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Slow electrical stimuli affect connectivity in cultured neuronal networks

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***Abstract*—Learning, or more generally, plasticity may be studied using cultured neuronal networks on multi electrode arrays. Many protocols have been proposed to change connectivity in such networks. One of these protocols, proposed by Shahaf and Marom, aimed to change the input-output relationship of a selected connection in a network. Although the results were quite promising, the experiments appeared difficult to repeat and the protocol did not serve as a basis for wider investigation yet. Here, we repeated their protocol, and compared our 'learning curves' to the original results. Although in some experiments the protocol did not seem to work, we found that on average, the protocol showed a significant learning effect indeed. Furthermore, the protocol always induced connectivity changes that were much larger than changes that occurred after a comparable period of random stimulation. Finally, our data suggest that network connectivity changes are induced more easily using stimulation at a fixed, than using randomly changing electrodes.**

I. Introduction

Several protocols have been proposed to study plasticity in cultured neuronal networks. One protocol, by Marom and Shahaf [1] aimed to train a culture to produce a predefined response upon stimulation. Although their results seemed quite successful, to our knowledge, they were never reproduced elsewhere and they did not serve as a basis for wider exploration yet. One of the difficulties in the interpretation of their results is the fact that it is not completely understood how and why slow electrical stimulation may alter network connectivity. Vajda et al. showed that repeated slow stimulation at a single electrode may indeed change connectivity, but in an uncontrolled manner [2]. In this study we tried to reproduce the results of Marom and Shahaf. Furthermore, we investigated the requirements that a driving stimulus should meet to induce (the desired) connectivity changes.

II. Methods

A. Cell cultures

We obtained cortical cells from newborn Wistar rats. After trypsin treatment cells were dissociated by trituration. About 400,000 dissociated neurons (400 µl suspension) were plated on a MEA precoated with poly ethylene imide. This procedure resulted in an initial cell density of approximately 5000 cells per mm^2.

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Neurons were cultured in a circular chamber (inner diameter: 20mm) glued on top of a multi electrode array (MEA) with 60 electrodes (Multi Channel Systems, Germany, see Figure 1). The culture chamber was filled with 700 µl R12 medium [3]. MEAs were stored in an

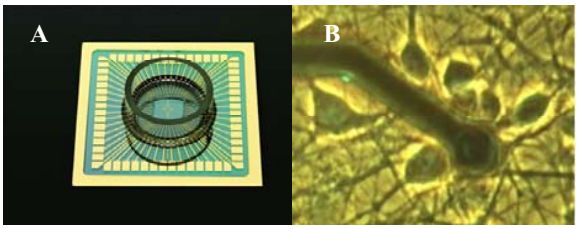

Figure 1. A: Multi electrode array (MEA), used to record neuronal activity in cultured networks of cortical neurons. It is based on a glass substrate with 60 embedded electrodes in the centre of the chamber, with 100 µm inter electrode distance. The glass ring glued on top was filled with glia conditioned growth medium and firmly sealed. **B**: close up of one of the electrodes and several neurons. Electrode diameter: 10 µm. Most electrodes did not pick up signals from more than one neuron.

incubator, under standard conditions of 37°C, 100% humidity, and 5% CO_2 in air. For recording, we firmly sealed the culture chambers with a watertight but CO_2 permeable foil, and placed the cultures in a measurement setup outside the incubator. During recording we maintained the CO_2 level of the environment around 5%. For details about the recording setup see [4]. All recordings were started after an accommodation period of at least 20 minutes.

After the measurements the cultures were returned to the incubator.

We used 11 different cultures for 21 experiments (see Table 1), which were performed 36±20 days after plating of the dissociated cells.

B. Training experiments

We first stimulated all electrodes at various amplitudes to select a stimulation electrode that frequently induced a network burst and (following the original training protocol) an evaluation electrode that responded to these stimuli at a ratio of ~0.1. We plotted curves of the number of action potentials at each electrode, as a function of the latency to the stimulus. Figure 2 shows an example of the probability to record an action potential at the evaluation electrode as a function of the latency after the stimulus (the 'responsiveness' of a selected electrode). Response curves of evaluation electrodes usually had a peaked shape, similar to that in Fig.2. The first peak around zero latency was probably caused by some residual stimulus artifact, or by non-synaptically transmitted direct responses, through retrograde stimulation of axons. We focused on the second peak, around 20 ms in the example in Fig.2

