Targeted attack on neuronal cultures

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Abstract: Here we study the behavior of *in vitro* neuronal networks whose neurons are sequentially eliminated, mimicking for instance the action of a stroke. Neurons are eliminated either in a random way or by targeting those that are central to network communication (hubs). For random removal, the results show an increase of overall network activity and a maintenance of the cohesion of the network. For targeted attack, two scenarios were considered depending on the definition of hub (node degree or betweenness centrality), in both cases their deletion led to networks to break apart. The results show the importance of central nodes in neuronal networks and could help improving the understanding on the behavior of functional connectivity alterations upon damage.

I. INTRODUCTION

Several attempts are being made to model and treat neurological diseases, either those related to progressive degeneration of neurons, such as Alzheimer's or Parkinson's, or those associated with trauma, such as strokes. It is already known that these sorts of diseases may cause the loss of crucial areas in the brain, affecting communication, the integration of information, and overall functionality [1]. These crucial areas are referred to as *hubs*, and can be viewed as nodes in networks that route a large amount of information. Particularly for Alzheimer's, current evidences show that important hubs for cognitive tasks are gradually lost with disease progression [2].

However, despite substantial efforts in biomedicine, there are currently not many experimental studies that investigate how the progressive loss of hubs affects the dynamics of a neuronal circuit. The sheer size of the brain and the difficulty to act on it has made very difficult to understand this problem. For this reason, researchers have focused mainly on *in silico* models that explore the robustness, and possible failures, of the structural connectivity of the brain [3], *i.e.* the synaptic map of connections among neurons that shape the physical architecture of the brain network. These studies are often accompanied with the analysis of the functional connectivity, *i.e.* the flow of information among neurons [4]. By putting together the two networks, structural and functional, scientists aim at understanding the fragility of the brain upon disease and how to stop it.

An alternative approach to numerical simulations is the use of *in vitro* neuronal circuits, *i.e.*, neurons grown in small areas that can be analyzed and disturbed in a controlled manner. In this direction, the present project is a *proof of concept* to cause progressive damage of hubs and to monitor the response of the network, shaping somehow an *in vitro* model for Alzheimer that can inspire further research.

Thus, here we examined the dynamics and functional connectivity of two–dimensional (2D) *in vitro* neuronal circuits formed by densely packed aggregates of neurons (*clusters*). The functional study reveals those clusters that exchange information more often. This functional connectivity may be very different from the structural one, which cannot be accessed, but provides a good proxy to network behavior. Our goal in the project was to shed light on the network resilience mechanisms and how it responds to targeted attacks on hubs in comparison with random attacks (also known as failures).

II. EXPERIMENTAL METHODS

A. Neuronal cultures and procedure

The following techniques were performed under the approval of the Ethical Committee of Animal Experimentation of the University of Barcelona (CEEA-UB) in Dr. Jordi Soriano's laboratory. Initially, the experimental process consisted on the dissection of embryonic cortices from Spargue–Dawley rats at 19–21 days of development. Straightaway, cortices were disaggregated by pipetting and deposited into 4–well culture plates. Every well contained the proper medium to assure the maintenance of the neurons and a coverslip of 13 mm in diameter. The coverslip was previously attached to a ring of polydimethylsiloxane (PDMS) with a central cavity 6 mm in diameter and 2 mm thick, where neuronal aggregates were settled down as a two–dimensional neural network (Fig. 1A).

In every experiment, cultures were considered mature when they reached day *in vitro* (DIV) 9 and were recorded up to DIV 19. Throughout this period of time they were active enough to study their dynamics. The wells were then selected for sequential damage of hubs. The main criterion to select the clusters to be damaged was decided according to their functional connectivity. As described later, we considered in one culture those clusters that had the highest betweenness centrality, and in another culture the highest degree. These sorts of damage are referred as targeted attacks. In the other prepared wells, clusters were attacked randomly and thus they are interpreted as failures.

