

Activity modulation elicited by electrical stimulation in networks of dissociated cortical neurons

Paolo Massobrio, Pieter Laurens Baljon, Alessandro Maccione, Michela Chiappalone and Sergio Martinoia

Abstract—Recent results indicate that cultures of cortical neurons exhibit large amounts of spontaneous modulation. It has even been suggested that results obtained earlier could be explained by spontaneous development, rather than to be due to the external manipulation. This stresses the importance of having detailed knowledge of how a culture responds to stimulation, in order to discern activity modulation from structural plasticity. In this paper we apply several promising techniques of electrical stimulation to describe global network modulation occurring in these preparations. The results allow to anticipate on integration of this work in goal-directed stimulus-induced plasticity.

I. INTRODUCTION

THE relationship by which activity and structure influence each other in developing networks of cortical neurons *in vitro* is the topic of intense study [1-6]. As this relationship operates on sub-cellular scales we must resort to indirect measurements from these networks to describe this relationship. However, in recent years these means to assess and probe the structure of a network through its activity have evolved rapidly [7-11].

Stimulating the culture either chemically or electrically during development affects activity and as a result, influences the connections that are formed [2, 8, 11]. In other words, carefully planned stimulation could provide a certain amount of control over the activity side of the aforementioned relationship between structure and activity. Furthermore the stimulation can be applied as a biologically plausible manipulation, as neuronal networks *in-vivo* are incessantly fed by sensory inputs [11].

In this paper we report on our recent results obtained using electrical stimulation. We study the effects of stimulation through changes in the evoked as well as spontaneous activity.

The paper concludes with placing the current descriptive work in the context of goal-directed plasticity in the sense of the network ‘learning’ as described in [12-14].

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P. Massobrio, P. L. Baljon, A. Maccione and S. Martinoia, are with the Neuroengineering and Bio-nano Technology Group, Department of Biophysical and Electronic Engineering, University of Genova, Via Opera Pia 11a, 16145, Genova, Italy. Email: paolo.massobrio@unige.it, pieter.laurens@unige.it, maccione@unige.it, martinoia@unige.it.

M. Chiappalone is with the Italian Institute of Technology (IIT), Department of Neuroscience and Brain Technology, Via Morego 30, 16163, Genova, Italy. Email: michela.chiappalone@iit.it.

II. MATERIALS AND METHODS

A. Cell cultures

Culture preparation is described in detail in [5]. Primary cortical cultures were produced from brain tissue of rats at embryonic day 18 (E18). Cells were dissociated using trituration following treatment with trypsin. Cells were then plated on Micro-Electrode Arrays –MEAs (MultiChannel Systems, Reutlingen, Germany), pretreated with adhesion factors poly-D-lysine and laminin. Glia growth was not suppressed. We maintained cells in a humidified incubator with 5% CO₂ and 95% O₂. We used a serum-free Neurobasal medium, supplemented with B27 and glutamax. Under the aforementioned conditions we were able to record stable electrophysiological signals over 4-6 weeks.

B. Data recording and pre-processing

Extracellular signals were recorded at 10kHz using a MEA60 system (MultiChannel Systems, Reutlingen, Germany). Single recordings lasted 300s. Spikes were detected off-line using a threshold-based algorithm.

For spike detection and other off-line analysis we used our software tool SpikeManager [15], which includes several tools developed in MatLab (The Mathworks, Natick, MA) for the analysis of electrophysiological data.

C. Experimental protocols

We used three main experimental protocols for electrically stimulating the cortical cultures: test stimulus, paired-pulse stimulation and frequency-sweep stimulation. Motivations and details are reported below.

1) Test Stimulus

Extracellular voltage stimulation used for testing the evoked network response was delivered from a specific electrode of the MEA. The train we used as a test stimulus consisted of 50 biphasic pulses, 250 μ s per phase, positive first, delivered at the frequency of 0.2Hz; The peak-to-peak amplitude was set at 1.5 V [16].

2) Paired-Pulse Stimulation

In order to investigate whether repetitive activation of neuronal circuits can induce changes in dissociated networks, we developed two experimental protocols based on paired-pulse stimulation [17]: trains of pulses with a delay of 125ms (8Hz paired-pulse stimulation) and a delay of 66ms (15 Hz paired-pulse stimulation). The two pulses were delivered by the same stimulating site and the time

interval between the two pulses was kept fixed for the duration of the stimulation phase.