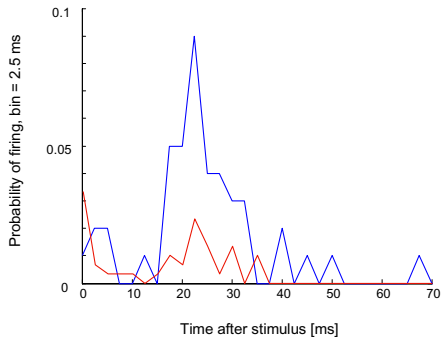

Figure 2. Red (lower) line shows the 'responsiveness' of a selected evaluation electrode before the training protocol. The time interval to detect responses was set at 10-40 ms, such that the summed probability before training was about 0.1. Blue (upper) line: the responses to the last 10 stimuli of the training protocol.

We selected evaluation electrodes that had a response ratio of ~0.1 in a time window around the maximum of the second peak. Time windows had a width of 20-50 ms and were adjusted to obtain this response ratio. In the example of Figure 2 the evaluation time window was set to 10-40 ms. We applied the following training protocol (slightly adapted from Shahaf and Marom [1]):

We stimulated the selected electrode until the evaluation electrode showed at least 2 responses to the last 10 stimuli or until the maximum stimulation time of 10 min was reached. When the threshold (or the maximum stimulation time) was reached, stimulation stopped automatically, followed by 5 minutes without stimulation. We repeated this cycle until the network wide response to the stimuli dropped below threshold in three consecutive cycles. This threshold was set to 80% of the average response to the first 5 stimuli.

We plotted the number of applied stimuli against the cycle number (a learning curve), and interpreted a decreasing number of stimuli as a learning effect.

C. *Training experiments vs. random stimulation*

We investigated if the training protocol had larger effects on connectivity than random stimulation at comparable frequencies and during periods of comparable duration. Therefore, in two experiments, we first recorded spontaneous activity for 1 hour, then applied a stimulation period of 5 hours during 40% of which the culture was actually stimulated. We stimulated at single electrodes which were randomly chosen before each next stimulus. Then, again, we recorded 1 hour of spontaneous activity. We estimated connectivity changes from the spontaneous recordings before and after manipulation and compared this to the connectivity changes after training sessions.

D. *Fixed electrode vs. random electrode stimulation*

Next, we investigated if the influence of single electrode stimulation differed from that of random electrode stimulation. Therefore we first recorded spontaneous activity, then we stimulated for ~1 hour, either at one single electrode, or at one electrode which randomly changed after each stimulus pulse. Then we recorded spontaneous activity again.

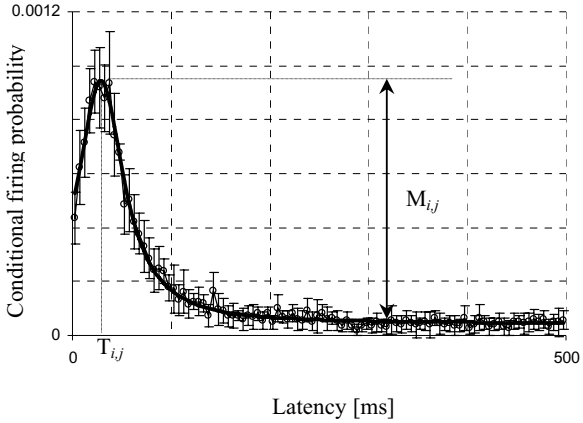

Figure 3. Example of estimated conditional firing probability (○, mean ± SD of 5 consecutive bins of 0.5 ms each). Solid line represents fitted equation, used to obtain values for strength ($M_{i,j}$) and latency ($T_{i,j}$) of the functional connection between a pair of electrodes (i,j).

E. *Connectivity analysis*

We used periods of spontaneous activity to analyze network connectivity. For all possible pairs of electrodes (60×59) we calculated conditional firing probabilities (CFP's) as the probability to record an action potential at electrode j at t=τ, given that one was recorded at electrode i at t=0. If a CFP curve was not flat, the two neurons were functionally connected. An example is shown in Figure 3. This functional connection may be described by two parameters: strength and latency [5]. These parameters may be used to follow the development of a functional connection in time [6].

III. Results

We applied the training protocol in 10 experiments. Figure 2 shows an example of the responses to the first 10 stimuli (red line) and to the last 10 stimuli (blue line) of the training protocol in a successful experiment.

