Recovery after focal damage in neuronal cultures

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Abstract: The aim of this thesis is to investigate the impact of localized laser ablation on the activity in neuronal cultures. We analyzed neuronal network's behaviour before and after the ablation, centering the study in a time window of about 10 minutes after damage. The results show that the global activity and functional connectivity decrease after the applied damage, together with the emergence of functional communities that indicate a fragmentation of the network. In addition, it seems that new functional links appear in the neighborhood of the targeted area, suggesting the strengthening of the surviving connections and hints at the fight of the culture against damage. The results could be interesting to model damage in the brain.

I. INTRODUCTION

Damage and recovery in neuronal networks are quite well understood phenomena from a biological point of view. In ictus, for instance, it is known that the damage in the brain is swiftly followed by response mechanisms that isolate the damaged region, prevent a cascade of neuronal death, and stimulate the damage locus to recover its function as soon as possible [1]. However, despite the profound neurophysiological knowledge, the study of focal damage in neuronal circuits within the framework of network theory is still an open question. Here, we aim to delve into this question by studying focal damage in neuronal cultures delivered through a laser pulse.

By comparing experiments with synthetic networks with similar wiring patterns, our experiments fall within the category of 'failure' or 'random attack', i.e, the deletion of an arbitrary node. This action contrasts with the removal of a central or important node, e.g., a node with many connections, what is called 'targeted attack' [2]. Much more nodes are required to silence a network in 'random attack' than in the targeted one, revealing that it is a less aggressive damage procedure. Thus, implementing this randomized damage to a node in our experiments, we can mimic the damage experienced by brain circuits under lesions or degenerative diseases.

Considering neuronal cultures *in vitro* help us to understand how connectivity arises between neurons in a control manner, without realizing *in vivo* experiments that reveals us poor and only qualitative information. In this way, the structure and dynamics of the neuronal cultures can be controlled in real time, providing us useful quantitative insights into the collective functioning of neuronal assemblies. It should be mentioned that network's theory is highly useful to understand how the culture restores the loss functions and in which timescale. Such timescales could be useful in studies at the scale of the human brain, to understand it better upon injury.

II. EXPERIMENTAL SETUP & METHODS

A. Neuronal cultures and procedure

Embryonic brains were dissected from Sprague-Dawley rat embryos at 18-19 days of development and cortical neurons dissociated by pipetting. Then, they were suspended in a polydimethylsiloxane (PDMS) mold with four cavities each one about 3.5 mm in diameter and 4 mm deep, containing the appropriate culture medium [3]. Neurons settled at the bottom of the cavities, forming compact aggregates (*clusters*) connected to one another (Fig 1A).

Cultures were prepared in Dr. Soriano's lab and recorded at the Institute of Photonic Sciences (ICFO). First, a pair of cavities with a comparable number of clusters and activity were selected. One of them was designated as *control* (undamaged culture), and the other as a *target culture* to study damage. These twin recordings were motivated to correct the data of the damaged culture if necessary, for instance due to temperature or other experimental manipulations different from damage [4].

A cluster in the 'target' culture was attacked with an ultra–short, near–infrared pulsed beam, killing all its neurons and disconnecting it from the rest of the network [4]. Activity in cultures was recorded for 30 min before and after damage. Most of the interesting results presented here are focused on a time window of 10 min after damage, to observe the recovery of the network.

B. Calcium fluorescence imaging

We monitored activity in cultures with fluorescence calcium imaging. We used Fluo-4-AM, a calcium indicator which consists in molecules that change in shape upon Ca^{2+} binding, becoming fluorescent. In neurons, electrical activity is always accompanied by an influx of Ca^{2+} ions. As Calcium is permanently present in neurons we can follow the routing of information flow, before and after the damage. As calcium fluxes are tightly linked

