly-D-lysine and laminin (Sigma) and were cultured in Dulbecco's Modified Eagle's Medium(DMEM) (Gibco) containing 5% FBS (Hy-clone), 5% heat-inactivated horse serum (Gibco), 2.5μg/ml insulin (Sigma), and 5-40U/ml penicillin-streptomycin (Sigma). The cultures were maintained in an incubator of 37 °C, 5% CO_2 and water-saturated atmosphere. Half of the culture medium was exchanged by the conditioned medium, twice a week for normal cultures. In the case of continuous monitoring of spontaneous activity, the medium was continuously perfused at a slow rate (0.1 ml/h).

Electrode-array: fabrication and recording

The MEA is a cell-culture dish with embedded micro-electrodes. We fabricated glass substrates with 64 recording and stimulation sites made of indium tin oxide (ITO), which was an electrically conductive and optically transparent material (5). The electrodes were located in an 8 x 8 square grid. The size of each terminal was 30μm x 30μm and the distance between them was 180μm (from center to center). The electrode patterns were fabricated using photo-lithography. After the patterning, a thin insulation layer was formed by a silicon-based photo-resist except the electrode terminals. The surface of the electrode terminals was further electrochemically coated with a thin layer of platinum black to reduce interface impedance, which was around 100 kΩ at 1 kHz.

The voltage between each surface electrode and a large platinum reference electrode was amplified, filtered (100 Hz - 10 kHz), and A/D converted. The extracellularly recorded neuronal spikes contain up to 6 kHz frequency compoments (13). Thus the 64-channel signals were sampled at 25 kHz with 16 bits, and stored on a hard disk. The stored data were analyzed using PV-WAVE (Visual Numerics Inc.). First, spikes were extracted based on a simple threshold method. The threshold level was set at 5 times of the standard deviation of the baseline. Then the extracted spikes were plotted on a two-dimensional plane. The amplitude and width of the spikes were selected as the two parameters. Under the hypothesis that spikes derived from a particular neuron show a constant waveform at a particular recording site, individual clusters on the two-dimensional plane correspond to individual single neurons. Normally, for matured cortical neurons, a few tens of neurons could be identified from the 64-channel recording data. The cultured cortical neurons on a MEA, 64 traces of recording signals, spike sorting carried out on a two-dimensional plane, and a raster plot of the extracted neurons, were illustrated in Figure 1.

Multi-site stimulation

Capability of multi-site stimulation is the major advantage of MEAs. In principle, we can select any combination of the substrate-embedded electrodes. Practically, however, we had to solve some technical problems. Typical amplitude of extracellularly detected neuronal spikes is around 100 μV, while that of the stimulation signals is up to 1 V. This large discrepancy causes huge stimulus-related artifacts, particularly at the stimulation sites. Because the evoked responses are initiated near the stimulation sites, we cannot see initial parts of the responses without avoiding this stimulus-related artifact problem. The simplest way is to switch off the inputs of the recording amplifiers during stimulus application. We combined a TTL-driven switch with each of the 64 pre-amplifiers. Then the next problem was that at what level the input of the amplifier should be clamped during stimulation. For long-term biocompatibility, inactive materials must be used for both recording and reference electrodes. The standard inactive materials,

