Abstract: Neuronal plasticity in the perspective of functional networks is a complex process that depends on several factors, specifically the connectivity of the neurons and their intercommunication. To set a controlled framework, here were studied homogeneous neuronal networks grown in vitro and investigated their recovery after delivering physical damage that split the culture in two. Data analysis was focused on the spontaneous neuronal activity, and comparing the state of the network before damage, just after it, and during network recovery. Results show that recovery was fast and favored by the tissue surrounding the damaged area. Also, functional connectivity analysis showed that the network had strong reconfiguration, transiting through different stages until reaching a final steady configuration.

I. INTRODUCTION

The mechanisms by which neuronal networks recover after physical damage are a complex issue that is still not completely well characterized. It is known that, in the brain, damage leads to irreversible changes in network dynamics [1]. It also has been seen that despite damage, and the loss of a substantial amount of neurons, neuronal networks are capable of reorganising and even strengthening their connections [2]. These processes are believed to prevent a cascade of failure that could completely destroy the brain. Thus, upon localized damage, neurons around the damaged region strongly activate and reconfigure the circuits. That is to ensure that damage stays under control and, despite the lost functions, the circuit can be reactivated as soon as possible.

Such recovery mechanisms are extremely difficult to visualize, understand and model directly in the brain. For this reason, in the present work we used in vitro neuronal cultures, which allows more controlled conditions than in vivo ones. Concretely, we studied the effect of a physical injury applied across a culture in which neurons cover homogeneously its surface.

Thus, the aim of the present investigation is to characterize the recovery of the culture from a network-dynamics point of view, obtaining a deeper knowledge on this subject and analysing the extent of the recovery, as some other studies suggest [3]. Additionally, and following the ideas presented in Teller et al. [3], we also investigated the influence that the tissue surrounding the damaged area had on the network during its recovery, specifically to see if it helped to stabilize the culture and restored it after the initial trauma.

II. METHODS

A. Culture preparation

Homogeneous cultures were prepared from Sprague-Dawley rat embryos cortical neurons and seeded on a polymer–like surface (poly-dimetil-syloxane, PDMS) prepared in the form of a disc and adhered onto a glass coverslip (Fig. 1A). Two different kinds of cultures were prepared: one with the PDMS neurons isolated form the rest of the culture (‘Culture 1’, Fig. 1A, dashed rectangle) and the other with the neurons in the disc connected to the neurons on the glass (‘Culture 2’). This permitted measuring the effect of the surrounding tissue on the recovery. The spontaneous neuronal activity was detected using an inverted microscope with an attached camera specially used for fluorescence imaging, as explained later.

The culture substrate PDMS disc was 6 mm in diameter and 2 mm high, and adhered onto a 13 mm diameter glass coverslip. The substrate was coated with poly-d-lysine (PDL) and left 24 hours before seeding the neurons. PDL is a protein that allows the neurons adhesion to PDMS and glass and permits the formation of homogeneous cultures [4]. After the coating, the cultures substrates were rinsed with double–distilled water (DDW).

The dissection procedure replicates the methods described in Ref. [3]. Cortical neurons were obtained from Sprague-Dawley rat embryos. Cortical tissue was mechanically dissociated by repeated pipetting, in order to obtain disconnected neurons and allow the later formation of a new, de novo network. Eight culture wells were prepared in each dissection, and the two most similar ones were selected to be studied. Every well contained about 500 neurons/mm². Neurons grew together with glia, a type of cells that procures support to neurons.

At 1 day in vitro (DIV) after the seeding, the neurons surrounding the PDMS from Culture 1 were mechanically removed (Fig. 1A, right panel). After that, and for all
Functional alterations in neuronal networks after physical damage

Sâlem Ayasreh Fierro

FIG. 1: Experimental procedure. A: Sketch of the main steps of culture preparation. Represented fluorescent images of the Culture 1 along the different stages of the experiment. The left image shows the activation of the entire culture before damage; the next image shows the damage areas (‘D’) and the loss of activity; and the last 3 images show the isolated activation of different areas after damage (hemispheres ‘A’ and ‘B’, surroundings ‘S’ and contour ‘C’, respectively). C: Sketch of the areas of the culture (‘A’, ‘B’, ‘D’,...). D: Raster plots of spontaneous activity of Culture 1 as a function of time. For clarity purposes, only 3 minutes from the total recordings are shown. Each point is the time activation of a region of interest (ROI). The rasters show the activity before damage and 24h after it. ROIs were reordered according to functional connectivity similarity. This plot illustrates the activity desynchronization between ‘A’ and ‘B’ areas after the damage.

cultures, neurons were infected with a virus that encoded for the Calcium indicator GCaMP6s [5], which allowed to visualize neuronal activity with the fluorescence camera, and on the same culture, along several days. Cultures were kept inside an incubator, at 37°C, 95% humidity and 5% CO₂ concentration. The medium was periodically replaced every 3 days [3].