In 50% of all experiments we found a learning curve as in the example of Figure 4A, which may be characterized by an initial decline (continuing for 14±8 cycles, mean ± SD), followed by a rise, roughly centered around trial Nr 20 (20±6). Eventually these learning curves reached a low level as in Figure 4A. The width of the increase around trial Nr. 20 varied and averaged 13±7 cycles.

In another 30% a stable low level was reached immediately, whereas 20% showed wild fluctuations without a clear trend. The average learning curve of all cultures, including the 20% without a clear trend, is shown in Fig. 4B. Strikingly, the first 10 trials yielded results very similar to the original results published by Shahaf and Marom [1].

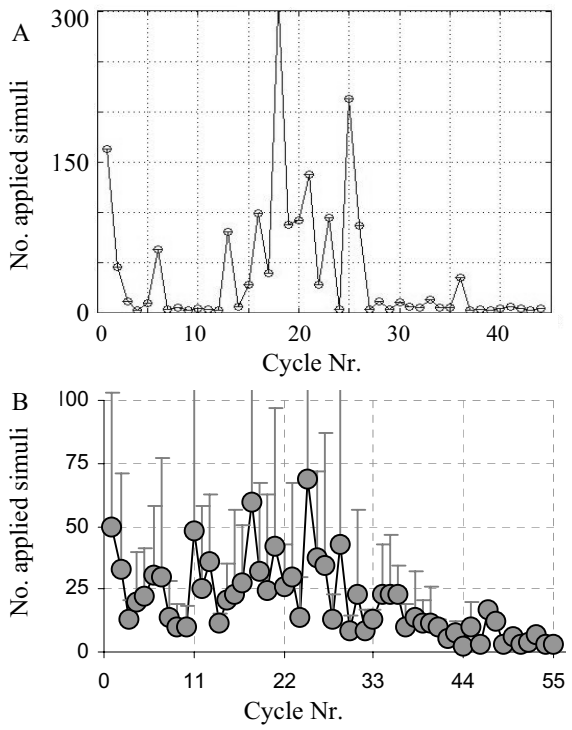

Figure 4. Learning curves resulting from training protocol. **A** shows a typical example of an individual learning curve, as observed in 5 of 10 experiments. **B** depicts average learning curve (●, mean ± SD) of all 10 experiments. No. applied stimuli decreases significantly with trial Nr. (Kendall's tau: Correlation coefficient: -0.33; P<0.01).

However, after the 10th cycle the average number of stimuli increased again. This phenomenon was seen in 5 individual experiments. Because the centre of the rise differed between experiments, on average the effect was somewhat blurred out, resulting in a lower and wider second peak in the averaged curve, as well as higher standard deviations (Fig 4B).

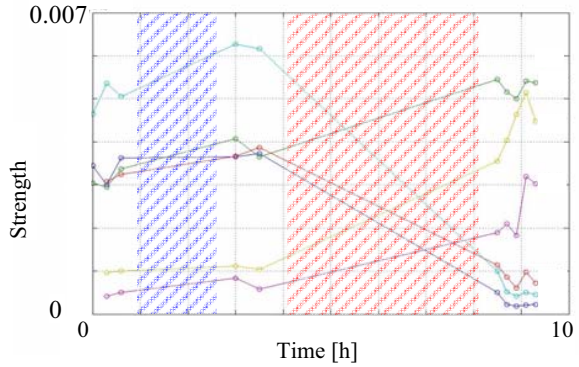

Figure 5. Strengths of persisting connections during one of our experiments. Experiments had 5 phases. White areas: spontaneous activity recordings. Blue bar: random stimulation (see section II.B). Red bar: training protocol (see section II.C). The graphs illustrate that the strength of most individual connections was affected by the protocol. In total, the strength of 64% of all persisting connections was significantly changed. The figure also suggests that global parameters like mean strength may not be affected by the protocol.

Finally, we used spontaneous activity recorded before and after the training protocol to investigate connectivity changes in the network. Occasionally new functional connections appeared or existing ones disappeared during the training protocol, but on average this number was small, compared to the number of persisting functional connections. The strength of 64% of all persisting connections was significantly affected by the protocol, either up or down. Figure 5 shows an example with only very few persisting functional connections. Usually there were many more persisting connections and the depicted example is not representative in this respect. However, the selected example clearly shows that the strength of functional connections changed substantially, both up and down Although the average strength did not change, the mean *absolute* change was 52±26%, clearly exceeding the spontaneous fluctuation during a comparable time span (~30% standard deviation, see [5]). The mean absolute change was not significantly higher than after random stimulation (Table 1). However, there were very few significantly affected functional connections after random stimulation, compared to 65% after training sessions.

