Abstract—We cultured rat cortical cells for 35 days in vitro to investigate the dynamics of neuronal networks. Multi-electrode arrays based recordings revealed that the network activity became saturated during 35 days cultivation. Immunofluorescence imaging revealed that the density of neurons decreased gradually and then showed constant value. In addition, both excitatory and inhibitory synaptic densities became saturated after initial increase throughout the long-term culture.

Keywords—Long-term culture, Synchronized bursts, Neuronal density, Excitatory and inhibitory synapses

1. Introduction

Cultured neurons in vitro have widely used to understand the fundamental mechanisms of the nervous system. Previous studies have investigated the neuronal dynamics at different stages of the network development. However, there have been few studies about the changes of the neuronal components with the electrical activity during long-term culture period.

Multi-electrode arrays (MEAs) are useful tool for long-term recording of neuronal network activity. Recently, we have developed the system based on MEAs to analyze the temporal dynamics of cultured neuronal networks. In addition, we have performed the immunofluorescence imaging of cultured neuronal networks on MEAs and analyzed spatial factors that affect neuronal dynamics [1]. This result indicates that immunofluorescence imaging of cultured neuronal networks in combination with MEAs-based recording of the network activity is effective to investigate the spatiotemporal dynamics of neuronal networks.

In the present study, the spontaneous electrical activity of cultured rat cortical networks was measured for 35 days in vitro (DIV). To clarify the developmental change of the network size, immunofluorescence staining of neuronal components (neurons, neurites, excitatory synapses and inhibitory synapses) was performed using antibodies. The changes of the network components were analyzed.

2. Materials & Methods

2.1. Cell culture

Cerebral cortices derived from Wistar rats on embryonic day 17 were dissociated and plated on a multi-electrode dish (MED) probe (Alpha MED Scientific, Osaka, Japan) or coverslip. The culture were filled with culture medium and incubated in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The culture medium consisted of Dulbecco’s modified minimum essential medium (DMEM) supplemented with 5% fetal bovine serum, 5% horse serum, 25 μg/mL insulin and 1% penicillin-streptomycin. A half of culture medium was replaced twice a week.

2.2. Spike and synchronized burst detection

The spontaneous electrical activity of each cortical culture was recorded using a MED64 extracellular recording system (Alpha MED Scientific) with a sampling rate of 20 kHz. The activity was recorded twice a week from 7 to 35 DIV. The spikes were determined when their amplitude exceeded a noise-based threshold in a window of 1 msec. Then the number of spikes (the total for all electrodes) was counted in the window. The number above the threshold of 100 spikes/window was defined as a synchronized burst.

2.3. Immunofluorescence staining

The cortical neurons cultured on coverslips were fixed with 4% formaldehyde at 7, 14, 21, 28 and 35 DIV. After permeabilization with 0.5% Triton X-100 in Phosphate-Buffered Saline (PBS) for 10 min, the cultures were incubated with PBS containing 10% goat serum and Triton X-100 for 30 min. The cultures were incubated with primary antibodies overnight at 4°C and then incubated with secondary antibodies for 1 h at room temperature. The primary antibodies were anti-microtubule associated protein 2 (MAP2) mouse IgG (a marker of neuronal somata and dendrites; Sigma-Aldrich, St. Louis, MO), anti-vesicular glutamate transporter 1 (VGluT1) rabbit IgG (a marker of excitatory synapses; Frontier Institute Co., Hokkaido, Japan) and anti-vesicular transporter of γ-amino-butyric acid (VGAT) guinea-pig IgG (a marker of inhibitory synapses; Frontier Institute Co.). The secondary antibodies were Alexa Fluor 405-labeled anti-mouse IgG (Molecular probes, Eugene, OR), 488-labeled anti-guinea-pig IgG, 546-labeled anti-rabbit IgG.

Fluorescence observation was performed using a confocal laser scanning microscope (FV1000D-IX81; Olympus, Tokyo, Japan). Image analysis was performed using Volocity software (PerkinElmer Inc., Waltham, MA). The density of neurons was determined by counting MAP2-stained somata. The densities of excitatory and inhibitory synapses were determined by counting VGluT-stained and VGAT-stained puncta, respectively.
3. Results & Discussion

3.1. Recording of electrical activity

Figure 1 shows the developmental changes of the array-wide firing rate and synchronized bursts rate of the cortical culture. Spontaneous electrical activity was recorded from 7 DIV and was found to increase from 10 to 28 DIV. This activity lasted for up to 35 DIV but varied in intensity from day to day. The synchronized burst rate resembled the firing rate in developmental change. These indicate that the network activity became saturated and exhibited synchronized bursts within 35 DIV.

![Fig. 1. Developmental changes of array-wide firing rate and synchronized burst rate in cortical culture. Firing rate data are shown as the mean+SEM, whereas synchronized burst rate data are shown as the mean-SEM.](image)

3.2. Immunofluorescence imaging

To investigate the developmental change of the survived neuronal density, we counted the MAP2-positive neuronal somata during long-term culture period. Fluorescence observation revealed that the density of survived neurons decreased gradually up to 35 DIV, and then showed constant value until 60 DIV.

To clarify the development and distribution of the excitatory and inhibitory synapse densities, double immunostaining of VGluT1 and VGAT was performed. The immunofluorescence micrographs show that both VGluT1 and VGAT puncta also increased around the dendrites and somata over 5 weeks (Fig. 2A). We counted the densities of VGluT1-labeled and VGAT-labeled puncta at different culture ages (Fig. 2B). The densities of both puncta increased gradually along culture ages from 7 to 21 DIV, whereas the densities of both puncta became saturated and did not increase at 28 and 35 DIV. Comparing the both synaptic densities with the electrical activity (i.e., the array-wide firing rate and synchronized burst rate) shown in Fig. 1, the firing rate and both synaptic densities show similar characteristics. This result indicates that the electrical activity was elevated along with the increase of both synaptic densities during the early development of cortical culture (up to 21-28 DIV). At 28 and 35 DIV, both the synaptic densities and electrical activity did not increase and became saturated. These results also suggest that the cortical neurons became network confluence during long-term culture after the initial construction of the networks. However, the distribution of excitatory and inhibitory synaptic terminals on target cell somata and dendrites varied between neurons even in the same cultures. Based on these results, we have begun to analyze the relationships between the network dynamics and the neuronal components in detail.

![Fig. 2. Immunofluorescence imaging of VGluT1 and VGAT on cultured neuronal networks. (A) Immunofluorescence micrographs of cultured cortical networks. (B) Developmental changes of the densities of VGluT1-labeled and VGAT-labeled puncta on neurons.](image)

References