Impact of substrate rigidity on the connectivity and dynamics of neuronal cultures

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Abstract: Neuronal cultures are powerful tools that allow for the study of a complex system such as the brain in a more controlled environment. In order to do so, the characterization of neuronal properties must be accurately studied. The main objective of this study is to describe how the rigidity of the substrate where neurons grow affects neuronal behaviour. Neurons are grown in the same conditions with alternating substrate rigidities. Stiff substrates present lower front propagation velocities than soft ones, indicating shorter axonal length. The results confirm the essential role substrate rigidity plays in shaping culture morphology and dynamics.

I. INTRODUCTION

Nerve impulses travel from the body to the terminal of the neuron through axons. Traditionally, they have been described as cables through which action potentials are transmitted. As a result, axonal morphology greatly impacts the propagation of said information [1]. In particular, axonal length plays a vital role in neuronal behaviour and communication. However, we are yet to understand exactly how they shape collective system dynamics, specifically the propagation of activity in a neuronal network.

Neuronal activity and, by extension, emerging complex phenomena in neuronal assemblies, are nearly impossible to study directly in the brain, i.e. *in vivo*. An alternative approach used to tackle this issue are *in vitro* neuronal cultures. Neuronal cultures have been proved to be reliable and accurate models in areas such as biophysics and bioengineering [2]. For instance, it is known that mechanical properties of the substrates in which cells grow have an impact on cellular mechanisms such as cell migration, differentiation and adhesion [3].

Additionally, it has been shown that neuronal culture dynamics depend on connectivity [4]. Therefore, to study how a certain property affects connectivity we can use indirect measures such as front propagation velocity.

Thus, the project at hand aims to investigate how substrate rigidity influences velocity of propagation, and in turn, axonal length. It is worth mentioning that upon reviewing the literature on this subject we found conflicting results, proving how important further research in this area really is.

II. METHODS

A. Neuronal culture preparation

Homogeneous cortical neuron cultures were prepared from Sprague-Dawley rat embryos and seeded over polydimethylsiloxane (PDMS) substrates with varying rigidities, which had been adhered to glass coverslips.

In preparation for the dissection, each 13 mm coverslip was either mounted with two 6 mm plain PDMS discs of equal stiffness or left bare. The latter represented the 100% rigidity control. For the PDMS preparation, the PDMS weight ratios of cross-linking (curing agent 'c') and pre-polymer ('p') were $(100 \cdot c/p)$: 1%, 3%, 10%, 15% and 30%. The higher the percentage of curing agent, the stiffer the resulting PDMS. Twenty-four hours prior to the dissection, all the substrates were treated with a poly-l-lisine solution (PLL) to ensure the even distribution of the neurons and avoid their aggregation. Once the cortical embryonic tissue had been retrieved it was mechanically disassociated and diluted in plating media. Immediately afterwards, 1 ml of the plating solution mixture was seeded in each of the wells and placed in the incubator. Two hours later, the cultures were infected with a virus encoding for the fluorescence probe GCaMP6S that enabled the monitoring of neuronal activity. Additionally, culture medium was changed periodically to ensure optimal conditions. Protocol is schematically shown in Fig. 1A.

Cultures were recorded at a young state, *day in vitro* (DIV) 6 and DIV 7. Recordings of spontaneous neuronal activity were obtained using a camera attached to the fluorescence microscope. Each recording had a duration of 10 minutes and was acquired at 33 frames per second.

B. Preparation of PDMS substrates

PDMS is a soft, elastic, hydrophobic polymeric material widely used for biomedical applications [5]. Its preparation consists of two steps. Firstly, the PDMS pre-polymer is mixed with the curing agent and afterwards it is placed in the oven to cure. As previously mentioned, in our study we used several concentrations of curing agent in order to obtain varying degrees of substrate rigidity. PDMS substrates were prepared in petri dishes and were therefore completely flat. After the prepolymer was mixed with the curing agent, it was placed in a vacuum chamber to remove any unwanted air bubbles. The mixture was then cured at 70°C for 1h.