3) Frequency-sweep stimulation

The frequency-sweep protocols were used to investigate the effect of the frequency of stimulation on the bursting activity. These protocols derive from those described in [11]. In the first protocol we stimulated one electrode subsequently at frequencies of 0.2, 0.5, 1.0 and 2.0 Hz (single-site stimulation). Stimulation recordings were interspersed with 5 minutes of spontaneous activity. For each culture, this protocol was repeated at 8 different stimulation sites. The stimulation pulse had the same characteristics as that used for the test stimulus.

In the second protocol we selected 7 electrodes for *distributed stimulation*, i.e. the selected electrodes were stimulated subsequently, yielding a network-wide stimulation frequency of 0.5, 1, 2, 4, 8 or 16 Hz. Again stimulated recordings were interspersed with spontaneous recordings. Stimulation pulses had the same characteristics as that used for the test stimulus.

D. Data analysis

1) Burst Analysis

During development cultures show a broad range of network-wide synchronized activity or *bursting* [3, 5, 18]. A burst typically contains a large number of spikes at many channels, densely packed together in time. The time between individual spikes in a burst, is generally of the order of several milliseconds. The burst itself has a duration ranging from hundreds of milliseconds to seconds [3].

The burstiness index [11] BI_{α} expresses the percentage of spikes present in the $\alpha\%$ most active time bins of a recording. We use a value $\alpha=15$ and a time resolution of 100ms.

2) Post-Stimulus Time Histogram

The Post-Stimulus Time Histogram (PSTH) [19], allows to investigate the mean response of a network to stimulation. The PSTH expresses the probability of firing as a function of time after a stimulus.

To construct a PSTH, the culture is repeatedly stimulated. The delay of spikes occurring within a given window after a stimulation is gathered and combined for all stimulations. The histogram of observed delays is the PSTH and expresses the mean response over all applied stimulations. We use a window size of 400-600ms, and a resolution of 4ms in the construction of the histogram. We use the mean area of the PSTH, averaged over all active channels, as a single measure to quantify the response to stimulation.

III. RESULTS

A. Spontaneous activity and response to stimulation

In vitro cortical networks are spontaneously active [5]. The firing rate changes during development and it is strictly related to the age of the network. We used cultures in the range of 19-35 DIV, usually showing a mix of bursting and

spiking activity (Fig.1). For our analysis we use the active electrodes, i.e. exhibiting a mean firing rate of at least 0.2Hz.

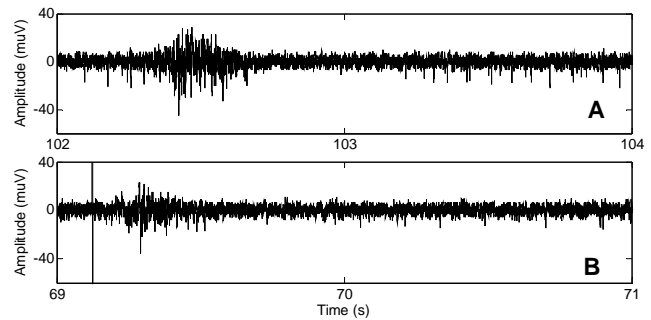


Figure 1. Both panels show 2s of activity from the same electrode. Panel A shows spontaneous activity comprising a burst in the first second, followed by a quiescent period of 0.5s and afterwards tonic firing. Panel B shows a stimulus-induced burst. The stimulus artifact is the vertical line in the leftmost part of the panel. The culture was 24DIV.

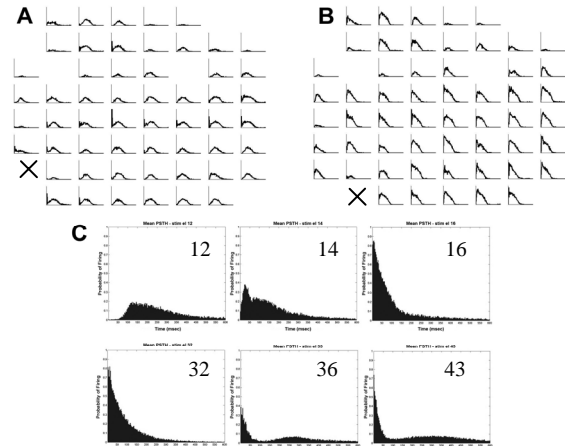


Figure 2. Post-Stimulus Time Histograms (PSTH) of the neural preparation. A, B) PSTHs for each recording site, arranged according to the actual topography of the MEA electrodes, in a typical experiment. In panels A and B, electrodes 17 and 28 are stimulated respectively (see text). The evoked response changes in both shape and magnitude when stimulation is delivered from different sites. C) Modulation of the mean responses obtained by averaging over the active electrodes in a sample experiment. Different shapes of the mean PSTH correspond to different stimulating electrodes (stimulation-electrode number in parentheses), while the same recording electrodes show a reproducible response.