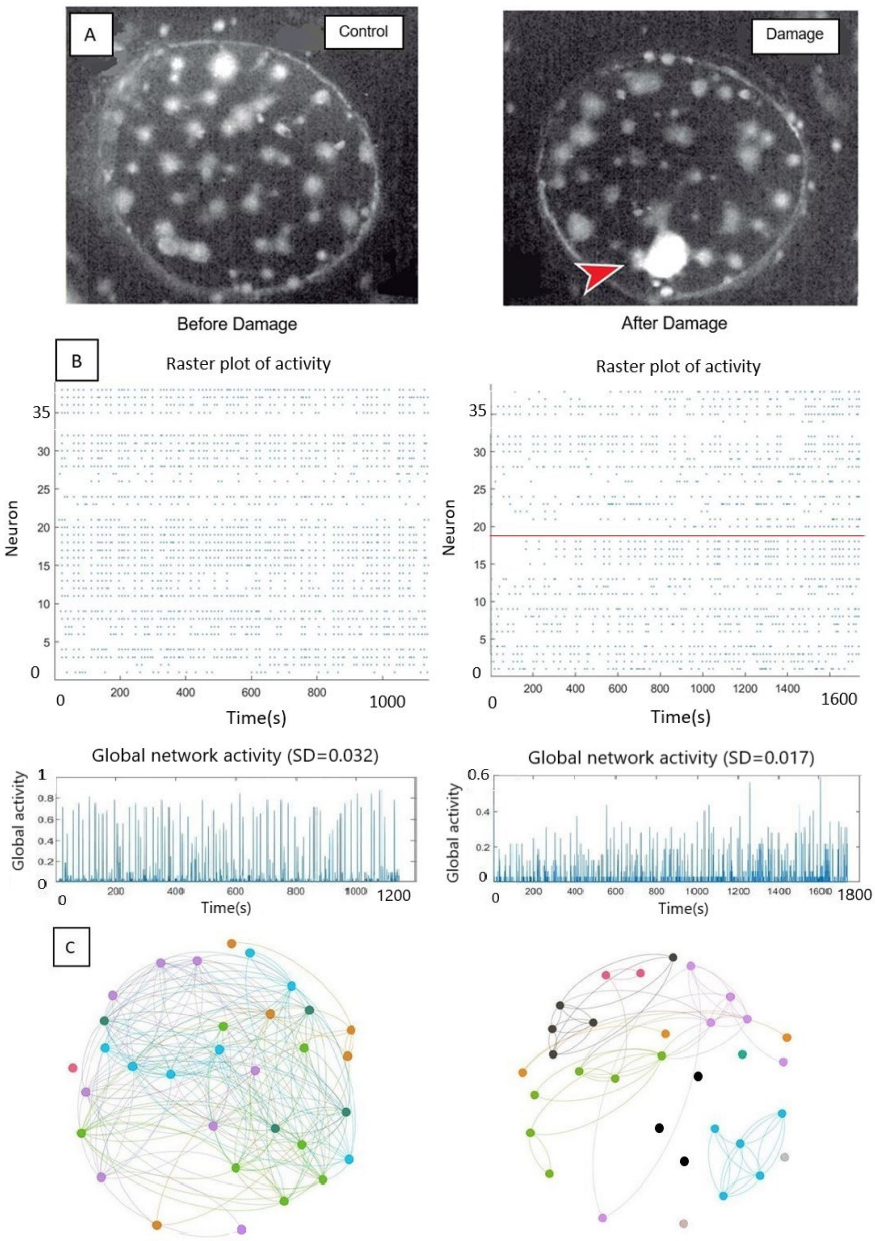

FIG. 1: **A**: Representative fluorescence images of the recordings. On the left, a typical control; on the right, a culture to be targeted. Red arrow marks the killed cluster. **B**: Corresponding raster plots of spontaneous activity for the targeted culture, before (left) and after (right) damage. The global activity of the network is shown on the bottom of the raster plots. These one's illustrate that, before damage, activity is rich and most of the clusters fire synchronously, while after damage the raster's activity substantially decreases. The neuron damaged corresponds to the number 19 indicated with a red line. **C**: Functional connectivity maps. On the left, all the culture is connected before damage. On the right, we can clearly see a white, non-connected region where damage was made, together with a region isolated from the rest. The central black dots are nodes that had no activity and were therefore discarded during functional connectivity analysis.

to neuronal electrical activity, recording free calcium dynamics provides a direct measure of network activity [5].