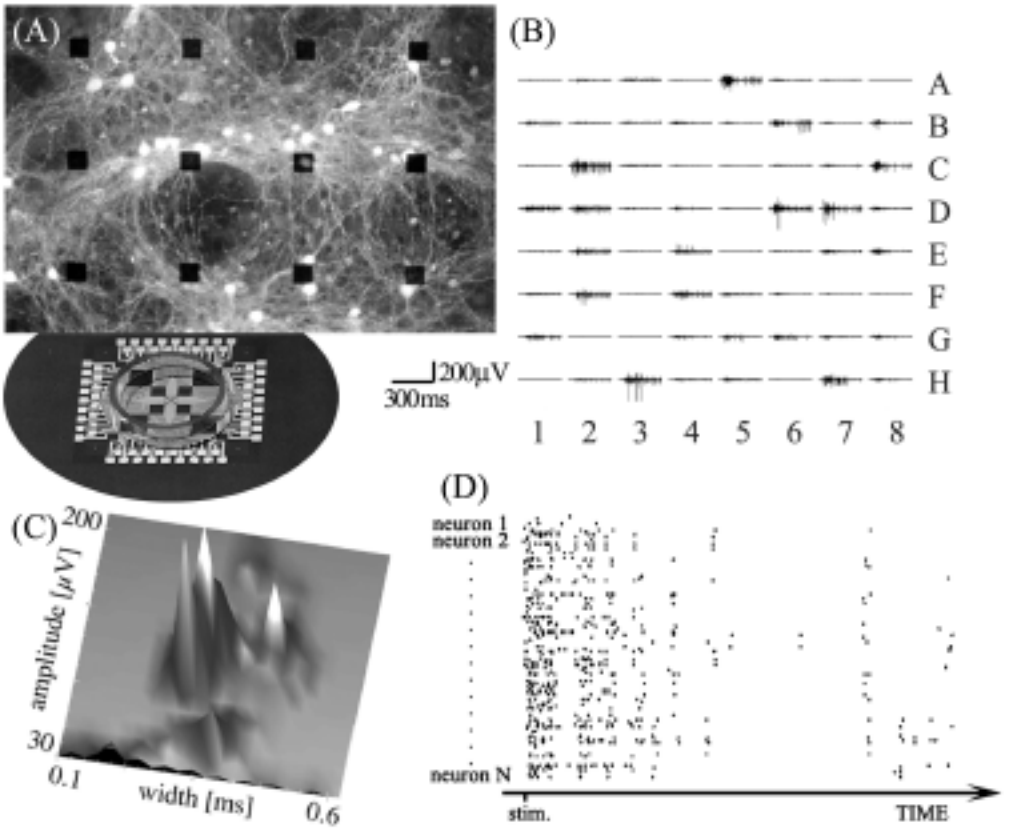

Fig. 1. - *MEA-based recording of neuronal activity.*
(A) Cortical neurons cultured on a MEA. (B) Neuronal spikes recorded at the 64 sites. (C) Spike sorting based on an amplitude-width plane. (D) Raster plots of evoked activity for N extracted neurons.

such as Pt and Au, are highly polarized in electrolyte solutions. DC offsets at the interface and their drifts are inevitable. Thus we have to track the offsets for each of the 64 electrodes and clamp the inputs of the amplifiers at this tracked level during stimulation. Otherwise, the switching itself causes another transient. The final step was to minimize electrical charges injected into the electrode/electrolyte interface by the stimulus itself. Biphasic pulses were used for stimulation and the residual charges (because of nonlinear properties of the interface, complete canceling of charge injection was impossible) were discharged through the low-impedance pathway for stimulation. The total interface circuit that we have developed is shown in Figure 2 (A). Three solid-state switches were integrated with a pre-amplifier and all the timings including stimulus-signal application and switching were controlled by a PC. Using these 64 integrated modules, we could achieve recording neuronal spikes within a few milliseconds after the stimulus application at the stimulation site (Fig. 2 (B)). The spatially propagating activities elicited from three different locations were visualized (Fig. 2 (C)).

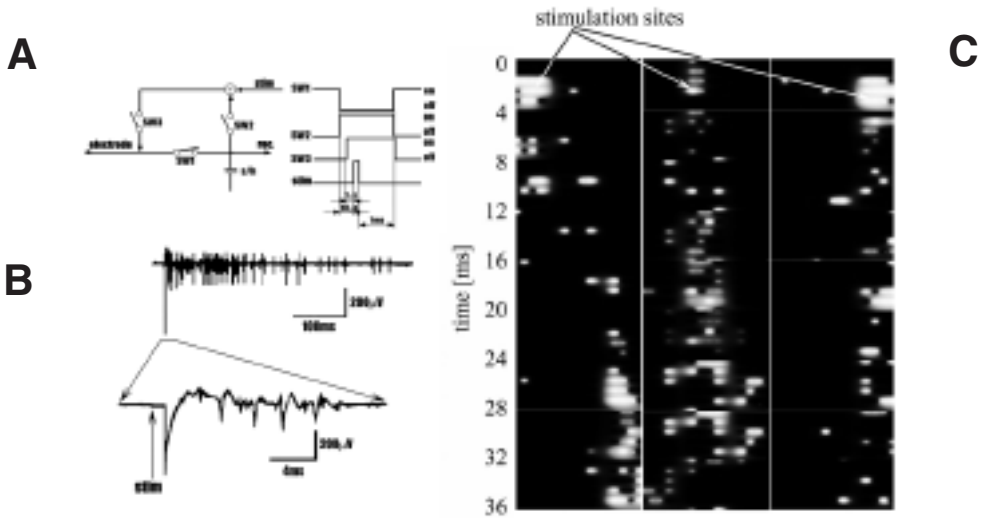

Fig. 2. - *MEA-based multi-site stimulation*.
(A) The interface circuit to reduce stimulus-related artifacts. (B) Evoked activity recorded at the stimulation site. (C) Spatial propagation patterns evoked by electrical stimuli from three different sites.

