Initiation and propagation of activity in neuronal cultures under connectivity alterations

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Abstract: Activity in living neuronal networks is governed by complex laws that are still poorly understood. To advance in this quest, we study the initiation and propagation of activity fronts in *in vitro* neuronal networks. We consider two major experiments in which we modify the connectivity of the network. In a first one, we physically cut a region of the culture. I a second one we apply a drug that changes the connectivity of the network. In both experiments, we determine the velocity of the activity fronts, their timing and the point of origin. We frame our results in the context of the *noise focusing* phenomena, a mechanism that explains the initiation of activity in terms of network connectivity, neuronal dynamics, and amplification of noise.

I. INTRODUCTION

Living neuronal networks are high complex systems whose dynamics is controlled by a large number of variables, most notably neuronal intrinsic activity, network connectivity and noise. These variables often shape *spontaneous activity*, i.e. the activation of the network without any external trigger. A pivotal example is the brain *resting state*, in which rich, spontaneous network dynamics appears without the need of an explicit task to drive the brain [1]. Given the importance of this spontaneous activity, neurons *in vitro* have emerged as one of the techniques to understand it. Neuronal cultures simplify the system and allow perturbations on them [2], which is not possible in the brain. An important feature of these cultures is that they exhibit excitatory and inhibitory connections. The first ones increase the membrane potential over the resting state, while the second ones reduce it. The balance between these two types shape the frequency of activity.

Spontaneous activity is characterized by a near–periodic activation patterns, in the form of collective neuronal firings termed *bursts*. The mechanism behind the initiation and maintenance of this activity are not completely understood, but is known that neuronal properties and network connectivity are involved [2–4]. It has been seen that there are regions of the culture that are more likely to start activity, identifying *burst initiation areas*. There is an interplay of neuronal dynamics, connectivity and *noise* (small, sporadic neuronal activations), making some regions optimal to nucleate a burst and propagate it through the system.

The *noise focusing* mechanism (a concept that Dr. Soriano and Prof. Casademunt developed) describes the behavior of noise amplification. The concept is explained as the amplification of noise through the network, leading to an implosive concentration of activity in a region (due to inputs from others cells). The amplification of the noise is so strong that is able to reach the threshold value to excite several neurons, resulting in an *ignition avalanche* that shapes the burst. The region with a confluence of noise–amplified paths is the burst initiation zone [5].
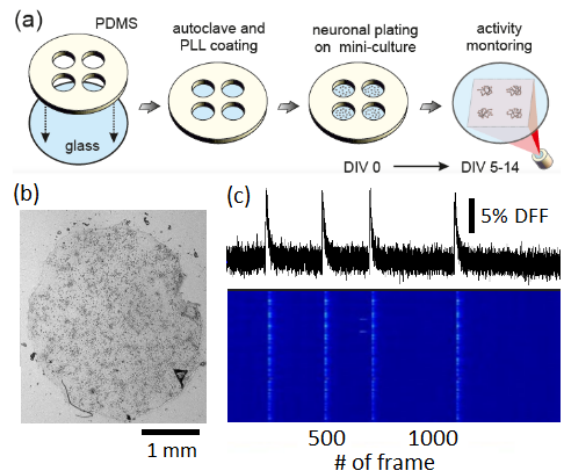

FIG. 1: (a) Sketch of the preparation of 4 mini cultures for imaging. (b) Typical homogeneous culture. (c) Fluorescence trace of a neuron and network overall behavior (raster plot). Bright areas correspond to an increment of the fluorescence due to bursts, showing that there are neurons with higher amplitudes in their peaks than others. Almost the entire population participates in the bursting episode.

II. EXPERIMENTAL SETUP

Neuronal cultures were prepared by Dr. Soriano' s group. Cortices from rat embryonic tissues of $18 - 19$ days of development were dissected and neurons plated on glass [6]. The glass substrate incorporated a perforated poly-dimethylsiloxane (PDMS) mask [see Fig.1(a)] that contained a set of 4 circular holes of 3 mm in diameter, limiting the area of neuronal growth to small regions. The combined PDMS-glass structure effectively provided 4 mini cultures to work.

The chemical properties of the substrate can create two

possible neuronal cultures: homogeneous ones if it contains adhesive proteins, or aggregated ones if the medium lacks these proteins, resulting in a natural aggregation of neurons. Our experiments are homogeneous cultures [see Fig.1(b)], although one of them have some aggregates inside.