We can detect this increase in the calcium fluorescence signal so that later, by adjusting a reference line, we can determine whether a neuron has activated or not, and assign them the value of 0 (no activity) or 1 (activity). Data is then shown as 'raster plot' (Fig. 1B, top). Afterward, we will be able to extract complex network measures such as global efficiency G_{eff} and the community statistic Q, allowing us to do a quantitative exploration of the data.

C. Basic data analysis

To study our recordings we use NETCAL's code from Soriano's Lab. Each cluster corresponds to a Region of Interest (ROI) over the images. For each cluster, we associate a number and we label them in order to extract he gray scale of each one and thus be able to quantify their intensity upon activation (how fluorescent they are). This is what we called 'extract traces'. This traces are then analyzed as said above to get the series of 0s and 1s and built the raster plots. Each row in the raster plot is a neuron. Along time, it shows the number of activations it had. In other words, the number of times the neuron has fired an electrical impulse.

In our output data we have listed each cluster and at what time it fired. If we count the number of times a cluster appears in the data file we will get the number of times it has fired. This corresponds to the computation of points in a row of the raster plot, and provides the cluster's individual activity. On the other hand, and more interestingly, we can extract the 'global activity' of the network by looking at a column in the raster plot, and that corresponds to the number of activations at a given time. The idea is to take a window of 1 s and count the fraction of clusters in the network that coactivated (Fig. 1B, bottom). This analysis gives an idea of the synchrony of the system. For instance, if all clusters fired together we would say that the system is activating in synchronous way. This analysis is important since we would expect a change upon damage.

D. Functional connectivity and network measures

We calculate the cross–correlation between the time series of clusters' pairs (row in the raster plot) to know how likely it is that they interact. The cross correlation is quantified by Person's correlation coefficients. If i and j are any pair of clusters:

$$r_{ij} = \frac{\sum_t (x_i(t) - \bar{x_i})(x_j(t) - \bar{x_j})}{\sqrt{\sum_t (x_i(t) - \bar{x_i})^2 \sum_t (x_j(t) - \bar{x_j})^2}}, \qquad (1)$$

where $x_i(t)$ and $x_j(t)$ are the train series ('0' for no activity, and '1' for activity) of clusters i and j, and $\bar{x_i}$ and $\bar{x_j}$ their average values.

However, we shall define a threshold in order to accept the correlation or not in comparison with a *randomized* raster plot, which would procure r_{ij}^R values. This threshold is given by the average value of r_{ij}^R distribution and two times the standard deviation SD^R. Therefore, the final correlation values are those that verify $r_{ij} > r_{ij}^R + 2\,\mathrm{SD}^R$. Accepted values are set to 1, and the rest to 0, respectively.

Once, we understand the correlation concept we can now apply it to the network's theory. We may reorganize the matrix putting the groups of clusters that share a similar correlation on the diagonal, which helps us visualize communities. With the help of the 'Brain Connectivity toolbox' in Soriano's Lab software we can get the global efficiency G_{eff} that tells us how connected is the network globally. It is given by:

$$G_{\mathrm{eff}} = \frac{1}{N(N-1)} \sum_{i \neq j} \frac{1}{d(i,j)}, \qquad (2)$$

where N is the total number of clusters and d_{ij} the shortest topological path length between any pair of clusters. Topological distance means the number of connections that are required to walk from a cluster to another. If there is no path connecting them, d_{ij} will be infinite and information flow for that pair of clusters will be zero. We note that the first factor in Eq. (2) corresponds to normalization, so that if all clusters connect to all, then the shortest path is always d_{ij}=1, and the overall efficiency will be maximum and equal to the total number of possible connections between two clusters, giving $G_{\mathrm{eff}} = 1$. Thus, in general, more connections between clusters imply a smaller d_{ij} and a higher G_{eff} [6].

On the other hand, we also considered the *Community Statistic* Q, which measures the tendency of clusters to preferentially communicate to one another in small groups. Fig. 2A and Fig. 2B provide, respectively, an example of a modular network and a non–modular one. If communities exist, then Q accounts for as the ratio between the number of links within communities and the number of links between communities. Q is mathematically defined as

$$Q = \frac{1}{2m} \sum_{ij} \left(A_{ij} - \frac{k_i k_j}{2m} \delta(c_i, c_j) \right), \qquad (3)$$

where A_{ij} represents the edge weight between nodes i and j, k_i and k_j are the sum of weights of the edges attached to nodes i and j. Also, m is the sum for of all the edge weights in the graph, i.e, A_{ij}, δ is the known Kronecker delta function and c_i and c_j are the communities of the nodes i and j [7].