RESULTS

Developmental changes in spontaneous activity

The recording setup used for continuous recording is illustrated in Figure 3 (A). Conditioned medium was continuously perfused at a slow rate (0.1 ml/h.) to keep constant culture conditions. Spontaneous activity started in a few days after plating. During the first week, only a few recording sites were active. The signal to noise ratio was quite low. The amplitude of the observed spikes was a few tens of microvolts while standard electrode noises were between ten and twenty microvolts (peak to peak). We could see neither synchronous activity nor spatially propagating activity in the first few days (Fig. 3 (B)). Then loosely coupled activity appeared. The duration of individual activity was long, sometimes more than a second. Two or three bursting activities were generated every one minute. We could clearly see the time-delays between the recording sites.

In the second week, the spontaneous activity was gradually synchronized. We could see spatially propagating activity. The time-delay between the recording sites was shortened and the networks showed synchronized bursts, which was typical in cultured cortical neurons (Fig. 3 (C)). These synchronized bursts were weakened in the end of the second week, and restarted after that. In the third week, the restarted activity was monotonously strengthened. Higher frequency, and tightly coupled bursts were observed in a wide area. After that, asynchronous components were mixed with the synchronized bursts.

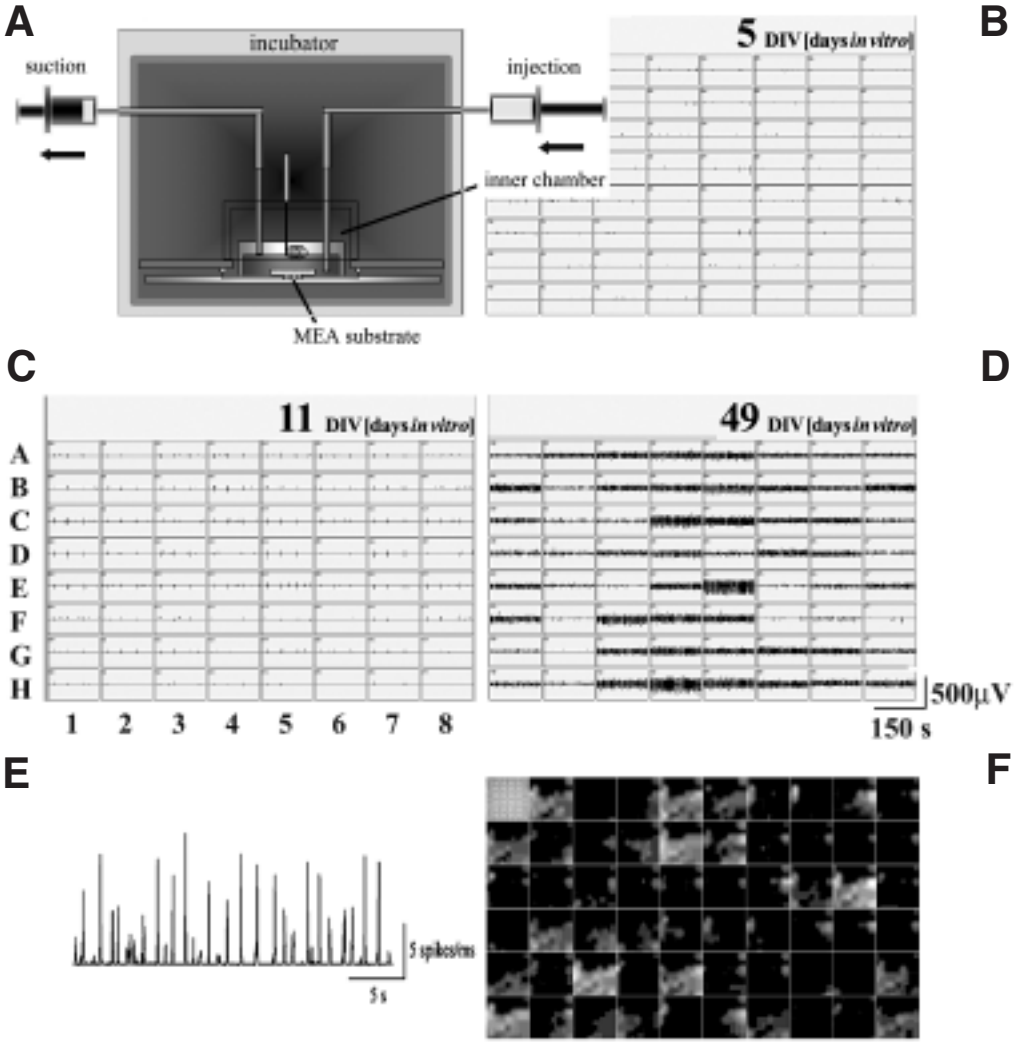

Fig. 3 - *Developmental changes of spontaneous activity in cultured cortical neurons*.
