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Temporal and Spatial Analysis of Astrocyte calcium waves

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Abstract— In the last decade new ideas were born about the temporal and spatial dynamics of intercellular calcium waves in astrocytes. In this paper we introduce a new approach to analyze the ways in which astrocytes communicate in cultures. We present a method to describe the spatial propagation of Ca^{2+} waves *in vitro* and a technique to compare the activity of different cells *in vivo* and *in vitro* under different stimulation conditions. The proposed method resulted to be an interesting way to distinguish different astrocyte clusters, which can be related to the communication characteristics in the network.

I. INTRODUCTION

Glial cells are pivotal for communication and coordination processes of neuronal activity. In particular, Ca^{2+} waves provide for a mechanism of integration and transmission of information [1]. The activity of astrocytes, the most abundant glial cell type in CNS, is regulated through signals travelling among cells as Intercellular Calcium Waves (ICW). They consist of localized increase in cytosolic [Ca^{2+}] that is followed by a succession of similar events in wave-like fashion [2]. These Ca^{2+} waves can be restricted to one cell or transmitted to neighboring cells.

Two main pathways contribute to the spread of ICWs; the first one involves the release of neurotransmitters like ATP or UTP which activate cell membrane Ca^{2+} receptors; the second one is based on the diffusion of inositol 1,4,5-triphosphate (IP3) and other diffusive factors from a stimulated cell through intercellular gap junctions [3][4]. Intercellular Ca^{2+} signaling has been observed in hippocampal astrocyte cultures and in hippocampal slice cultures in response to chemical and mechanical stimuli [5][6][7][8]. In many works, eg. [9], it was reported that the spatial propagation of such waves is endowed with a spyral shape.

Moreover, it has been demonstrated that the passage of a wave modifies some calcium dependent mechanisms in astrocytes [2]. In some cases, the wave propagation could lead to the activation of astrocytes, thus leaving a "hallmark" that persists and modifies forthcoming astrocytic responses, setting the cellular basis for plasticity in glial cells and, perhaps, memory [10].

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In this paper we employed confocal fluoroscopy recordings of calcium activity in astrocyte networks to describe the spatial dynamics of ICW. We also studied the correlations among cells under different stimulation parameters (pre *versus* post- stimulation, vivo *versus* vitro).

II. MATERIALS AND METHODS

A. Cell culture

Calcium imaging movies from pure (or semi-pure) in-vitro cultures of primary astrocytes from P2 rat hippocampus were recorded at the Università Vita e Salute San Raffaele of Milan, Malgaroli's neurophysiology laboratory. Cultures and recordings were achieved by M.R. Primary cultures of P2 rat hippocampal were prepared as reported by others, eg. [11], with minimal modifications.

The imaging was exploited through calcium fluoroscopy, by employing the Fluo-4 AM fluorescent dye. Cultures were incubated for 15 minutes with 10μM Fluo-4 AM dissolved in Tyrode solution (Tyrode: NaCl 119mM, KCl 5mM, Epes 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2mM and Glucose 6gr./liter.); 0.01% Pluronic acyd f-127 was added to increase dye permeability. After incubation cells were washed out for 5 minutes with Tyrode solution. During recording cells were maintained in Tyroide in 100% O_2, 24°C. During stimulation protocols, in zero-calcium extracellular conditions, a single astrocyte was excited by administration of Ionomycin 1μM (in Tyrode) [12] provided directly on the cell membrane through a micropipette (diameter 6μm).

We performed 15 recordings with a Zeiss LSM 510 confocal microscope and by employing a Zeiss FITC Narrow Band Laser with central excitation band 488nm (BP 505-530 nm), nominal current 3.1A, used at the 25% of its nominal power. Acquisitions were performed by using a 40x Zeiss Objective, NA 0.8 in Water.

The field of view of each recording was a square of 1302.7μm side, scanned by the laser light (pinhole 280μm) in 1.5sec with gain = 904 by obtaining a 512 x 512 output matrix at 12 bit. The total acquisition time was 360sec, for a grand total of 240 frames. All cultures were recorded both in basal and in after-stimulus conditions. This resulted to be necessary to compare cells activity before and after stimulation.

Images were analyzed and processed with ImageJ® and Matlab® software.

B. Spatial Analysis Procedure

We analyzed 15 240-frame movies reporting calcium activity after Ionomycin stimulation. Our primary aim was the study of ICW spatial propagation dynamics, looking for curvilinear and spiral wavefronts. As first step we calculated the $\Delta F/F_0$ of all the temporal slices [13]. Then, we

segmented and identified the cells by applying a threshold-based approach on: 1) pixels intensity; 2) presumed cells area range; and 3) circularity. The threshold values were trained on the recordings, in order to maximize the number of recognized cells. In the definition of area and circularity, in particular, we referred to values reported in literature [14], while intensity threshold was settled empirically. This protocol of analysis was used for 15 recordings. In this way, we were able to recognize and count the number of cells activated in each frame.