Activity was monitored through high–speed, single–cell resolution calcium fluorescence imaging. When a neuron fires, there is an intake of calcium, which changes the intracellular calcium concentration of the cell. These changes are detected through genetically–encoded fluorescent calcium indicators present in the neurons. Spontaneous activity was recorded by a fluorescence camera as image sequences of $30-60$ minutes in duration [Fig.1(c), top] . In the present study I analyzed the images obtained from experiments, that were processed to obtain fluorescence intensity of 1000 individual neurons as a function of time [Fig.1(c), bottom]. The data provided was useful to reconstruct the neuron's ignition sequence, the propagation velocity of the activity avalanche (burst), and finally its nucleation center.

Two major experiments shape the study, namely a *cutting protocol* and *inhibition–blockade* perturbation. For the first one, a mature culture at *day in vitro* (DIV) 12, with both excitation and inhibition ('E+I') active, was first recorded for 30 min at 83.3 frames/s, then a small section of the culture was removed, and activity recorded again. For the second one, a DIV 9 culture in 'E+I' conditions was recorded for 40 min at 66.6 frames/s, then the inhibitory synapses were blocked with 40μM bicuculline, a GABA$_A$ receptor antagonist, and activity with solely excitatory neurons measured again for 60 min.

III. PROCEDURE & DATA ANALYSIS

Our goal is to characterize if there is a privileged region were activity nucleates thanks to the interplay of connectivity, dynamics and noise. Our efforts start by analyzing the videos of the experiments and obtain useful data.

The original video of the experiment was separated as a train of images (frames) which are loaded onto a program developed by Dr. Soriano's group called NeuroImages. First, we have to mark manually each neuron as a squared region of interest (ROI) with a typical lateral size of 4 pixels (about $34\mu m$). Since this task is difficult, we took the alternative solution of placing a grid of ROIs over the images, with a total of about 1000 ROIs.

Fluorescence intensity traces $F(t)$ were then be obtained with this program, analyzing the average gray level in each ROI along the entire acquired image sequence, finally providing the intensity for each neuron as a function of time or frame number [Fig.1(c)].

This data is the core of our analysis, which allows us to identify what neuron (or region) was the first one to be activated, and the velocity of the burst. To have an overview of all the traces and to get the average signal of the culture, a provided Matlab program was used, identi-

fying the best firing neurons of the system, being a valuable information, as we can see in Fig.1(c). In this step, fluorescence trace was normalized for each neuron to correct its background brightness level as

$$\widetilde{F}(t) = \frac{F(t) - F_0}{F_0} = \frac{\Delta F}{F_0} \times 100 \qquad (1)$$

where F_0 is the average amplitude of the background fluorescence signal at rest. $\widetilde{F}(t)$ is expressed as DFF(%).

The data from NeuroImages was split into smaller files to make it more manageable, due to the number of ROIS ($\simeq$1000) and the large number of frames. These files were loaded onto another provided program called Neurodynamics, which analyzes all observed bursts and, for each one, extracts the time of activation of all neuron participating in it.
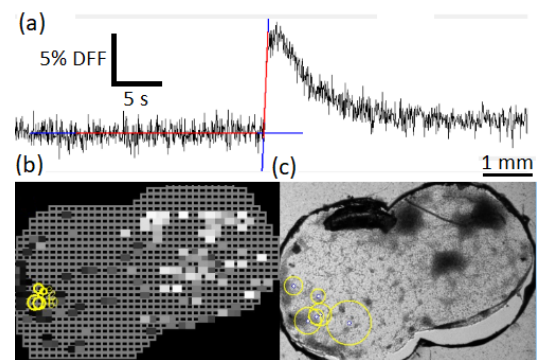

FIG. 2: Data analysis. (a) Analysis of the fluorescence trace of a representative neuron in NeuroDynamics. Linear fits are shown on the pre–peak noise activity (zero slope), and on the growth of fluorescence upon activity. These fits are the two important data which uses the program to identify the onset of activity as the crossing of the lines. (b) Display of the network that we worked with in Neurodynamics. Each square is a ROI with a linked fluorescence trace. Firing ROIs are shown in black–to–white squares, where black are the ones where the avalanche starts and white where it ends. In this image the wave goes from left to right. Yellow circles are the regions where the program computes the nuclei of activity initiation, and taking in account different quantities of ROIs. (c) Location in the neuronal culture of the identified bursts. The position of the nucleation points is the center of the yellow circle, and the radius is the error in their determination.