Before any attack, cultures were recorded for 20 min-

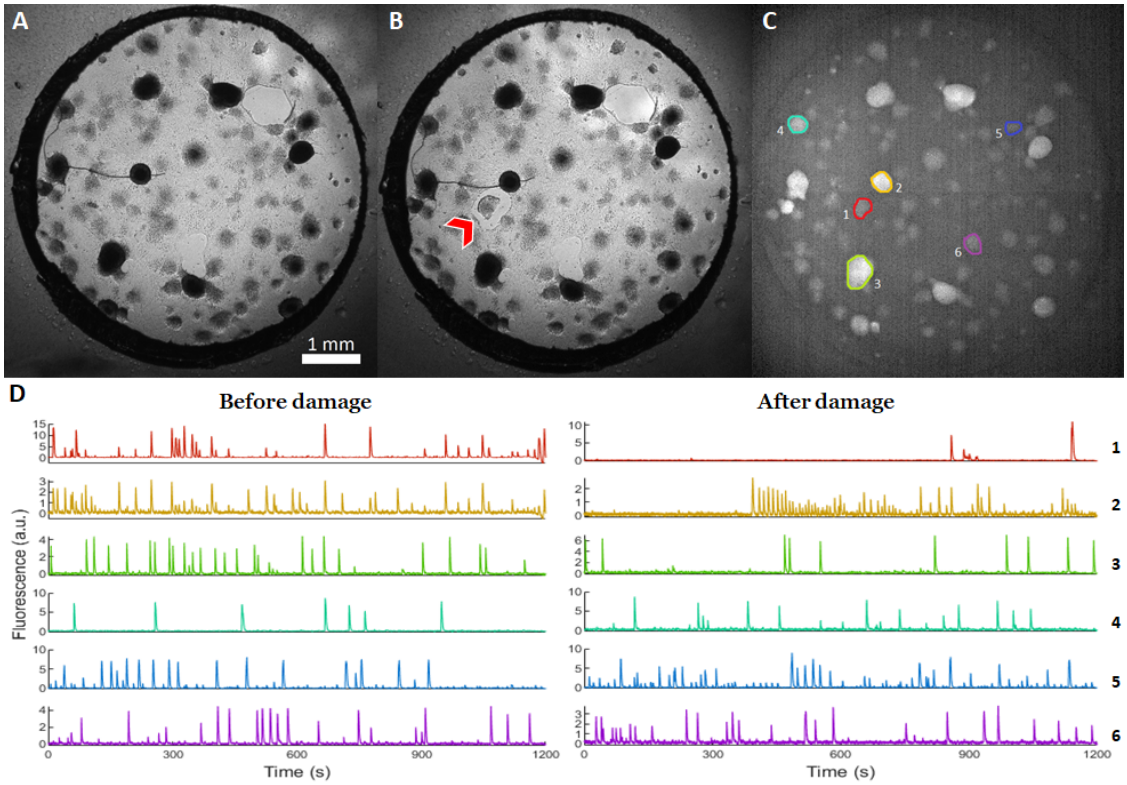

FIG. 1: Damage on a neuronal culture and fluorescence analysis. **A**: Bright-field image of the culture before damage. The darkish and greyish rounded shapes are the neuronal clusters and the fibres are unions of axons. **B**: Bright-field image after targeted attack on degree. The cluster attacked is indicated by an arrow. **C**: Fluorescence image of the culture after attack. The outlined clusters are the ones whose activity traces are showed in panel D. **D**: traces of the ROIs before and after damage.

utes at 50 frames per second. After the attack, cultures were left in the incubator for about 25 minutes while the data was processed to identify the hub. Once it was identified, the cluster was disconnected from the network using a needle (Fig. 1B) and the culture was immediately recorded again. In addition, between each damage the culture was allowed to rest for about 24 hours in order to assure the recovery of the network.

B. Fluorescence calcium imaging

We used a microscopy technique called fluorescence calcium imaging to record the spontaneous activity of the networks. This technique permits to observe changes in Calcium ions (Ca^{+2}) concentration thanks to the binding of Ca^{+2} with the genetically encoded indicator GCaMP6s.

The indicator is transferred to neurons via viral infection, and activates only when binds Ca^{+2}, which is taken by the cells upon activation. The Calcium changes the conformation of the indicator and becomes fluorescent. Consequently, it can be excited by blue light and the corresponding emission in green can be captured by a fluorescence camera (Fig. 1C). Recordings were acquired and converted into movies using the Hokawo 2.5 software.

C. Data analysis

We used NETCAL, a MATLAB-built software produced by the Neurophysics Group of Dr. Jordi Soriano, to analyze the recordings. In the first place, the preprocessing stage assesses that there are not missing frames in the recording. Secondly, all clusters are classified manually as regions of interest (ROIs). Approximately there were 100 ROIs in each experiment. Subsequently, we obtain the fluorescence signals for each cluster as a function of time. As shown in Fig. 1D, there is a fast increase of fluorescence upon the activation of a cluster followed by a slow decrease. The decay of fluorescence is due to the unbinding of Ca^{2+} ions in the channels of the membrane.