Our purpose was to follow the spatial propagation of the wave frame-by-frame. In order to achieve that, we implemented an algorithm which creates a movie reporting only the new cells activated in the upcoming frame but preserving the cells activated during the previous slices. Fig. 1 shows the algorithm used to follow cells activation and to define dimension and shape of the ICW.

Finally, we merged the obtained result to the original image, as fig. 2 exemplifies.

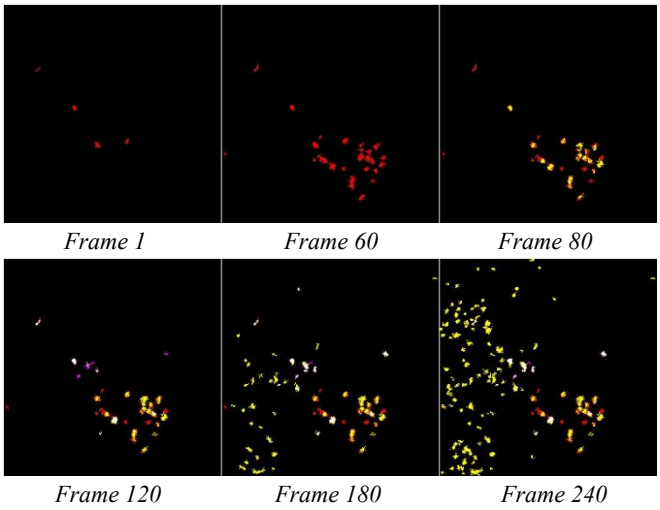

Fig.1 Example of the result obtained after using the algorithm described in the paper. Frame by frame all the new cells activated are added to the cells activated during the previous frames. The new cells activated are highlighted in different colors to improve the visualization (512 x 512, 8 bit, acquisition time:1.5sec).

C. Temporal Analysis Procedure

For each cell the fluorescence signal was quantified by measuring the mean pixel value of a manually selected area corresponding to an astrocyte. This was done for each frame of the image stack using ImageJ® software. We calculated cross correlation between the tracks obtained from the more visible cells in the culture. After that we computed the maximum value of correlation:

$$Peak = \max_k \left| \frac{1}{N-k-1} \cdot \sum_{n=0}^{N-k-1} cell_i(n) \cdot cell_j(n-k) \right|, \quad k = 0 \div N-1$$

where N is the number of frames in the movie.

We repeated this procedure for movies acquired under different conditions, comparing the correlation of couple of cells before and after the Ionomycin stimulation and the differences between *in vivo* and *in vitro* behavior. In the latter case we concentrated on the astrocytal basal activity.

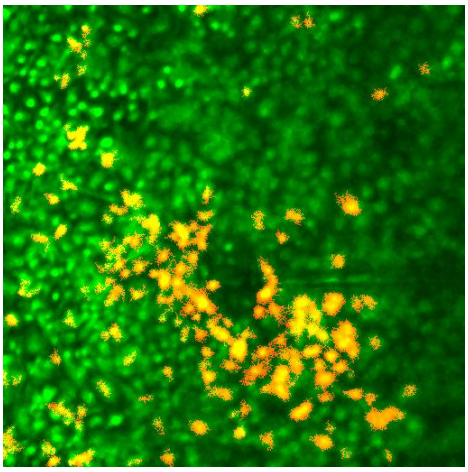

Fig.2: The segmentation of cells is added to the original image.

In order to compare our *in vitro* results to *in vivo* activity, we exploited the *in vivo* recordings kindly provided as supporting material by [15]. The main difference between our acquisition method and the one supplied by Hirase et al. is the length of the acquisition (2400 frames at 2 Hz *versus* 240 frames at 1/1.5 Hz), while the culture extent is comparable (1200 µm for both). Thus, to make possible a comparative analysis, we took into consideration only 240 temporal slices of the *in vivo* movie, spaced 1 second each other.

III. RESULTS

A. Spatial propagation

Fig. 3 shows the typical propagation of ICW in an astrocyte culture after Ionomycin stimulation. The red spot represents to point of puff application, while the red arrows represent the wave propagation. As expected, it is possible to observe a spiral propagation of the ICW.

B. Temporal analysis

We calculated the absolute value of the correlation peaks between couples of cells and then we averaged those peak values for all the cells within the culture.