Q varies between 0 when the whole network forms a single community (everything is connected) and 1 where each neuron is a community (they are isolated). Typically, we will say that communities exist for $Q \gtrsim 0.3$.

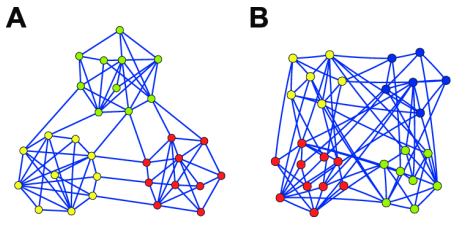

FIG. 2: An example of a small network with modular structure (A) and its randomly rewired network (B).

III. RESULTS AND DISCUSSION

A. Network's evolution upon damage

Network theory gives us a way to quantify the connections and communities that exist between the clusters of neurons that form our cultures, and helps us to make a quantitative analysis of what is happening to a neuronal culture. Despite this, once we have obtained our results it is up to us to interpret them from a biological point of view by reasoning and understanding logically what is happening.

In this work we have focused on the damage with laser ablation, but there are other ways to harm a network such as supplying a drug called CNQX that weakens the connections between them. Later we will make a brief comment on this type of damage also to compare it with focal damage and to put in context the consequences of damaging a network.

For focal damage, we analyze the change between Fig. 1B (left, before damage; right, after damage), where we see in the raster plots that the global activity decays. Indeed, although it is difficult to see, some clusters have a lower density of dots, and that some clusters has stopped firing (such as the number 20). However, the plots that best illustrate what is happening are the functional networks of Fig. 1C, where we see that clearly the network has been broken into two communities, leaving one part of the network isolated from connecting to the other.

For the experiments in which we add a drug, we have computed the values of the global network activity, GNA, G_{eff} and Q, as shown in Fig. 3. In total we had 4 series with increasing concentration of CNQX (grey curves), and average the results among them (black curve). We see in Fig. 3 that both GNA and G_{eff} first decay to then slightly recover. This suggests that recovery may exist in networks. However, for Q, what happens is that it first increases as the network splits into communities to later decay as the network mildly recovers. An important result present here is the existence of a stage of recovery of the number of connections and links between neurons, highlighting the struggle of the culture to fight back perturbations before totally losing activity.

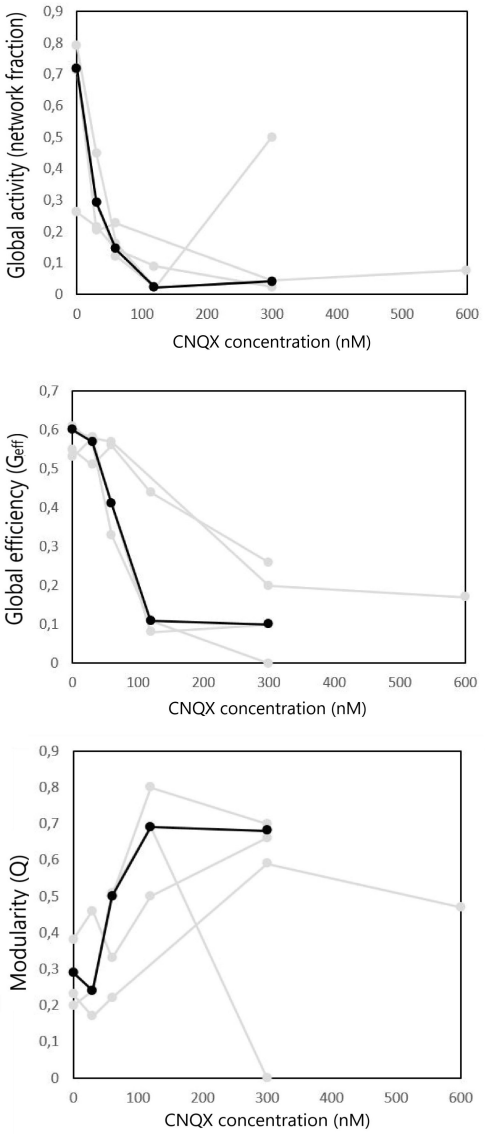

FIG. 3: Coactivation size (top), Global Efficiency G_{eff} (center) and modularity Q (bottom). The grey dots are the different individual experiments for each of the drug's concentration. The dark dots are the average of four experiments.