A small sketch of the process in Neurodynamics is as follows. For simplicity, we took the approximation that all ROIs represent neurons, although some ROIs will be just background brightness. We first selected a good firing neuron with the extracted information of the Matlab program, and we analyzed each peak of the trace [see Fig.2(a)]. For this, we set two fitting lines, a first one with zero slope for the pre–peak noisy area, and a second one shaping the growth of fluorescence during the activation of the neuron. The crossing point sets the *activation time* for the selected neuron. When this is done,

the program identifies with different algorithms the other neurons that participated in the same burst and retrieves the information. The final outcome is the spatial location of the initiation of the burst, its velocity, and the direction of propagation [see Fig.2(b)]. We could play with the correlation and slope threshold percentage to suppress or add some firing neurons to help the program to arrive to an acceptable solution. For better results, the program calculates the nucleation zone taking in account the first group of firing neurons (with 1, 2, 3, 5, 10 ROIs). We accept a burst when the circles of the different calculations are in the same small region, although sometimes the program could not find the nucleation zone with the precision of Fig.2(b). Each accepted burst is registered in a data text with its location in the culture and the velocity in mm/s, as we can see graphically in Fig.2(c). The error of the final location of a burst can be imagined as a small gaussian distribution in all directions, due to this variation when the program takes in account different groups of initially firing neurons.

The time between bursts, or *inter–burst interval* (IBI) was also obtained, and was computed by identifying the frame in which a burst occurred and converting it into seconds. To compare results before and after a given perturbation, we calculated the mean μ and the standard deviation σ of the IBIs.

IV.   RESULTS & DISCUSSION

Here we expose the final results for the two types of experiments. In each experiment we cover different perspectives to understand which variables affect more prominently the noise focusing effect. We note that, for the cutting experiment, we physically alter the connectivity between neurons. For the inhibition–blocking one, we modify the dynamics by silencing the inhibitory neurons.

A.   Cutting experiment

The data is shown in Fig.3. We separated the data in two groups, corresponding to fast propagation and slow propagation, and build a map showing the location of burst initiation and the neuronal density (as grey–scale image). Before the cut, we can observe that, although the localization of the avalanches do not always occur in the same place, there is a zone of nucleation that have more activations than the others, located at the center–bottom part of the culture. There are also activations at the center–left zone, but very minor. The velocity and density of neurons seem to have a weak correlation. One would expect that a high density would favor more connections and a higher velocity, but it is not clear from the data.

After the cut, it is evident a big change in the behavior of the nucleation in the system. All the initiations of

the lower part have been moved to the upper–left corner of the culture, where before the cut we had very few initiations. The fact that the cut on the right of the culture affect the initiation on the left (about 2.5 mm apart from each other) indicate that burst initiation is strongly influenced by long–range connections, i.e. it is not a local phenomena. This long–range connections most likely help to amplify and transport small activations from distant neurons onto the nucleation area. We note the length of an axon is up to 1.1 mm, a third part of the diameter of the system. Hence, long-range connections are crucial for having nucleation in an area. By simply removing a small piece of the culture, those neurons lost a big number of connections, so that they do not get enough inputs to fire in the first place.
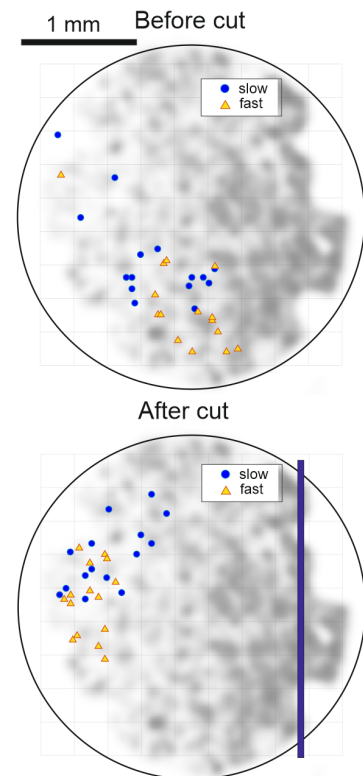

FIG. 3: Cutting experiment. Nucleation points obtained with Neurodynamics after the process in Fig.2. For better visualization, only the 15 fastest and 15 slowest activations in each condition are marked.

Around the area where there was fast bursts before the cut, now we have a region where most of the fast and slow bursts are concentrated. In addition, we have 5 fast bursts in the lower part of the nucleation area, with good density of neurons, but in the upper part we also have high density of neurons but with slow bursts. Thus, with this experiment we can not conclude that the higher the density, the higher the speed.

Fig.4(a) shows the overall dynamics of the system before and after the cut. We can observe that before the cut, the bursting and the amplitude of the peaks is mostly

regular. After the cut, overall activity decreases, and amplitude of peaks vary.