By analyzing basal activity *in vitro*, we found average correlation peaks value of 0.187 ± 0.026 (avg ± std) between cells. Thus, our *in vitro* recordings showed absolutely no correlation among cells.

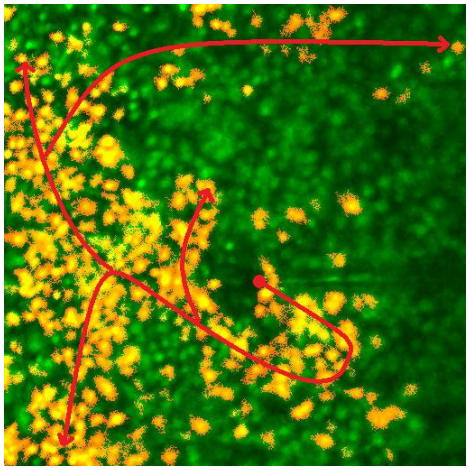

Fig 3: Typical spiral shape of calcium wave propagation in astrocyte culture (RGB image, 512x512 pixels, 12 bit).

On the other hand, the values we observed *in vivo* were completely different. The average correlation peak value resulted 0.413 ± 0.084 (avg ± std). So, even without stimulation, we could find high correlation levels among cells, with peaks of correlation reaching 0.7.

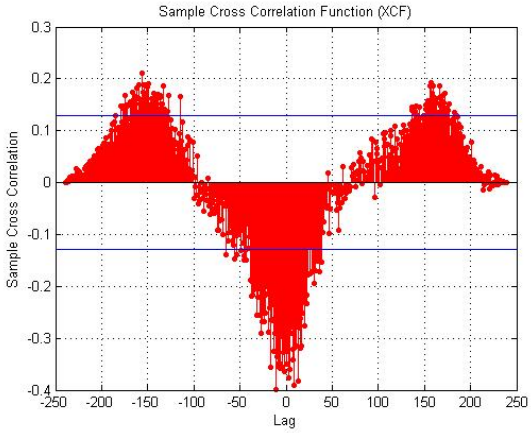

Fig 4: Example of negative high correlation among a couple of cells in basal conditions (without stimulation).

In the comparison between pre and post-stimulation correlation (*in vitro*) we found no correlation among cells in pre-stimulation epoch (avg ± std: 0.2329 ± 0.0095). In post–stimulation conditions we found correlation peaks over 0.5 (avg ± std: 0.3846 ± 0.08629) (Fig.5). Nevertheless, synchronization events did not interest all the cells within the culture, but small clusters of them only.

IV. DISCUSSION

Through spatial analysis we followed ICW propagation in order to verify cell activation dynamics in astrocyte cultures. We observed typical spiral shape as literature reports [9]. This kind of propagation was evident in most of the recordings acquired, mainly in the most mature cultures.

As for the temporal analysis we were able to identify an indicator which highlights the differences between *in vivo* and *in vitro* behavior. In every movie obtained *in vitro* we couldn't find any sign of correlation in basal conditions, while the correlation value is high in *in vivo* images stack. The cultures we recorded have been deprived of the neuronal counter-part of astrocytes. Moreover, the lack in the physiological cell morphology and gap junctions distribution could be the explanation of why astrocytes seem to work in total asynchronous way. As postulated by [1], the spread of ICW leaves a 'hallmark' which influences astrocytes activity. This is probably the reason why we found peaks of correlations after the Ionomicyn stimulation. But the absence of neurons in the network or the immature state of the cultures may limit the correlation to small number of cells, belonging to the same cluster.

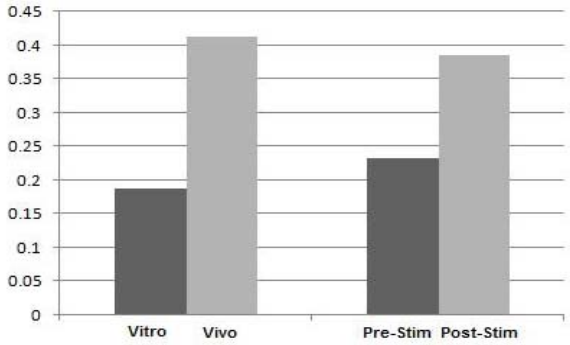

Fig. 5: Average level of cross-correlation peaks under different stimulation conditions. This picture summarize the results obtained through the temporal analysis. As represented, average level of cross correlation peak is low in in-vitro recordings. On the contrary, we obtained high peaks of correlation in vivo. In the same way, we found low level of correlation in pre-stimulation epoch and correlation level after stimulation.