The ensemble of these signals for all clusters forms the so–called traces. The traces illustrate the relative change in fluorescence with respect to basal levels for every cluster. For each unit of time, the sharp peak is interpreted as '1', whereas a '0' indicates no activity. Once all the activation events were detected, data was represented as raster plots (Fig. 2A). Raster plots are the main datasets to extract more information about the dynamics of the network, specifically network dynamics and functional connectivity.

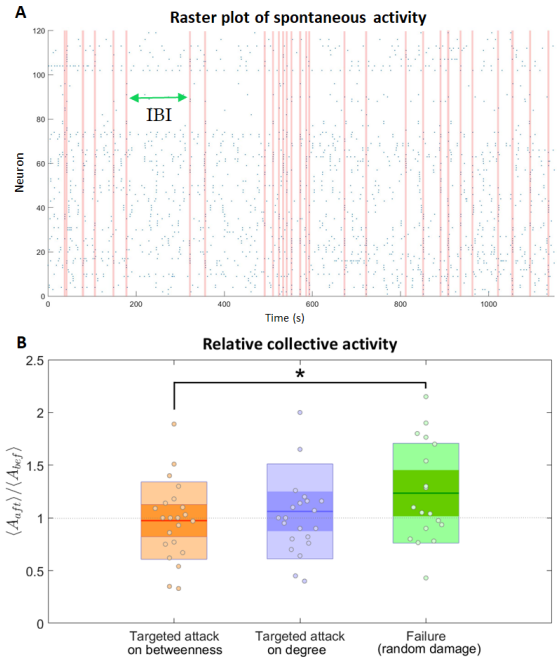

FIG. 2: **A**: Raster plot of spontaneous activity. Every vertical line designates a collective burst. **B**: Box plot of the collective activity ratio between after (A_{aft}) and before (A_{bef}) damage for the three different kinds of attack. The significance between targeted attack on betweenness centrality and failure is moderate ($*\text{p} = 0.0191 < 0.05$, Student's t–test).

III. THEORETICAL METHODS

A. Functional connectivity

To better comprehend the functional organization of neuronal networks upon damage it is essential to compute the functional connectivity. This is why we used Transfer Entropy (TE) [5]. It is based on two concepts: mutual information and Granger causality. Conceptually, TE assesses the influence between node X and node Y. This means that, if it is possible to predict the evolution of node Y thanks to the information from node X, then we can say that there is a causal relationship between X and Y, *i.e.* that X is influencing Y, or that there is a transfer of information from X to Y. This transfer of information is associated to a functional connection.

TE can faithfully measure the strength of this connection (amount of information transferred). The higher the TE score, the stronger the connection. Nevertheless, we must be aware that we are studying a non–linear system, so a strong connection does not imply the presence of a physical synaptic connection, although it is highly probable. Additionally, we must note that even if two clusters are actually connected, the information transmitted between them can be weak or non-existent since information does not necessarily flow all the time across all possible connections. Furthermore, if signals are either random or identical, meaning there is a simultaneous activation, it is not possible to predict an exchange of information, hence the TE score is zero.

For the purpose of the present work, functional connectivity was computed for each pair of clusters using software already present in Soriano's group, leading to functional connectivity matrices that were directed (direction of information flow) and weighted (amount of information transferred).

B. Network measures

Functional connectivity matrices were analyzed to obtain information about the network. A first measure of interest is the *Global Efficiency* (G_{eff}) that captures the ability of the network to integrate the information. In other words, it reveals the capacity to distribute the information to the different regions of the network. It is mathematically expressed as [6]:

$$G_{\text{eff}} = \frac{1}{N(N-1)} \sum_{i \neq j}^{N} \frac{1}{d_{ij}}, \tag{1}$$

where N is the number of nodes and d_{ij} is the minimum amount of steps that communicate nodes i and j, the so–called shortest path length. The global efficiency is equal to 1 when the network is completely interconnected, whereas it is 0 when is totally detached.

A second measure is the *Modularity* (Q). Networks with high modularity have dense connections among nodes within each module and few connections between modules; this results in a segregated network. In contrast, lower values of the modularity depict an integrated network so that modules are so interconnected that shape an almost unique network. Q is defined as:

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

where A_{ij} is the adjacency matrix that contains the weight of the connection between nodes i and j, k_i and k_j are the sum of weights bound to nodes i and j, m is the sum of all of the edge weights, δ is the Kronecker delta function and c_i and c_j are the communities whose nodes i and j belong to, respectively.