These change in dynamics can be analyzed with the histograms of Figs.4(b,c). The system without the cut follows a moderately Gaussian dependence on velocity, until it is distorted with the cut, breaking this dependence, although if we look at the means, the system more or less follows the same behavior in the velocity, even though its dispersion is greater. The same happens with the inter–burst intervals. If we only analyze the histogram, we obtain that they follow the same behavior with some long intervals without activations.
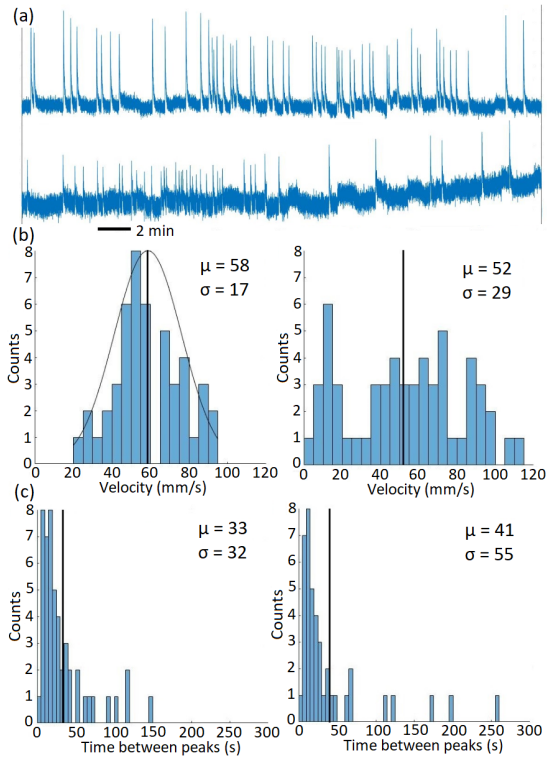

FIG. 4: Cutting experiment. (a) Comparison of fluorescence signal from 19 min recording of spontaneous activity before (top) and after cut (bottom). (b) Comparison of histograms of the velocity before (left) and after (right). (c) Corresponding histograms of the inter–burst intervals (IBIs).

B.   Inhibition–blockade experiment

Here we analyze the phenomenon of noise focusing from another perspective, namely the dynamics of the system when inhibitory synapses are affected. This neuronal culture [see Fig.5(a)] is larger than the previous one, with a length of about 5 mm, so we have many more neurons involved, although we have 3 large neuronal aggregates on the top.

In the first part of the experiment, which corresponds to measures with both excitation and inhibition active, we can visualize that there is a concentration of burst ini-

tiation areas at the center–right of the culture, although we do not achieve a moderately punctual place. We cannot discern any difference between high and low speeds. Interestingly, the aggregates do not seem to facilitate nucleation, since the area with more initiations is precisely where there is uniformity of neurons. This indicates that neuronal density (and density of connections) itself is not sufficient to explain nucleation.
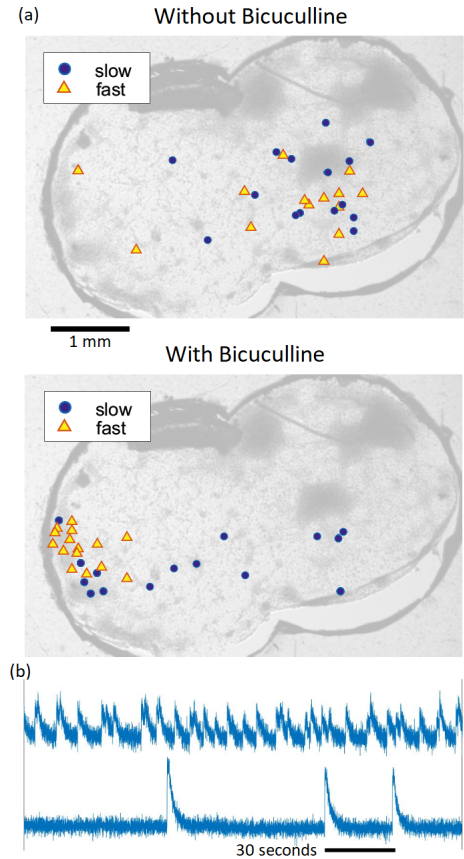

FIG. 5: Bicuculine experiments. (a) Nucleation points obtained with Neurodynamics after the process in Fig.2, in a neuronal culture of 5 mm long. For better visualization, only the 15 fastest and 15 slowest in each phase are marked. (b) Fluorescence traces of (E+I) cultures (top) and E cultures (bottom trace) for 3 min.