Next we investigated the effect of slow stimulation on network connectivity. In 6 experiments we stimulated at one electrode using a low frequency (0.2-0.33 Hz), whereas in 3 experiments, we used similar stimulation at random electrodes. Compared to the random electrode experiments, the number of significantly changed functional connections doubled when a single electrode was stimulated. Table 1 depicts the results of these experiments.

Table 1. Effects of single electrode vs. random electrode stimulation.

Experiment:	N	Stimulation period [min]	FSCS [%]	$\overline{\Delta}$ [%]	$\overline{\lvert\Delta\rvert}$ [%]
Training	10	317±160	65±24	-19±49	55±33
Random electrode	2	300±0	6±2	-3±44	34±28
Single electrode	6	73±52	20±8	-24±32	38±21
Random electrode	3	60±0	10±6	0±42	36±27

N: No. experiments; FSCS: Fraction of functional connections with Significantly Changed Strength; $\overline{\Delta}$: mean strength change of significantly affected functional connections.; $\overline{|\Delta|}$: mean *absolute* strength change of sign. affected connections. All values are expressed as mean ± SD.

Table 1 shows that the average mean changes in both types of experiments were much smaller than the mean absolute change in the training experiments. However, the periods of stimulation were also much shorter in these experiments than during training sessions (317±160 minutes). In all types of experiments $\overline{\Delta}$ did not significantly differ from zero (t-test, p>0.1)

IV. Discussion

The applied training protocol was effective in 50% of our experiments. Possibly, the protocol was also effective in the 30% of experiments that immediately reached a stable low level. However, in these experiments, the results were more difficult to interpret because the 'learning curves' did not show any improvement. In 20% the training protocol did not reduce the number of input stimuli needed to reach the desired output. Still, in all experiments, including these unsuccessful ones, analysis of spontaneous activity before and after the protocol showed that the strength of a major part of all functional connections had been changed. This means that the training protocol always did affect network connectivity, even though it sometimes failed to induce a chosen modification.

One possible explanation for the highly varying success of the training protocol is the balance that cultured networks may develop between activity and connectivity. Because activity patterns arise from certain connectivity, and activity, in turn, influences connectivity, the finding that networks develop stable activity patterns [5, 7] may be interpreted as an established balance between activity and connectivity.

If external stimulation pushes the network out of balance, it may develop towards a new equilibrium which may or may not include the selected connection.

Thus the choice of the connection to be trained may determine the success of the protocol, but one cannot predict whether or not a selected connection will lead to a success. On average, however, we did find a significantly 'improved' response to electrical stimuli after the training protocol.

Figure 5 suggests that random stimulation (blue bar) hardly affected connectivity. However, on average, the random stimulation period lasted much shorter than the training protocol (317±160 minutes). During training sessions, stimulation was switched on for 10 minutes or less, followed by 5 minutes without stimulation. Thus, during the training protocol, stimulation was switched on between ~10% (desired response reached quickly) and 67% (desired response not reached) of the duration. To compare the effects of training sessions to those of random stimulation, we applied a 5 hour stimulation period between two spontaneous recordings. During 40% of these 5 hours, stimulation was switched on. We found that the fraction of significantly changed functional connections was far larger after the training protocol (65±24%) than after random stimulation (6±2%), see Table 1. Also, the magnitude of changes tended to be larger after the training protocol, but this difference was not significant.

Finally we compared connectivity changes after slow stimulation through a randomly varying electrode to those after slow stimulation at one selected electrode. Table 1 shows that the number of significantly changed functional connections doubled when we stimulated one single electrode. The magnitude of significant changes did not differ.

Our data suggest that slow stimuli may only change network connectivity if they are applied through the same electrode every time. It seems unlikely that the stimulus it self will have such an impact on connectivity, rather, the resulting network bursts may change connectivity. By starting the bursts at the same point every time, we may support one typical burst pattern, which may lead to a certain activity-connectivity balance. If this is true, then the changes induced by long term stimulation at one electrode will decrease with time, leading to the balanced situation. Eventually, stimulation will not lead to further changes. Stimulation at a different electrode however, would start the search for an equilibrium again. This, however, remains open for further investigation.

V. Acknowledgment

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