Regarding the definition of a hub, *i.e.* the centrality of a node, we considered two possible measures: the *degree* and the *betweenness centrality*.

The degree measures the number of connections of every node and, hence, it is expressed as:

$$k_i = \sum_j A_{ij}, \tag{3}$$

being A_{ij} the adjacency matrix. Indeed, as the graphs studied are directed, the total degree is composed by the ingoing $k_i^{in} = \sum_j A_{ji}$ and the outgoing links $k_i^{out} = \sum_j A_{ij}$. In the end, $k_i = k_i^{in} + k_i^{out}$.

The betweenness centrality of a node is determined as the ratio of the shortest paths between any pair of vertices that pass through the studied node. An aside before continuing, it is necessary to emphasize that we are assuming information travels through the shortest path length [7]. The mathematical definition is:

$$b_i = \sum_{j \neq k}^{N} \frac{n_{jk}(i)}{n_{jk}}, \tag{4}$$

where n_{jk} is the amount of shortest paths that attach j to k, and $n_{jk}(i)$ is the number of shortest paths connecting j with k that travel through i.

In addition to these measures, we also considered the network activity as a way to quantify damage. Concretely, we considered the average number of collective activations (bursts), *i.e.* where at least 10% of the clusters fired. Network activity was then obtained by dividing the duration of the recording (1200 s) over the average inter–bust interval (IBI), which is the time between consecutive bursts as Fig. 2A displays.

IV. RESULTS AND DISCUSSION

A total of 9 experiments were conducted, which means a data set of 3 experiments for each type of attack. In Fig. 2B we can observe how the collective activity is affected by damage. In a broad outline, it is easy to observe a difference between targeted attack and failure. The latter shows an increase of activity after damage, which could be related to a response mechanism of the culture to balance the perturbation caused by the damage and recover its loss functionality. However, for targeted attack, the activity remains practically constant for both degree and betweenness actions. This suggests that the damage on a hub node weakens the capacity of a network to activate response mechanisms. Additionally, since the data distributions for the two targeted attacks are very similar, we conclude that both types of attack produce the same effect in the loss of a hub.

After analyzing activity we focused on the disruption of information traffic, which is captured by the global efficiency G_{eff}. In Fig. 3A we can easily observe that, after every targeted attack on betweenness, the capacity of exchanging information is reduced. However, the network recovers just after one day. As an exception, the eighth damage is negligible due to the network was really aggregated and its performance was unusual. In contrast, Fig. 3B shows the evolution of G_{eff} for random attack.

In this situation, we observe that damage can help in the communication among clusters by means of an increase of the global efficiency. To understand this observation, it is important to emphasize that a neuronal network contains excitatory and inhibitory connections, which means that there are some clusters whose role is to initiate the activity whilst there are others that tend

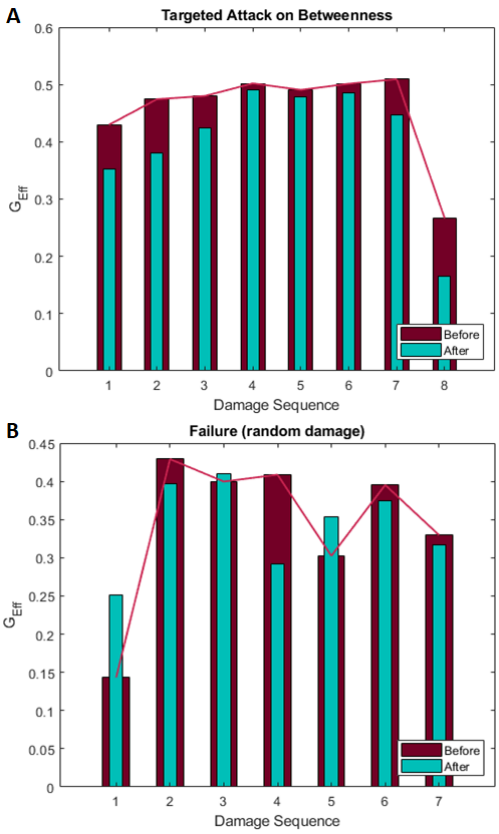

FIG. 3: Bar plots representing the evolution of the global efficiency at every attack. **A**: Behavior when attacks are targeted to the node with maximum betweenness centrality. **B**: Behaviour when the culture is attacked randomly (failure).

to restrain it. Therefore, an improvement of communication can be related to an elimination of a cluster that contains several inhibitory connections.

