Study of hypothermia on cultured neuronal networks using multi-electrode arrays

Liel Rubinsky a,*, Nadav Raichman b, Itay Baruchi b, Mark Shein b, Jacob Lavee c, Hanan Frenk a, Eshel Ben-Jacob b

a School of Social Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel
b School of Physics and Astronomy, Raymond & Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel
c Department of Cardiac Surgery, Sheba Medical Center, Tel Hashomer 52621, Israel

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Abstract

Efficient and safe use of hypothermia during various neuro-medical procedures requires sound understanding of low temperature effects on the neuronal network's activity. In this report, we introduce the use of cultivated dissociated neuronal networks on temperature controlled multi-electrode arrays (MEAs) as a simple methodology for studying the long-term effects of hypothermia. The networks exhibit spontaneous activity in the form of synchronized bursting events (SBEs), followed by long intervals of sporadic firing. Through the use of our correlation method, these SBEs can be clustered into sub-groups of similar spatio-temporal patterns. Application of hypothermia to the network resulted in a reduction in the SBE rate, the spike intensity and an increase in inter-neuronal correlations. Within 2 h following the cessation of hypothermia, the cultured network returned to its initial spatio-temporal SBE structure. These results suggest that the network survived cold exposure and demonstrate the feasibility of long-term continuous neural network recording during hypothermic conditions.

Keywords: Hypothermia; Neuronal networks; Multi-electrode arrays; Synchronized bursting events

1. Introduction

Hypothermia of the brain is commonly used to reduce metabolism for the protection of the brain immediately after trauma, during surgical procedures and in recovery of patients (Sessler, 2001; McIntyre et al., 2003; Varathan et al., 2001; Kumral et al., 2001). The therapeutic effects of hypothermia in reducing cerebral injury are well documented (Barone et al., 1997; Muller et al., 2004). Low temperature is now routinely used in surgical procedures, such as deep hypothermic circulatory arrest (DHCA) where the body and brain temperature of patients are lowered to 18–20 °C, allowing for “safe” ischemic windows of approximately 60 min (Kumral et al., 2001; Bissonnette et al., 1999; Myron, 1989).

Despite the important medical applications of hypothermia stated above, the effect of low temperature on the brain during these procedures is not fully understood or optimized. For instance, DHCA still results in up to 10% of patients having documented postoperative neurological morbidities, including dysfunction in memory and learning (Muller et al., 2004; Bissonnette et al., 1999; Myron, 1989). Furthermore, while low temperature has a beneficial effect on protecting the injured or treated brain, an extended period of hypothermia can by itself result in health complications including apoptosis and necrosis of neurons (Bell et al., 1989; Ditsworth et al., 2003).

In order to better understand the process of hypothermia at work, it is important to develop the proper methods to study its effects. One technique that allows systematic and easily controlled investigations from the basic micro-level cell network up to the macro-level of the brain cortex is the use of cultured neuronal networks (Bossenmeyer-Pourie et al., 2000; Marom...
and Shahaf, 2002). Cultured neuronal networks cultivated on multi-electrode arrays (MEAs) offer an ideal in vitro platform for investigating synchronization and coordination of individual neurons as well as the collective dynamics of the network as a whole (Marom and Shahaf, 2002).

In recent years, our research group has developed a general methodology for the study of cultured neuronal network activity and function. Specifically, we grow neuronal networks on MEAs and characterize the electric activity of the network using methods based on correlation analysis (Hulata et al., 2002). The technique allows for the long-term study of the spatio-temporal correlations between neurons and between regions in the network (Segev et al., 2004).

In the current study, we developed a new method for monitoring the effects of hypothermia on an in vitro neuron culture by comparing the intensity, pattern correlation and culture state before, during and after hypothermia. Here we report on our ability to evaluate our recording setup and analysis tools, to identify temporal effects of hypothermia on the network activity, and to generate new insight into the effects of low temperature on the collective dynamics of the neurons.

