Introduction

Neuronal networks are widely used in the study of synchronized burst activity and signal propagation phenomena. Recently, a new level of complexity was added by usage of patterning techniques. This allowed researchers to manipulate the cells positions and their connections, which enables a new understanding of signal transmission inside neuronal networks.

Since signal propagation in simple two-cluster cortical neuronal networks was demonstrated for the first time, there has been a fundamental question: How do the number and size of clusters and the connections between them influence the collective dynamics of the network? However, the answer to that question is still not clear. In order to answer it, an investigation of extracellular potential traces of multi-cluster neuronal networks is presented in this work. Spontaneous and evoked electrical activity analysis allowed us to find the initial bursting cluster as well as the signal propagation direction and passages in our neuronal networks.

Methods

Cell Patterning: In order to construct the isolated sub-networks, we applied our chemical patterning method (Suzuki et.al., Biomaterials, 5210-5217, 2013), which modified the cell adhesiveness of the poly-D-lysine to MEA by photolithographic method using vacuum ultraviolet light. In this study, we designed anew the pattern geometry. In addition mechanical manipulator was used to reshape certain cortical sub-networks, as well as for pattern isolation.

Cell culture on MEAs: Cerebral cortices derived from Wistar rats at embryonic day 17 were dissociated on chemically-patterned multi-electrode dish (MED) probes. The cells were filled with culture medium and cultured for 1 month in CO2 incubator. (Ito et al., Neuroscience, 50-61, 2010)

Electrical signal recording: Data was recorded from sub-network clusters on the electrodes by using MED64 (Alpha MED Scientific, Osaka, Japan) extracellular recording system at a sampling rate of 20 kHz. Spontaneous electrical activity was recorded each time before the external stimulation in order to obtain reference signal. External electrical stimulation with different signal parameters, such as amplitude, shape, length and period was used.

Fluorescent 3D imaging: After cultivation, cells were fixed and stained with MAP2 and NF200 antibodies to identify neuronal cell bodies, dendrites and axons. Cell nucleus were stained with hoechst33342. The fluorescence was observed with confocal laser scanning microscopy (IX81/FV1000D, Olympus).

Results and Discussion

Neuronal clusters and network connections

Cluster formation can be observed as early as 24 hours after the seeding. Neuronal cells start agglomerating on the PDL islands above the electrodes. However, those first clusters are highly instable and can disappear after a few days and reappear later. Also, due to cell adhesion, weakly attached clusters can migrate around the electrode area in the first week of the experiment. Almost no new clusters are forming after 7 days in vitro (DIV), and no migration is observed anymore – the initial clusters’ formation phase is over, and henceforth clusters only continue to grow in size.

The formation of the axon connection network starts around 3 to 7 DIV. The network formation is usually finished after 15 DIV, as after that only minor changes were observed, mostly with regard to the thickness of the axon passages. Due to the specifics of patterning experiments, axons do not only grow on the designated passages between PDL islands with clusters, but also sometimes in random directions, which can be fixed later by manual cutting. Ideally, one cluster should have no more than 4 direct connections to its closest neighbors. It is important to mention that one direct connection consists of several axons. When the network reaches 21 DIV, it is considered mature. Cluster growth usually also stops around that time.

In the present study, 2 types of clusters were observed: neuronal (consisting mainly of neurons) and neuro-glial (a mix of neuronal and glial cells, with predominance of the latter). Those two types could be distinguished even
before immunocytochemistry. Neuronal clusters are smaller in size and brighter in color. Their sizes varied from 50 to 150 µm for neuronal clusters and from 150 to 500 µm for neuro-glial clusters. Furthermore, neuronal clusters had larger amounts of neurons per cluster, 25-30 in average compared to 10-15 in neuro-glial ones.

Neuronal and neuro-glial clusters also showed differences in electrical activity and behavior during external stimulation. Neuronal clusters were in general more active and had a bigger amplitude of extracellular potential traces, they were easier to evoke and faster to reply (figure 1).