Last but not least, another way to quantify the interconnectivity of the network is by studying the modularity. In Fig. 4B there is an example of a graph that exposes the effect of removing the maximum betweenness centrality node. As this kind of hub has the role of connecting various communities through their shortest paths, its deletion produces a segregation of the diverse populations of clusters, which means that the modules are more isolated and the integration of information is poorer. This leads to a decrease in communication capacity that is reflected in an increase of the modularity. Indeed, this is precisely what is pictured in Fig. 4C.

We note that, after considering all the experiments in Fig. 4A, both types of targeted attack tend to increase modularity, something expected since hubs are removed. These results were also observed by Kabbara *et al.* [1]. The opposite case occurs in random attacks. As it is more probable that a failure affects a non–hub cluster situated within a functional module, it may occur that the deleted node was strongly inhibitory, effectively causing a reinforcement of the integrability of the network.

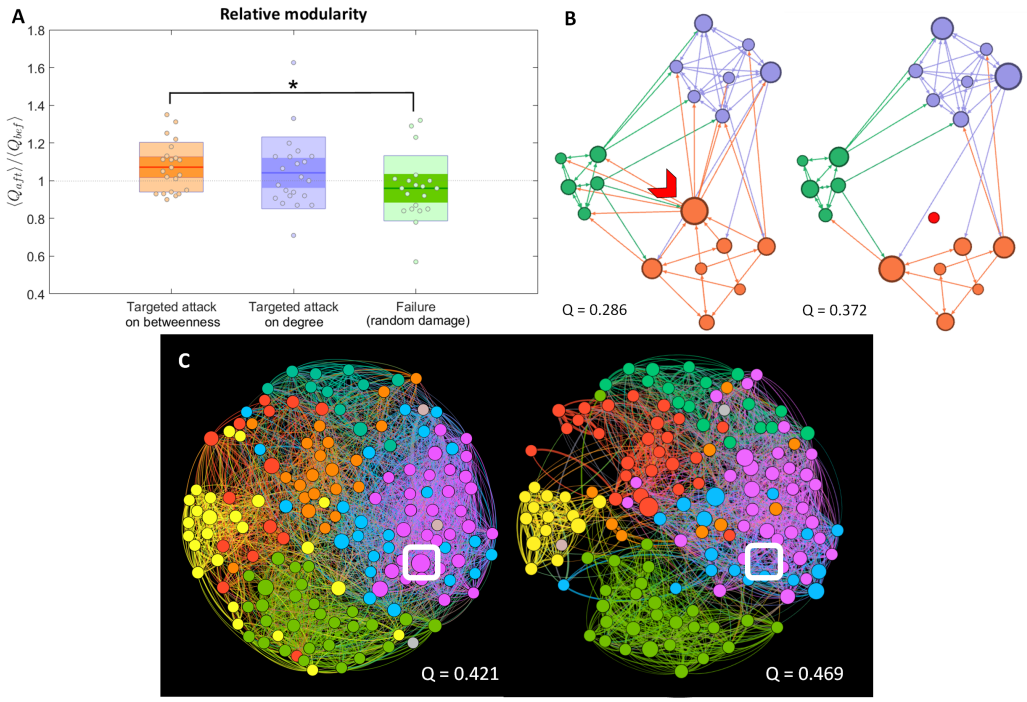

FIG. 4: Modularity upon damage. **A**: Box plot of the modularity ratio between after (Q_{aft}) and before (Q_{bef}) damage for the three different kinds of attack. The significance between targeted attack on betweenness centrality and failure is moderate ($*p = 0.0321 < 0.05$). **B**: Example of how modularity increases after removing the hub with maximum betweenness centrality (red arrow). **C**: Functional connectivity maps of an experiment before and after a targeted attack on betweenness centrality.

V. CONCLUSIONS

- There are differences in the response mechanisms after damage between random and targeted attacks, especially when the attack is on the cluster with maximum betweenness centrality. For the case of failure, we observed a clear increase in activity in order to rewire the connections and to counteract the loss of a node.
- Regarding modularity, the elimination of a hub results in a segregation of the network, a fact that hinders the transfer of information between communities. While, on the contrary, a failure leads to a more integrated network.
- In spite of the necessity of more experiments, it appears that a targeted attack on either degree or betweenness centrality produces the same effect.
- This proof of concept shows potential in unveiling the behavior of neuronal networks under successive damage. Results could be understood much better with the support of numerical simulations.

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