By using localized extracellular voltage stimulation by a single electrode of the array, it is possible to control the rhythm of electrophysiological activity [20]. In Fig. 2 the PSTHs, obtained from signals recorded from all the array electrodes, are shown. Samples from the responding microelectrodes and occurring in the 600 ms-window after the stimulus were used to compute the PSTHs. The histograms are arranged over an 8x8 grid (the MEA layout), in two different conditions: under the stimulation from site 17 (first column, seventh row, Fig. 2A) and under the stimulation from site 28 (second column, eighth row, Fig. 2B).

The network responds to the stimulus in two different ways. In one case (Fig. 2A) the network shows primarily the

so called *delayed response*, at about 50-100ms after the stimulus. This delayed response could be interpreted as the propagation of activity through the entire network using numerous recurrent synaptic connections. By changing the stimulating site (i.e. 28 instead of 17, Fig. 2B), an *early response* is observed.

In general, in different preparations it is possible to obtain different responses, from the pure early response (the majority of the obtained responses) up to the pure delayed response, with a multitude of intermediate combinations of these two extremes. Fig. 2C shows the mean of PSTHs after stimulating 6 different electrodes. These mean network responses show different mixtures of early and late response.

B. Paired-Pulse Stimulation

Since delayed evoked responses usually appear at a time distance between 50-100 ms from the stimulus (see par. III.A), we investigated what happens when delivering a second pulse during the first or the final part of the evoked bursts.

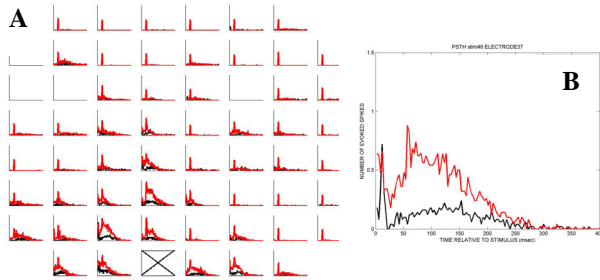


Figure 3. Facilitation with paired-pulse protocol (15Hz stimulation). A) The superimposed profiles of the PSTH 8x8 map show how the PSTH increases during application of the protocol (red trace) with respect to a single stimulation (black trace). B) Zoom of the responses obtained at one channel (37) in one specific experiment.

The stimulation with the paired-pulse protocol results in changes in activity as can be seen in Figures 3 and 4. A higher-frequency stimulation results in a facilitation of responses to the second pulse (see Fig. 3). If the delay between the pulses is increased, facilitation of the response to the second pulse is reduced (see Fig. 4).

C. Frequency-sweep stimulation

Following the approach proposed in [11], we tested the effect of high-frequency stimulation on burst activity. We quantify the effect of the two protocols presented in Sec. II.D by studying the PSTH area (normalized to the lowest-frequency response) and the burstiness index.

Fig. 5A shows the normalized area of the PSTH when varying the stimulation frequency. It is evident that for higher frequency of stimulation, the size of the response decreases. Similar results can also be shown by evaluating the burstiness index in Fig. 5B. Fig. 5C is based on the distributed-stimulation protocol, and in accordance with [11] we obtain a decrease in the burstiness level for increasing frequency of stimulation.

