Study of spontaneous bioelectrical activity of two hierarchically connected neural networks in vitro.

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Abstract. Synchronization of neuronal subpopulations and spiking pattern propagation are essential features for bioelectrical activity of the brain. In this study, we proposed an engineering method to grow hierarchically connected networks in vitro through the microchannels and investigated the relations of local spiking propagation through the microchannels with integrated bursting events in the chambers.

1. Introduction
A synchronization and information transfer between connected neural networks are considered to be the most notable features of the brain activity, which form various functions of a healthy brain (cognition, memory, sleep) and its disorders lead to pathological states (epileptiform activity) [1]. Neural networks in vitro spontaneously generate spiking activity in a form of periodic and synchronized discharges e.g. bursts after several days (DIV) and are often used to study such fundamental mechanisms [2, 3]. Recent advances in microfluidic methods allowed to culture neurons in separate compartments connected by microchannels with an asymmetric shape that defined a direction of a connectivity formation between the neural networks [4, 5]. Using extracellular electrophysiology of multielectrode arrays, we study the propagation of the spiking activity through the microchannels and following synchronization of the bursting activity in the networks.

2. Materials and methods
Microfluidic chips were fabricated from PDMS (Sylgard 184, Dow Corning) by “soft lithography” method [4] and were manually mounted onto the surface of a planar microelectrode array (MEA, Multichannel Systems, Germany). The chip consisted of two chambers for neuronal cells culturing and 16 microchannels for neuritis growth. The microchannels consisted of three segments, two segments had a narrow triangular shape (200 μm length, 40 μm width) and one segment had a large triangular shape (200 μm length, 150 μm width). The large segment had two traps to capture the axons of neurons
from the Target network (figure 1(a)). The width of the bottlenecks between triangular segments was 7 μm. The asymmetric design of microchannels provided the axonal growth from the Source to the Target chamber and reduced growth in the opposite direction (figure 1(b)).

Hippocampal neuronal cells were dissociated from embryonic mice (E18) and plated into the chambers of microfluidic chips at a density of approximately 8,000 cells/mm². Details for plating procedure can be found in [4].

Phase contrast images were obtained from neurons growing on coverslips. Data were collected sequentially from multiple sites at 20 minutes intervals using a Cell IQ system (ChipMan Technologies, Finland) with x20 objective (Nikon CFI Plan Fluorescence ELWD ADL). Neurite outgrowth dynamics were analyzed in 20 microchannels of 4 chips from 1 to 4 DIV.

The electrophysiological activity was recorded from 59 (1 reference) TiN electrodes of the MEA system (Multichannel Systems, Germany) at a sample rate of 20 kHz on 10, 15, 20, 25 days in vitro. Electrode diameter was 30 μm, an inter-electrode distance was 200 μm. Details of the spike and the burst detection can be found in our previous study [4].

To estimate a direction of spike propagation through axons in the microchannels we analyzed spike pairs recorded in two adjacent electrodes with physiologically relevant delay (figure 2(a)). As consistent with the literature, the velocity of the spike propagation along an unmyelinated axon was found to be in the range of 0.2–1 m/s [6-8]. According to 200 μm distance between the microelectrodes, the physiological delay between the spikes was in range of [0.2-1] ms. A histogram for all spike pairs represented one or several maximums corresponding to spike propagation from source to target (figure 2(b), Time > 0) or opposite (figure 2(c), Time < 0). We considered local maxima with at least 10% more spike pairs within the nearest local minimums as a spike propagation peak. Spike pairs within each peak ±0.2 ms for Time>0 (figure 2(b)) and on each active channel (out of 8) was summarized and normalized by the total number of received spikes in target electrode (figure 2(a), 2). Similar procedure was done for Time<0, for opposite spike transfer measure (figure 2(c)) where spike pairs were normalized on the spikes in source electrode (figure 2(a), 1). Both measures represented a probability of forward or backward spike propagation within the channels.

To estimate burst propagation we measured a number of the bursts in the Target chamber that followed the bursts in the Source chamber and vice-versa [4]. A probability of the burst propagation in a forward direction and opposite direction was estimated as the number of bursts propagated from Source to Target divided by the bursts number in Source and bursts propagated from Target to Source divided by bursts number in Target, respectively.

3. Results and discussions

The axons grew through the microchannels from the Source chamber to the Target chamber within 5 days after cell plating. To determine the direction of the axons in microchannels, we estimated a time of axons to grow to a bottleneck between 2nd narrow and 3d large segments (figure 1(c)). In 65% of the cases, the axons from the Target network did not reach the bottleneck during 4 days. At the same time, the axons from the Source network filled the first two segments. For the remaining 35%, the difference in the time of reaching the bottleneck for the axons from the Target and the Source network was 37.6 ± 23.9 hours (Mean ± standard deviation), a positive value indicated that axons from the Target chamber grew longer). Thus, the neuronal processes grew slowly in the large segment. This observation is consistent with the fact that the growth cone velocity decreased in the expansion of microchannels [9]. The difference in the growth cone velocity of processes from the Source and the Target networks could contribute to filling the space in bottlenecks between sections 2 and 3 of the microchannels mainly by axons from the Source chamber, which is essential for the constructing directionally connected networks.
Figure 1 (a, b, c). Microfluidic chip design. (a) Scheme of two chambers connected by 16 asymmetric microchannels. (b) Schematic view of neural network connectivity in the device with two chambers and microchannels. The shape of the microchannel provides axon growth from the Source to the Target chamber. (c) Example of neuritis outgrowth. Neuronal branches grow from the Source chamber to the Target without a resistance in contrast to the opposite direction. Branches from Target grow into “trap” structure. Synapses form in the third segment of a microchannel.