After blockade of inhibition, i.e. with only excitatory neurons active, we visualize an important change in the location of the activations, which are mostly localized on the left side of the system. Most of the high speed fronts concentrate here. We observe a much higher variability for the slow velocities. We could say that the neutralization of the inhibitory neurons affects much more the high speed activations. A possible hypothesis to these results could be that, on the left side of the culture, there was a greater amount of inhibitory neurons, which difficult the arrival of sufficient inputs for the neurons to fire. Hence, before the silencing of inhibition, at the central–right area there were more possibilities to reach sooner

the threshold value of -30 mV to fire.

By analyzing the dynamics with Fig.6(a), we can first observe that velocity increases by a factor 2 when inhibition is blocked. Since the blockade of inhibition effectively increases connectivity, this result suggests a relation between connectivity and velocity, although more experiments would be necessary. On the other hand, Fig.6(b) shows that the system is initially very active and fires periodically, at intervals that are around 7 seconds on average. Once the inhibitory neurons are suppressed, the system fires approximately 7.4 times less often than at the beginning.

These results indicates that the presence of inhibition is important to help a system to fire more often. Based on the literature [5], we hypothesize that the inhibitory neurons play a major role to remain the neuronal culture healthy, stabilizing it so it can shoot more often. The disappearance of inhibition causes a large discharge, making necessary a long time to rest and re-shoot. A bit of inhibition helps regulating the system, since the recovery time is much faster and therefore the system can fire more often.
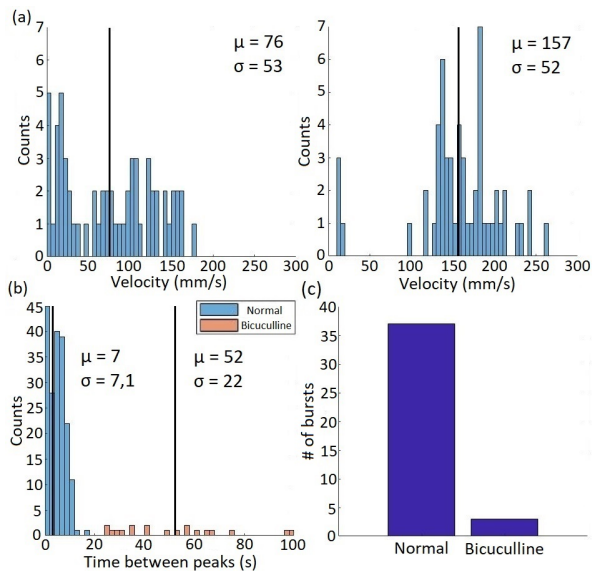

FIG. 6: Bicuculine experiments. (a) Velocity histograms. 'E+I' on the left, and 'E–only' on the right (b) Comparison of histograms of the time between peaks. (c) Comparison of the number of bursts in each condition in 3 min.

V. CONCLUSIONS

With the results and discussions exposed in this project, we can conclude that role of connectivity in dynamics is rich and complex. The combination of short and long–range connections, together with excitation and inhibition, shapes in grand manner the dynamical traits of the network. The cocktail of variables is complex, making it difficult to understand with our present knowledge, even though there have been major steps in this direction. The mechanism of noise focusing [5] indeed helped us to figure out at least that connectivity, activity and noise have a non–trivial interplay.

More experiments with cultures of the same and different shapes are needed to have enough statistics to fully understand the interplay of the above agents, compacting the new experiments in richer configurations (homogeneous/aggregated networks) and behaviors ('E+I' versus 'E', and even different levels of 'E') and, step by step, including more complex architectures. Furthermore, simulations *in silico* will be very important, providing a direct comparison with experiments. Simulations indeed, such as in [5] would provide additional information of the system, such the relative importance of the different actors at play to shape activity, or the impact of complex architectures beyond the homogeneous case.

[1] Deco, G., Jirsa, V.K., McIntosh, A.R., Trends in Neurosciences **36**, 268-274 (2013).
[2] Soriano, J., Casademunt, J. *"Cultius neuronals: un sistema model per entedre la complexitat del cervell"*. Revista de Física (2015). Doi: https://dx.doi.org/10.2436/20.2001.01.11
[3] Stetter, O., Battaglia, D., Soriano, J., Geisel, T. PLoS Comput Biol **8**, e1002653 (2012).
[4] Teller, S., Granell, C., De Domenico, M., Soriano, J, Gómez, S., *et al.*, PLoS Comput Biol **10**, e1003796 (2014).
[5] Orlandi, J. G., Soriano, J., Alvarez-Lacalle, E., Teller, S., and Casademunt, J., Nat Phys **9** 582–590 (2013).
[6] Tibau, E., Valencia, M., Soriano, J. Front. Neur. Circ. **7**, article 199 (2013).