STABILITY OF SPONTANEOUS ELECTRICAL ACTIVITY OF NEURAL NETWORKS *IN VITRO*

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Using brain-computer interfaces, one can both read data from and transmit them to the brain. However, these data are only a set of sensor system signals and not the knowledge or experience. Neural networks are a basis for cognitive activity and can simulate processes similar to learning *in vitro*. In this work we tested the hypothesis of a neural network's ability to learn by detecting deviations from its stereotypical activity and modifying them in a way that allows it to get rid of external electrical stimulation. Spontaneous activity of several neuronal cultures *in vitro* was analyzed by clustering method. The results showed that activity of untrained cultures remained stable for a long time, and external electrical stimulation led to switching between various spontaneous activity patterns.

Keywords: neuronal cultures, neural networks, learning, spontaneous activity, cluster analysis, bursting activity analysis

Funding: this work was supported by the Russian Science Foundation, grant no. 15-11-30014.

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Received: 01.04.2016 **Accepted:** 07.04.2016

УСТОЙЧИВОСТЬ СПОНТАННОЙ ЭЛЕКТРИЧЕСКОЙ АКТИВНОСТИ НЕЙРОННЫХ СЕТЕЙ *IN VITRO*

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С помощью нейро-компьютерных интерфейсов можно как считывать информацию с мозга, так и передавать ее в мозг. Однако эта информация — сигналы сенсорных систем, а не знание и опыт. Нейронные сети, представляющие собой основу когнитивной деятельности, способны *in vitro* воспроизводить процессы, аналогичные обучению. В работе проверена гипотеза о том, что нейронная сеть реализует обучение путем обнаружения отклонения от своей стереотипной активности и модификации ее таким образом, чтобы избавиться от внешней электрической стимуляции. Спонтанная активность нескольких нейрональных культур *in vitro* была проанализирована методом кластеризации. Результаты показали, что активность необученных культур остается стабильной на протяжении длительного времени, а внешняя электрическая стимуляция приводит к переключению между паттернами спонтанной активности.

Ключевые слова: нейрональные культуры, нейронные сети, обучение, спонтанная активность, кластерный анализ, анализ пачечной активности

Финансирование: работа выполнена при поддержке Российского научного фонда, грант № 15-11-30014.

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Статья поступила: 01.04.2016 **Статья принята к печати:** 07.04.2016

In medicine, brain-computer interfaces are used for the development of neuroprostheses comparable to healthy organs in their responsiveness to user's mental commands [1–3]. Data can also be transmitted in the reverse direction, from the computer to the brain; for example, lost sensory functions, such as auditory [4] and visual [5], can be recovered using electrical stimulation. However, what's more challenging for a researcher is transmission of information as such, i.e., knowledge and experience. It has been shown that using multi-input/multi-output non-linear dynamic model allows for transmitting a certain spatiotemporal pattern detected in the hippocampus of one rat to the hippocampus of another rat, which leads to the statistically reliable alterations in the behavior of the second animal [6].

It is known that neuronal networks that form a basis for cognitive activity do not have a distinct location in brain structures, but are distributed throughout them [7, 8]. From that, a need to reprogram neuronal networks ensues. Publications on the patterns patterns of neuronal network spontaneous activity *in vitro* and on the methods of its external modification [9–13] prove that networks of dissociated primary neuronal cultures of cortical and hippocampal cells on multi-electrode arrays (MEAs) can control external stimulation by changing their activity on the selected experimental electrode. Such neuronal network learning was first demonstrated by Shahaf and Marom [14] and was successfully reproduced by other researchers thereafter [15–18].

As the network is gradually developing from the dissociated neuronal culture, it starts to exhibit spontaneous bioelectrical activity recorded by array electrodes. Thus, in the absence of external stimulation in the first days of culture growth, only single action potentials are registered, but after a while they cluster into bursts [19]. In this work we hypothesize that spontaneous burst activity comes down to a small number of stereotypical patterns, and cluster analysis can help identify one or several dominating patterns. Since the external stimulation breaks the existing activation sequence, the network changes its activity pattern to switch off stimulation and go back to the typical pattern. Then, cluster analysis performed after the stimulation (training) can detect the resumption of the initial activity pattern.

METHODS

Cell cultures

Primary cell culture was prepared from the hippocampal tissue of newborn rats of C57BL/6 breed. Experimental animals used in the study were managed according to the guidelines specified in Order no. 267 of the Ministry of the Russian Federation, dated June 19, 2003, "On the approval of rules for good laboratory practice". The experiment was approved by the local Ethics Committee for Biomedical Research of the National Research Center "Kurchatov Institute" (protocol no. 1, dated July 9, 2015).