**Signal propagation analysis**

In general, a signal in a neuronal network can be transmitted from one neuron through several axon terminals connected via synapses to dendrites on other neurons. In case of a cluster network, the situation is similar. Since one cluster is considered as an organic whole, axon connections were also considered as single signal propagation pass between two clusters, regardless to which specific neuron in the agglomerate it belongs. In the present study, 2 types of neuronal sub-networks are discussed: simple (3 to 4 clusters) and complex (more than 5 clusters). Two cluster sub-networks, as well as the single isolated clusters, were not considered.

Every synchronized burst activity event has an initial cluster – the cluster which bursts first. In simple sub-networks, the initial cluster rarely changes, but in complex sub-networks, there are usually two or three clusters which can start a synchronized burst event. However, even in this case, one cluster would be the most active, starting more than 50% of bursts. In the present work, we called this type of initial clusters “Leaders” (or Leader cluster).

Study had shown that around 80% of Leaders were neuronal clusters. In general, neuronal clusters were more active and produced bursts with bigger amplitude. Naturally, it was easier for those clusters to initiate firing within the sub-network.

However, the full theory behind the synchronized burst propagation mechanism in patterned cluster neuronal networks is still unclear. This phenomenon requires further study. The combination of MED recording and firing visualization with Ca imaging could be used as a powerful tool for much more precise analysis. This will be very useful especially in case of complex sub-networks with multiple connections. Also, Ca imaging will allow us to understand from which part of the cluster the burst starts and how it spreads within. It is very interesting to know, whether there are silent neurons, which never fire in the clusters or not. And if there are, then what role do those silent neurons play.

**Figure 1.** Extracellular potential traces (a) Neuronal cluster, (b) Neuro-glial cluster. Scale bar – 500 µm. Blue line – time of the external impulse.

**Mature network reshaping**

Patterning experiments have some difficulties due to the cells adhesion to the chemicals (PDL) above the electrodes. In some cases, clusters may form connections to the surrounding neuronal network, formed around the electrode area or within the sub-network. Also, glial cells are another problem of in vitro experiments with neuronal cells. Glia is the connective tissue of the nervous system, consisting of several different types of cell associated with neurons. This connective tissue can also contribute to burst activity.

To avoid interference from glia and unwanted connections in the mature culture, manual reshaping or cutting can be used. The experimental setup consists of a sharp glass blade with a cutting area of around 50 µm in diameter, a mechanical manipulator and a microscope. The mechanical manipulator allows control of the fine movement of the blade and the penetration depth. Precise control is very important, since there is always a possibility to destroy cell-electrode connections, axons between clusters, or passages for electrodes on the MEA substrate.

As an example, one can take a look at a mature (27 DIV) 3-cluster neuronal sub-network (fig. 2). The upper two clusters are spherical, and the lower one has an eight-shape. This is significant for the experiment. Mostly, in patterning experiments, spherical clusters are obtained. Their burst activity is close to the ordinary dissociated neuronal network. However, neuronal clusters with complicated geometries can show unique burst patterns.

For each experiment, both before and after cutting, an initial burst cluster, which we call the leader cluster as
the majority of bursts originate from it, could be found. If the Leader was on channel 34 for 75% of all bursts before cutting, after, the leader would have switched to channel 57 in 65% of all bursts compared to only 2% before. Also, the amount of initial bursts for ch. 34 was reduced and became only 27%. The amount of total bursts was also reduced by 57%. In addition, a new response waveform appeared. The burst count data and the different response waveforms are shown in figure 3.