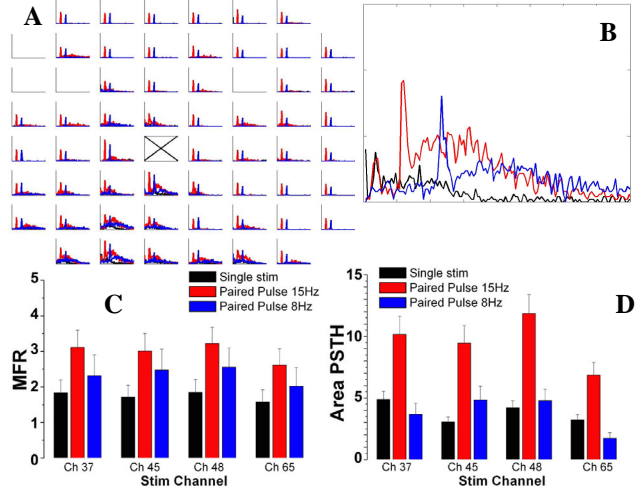


Figure 4. A,B) Facilitation and depression with paired-pulse stimulation (15Hz, red and 8Hz, blue). The profiles of the PSTH show how the low-frequency response (blue) decreases with respect to high-frequency response (red) and apparently returns to that of a single stimulation (black). Horizontal timescale is 400ms. Results are quantified by the mean firing rate (C) and mean area under the PSTH (D) over all active electrodes. Results shown are from a typical culture out of $n=4$ in the total experiment.

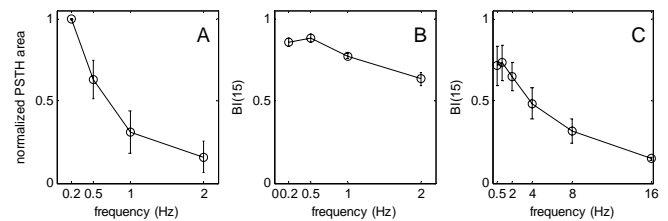


Figure 5. normalized PSTH area (A) and burstiness index (B) when a single-site protocol is applied, and (C) burstiness index in the case of distributed stimulation. Results from both protocols are based on $n=4$ cultures (8 in total). PSTH area is computed over 450ms and normalized to the response at 0.2Hz.

IV. DISCUSSION AND CONCLUSION

The results illustrate the existence of qualitatively different responses to stimulation, ranging from an early to a late response in the PSTH. Using the same descriptive language of PSTHs our results indicate the existence of a clear facilitation of responses in the protocol of paired-pulse stimulation. Since this latter protocol has been used often in attempts to induce plasticity, though primarily outside the field of MEAs, knowledge of the behavior of the culture during such a protocol is indispensable.

The frequency-sweep protocols investigated the influence of stimulating frequency on network activity. These results are relevant when designing protocols to induce changes through electrical stimulation. In particular this type of protocol can be used as background stimulation for example to suppress bursting in the culture. In that way it may also provide insight in the maturation of networks in vivo, where bursting ceases naturally, possibly caused by external sensory stimulation. We successfully replicated earlier results in reducing bursting through stimulation.

These results describe in more detail the activity in a

culture when stimulated. When inducing plasticity through electrical stimulation one tries to control the activity part in the reciprocal relationship between structure and activity as mentioned earlier. As our results clearly show, what type of activity a protocol of electrical stimulation induces is far from trivial. It depends on the location of stimulation as shown by the first protocol. Furthermore the frequency of stimulation influences the response by facilitating local responses to a stimulus arriving shortly after the first. But when stimulation persists, a global effect can be seen in the network-wide bursting behavior.

A hypothesized mechanism causing plasticity generally entails one or several particular patterns of activity, which are contrasted to a control condition, the latter often without stimulation. The results of this paper allow to better tailor a protocol of stimulation eliciting precisely that activity required for activity-induced plasticity. Moreover it allows to formulate the additional conditions controlling for the concomitant effects of stimulation.

Furthermore, though we have several methods for analysis available, it is clear that beside the need for novel protocols of stimulation able to induce plasticity, a potential gain resides in improving our ways to describe structure and plasticity. In recent papers [5, 10] several authors have tried to generalize from correlation-based metrics –such as the PSTH, and to use fewer parameters that describe more efficiently the observed phenomena.

In future work we plan to combine experimental work on stimulation with modeling techniques in order to be able to pinpoint those parameters that coincide optimally with development and changes in activity. These parameters will then form the basis for the design of experiments that aim to induce goal-directed plasticity.

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REFERENCES

[1] S. Marom and D. Eytan, "Learning in ex-vivo developing networks of cortical neurons," *Progress in Brain Research*, vol. 147, pp. 189-199, 2005.

[2] M. A. Corner, J. Van Pelt, P. S. Wolters, R. E. Baker, and R. H. Nuytink, "Physiological effects of sustained blockade of excitatory synaptic transmission on spontaneously active developing neuronal networks - an inquiry into the reciprocal linkage between intrinsic biorhythms and neuroplasticity in early ontogeny," *Neuroscience and Biobehavioral Reviews*, vol. 26, pp. 127-185, 2002.

[3] D. A. Wagenaar, J. Pine, and S. M. Potter, "An extremely rich repertoire of bursting patterns during the development of cortical cultures," *BMC Neuroscience*, vol. 7, 2006.