The analysis of correlation peaks resulted a way to classify neurons within the network in post stimulation *in vitro* recordings as well as in *in vivo* recordings. It was evident that cells belonging to different clusters were characterized by low correlation levels. Otherwise cells communicating each other showed correlation levels over the average value of the network. This classification of clusters is not spatially evident. Cells communicating together are not always close each other and could be on opposite sites on the culture. We also tried to plot the correlation peaks versus the distance between cells: we found that there is not are not direct links between distance and correlation.

We also found that correlation peaks could be positive or negative. As showed in Fig. 4, when the peak is negative there are also two positive peaks of lower value (and *vice-versa*). This means that cells oscillates in anti-phase, as [16] reported. The mechanism controlling the phase locking

among cells needs still a further and deeper investigation.

V. CONCLUSIONS

The results presented in this work suggest a method for the analysis of statistical characteristics of astrocytes network activity. Particularly significant is the temporal analysis of cultures.

Indeed we exploited the values of correlation peaks to distinguish different clusters of cells within the network. This kind of approach resulted a simple way to prove the different behavior showed by atrocytes between *in vitro* and *in vivo* recordings. We found small correlation peaks in the first case, demonstration of slow 'talking' level between astrocytes, while high correlation levels were evident in the second case, suggesting strong coupling *in vivo.*

Moreover, the proposed method introduced confirmed well known theories about the influence of calcium waves in the basal activity of cells. Indeed, the spread of calcium waves induced a synchronization which was maintained for several minutes after the stimulation. On the contrary, no sign of correlation was present in any of the recordings before stimulation.

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VII. REFERENCES

[1] A. Iacobas, O. Suadicani, C. Spray, E. Scemes, "A Stochastic Two-Dimensional Model of Intercellular Ca^{2+} Wave Spread in Glia", *Biophysical Journal*, Volume 90 January 2006 24–41.

[2] E. Scemes , C. Giaume, "Astrocyte Calcium Waves: What they are and what they do", *Glia*. 2006 November 15; 54(7): 716–725. M. J.

[3] Berridge, "Inositol phosphate and calcium signalling," *Nature*, vol. 361, pp. 315-325*, 1993.*

[4] R. Ashworth, S. R. Bolsover, "Spontaneous activity-independent intracellular calcium signals in the developing spinal cord of the zebrafish embryo", *Developmental Brain Research* 139 (2002) 131–137.

[5] A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper and S. J. Smith, "Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling," *Science*, vol. 247, pp. 470-473, 1990.

[6] A. C. Charles, J. E. Merrill, E. R. Dirksen and M. J. Sanderson, "Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate," *Neuron*, vol. 6, pp. 983-992, 1991.

[7] A. H. Cornell-Bell and S. M. Finkbeiner, "Ca2+ waves in astrocytes," *Cell Calcium*, vol. 12, pp. 185-204, Feb-Mar. 1991.

[8] M. Falcke, D. Malchov, "Understanding Calcium Dynamics", Ed. Springer, 2003.

[9] M. E. Harris-White, S. A. Zanotti, S. A. Frautschy, A. C. Charles, "Spiral Intercellular Calcium Waves in Hippocampal Slice Cultures", *J Neurophysiol* 79:1045-1052, 1998.

[10] L. Pasti, T. Pozzan and G. Carmignoto, "Long-lasting Changes of Calcium Oscillations in Astrocytes," *J. Biol. Chem.*, vol. 270, pp. 15203-15210, 1995.

[11] K. D. McCarthy and L. M. Partlow, "Preparation of pure neuronal and non-neuronal cultures from embryonic chick sympathetic ganglia: a new method based on both differential cell adhesiveness and the formation of homotypic neuronal aggregates", *Brain Res.,* vol. 114, pp. 391-414, Sep 24. 1976.

[12] D. Thomas and M. R. Hanley, "Pharmacological Tools for Perturbing Intracellular Calcium Storage," *Methods Cell Biol.*, pp. 65-65, 1994.

[13] Q. Wu, F. Merchant, K. R. Castleman, *Microscope Image Processing*, AP, 2008.

[14] F. Esposti, "Analysis of the long-term memory characteristics of neuronal activity", PhD thesis, 2009, Politecnico di Milano.

[15] H. Hirase, L. Qian, P.Barthò, G. Buzsàki, "Calcium Dynamics of Cortical Astrocytic Networks in Vivo", *Plos Biology,* Volume 2, Issue 4, April 2004.

[16] T. Hofer, L. Venance and C. Giaume "Control and Plasticity of Intercellular Calcium Waves in Astrocytes:A Modeling Approach", *The Journal of Neuroscience*, June 15, 2002, *22*(12):4850–4859.