(A) Recording setup for long-term continuous monitoring. The MEA substrate was kept in an inner chamber inside the standard CO_2 incubator. Conditioned medium was continuously perfused at a slow rate using a syringe pump. (B) Spontaneous activity at 5 DIV. Small signals are observed at a few recording sites. (C) Typical synchronized bursts observed during the second week. (D) Complex activity observed in mature cortical networks. (E) Spike density versus time for mature networks. Total numbers of spikes detected at all the 64 sites were calculated for each millisecond. The activity consists of concentrated bursts and quiescent periods. The bursting frequency is around 1 Hz. (F) Spatial patterns of spontaneous activity in mature networks. The numbers of detected spikes were calculated for each recording site and displayed as brightness.

The activity reached an almost stable state in about one month (Fig. 3 (D)). The spontaneous activity at this state consists of synchronized bursts and asynchronous components. Synchronized bursts were generated almost every second, but the ini-

tiation sites were not unique but a couple of propagation patterns could be observed (Fig. 3 (E, F)). At this matured state, signal to noise ratio was much higher than that in the initial state. Spike signals with amplitude of more than 400 μV were often observed. The recording was carried out until two months *in vitro*, and we confirmed no observation of significant changes after one month. For this mature state of cultures, we could easily elicit reproducible evoked activities by electrical stimulation.

Tetanus-induced modification of evoked activity

Electrical stimulation could elicit activity in immature cultures, but reproducibility of the evoked responses was not good. To get responses, rather high intensity and long inter-stimulus interval were required. For mature cultures of more than one month *in vitro*, evoked responses could easily be elicited by weaker stimulation at shorter intervals. The reproducibility of the responses was quite good. Thus in this experiment, mature cortical cultures were used.