B. Calcium imaging

Calcium signalling is one of the main methods that neurons use to transmit information along the cell. When the electric pulse inside the neuron (action potential) reaches the neuronal emitting terminal (axon), the voltage change that generates is detected by some channels in the neuron cellular membrane, letting Ca²⁺ ions to flow inside the neuron for a short period of time. This sudden increase in calcium concentration triggers the emission of neurotransmitters to the synaptic cleft, which activate the downstream connected neurons. The fast increase of Ca²⁺ reveals activity, and can be detected with the sensor GCaMP6s, a protein that becomes fluorescent upon Ca²⁺ binding (Fig. 1B). Thus, whenever calcium ions are bound, this protein is susceptible of fluorescent emission...
at 510 nm (green) when excited with quasi-UV radiation of 460 nm (far blue).

To measure this radiation, an inverted fluorescence microscope was used (Zeiss Axiover 25C). The fluorescent images were recorded with a high–resolution camera attached to the microscope. All the recordings were performed at 33 frames/s and a 2.5x objective, providing a field of view of 7.2 × 7.2 mm², sufficient to visualize the entire culture.

C. Culture recording and damage action

The experiments started at 15 DIV, when neurons had strong spontaneous activity. At this stage, the neurons formed an interconnected homogeneous layer, with the neurons in fixed positions. A total of four cultures, two for each type (Culture 1 and Culture 2) were selected for the experiment, one as the subject of damage and the other as control. Before applying the damage, both cultures were recorded separately for 15 minutes, with 30 minutes gap between them. In intact cultures, activity was characterized by the fast activation of the entire culture in a short time window (Fig. 1B, left).

The physical damage was done with a scalpel (Fig. 1A, right panel). A 1 mm depth cut over the PDMS disc was performed. Immediately after the damage both cultures were recorded for 30 minutes each. Then, cultures were recorded during 15 minutes at: 2 hours, 6 hours, 24 hours, and 3 days after the damage (Fig. 1B).

During the recording, cultures were kept in a glass “micro-incubator” that allowed the recording to be carried out in the same conditions as in the normal incubator, i.e., 95% humidity, 5% CO₂ concentration. The temperature during the experiments was set at 25°C, smaller than the usual incubator conditions to favour spontaneous activity.

D. Image analysis

The obtained videos, acquired with Hokawo 2.5 software, were analysed using NETCAL software [6]. This program analyses the raw signal data to correct the artifacts caused by basal fluorescence, light fluctuations and drifts. Processed data was discretized into a 40 × 40 circular grid. Each element of the grid represented regions of interest (ROIs) from which average fluorescence levels were extracted. A total of 1390 ROIs were present within the circular grid. The fluorescence level of each individual ROI of the grid was transformed into a binary signal (spike train) using the Schmitt trigger method [7]. Conceptually, if there is neuronal activity at time t, the signal gets a sudden increase in form of a spike and the ROI state is set to 1 for that time t. Oppositely, if there is not a relevant signal increase, then the ROI state is set to 0. The complete data is then represented as ‘raster plots’, where dots indicate the presence of activity (Fig. 1D).

Discretized activity data was then processed with another software, developed by Soriano’s Lab termed Visualize Front. The software was used to focus on global firing events, where at least 90% of ROIs are active, and that correspond to the vertical bands in the raster plots.

E. Activity analysis and functional connectivity

All the measurements were made considering the 5 main regions of the culture, which can be physically identified (Figs. 1A (right) and 1C). After damage, the neurons over the PDMS were classified in 3 regions: the two hemispheres (‘A’, ‘B’) and the damaged tissue (‘D’). Additionally, we considered a ring around the walls of the disc with small aggregated clumps of neurons, called contour (‘C’). Finally, we also considered the glass surroundings (‘S’) around the PDMS, an area that is almost empty of neurons in Culture 1. We note that not all the ROIs were used to make the computations, as in some cases ROIs could fall between two physical regions. Only if at least 80% of the ROI was inside the area, it was considered valid.

Spontaneous neuronal activity was measured from the data processed with the Lab–made software Visualize Front. This software provides the time between consecutive global firing events (inter–burst interval, IBI). For every recording, this magnitude represents a distribution of points, which was averaged with the median. This average value represents the typical time between every activation. Since neuronal activity can be understood as the firing frequency of the culture, such an activity could be simply computed as the inverse of the average IBI.