**Echo-effect model**

Most of the clusters do not change their geometry after the initial stage of growing. However, in case of cluster ch. 57, we observed a coalescence of two separated clusters in the mature sub-network. Before the final coalescence, the cluster ch. 57 had already absorbed one smaller cluster. This caused a new type of firing activity and interactions within the cluster. In simple clusters, firing starts from a group of neurons and spreads to the whole cluster in milliseconds. Thus, we observe a burst in the localized cluster. However, in specific case with cluster ch. 57, we have three clusters combined into an organic whole. The process could be described as an echo behavior – when firing starts in one sub-cluster, it spreads to the second one, causing it to burst. This echo effect can be observed if the firing activity in the first sub-cluster is finished by the start of the bursting activity in the second one. Depending on which sub-cluster was the initial one, we can observe double or triple echoes.

Before cutting, there was no triple echo effect observed. We can thus try to explain the phenomenon according to the connectivity map. By echo effect (fig.4), we mean an incident where there is no further possibility for the signal to propagate outside the cluster, and the activity wave reflects back into it. Before cutting, cluster ch. 57 had at least two different strong axon connections from 2 sub-clusters. Thus, only the third sub-cluster can be responsible for the echo. After cutting, there was only one connection passing on the left side of the central sub-cluster. Thus, there are 2 potential sub-clusters which could cause the echo effect.

As was discussed above, the characteristic waveform changed after the cutting for ch. 57, together with the firing intervals of the sub-network in general. In the case of external stimulation, the response obtained from channel 57 could be interpreted as a spontaneous event, since it was approximately a second late. However, during stimulation, no double or triple waveforms were detected. Thus, we have strong reasons to further investigate this phenomenon.

One of the reasons for the lack of double and triple response patterns, however, could be found in the echo effect mechanism. If we suggest, that an incoming impulse can block the echo within the complex cluster, we fully observe ordinary spontaneous burst activity, typical for an isolated cluster.

To support or refute each of these theories, unfortunately, additional experiments with adopting at least a Ca-imaging technique are required. Such experiments could possibly clarify not only the phenomenon of proposed echo-effect model, but also support or refute the theory of the role of glial cells in complex signal transition. Also, the gene analysis could be used to justify impulse block theory in coalescent complex clusters.

In general, present study is the first step to understand a complex firing behavior of the coalescent neuronal clusters. This phenomenon could help to understand the interaction between closely located computational neuron agglomerates in our brain.

**Summary**

The main propose of this study was to investigate the significant aspects of clusters formation and their properties. According to the experimental data, there are two types of clusters – smaller sized neuronal and bigger sized neuro-glial ones. They have different firing amplitudes, which is visible during extracellular
potential traces recording, and different colors, visible by light microscopy, and thus could be distinguished even before immunocytochemistry. The cluster size varied from 50 µm to 500 µm, and the number of neurons per cluster varied from 10 to 50 with an average of 20. It is important to mention, that in general, neuro-glial clusters had less neurons and included large amount of glial cells, which could be the cause of smaller measured amplitudes of the waveforms.

Clusters were considered as an organic whole, and thus the axon connections between them were also considered as single signal propagation passages. Two different types of axon passages were distinguished – weak (containing few axons) and strong (containing more than 5 axons). In general, during synchronized activity, the bursts were propagating preferably through strong axon connections.

Two different sub-network types were studied – simple (up to 4 clusters) and complex (more than 5 clusters). Each sub-network had an initial cluster – the cluster which fired first and from which the signal was spreading through the system. The initial cluster in simple sub-networks mostly remained unchanged, while in complex sub-networks, there were usually two or three initial clusters per recording. Also, for each network, a Leader cluster (more than 50% of initial bursting) could be defined. According to our experimental data, the Leaders are usually neuronal clusters.

Since the main topic of our present research is the study of patterned neuronal networks, manual reshaping, an additional patterning technique, was used. This process had a great influence on the mature sub-networks. First, the amount of synchronized bursts was reduced by half. Second, it was shown, that the sub-networks connectivity change affects the distribution of the initial bursts among the clusters, which can lead to Leader changes. Also, we showed that the cluster geometry is substantial for the shape and amplitude of the waveforms and that complex or coalesced clusters can result in an echo-effect in bursting activity.