2. Materials and methods

2.1. Network preparation

The neuronal network used in this study was prepared from dissociated cortical cultures of neurons and glia of Sprague–Dawley rats in their 18 embryonic day (E18), prepared and maintained according to the protocol described in (Segev et al., 2002), and grown on a multi-electrode array. The MEA consists of 60 microelectrodes (Multi-Channel Systems, Germany). The electrodes are 30 μm in diameter and are arranged in a square grid with a distance of 200 μm between electrodes. Before plating, the MEA is first coated with a poly-D-lysine (PDL, Sigma P-7886, Israel) substrate and left overnight. The neurons are then plated at a density of 2 × 10⁶ cells/dish on the PDL substrate. The culture is maintained in a culture solution of growth medium containing 25 ml Horse serum (Beith Haemek, Israel), 100 μl Gentamycin (Beith Haemek), and 10 ml Glucose 1 M to 464 ml of MEM (Beith Haemek) to which was added 125 μl of Glutamine (Beith Haemek). The culture is kept in an incubator at 37°C with 5% CO₂ and 95% humidity. Cultures are incubated for a minimum of 14 days to mature into self-organized connected and active neuronal networks. Twice a week 1 ml of medium is replaced.

2.2. Data acquisition

The electrical signals recorded from the neurons are local action potentials from cells that have formed capacitive coupling with the electrodes. At 14 days in vitro, the network is placed in an external recording chamber that maintains the same physiological conditions as those found in the incubator. The network electrical activity is non-invasively recorded by a set of 60 amplifiers (Multi-Channel Systems) with a sampling rate of 12 kHz and transferred to a computer and saved to disk using Alpha Map data acquisition software (Alpha Omega Engineering, Israel). Spike detecting and sorting of the recorded action potentials were performed by our wavelet packets decomposition method as described in (Hulata et al., 2002).

2.3. Hypothermia application

Before application of hypothermia, the neuronal activity was recorded for 5 h under normal physiological growth conditions in order to establish a base line for normal neuronal activity. The recording was continued while deep hypothermia was then applied by pumping chilled water stored in a plastic container submerged in a Neslab RTE-221 cooling bath (Neslab RTE-221, Thermo Electron Corporation, U.S.A.) through channels in the walls of the recording chamber. This effectively reduced the temperature to 19 ± 1°C. Temperature was verified using a thermocouple-measuring device (Digi-Sense Thermometer, Cole Parmer, U.S.A.). The thermocouple measurements indicate that cooling of the chamber began immediately and the designated temperature was achieved within 5 min. The cultures were maintained in this hypothermic state for 20 h.

Following 20 h of deep hypothermic conditions, the cooling system was shut down and the temperature was returned to 37°C and continuously recorded for a minimum of 5 h. Thermocouple measurements show that the temperature was re-achieved within 5 min.

2.4. Identification of sub-classes of synchronized bursting events

At 14 days in vitro, the cultured network exhibit rich spontaneous dynamical behavior characterized by the formation of synchronized bursting events (SBEs). SBEs are short time windows (200–1000 ms) during which most of the recorded neurons participate in relatively rapid firing of action potentials (Fig. 1). The SBEs are separated by long intervals (above seconds) of quiescence with few sporadic neuronal firings. A closer inspection reveals that each SBE has a distinctive spatio-temporal activity pattern between the neurons (Fig. 1).

We noticed that in a single culture several different SBE spatio-temporal patterns may appear, and these patterns repeat themselves overtime. We applied a clustering method based on correlation analysis in order to classify these SBEs into sub-groups of similar spatio-temporal activity patterns. The mathematical details of the method used to analyze the results were described in detail in several publications (Segev et al., 2002, 2004).

In this method, we first represent each SBE by a two-dimensional binary spatio-temporal image D where the x-axis is the time in units of 5 ms and the y-axis are the indices of the recorded neurons. We set D_{ij} = 1 if the jth neuron fired during the ith time bin, and equal to zero otherwise. The image of each SBE is centered on the time bin where the SBE had the most spike activity and the time width around the center was 1500 ms.

In order to cluster the SBE images into sub-groups of similar spatio-temporal patterns, we calculated the inter-SBEs correla-
tion matrix $C$ where
\[ C(n, m) = \max \left( \sum_{i=1}^{N} D_n^i(t) \otimes D_m^i(t - \tau) \right) \]
and $\otimes$ denotes the standard normalized correlation function between two vectors as a function of the displacement $\tau$, $N$ the number of neurons and $n$ and $m$ are SBE indices. Each row/column in $C$ represents the resemblance between spatio-temporal patterns of a single SBE and all the other detected SBEs. Next, we applied a standard dendrogram clustering method (Matlab® Statistics Toolbox) to the correlation matrix in order to group together SBEs with higher spatio-temporal resemblance. The dendrogram method calculates the Euclidean distance between the correlation vectors in $C$ and reorders the matrix in a hierarchical manner to bring vectors with minimal distance next to each other. When plotting the reordered correlation matrix, sub-groups of SBEs are identified as square blocks that are highly correlated within the set and have lower correlation with SBEs outside the set (Fig. 3A).