Then we investigated the direction of spike propagation in the microchannels. We recorded the spiking activity from the MEAs starting from 10 DIV until 25 DIV every 5 days. Figure 2(b, c, d) shows examples of spike propagation analysis using signals from three electrode pairs (See Methods). The histogram for each spike pair shows a clear peak suggesting that the spike transmit several axons in various directions. Figure 2(b) shows an example where the histogram has only one peak propagated from Source to Target network with a time delay of 0.4 ms. In some channels we observed peak that has negative delay, indicating propagation in the opposite direction (figure 2(c)). In figure 4(d), there are two peaks with time delays of -0.4 and 0.6 ms that are corresponding with spikes propagated in both directions. In rare cases, the peaks were not detected due to statistically poor or noisy recorded signal.

The spontaneous bursting activity appeared in the neural networks in 7-10 days in vitro. Some of the bursts propagated from one network and evoked burst in the second network with delay less than the initiating burst duration. In 3 out of 5 cultures, we found the burst propagation, where the direction of spikes propagation in the microchannels was predominantly from the Source to the Target chamber (figure 2(e)). This tendency maintained with the development of neural networks. In other 2 cultures spikes propagated in both directions (figure 2(f)) and the bursts didn't propagate from one sub-population to another.

Next, we estimated the fraction of the bursts generated in the Source chamber, which propagated through the microchannels and evoked the burst in the Target chamber. Example of raster activity is illustrated in figure 3(a). The percentage of the bursts propagated to the Target chamber was equal to 34% on 25 DIV (figure 3(b)); the bursts also propagated in backward direction from the Target to the Source chamber and was relatively small, ~10%.
Figure 2 (a, b, c, d, e, f). Analysis of the propagation of individual spikes along axons. (a) The relayed (1) and received (2) electrodes in a microchannel. (b) - (d) Histograms of spike patterns delays from three different channels. (e) Burst propagation (left, raster plot) and spike propagation within the channels (right). Histograms from eight electrode pairs and the raster of one burst propagated from Source to Target chamber. (f) Same analysis for a culture where burst propagation was not obtained. Histograms from eight electrode pairs and the raster of one burst propagated from Source to Target chamber.

In two cultures where we observed propagation of spikes in both directions, the bursts did not propagate from one sub-population to another (figure 2(f)). In other 3 cultures we found that the probability of burst propagation in the forward direction was several times higher than the propagation in the backward direction. The average probability of burst propagation in the forward direction reached a maximum value of 61 ± 13% (n=3) at 20 DIV. The average probability of burst propagation in the backward direction reached a maximum value of 6.6 ± 7% (n=5) on 25 DIV. Statistically significant changes in the probability of burst propagation was found on 20 DIV (p<0.05, T-test).

The delays between burst beginnings in the Source chamber and the response burst in the Target chamber were higher at the beginning of network development and tended to decrease. Statistically significant changes were found between 15 and 20 DIV (p<0.05, T-test). The percentage of spikes propagated in the forward direction increased during development (figure 3(d)) and was several times higher than the propagation in the backward direction.

4. Conclusions
In this study, we investigated a synchronization of two neural networks coupled with directional connectivity. Asymmetric microchannels formed unidirectional synaptic Source-Target connectivity and then allowed to study spike propagation in individual channels using MEA. We found that spikes propagated in the desired direction with high efficiency (figure 3(d)) and with physiological velocity the delays were up to several milliseconds. On the scale of the network, the bursts delays between two cultures were up to tens of milliseconds, which was also shown in the studies of modular networks in vitro [6,10,11] and in vivo [12]. Considering such delays, we propose that the synapses in the last section of the microchannel were involved to generate population response in the Target chamber. Manipulation of that structure may uncover basic synchronization principles and information transfer in neural networks with heterogeneous and modular connectivity.
Figure 3 (a, b, c, d). Burst propagation. (a) Raster plot of the spontaneous activity. Bursts propagate from the Source to the Target chamber with a delay. (b) An average firing rate profiles indicate spontaneous bursts propagated from the Source culture through the microchannels to the Target culture (left) and in the opposite direction (right). (c) An average number of bursts in the Target culture induced by the Source culture (black), and in the Source induced by the Target (grey) during development (n = 3 cultures). (d) An average percent of spikes propagated in the forward direction (black) and backward direction (grey) during development (n = 3 cultures).

We showed that spike propagation through the microchannels increased linearly from div 10 to div 25 together with monotonous increase in burst duration and burst spiking rate. During that, development stage a number of the axons within the microchannels increased and the bursts became longer which may explain such propagation increase.

However, the burst propagation between two chambers increased much faster from 10 to 20 div and then sustained on div 25 (figure 3(c)). During synaptogenesis at that maturation period, it was found that number glutamatergic terminals on the dendrites followed the same pattern while gabaergic terminals monotonously increased [13]. The results suggest that synchronization and population activity propagation between two unidirectionally connected modular networks may be strongly modulated by excitation/inhibition balance within the networks.

Acknowledgments
Experimental results of neuritis growth and the propagation of individual spikes along the microchannels were supported by Russian Science Foundation (project №19-75-00095); the study of the burst propagation and synchronization was funded by RFBR according to the research project № 18-29-10068.

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