On the other hand, functional connectivity permits us to characterize information flow, and whether or not two neurons are functionally connected in the network. We considered Pearson’s correlation to obtain the functional connectivity between all pairs of ROIs, computed as:

$$r_{ij} = \frac{\sum_i(x_i(t) - \bar{x}_i)(x_j(t) - \bar{x}_j)}{\sqrt{\sum_i(x_i(t) - \bar{x}_i)^2 \sum_j(x_j(t) - \bar{x}_j)^2}},$$

where $x_i(t)$ and $x_j(t)$ are the spike trains of ROIs i and j, with respective mean values $\bar{x}_i$ and $\bar{x}_j$. With that, a 1300 × 1300 matrix of cross correlation between ROI pairs was obtained, $r_{ij}$. Then an equivalent matrix, $\chi_{ij}$, was computed with randomized values of $x_i(t)$ and $x_j(t)$, but maintaining the original activity, i.e., the number of ‘1’ values in each ROI signal. Then, for every $i − j$ pair, actual and random correlation were compared in order to construct the connectivity matrix, $w_{ij}$. If $r_{ij} > \chi_{ij}$, the $i − j$ ROIs were considered to be functionally connected and the value $w_{ij}$ was set to 1; otherwise it was set to 0. The ROIs connectivity matrix, $w_{ij}$, was finally transformed into a much connectivity matrix for the areas of the culture (‘A’, ‘B’, ‘D’...), termed $w_{xy}$. This was done by summing all the correlations between every couple of areas $x − y$.

Sàlem Ayasreh Fierro

Treball de Fi de Grau 3 Barcelona, January 2022
Interestingly, from the perspective of network activity as a whole, the neurons in the vicinity of damage (‘C’ and ‘S’ areas) seem to be the main factor to explain the short–term activity restoration, an observation that is in agreement with the study of Teller et al. [3].

In Fig. 2, top, a sudden drop of activity appears for the control of Culture 1, which could not be explained. The presented experiments are hard to reproduce systematically, as many variables are difficult to control (neurons origin, rat embryo, cell density, cell attachment,...). This evidences the need of more experimental replicas, in order to obtain less biased conclusions, which was not possible to implement due to the high difficulty of the experiments and the short duration of the project.

However, spontaneous activity did recover. The more neuronal tissue surrounding the damage, the faster recovery was. Thus, from this point of view, our results agree well with previous studies using aggregated neuronal networks [3]. Our experiments can be even seen as a better model, since we can almost visualize independent neurons, not aggregates of them.

### III. RESULTS AND DISCUSSION

#### A. Activity evolution

In homogeneous cultures, the neuronal pulses propagate along the network as a fast avalanche [8]. From a practical point of view the neurons activate synchronously in a short time window. The more interconnected the circuit is, the higher are the chances to have global activations [9], i.e., to see a large number of activity events. By damaging the network, the loss of connections causes a sudden dramatic drop of activity. The regions with most connections directly damaged are those that are expected to have more severe drop in activity and a slower short–term restoration.

The results of network activity are shown in Fig. 2. Activity is high before damage and suddenly falls to zero upon damage. During recovery, differences between Culture 1 and Culture 2 can be seen. Culture 1 (Fig. 2, top) has a slower short–term recovery, and the final activity is very similar to pre–damage levels. Instead, the activity recovery in Culture 2 (Fig. 2, bottom), enhanced by the activity of contour ‘C’ and surrounding ‘S’ areas, rapidly reaching pre–damage activity levels, and even surpassing them.

---

**FIG. 2:** Mean activity evolution plot for Culture 1 and Culture 2. Activity is represented for every relevant region of the damaged culture and the mean activity of the control. Damage is represented as a dashed red line. There is no data for control of Culture 2 before damage.
hypothesis could not be made.

The connectivity analysis has shown that despite the activity recovery, disconnected regions could not bound back together, either by new physical connections or dynamically, using the network plasticity. This shows a dynamical reconfiguration as Ref. [2], [3] suggests. The Culture 1 and Culture 2 comparison has been useful to portray the big impact of the surrounding tissue along the recovery process. It has been seen, though, that the final permanent stage is very similar for both situations. This can also suggest the possibility to obtain a general model for equilibrium states.

IV. CONCLUSIONS

Our study shows that it is possible to track and characterize the recovery process of a neuronal network using a relatively simple in vitro experiment.

After the physical injury, the culture did not collapse with a massive cascade fail. Instead, it stopped the damage progression and recovered activity to the level before the trauma, or even exceeded pre-damage levels. The recovery process was assisted by the tissue around the damaged area, which helped to stabilize the culture.

Although activity levels recovered well, the functional organization did not. Some rewiring took place, but the network reconfigured in a way that was different from pre-damage state. We hypothesize that functional restoration may depend on the topology of the network, i.e. some structural connectivity may be more favorable for recovery than others.

Acknowledgments

I would like to thank my advisor, Dr. Jordi Soriano, for all the help, enthusiasm and guidance that gave me along the project. I want to thank Clara Fernandez, who taught me all laboratory knowledge I have. Also thanks to my family and friends for their help and support.