We also applied the Principal Component Clustering Analysis (PCA, Matlab® Statistics Toolbox) on the inter-SBEs correlation matrix using the method described in (Segev et al., 2002). Fig. 3B shows the scattering of the SBE correlation values on the first two principal components, revealing the division of the data into clusters, identifying patterns of similar neuron activities.

### 2.5. Inter-neuron correlation

For each SBE, we calculated the average inter-neurons correlation $S$. We did so by first calculating the temporal correlation for each pair of neurons in the burst:
\[ S_{i,j}^n = \frac{\langle (D_n^i - \bar{D}_n^i) \times (D_n^j - \bar{D}_n^j) \rangle}{\sigma(D_n^i)\sigma(D_n^j)} \]
where $i$ and $j$ are the neuron index in the $n$th burst, with the symbol $\langle \rangle$ denoting averaging, and $\sigma$ as the standard deviation. The SBE average inter-neuron correlation is then defined as:
\[ S^n = \langle S_{i,j}^n \rangle_{i,j} \]
averaged over all the pairs of neurons. The resulted $S$ is a measure of the synchronization between the neurons in the SBE time window, where $S=1$ if the neurons fire in perfect synchrony, and $S=0$ if the neurons do not spike at the same time at all.

### 3. Results

At 14 days in vitro, the cultured network exhibit rich spontaneous dynamical behavior characterized by the formation of synchronized bursting events. SBEs are short time windows (200–1000 ms) during which most of the recorded neurons participate in relatively rapid firing of action potentials (Fig. 1). The SBEs are separated by long intervals (above seconds) of quies-
cence with few sporadic neuronal firings. A closer inspection reveals that each SBE has a distinctive spatio-temporal activity pattern between the neurons (Fig. 1).

In three separate cultures, deep hypothermia was applied by lowering the culture temperature from normal conditions of 37 °C to 19 °C. Each culture remained in hypothermic conditions for 20 h, after which the normal temperature was returned. In all three cultures, the application of hypothermia leads to similar results as described below.

The most pronounced and immediate observation was a drastic decrease in the SBE rate (Fig. 1) and in the number of spikes per burst (Fig. 2A), seen within a few minutes after application of hypothermia. The rate of SBEs dropped from a burst every 15–30 s to every 2 min on average (Fig. 1B). These low levels of activity remained the same throughout the whole duration of hypothermia application. Moreover, during hypothermia there was a gradual decrease in burst width and burst intensity (number of spikes per burst and number of neurons per burst) (Fig. 2).

We also observed a slight increase in the averaged inter-neuron correlation (Fig. 2). The latter could be a direct result of the sparse neuronal firing that lead to sharp narrow bursts (Fig. 1B), and therefore a more synchronized activity. The persistent synchronized activity during hypothermia, though at a lower rate, demonstrates that the network connectivity stays intact. The lower number of participating neurons most likely led to a less efficient transmission of action potentials and narrower SBEs.

Immediately following hypothermia the network exhibited a dramatic increase in activity, with a temporary overshoot reaching three times the SBE rate compared to the activity level prior to hypothermia (Fig. 1C). This overshoot was seen in all culture repeats and lasted for over an hour. This result could most likely be due to the sudden termination of the cooling fluid on the network, and the rapid return to normal temperature within 5 min. It is assumed that if the network had been gradually returned to 37 °C over the course of several hours this overshoot would most likely not be evident. The overshoot itself can be explained by an increase in the network excitability, as compensation for the strong inhibition of the network activity during hypothermia.

Fig. 3. Classification of SBEs from before and after hypothermia. A: Inter-SBEs correlation matrices where calculated for two data sets—300 SBEs taken from before hypothermia and 300 SBEs taken from 5 h after hypothermia. Clustering analysis was then applied over the two correlation matrices. The resulting clustered matrices show a clear separation of the data into three sub-groups that contain high values of correlation. Areas of the matrix in between the three sub-groups have lower values of correlation. The third matrix shows the inter-SBEs correlation values as calculated from the combined data of the six sub-groups identified in the previous matrices. Sub-groups #1 and #4 and sub-groups #2 and #5 share high values of correlation. B: PCA plots of the three sub-groups identified in each of the two clustered matrices. Distinction between SBEs of different sub-groups is color-coded. After hypothermia the separation between the sub-classes becomes more pronounced. C: Raster plots of the average neuronal activity in each SBE sub-group shown in A. Red/blue colors represent high/low spike rate.
Approximately 2 h after termination of hypothermia application, the network activity gradually returned to its pre-hypothermia SBE rate and activity level (Fig. 1D). This was also seen in other measurements, including burst duration, intensity and in the inter-neuron correlation (Fig. 2).