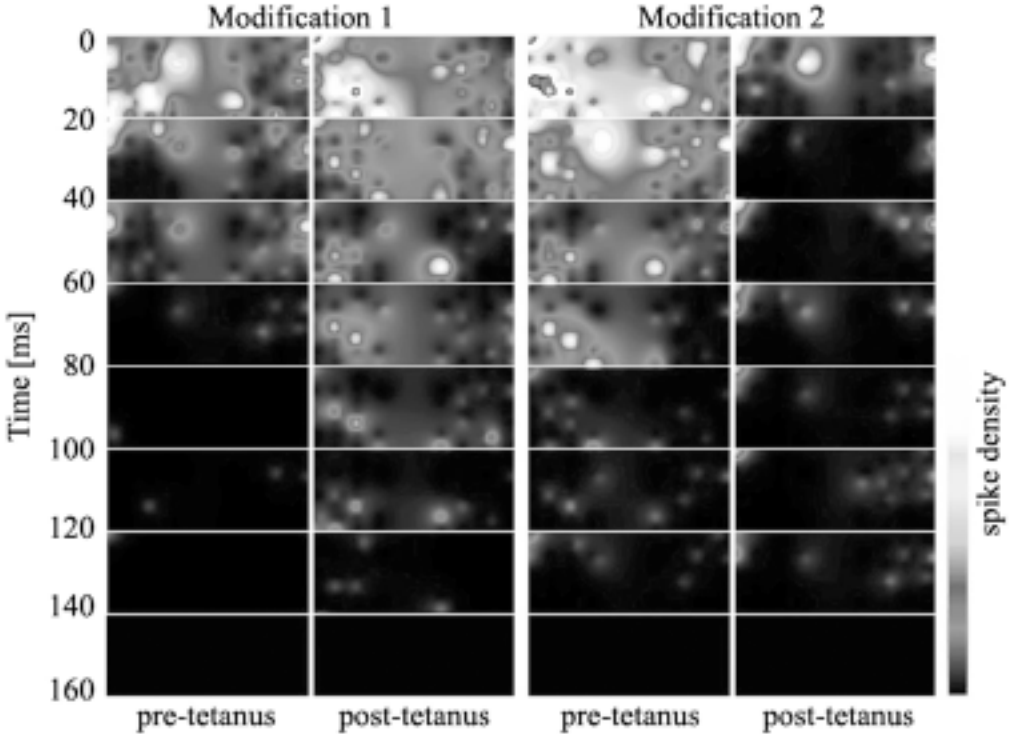

Fig. 4. - *Tetanus-induced modifications of evoked activity displayed as spatial propagation patterns*. Pre-tetanus and post-tetanus patterns are illustrated for potentiated and depressed responses. In the case of potentiation (modification 1), more intense and long-lasting activity was observed after tetanus. In the case of depression (modification 2), strong evoked activity at the initial state almost disappeared after tetanus.

First, test stimuli were applied from a single site of the 64 substrate electrodes. The stimulation site was sequentially switched for all the 64 sites and corresponding 64 evoked responses were recorded. This process was repeated ten times to confirm reproducibility. Then, one of the 64 sites was selected for tetanus application. Tetanic stimulation consisted of 20 trains of 10 pulses of the same intensity and duration as the test stimulus at 20 Hz. After that, exactly the same test stimulus procedures were applied again and another 64 sets of evoked responses were recorded. Spike-sorting procedures described before were applied to analyze the recorded activity. The responses of all the extracted neurons were displayed as raster plots and the activities between pre- and post-tetanus were compared.

The tetanus-induced changes were summarized as the following three aspects: (1) Some of the 64 evoked responses were strengthened, some of them were weakened, and the rests were unchanged. (2) Responses of individual single cells were neither strengthened nor weakened. A particular cell generated more spikes to the stimulus from some sites after tetanus, while less spikes to some other sites. (3) For some particular stimulation sites, most of the cells showed common changes, either increased or decreased activity. Figure 4 shows spatial propagation patterns of potentiated and depressed pathways. The initial and final states were illustrated.

DISCUSSION

Critical period of spontaneous activity transition

Spontaneous activity in cultured cortical neurons started a few days after plating. During the first two weeks, frequency of the activity as well as its synchronicity increased. These changes are probably due to the increase in density of synapses and electrically active molecules, such as ion channels and receptors. The neurons are more tightly connected and the activity initiated at a certain site often propagates to a wide area of the networks. The periodical synchronized burst, which is a characteristic property of cultured cortical networks, was clearly observed in 11 DIV cultures. Finally, in about one month, the networks *in vitro* reached a steady state, where burst-like activity was generated at 1 Hz with some asynchronous components. At present, there is no common explanation if this spontaneous activity is physiologically normal or not. What we can conclude is that mature dissociated cortical networks have a potential to generate tightly synchronized activity in a wide area.

These developmental changes in spontaneous activity were not monotonous. The cultures at about two weeks *in vitro* showed a quite low-level of activity. This suggests that expression and maturation of electrically active components is a complex process. It is well known that inhibitory synapses are formed in a later period than that of excitatory one. In an early developmental stage, excess expression of NMDA receptors has been reported. Two weeks might be a critical period of spontaneous activity transition. More data of this particular period should be collected and ana-

lyzed. Histochemical methods as well as measurements including second messenger system should be applied.

Plasticity in neuronal networks

Focal tetanic stimulation modified properties of evoked responses. Both potentiation and depression were induced in the same single neuronal network. What was the key factor that governed this bi-directional change? It was reported that evoked responses in cortical networks *in vitro* consisted of two components, early and late ones. Detailed analysis of raster plots suggested that potentiation was dominant in early components, while depression was more significant in late components. Generally speaking, early components show better reproducibility than late components. Then correlation analysis between each of the 64 evoked activities and the tetanized activity was carried out. The results suggested that closely correlated components were more strengthened. The peripheral components in the correlation spectra were depressed. This tendency was common to most of the 64 pathways. Thus we can conclude that repeatedly activated pathways are strengthened and the induced changes are reflected in the activity of other pathways. Directions of the changes are determined by how closely the pathway was correlated with the tetanized pathway. The total modification is not cell specific but pathway specific. Then, the next problems are that how long these induced changes last and that how additional strong activity interacts with the induced changes. It might be achieved that repetitive application of spatio-temporally correlated stimulus affects network properties in an association-like manner.