Cells were cultured on 60-channel multi-electrode arrays 60StimMEA200/30-ITO (Multichannel Systems, Germany). Prior to the experiment, plates were coated with poly-L-lysine for better cell adhesion. The initial culture density was 300,000 cells per mm^3. Their dissociation was achieved by using 0.25 % trypsin (Invitrogen 25200-056, USA). Neuronal viability was maintained in NeurobasalTM culture medium (Invitrogen 21103-049) in the complex with the bioactive additive B27 (Invitrogen 17504-044), glutamin (Invitrogen 25030-024) and penicillin-streptomycin (Life Technologies 15140122, USA) in GALAXY 170S incubator (New Brunswick Scientific, USA) under stable conditions: temperature of 37 °C, humidity of 100 % and 5 % CO_2 air concentration. Glial cell growth was not inhibited, because glial cells were necessary to ensure long-term culture viability *in vitro*. Half of the medium volume was replaced every three days.

Bioelectrical cell activity was recorded with MEA1060-Up-BC-Standard system (Multichannel Systems). For data acquisition, the bundled software was used.

Dynamics of spontaneous burst activity were observed in two cultures. Recording was performed from the 4^{th} day *in vitro* (DIV) until culture death. For further processing, we used data obtained in the interval between the onset and offset of burst activity. For training, one 24-DIV culture was used.

Protocol of neuronal culture training *in vitro*

1) 1-hour background recording of culture bioelectrical activity .

2) Stimulation by single bipolar rectangular pulses of ± 300 mV on each electrode (one at a time) to select the electrode that evoked the most intense culture response.

3) 5 stimulation cycles on the electrode selected in step 2. Every cycle consisted of a 5-minute series of rectangular pulses with 2-minute breaks. In every experiment, the pause between the pulses was adjusted so that every pulse could induce burst activity. Based on stimulation results, a recording electrode was chosen, for which electrical activity within 30–80 ms after the signal was the lowest.

4) 1-hour recording of culture spontaneous bioelectrical activity.

5) 20 training cycles. Training was considered successful if a twofold increase in the probability to record spike activity within the preset time interval on the electrode chosen in step 3 was observed. Training consisted of stimulation described in paragraph 3, given that as soon as the success criterion had been reached, stimulation was discontinued and a 2-minute break was provided.

6) 1-hour recording of culture spontaneous bioelectrical activity.

Detection of spikes and burst events

The initial signal was digitally processed by a second-order high-pass Butterworth filter with a passband of over 200 Hz, which allowed for the exclusion of low-frequency noise. Action potentials were detected if signal amplitude exceeded 4 standard deviations. In that case, the maximum amplitude was considered time of spike onset.

A burst event (burst) occurs on one electrode and is characterized by a short-term explosion-like impulse generation (0.1–3.0 s depending on culture age and its stocking density), and is usually accompanied by the low-frequency (1–5 Hz) signal component. Detection of bursts was based on the identification of the low-frequency component in a given interval and on spike detection in the vicinity of the component. Time when the first and the last spikes were generated was considered the burst onset and offset, respectively.

Population burst events are bursts that are observed simultaneously (with small delays of about 0.002–0.05 s) on more than a half of all active electrodes. The onset of the population burst is time of the first burst event onset.

Pattern analysis

As a feature V_k of a burst event, activation pattern was used [10]. Vector V_k dimensionality is equal to the number of active electrodes, i.e., electrodes on which at least one burst was observed:

$$V_k = \{t_k(i) - t^k_{start}\}^N_{i=0} = (c_{k0}\ldots c_{kN}), \quad (1)$$

where $t_k(i)$ represents activity onset on the ith electrode, and t^k_{start} represents time of population burst event onset. If no activity was recorded on the electrode during a given burst, but it was present during other bursts, the corresponding vector component takes the averaged value of other vector components.

As a metric, Pearson correlation coefficient was used. For cluster analysis, the weighted pair-group method with arithmetic averaging was used [20].

To obtain clusters, distances between neighboring vectors in the ordered feature vector sequence were found; then, based on the obtained distances, threshold value *th* was computed. Neighboring vectors, the distances between which were less than the threshold value, formed clusters.

Threshold value *th* was computed as follows. We built the graph representing the dependence of the maximum distance *(D)* between clusters on the number of clusters *(n)* in the order of increasing. Thus,

$$th = D(argmax(\frac{d^2D}{dn^2})+2), \quad (2)$$

where *argmax* is a function that computes the maximum value.