[4] J. Van Pelt, I. Vajda, P. S. Wolters, M. A. Corner, and G. J. A. Ramakers, "Dynamics and plasticity in developing neural networks in vitro," *Progress in Brain Research*, vol. 147, pp. 171-188, 2005.

[5] M. Chiappalone, M. Bove, A. Vato, M. Tedesco, and S. Martinoia, "Dissociated cortical networks show spontaneously

correlated activity patterns during in vitro development," *Brain Research*, vol. 1093, pp. 41-53, 2006.

[6] T. Tateno, A. Kawana, and Y. Jimbo, "Analytical characterization of spontaneous firing in networks of developing rat cultured cortical neurons," *Physical Review Letters E*, vol. 65(5 Pt 1), p. 051924, 2002.

[7] L. Berdondini, M. Chiappalone, P. D. van der Wal, K. Imfeld, N. F. de Rooij, M. Koudelka-Hep, M. Tedesco, S. Martinoia, J. van Pelt, G. Le Masson, and A. Garenne, "A microelectrode array (MEA) integrated with clustering structures for investigating in vitro neurodynamics in confined interconnected sub-populations of neurons," *Sensors and Actuators B: Chemical*, vol. 114, pp. 530-541, 2006.

[8] T. Tateno, Y. Jimbo, and H. P. C. Robinson, "Spatio-temporal cholinergic modulation in cultured networks of rat cortical neurons: evoked activity," *Neuroscience*, vol. 134, pp. 439-448, 2005.

[9] J. van Pelt and A. Schierwagen, "Morphological analysis and modeling of neuronal dendrites," *Mathematical Biosciences*, vol. 188, pp. 147-155, 2004.

[10] J. le Feber, W. L. C. Rutten, J. Stegenga, P. S. Wolters, G. J. A. Ramakers, and J. van Pelt, "Conditional firing probabilities in cultured neuronal networks: a stable underlying structure in widely varying spontaneous activity patterns," *Journal of Neural Engineering*, vol. 4, pp. 54-67, 2007.

[11] D. A. Wagenaar, R. Madhavan, J. Pine, and S. M. Potter, "Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation," *The Journal of Neuroscience*, vol. 25, pp. 680-688, January 19, 2005 2005.

[12] D. A. Wagenaar, J. Pine, and S. M. Potter, "Searching for plasticity in dissociated cortical cultures on multi-electrode arrays," *Journal of Negative Results in BioMedicine*, vol. 5, 2006.

[13] G. Shahaf and S. Marom, "Learning in networks of cortical neurons," *The Journal of Neuroscience*, vol. 21, pp. 8782-8788, 2001.

[14] Y. Jimbo, T. Tateno, and H. P. C. Robinson, "Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons," *Biophysical Journal*, vol. 76, pp. 670-678, February 1999 1999.

[15] M. Chiappalone, A. Novellino, I. Vajda, A. Vato, S. Martinoia, and J. van Pelt, "Burst detection algorithms for the analysis of spatio-temporal patterns in cortical networks of neurons," *Neurocomputing*, vol. 65-66, pp. 653-662, 2005.

[16] D. A. Wagenaar, J. Pine, and S. M. Potter, "Effective parameters for stimulation of dissociated cultures using multi-electrode arrays," *Journal of Neuroscience Methods*, vol. 138, pp. 27-37, September 30 2004.

[17] H. Markram, J. Lübke, M. Frotscher, and B. Sakmann, "Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs," *Science*, vol. 275, pp. 213-215, 1997.

[18] J. van Pelt, P. S. Wolters, M. A. Corner, W. L. C. Rutten, and G. J. A. Ramakers, "Long-term characterization of firing dynamics of spontaneous bursts in cultured neural networks," *IEEE Transactions on Biomedical Engineering*, vol. 51, pp. 2051-2062, November 2004 2004.

[19] G. L. Gerstein and N. Y. S. Kiang, "An approach to the quantitative analysis of electrophysiological data from single neurons," *Biophysical Journal*, vol. 1, pp. 15-28, 1960.

[20] H. P. C. Robinson, M. Kawahara, Y. Jimbo, K. Torimitsu, Y. Kuroda, and A. Kawana, "Periodic synchronized bursting in intracellular calcium transients elicited by low magnesium in cultured cortical neurons," *Journal of Neurophysiology*, vol. 70, pp. 1606-1616, 1993.