SUMMARY

Based on the advantages of MEA-based recording, developmental changes of spontaneous activity and tetanus-induced modification of evoked activity were studied. Rat cortical neurons were cultured on MEAs and the spontaneous activity was continuously monitored for two months. The activity started a few days after plating. During the second week, the cultures generated periodic synchronized bursts, which were the characteristic properties of cortical neurons *in vitro*. In about one month, the cultured networks reached a steady state. Between these two, we found a critical period during which only weak activities were generated. This critical period might reflect the transition from immature networks to mature networks including precisely controlled excitatory and inhibitory synapses. We could elicit clear evoked responses with high reproducibility in mature cultures. A focal tetanic stimulation was applied to the mature cultures and how the tetanus affects 64 kinds of evoked activity was studied. The evoked responses showed bi-directional changes in their propagation patterns, potentiation and depression. These induced changes reflected the correlation properties with the tetanized activity pattern. The next step will be the combination of long-term recording and multi-site stimulation. How long does the induced change last, as well as how additional strong activity affects the previously induced changes, will be studied.

Acknowledgements. This work was funded by Grant-in-Aid Scientific Research (No. 16300150, 17650129) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. BEAR, M.F. AND MALENKA, R.C. Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol*., **4**: 389-399, 1994.
2. BLISS, T.V.P. AND LØMO, T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol*., *Lond.*, **232**: 331-356, 1973.
3. GROSS, G.W., RIESKE, E., KREUTZBERG, H.W. AND MEYER, A. A new fixed-array multi-microelectrode system designed for long-term monitoring of extracellular single unit neuronal activity in vitro. *Neurosci. Lett*., **7**: 101-105, 1977.
4. GROSS, G.W., WEN, W.Y. AND LIN, J.W. Multielectrode analysis of coordibated, multisite, rhythmic bursting in cultured CNS monolayer networks. *J. Neurosci*., **6**: 1583-1592, 1986.
5. GROSS, G.W., WEN, W.Y. AND LIN, J.W. Transparent indium-tin oxide electrode patterns for extracellular, multisite recording in neuronal cultures. *J. Neurosci. Meth*., **15**: 243-252, 1985.
6. ITO, M. AND KANO, M. Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. *Neurosci. Lett*., **33**: 253-258, 1982.
7. JIMBO, Y., TATENO, T. AND ROBINSON, H.P.C., Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons. *Biophys. J*., **76**: 670-678, 1999.
8. JIMBO, Y., KAWANA, A., PARODI, P. AND TORRE, V. The dynamics of a neuronal culture of dissociated cortical neurons of neonatal rats. *Biol. Cybern.*, **83**: 1-20, 2000.
9. JIMBO, Y., KASAI, N., TORIMITSU, K., TATENO, T. AND ROBINSON, H.P.C. A system for MEA-based multis-site stimulation. *IEEE Trans*., BME-50: 241-248, 2003.
10. MARKRAM, H., LUBKE, J., FROTSCHER, M. AND SAKMANN, B. Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. *Science*, **275**: 213-215, 1997.
11. MEISTER, M., WONG, R.O., BAYLOR, D.A. AND SHATZ, C.J. Synchronous bursts of action-potentials in ganglion-cells of the developing mammalian retina. *Science*, **252**: 939-943, 1991.
12. MUKAI, Y., SHIINA, T. AND JIMBO, Y. Continuous monitoring of developmental activity changes in cultured cortical networks. *Elect. Engng. Jp.*, **145**: 28-37, 2003.
13. NAJAFI, K. Solid-state microsensors for cortical nerve recordings. *IEEE EMBS Magazine*, **13**: 375-387, 1994.
14. PINE, J. Recording action potentials from cultured neurons with extracellular microcircuit electrodes. *J. Neurosci. Meth*., **2**: 19-31, 1980.
15. SHAHAF, G. AND MAROM, S. Learning in networks of cortical neurons. *J. Neurosci*., **21**: 8782-8788, 2001.
16. VAN PELT, J., CORNER, M.A., WOLTERS, P.S., RUTTEN, W.L.C. AND RAMAKERS, G.J.A. Longterm stability and developmental changes in spontaneous network burst firing patterns in dissociated rat cerebral cortex cell cultures on multielectrode arrays. *Neurosci. Lett*., **361**: 86-89, 2004.
17. WHEELER, B.C. AND NOVAK, J.L. Current source density-estimation using microelectrode array data from the hippocampal slice preparation. *IEEE Trans*. BME-**33**: 1204-1212, 1986.
18. YANG D., AND MU-MIN P. Spike Timing-Dependent Plasticity of neural circuits. *Neuron*, **44**: 23-30, 2004.