Homeostasis of the network activity before and after hypothermia was also seen through the inter-SBEs correlation matrix. We selected from one experimental recording 300 SBEs from before the application of hypothermia and 300 SBEs from 5 h following hypothermia. For each of the two sets of SBEs (before and after hypothermia) an inter-SBEs correlation matrix was calculated. Clustering analysis of the correlation matrix of each of the two SBEs sets resulted in a clear separation of the data into three-square areas (sub-groups) that contained high values of correlation (Fig. 3A). Areas of the matrix in between the three sub-groups have lower values of correlation. The separation into three sub-groups is also evident when seen in the PCA plots (Fig. 3B).

Each sub-group represents a collection of highly correlated SBEs with a distinct spatio-temporal pattern of neuronal firing (Fig. 3C). In order to test whether the three sub-groups identified in the two sets of correlation matrices, before and after hypothermia, are identical (i.e. the SBEs maintain the same spatio-temporal structure after application of hypothermia as they did before), we calculated the inter-SBEs correlations between SBEs taken from the combined data of the six sub-groups (Fig. 3A). The resulting combined correlation matrix shows that sub-group #1 (before hypothermia) is highly correlated to sub-group #4 (after hypothermia), indicating that the spatio-temporal structure did not significantly change due to the application of cold. The same applies to sub-groups #2 and #5. The two smallest sub-groups, #3 and #6, were not as highly correlated, indicating that the SBEs of these sets did not share a very similar spatio-temporal structure. These results are also evident in Fig. 3C, where we show the average spatio-temporal structure of the SBEs taken from the three sub-groups before and after hypothermia.

Since the spatio-temporal patterns of SBEs are strongly related to the network connectivity, this last result may indicate that the neuronal connections did not change significantly during the 20 h of hypothermia. It was also observed that the separations between the SBE sub-groups in the PCA plots became more pronounced after hypothermia application, with a narrower statistical distribution of each cluster around its mean. Similar findings were observed in the two other cultures.

All the above results demonstrate that the network survived hypothermia without major changes to the underlying neural network activity.

4. Discussion

Several typical patterns of neuronal network behavior during and following hypothermia were observed within this study. First we found that the network as a whole can recover from deep hypothermia of 20h. This is seen in the return to activity levels observed before the introduction of deep hypothermia.

Moreover, we have found that after the exposure to deep hypothermia, the spatio-temporal SBE patterns did not significantly change, meaning that the network generally remained in its original connectivity pattern. This is a surprising finding since indications in our unpublished results using XTT (a method for accessing cell viability) show that above 25% of the cultured neurons die following 20 h of deep hypothermia. These results can be taken as evidence of the ability of the network as a whole to survive the effects of cold and indicates the value of this methodology in studying low temperature effects on neuronal systems for extended periods of time.

The more pronounced separation between SBE sub-groups following hypothermia, as seen in the PCA plots might be a result of an intensification of the neuronal connections as a response to the activity inhibition. Another explanation might be that extensive cell death may reduce the fraction of short-distance connections, and thus an enhancement of the expression of large-scale network connectivity.

The strong inhibition of our network activity during hypothermia may support and may help explain recent experiments in which cooling was used to arrest epileptic seizures in both human and animal experiments (Karkar et al., 2002; Yang et al., 2003). This cooling of the brain might have reduced the neuronal activity while leaving the general connectivity intact, as seen in our model. However, it must be taken into consideration that the effects of hypothermia in vivo experiments may involve processes, such as blood flow, oxygen supply and extended cooling/re-warming times, not found in our in vitro model.

In addition, this model may be of use in the study of the effect of hypothermia on memory. The findings show that the network maintained its spatio-temporal patterns before and after hypothermia. Each spatio-temporal pattern could be regarded as representing a different active “state” of the network. The maintenance of these patterns suggests the possibility that memory in neuronal networks may be retained even following strong activity inhibition.

In this work, we demonstrate that in vitro neuronal networks cultured on multi-electrode arrays can be used as a novel, relatively simple, self-contained system for examining the effect of hypothermia on spontaneous neuronal network activity. A spatio-temporal analysis of our recorded data from the arrays has been performed, and the properties of the network, such as its activity and cross-correlations between SBEs have been quantified. Our results show quantifiable effects of temperature on the neuronal network.

Conflict of interest

The authors declare they have no competing financial interests.

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