B. Coactivation size evolution for laser attack

Now, let us discuss about the evolution of GNA for the 'random attack' experiments. GNA has been computed averaging over two experiments (controls and targeted). It should be remembered that we have always worked with a healthy network and a damaged one. Here we only show the evolution of the targeted one before and after damage. We can clearly see in Fig. 4 that the cultures after damage lose global activity and the capacity of the clusters to activate in a synchronous manner, which causes a drop in GNA.

C. Evolution of global efficiency and connectivity statistic Q for laser attack

As for the evolution of G_{eff} and Q, these quantities have been averaged for two different experiments again. In Fig. 5 we realize that G_{eff} again declines after performing the damage as the global connectivity has been affected. In Fig. 6, we see that the modularity Q grows, highlighting the breaking of global links. Overall, the fact that two independent cultures lead to similar results in GNA, G_{eff} and Q indicate that experiments are reproducible, although many more would be needed to get robust statistics.

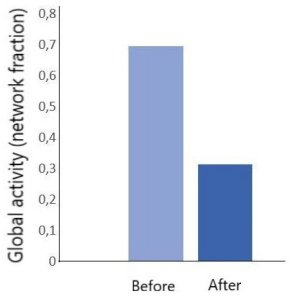

FIG. 4: Coactivation size evolution before (blue column) and after (blue dark column) averaged upon the duration of the recording. We also have averaged the coactivation size for two different experiments of the same type and condition.

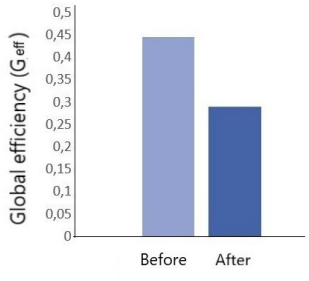

FIG. 5: Global efficiency before (blue column) and after (blue dark column) averaged over two different experiments of the same type and conditions.

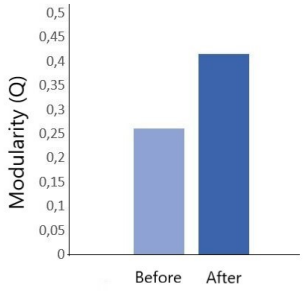

FIG. 6: Modularity before (blue column) and after (blue dark column) averaged over two different experiments of the same type and conditions.

IV. CONCLUSIONS

Studying the evolution and functioning of neural networks is not an easy task. Here we have tried to give a quantitative explanation with the parameters of network theory on how a neuronal culture reacts to damage. This study can also help us to understand from a medical point of view, how certain diseases act on the brain and develop efficient therapies to treat them.

By means of a mathematical, statistical and computational formulation we have been able to quantify this process. An important thing to highlight from our results is that most of the results have been obtained in a window of 10 min after damage, although the recordings were of 30 min. We proceeded in this way because activity after damage is stable, but changes possibly due to the initiation of recovery mechanisms. To investigate the stages of pre–damage, post–damage, initial recovery, and full recovery would require a vast amount of analysis beyond the scope of the present work. Such an effort was carried out in Ref. [4].

In short, we have obtained conclusive results with what our common sense tells us to happen when damage is applied to a neuronal network, either with random damage or with a drug. We see that global connectivity and activity is declining, and functional links between neurons are broken in the neighborhood around damage. Despite this, what is being observed is that there is a slightly recovery of the network and even a fight against neuronal death. This could be caused, for example, by strengthening the links between neighboring clusters or by the appearance of new ones, causing an increase in neuronal activity. The study, extended in detail, could be used as biophysical model to understand patients' behaviour upon ictus or degenerative diseases [4].

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