RESULTS

Cluster analysis of spontaneous burst activity in two neuronal cultures of 10…30 DIV (fig. 1) showed that in both cases over 50 % burst activation patterns belonged to the same dominating cluster (cluster 7 in fig. 1, A and cluster 5 in fig. 1, B). The majority of other population bursts (40 %) were distributed in two clusters that were equal in size, namely, clusters 6 and 9 (fig. 1, A) and clusters 4 and 6 (fig. 1, B). Dominating patterns of neuronal bursts were stable, despite of external factors related to medium replacements and to moving culture from the incubator to the recording device and back

In the neuronal network training experiment, electrode 22 was chosen for stimulation (see paragraph 2 of the training protocol); stimulation was terminated on electrode 12 (see paragraph 3 of the training protocol). A prerequisite for terminated stimulation was detection of 5 or more spikes in the interval of 50–80 ms after stimulation was applied.

Results of cluster analysis of spontaneous burst activity recorded at stages 1, 4 and 6 of the training protocol are presented in figure 2. Before stimulation was applied to the culture, population bursts were formed by two big clusters, namely, 15 and 18. After stimulation with no feedback (stage 4), the number of bursts decreased in clusters 15 and 18 and increased in clusters 2 and 4. After stimulation with feedback (stage 6), the number of bursts in dominating clusters 15 and 18 remained on the intermediate level. The rest of the activity shifted to cluster 3 from clusters 2 and 4.

The patterns of the dominating spontaneous burst activity registered before and after stimulation were different (fig. 3). In that respect, clusters 3 and 4 were alike, but both differed from clusters 15 and 18, which overlapped to a great extent. Cluster 2 combined features of both sets of clusters.

DISCUSSION

According to our hypothesis, spontaneous burst activity of neuronal networks *in vitro* must be characterized by self-organization and repetitive activity patterns. Our experiment and results obtained by other researchers [13, 21, 22] confirm

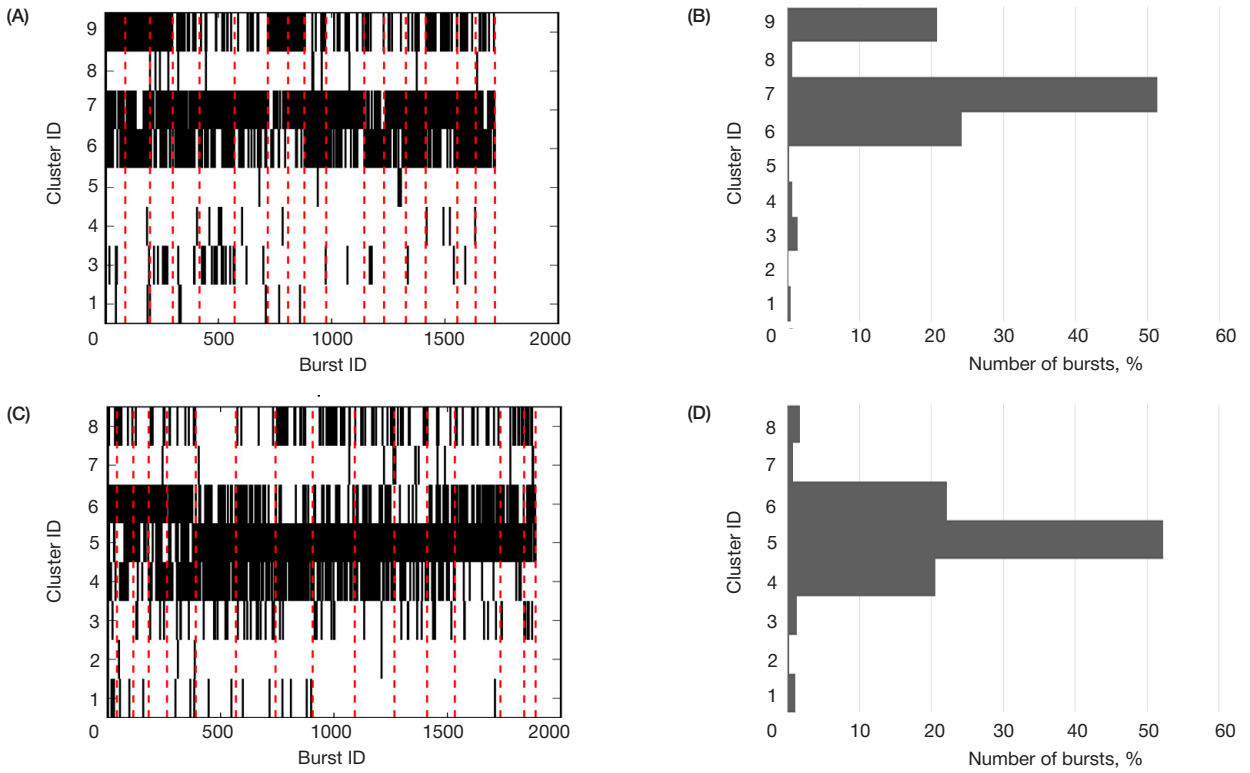

Fig. 1. Clustering of spontaneous burst activity of two neuronal cultures *in vitro* with the following IDs: 3035 **(A)** and 3040 **(C)**; percentage of bursts in clusters with the following IDs: 3035 **(B)** and 3040 **(D)**. Bursts are presented as they appeared. Red dotted lines represent days of culture development

that with culture growth, self-organization of neurons results in the emergence of the limited number of dynamic modes; each of those modes is characterized by its own activity pattern, i.e., an attractor. Thus, neurons *in vitro* can produce and maintain some activation sequence, which is necessary for memory trace retention.

We also made a supposition that patterns of spontaneous burst activity are resistant to external impact, including external electrical stimulation. Results of our study demonstrate that patterns do not depend on the presence of feedback during stimulation; what changes is the frequency of their occurrence. It allows us to make an assumption that in live neuronal networks, learning can be a result of variations of existing patterns with subsequent selection of a template used to solve the "problem". In the protocol used in this study, the "problem" was identified by means of external stimulation that can be switched off to provide a solution.

CONCLUSIONS

Study of spontaneous burst activity of neuronal networks *in vitro* showed that dynamics of network activity come down to a small number of attractors. Changes in burst activity registered after applying external stimulation showed that the dominating attractor of spontaneous activity does not disintegrate, but the variety of patterns increases. We can assume that learning is mediated by switching between the existing dynamic attractors of neuronal activity.

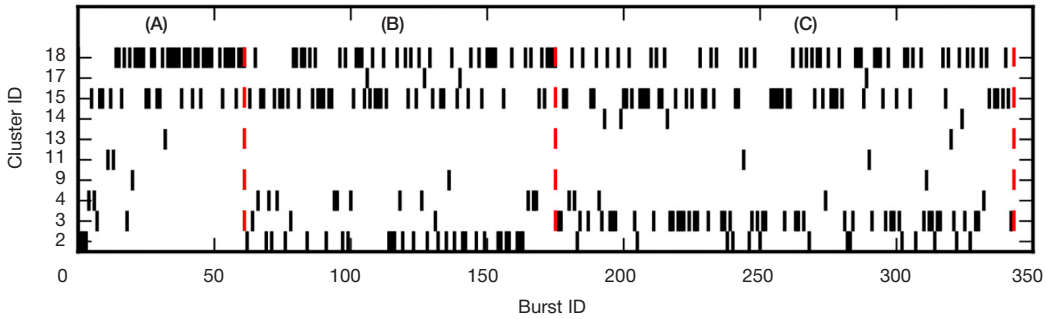

Fig. 2. Switching of activity patterns after stimulation without feedback and with feedback. **(A)** Before stimulation. **(B)** After stimulation without feedback. **(C)** After stimulation with feedback

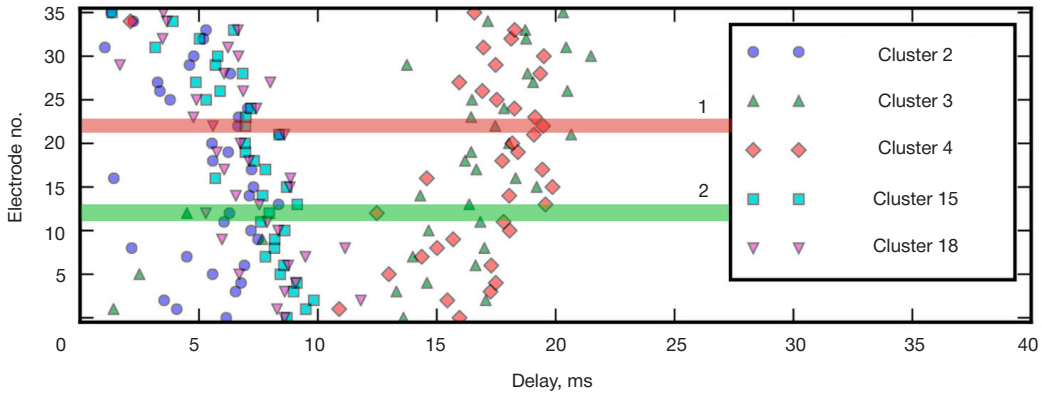

Fig. 3. Averaged burst feature vectors for clusters containing at least 5 % of all events shown in fig. 2. Green line (1) represents electrode 12, on which the culture was trained. Red line represents electrode 22, on which stimulation was performed

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