Regarding its mechanical properties, it has been shown

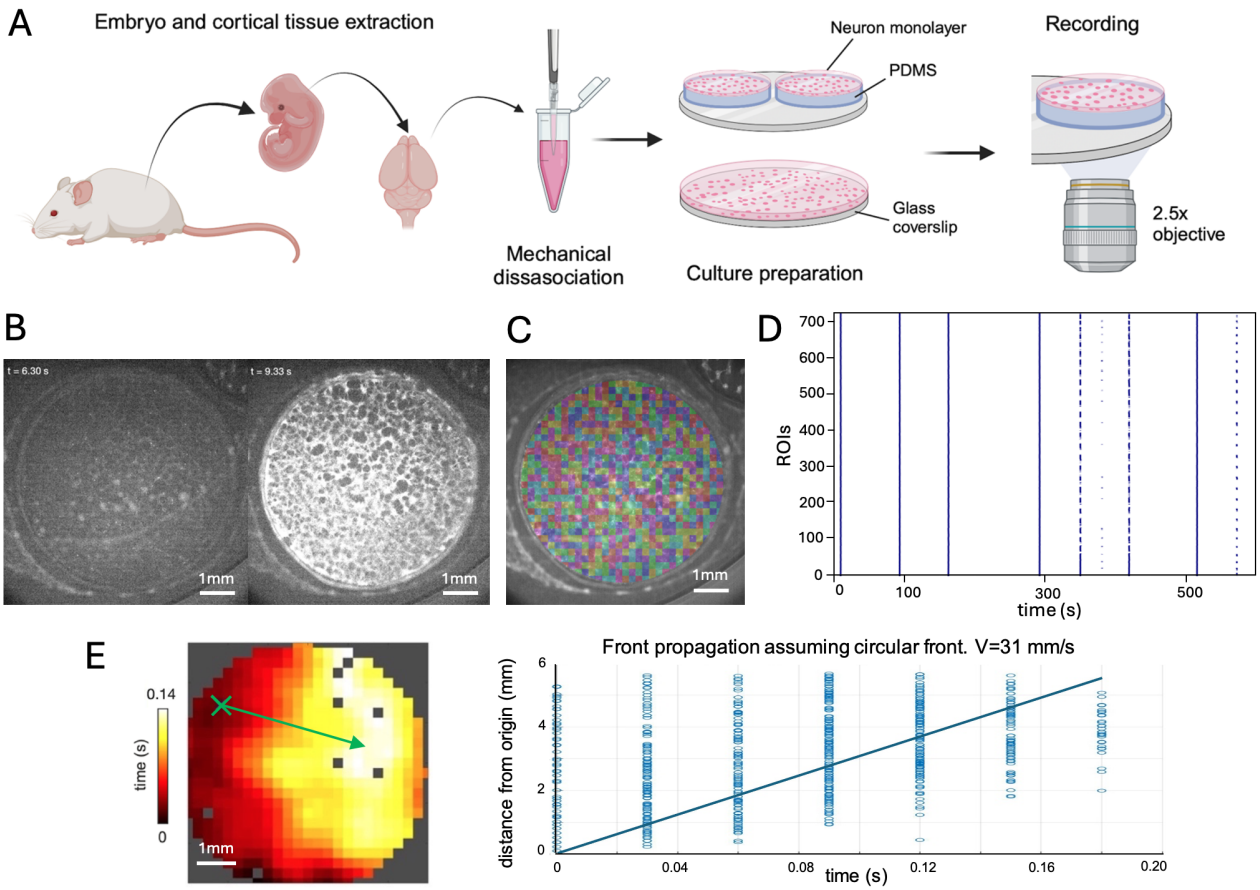

FIG. 1: Experimental procedure. **(A)** Diagram of the main experimental steps carried out in the laboratory, from the dissection to the recording of the cultures. Created with *BioRender.com*. **(B)** Grid of ROIs used for all experiments. **(C)** Fluorescence of the culture before and after a firing event. **(D)** Raster plot of spontaneous activity as a function of time. Each vertical line represents a collective firing event. **(E)** Front propagation, where the cross is the origin, and the green arrow the direction of propagation. **(F)** Distance from the origin to the front as a function of time, with its corresponding linear regression.

Percentage c/p of curing agent (%)	1	3	10	15	30
Elastic modulus (MPa)	0.2	0.6	2.0	3.0	6.0

TABLE I: Elastic modulus for all the PDMS curing agent to pre-polymer weight ratios. Computed using $E = \frac{20MPa}{n}$ as described in Ref. [6], where $n = p/c$ represents the PDMS pre-polymer to curing agent weight ratio.

that the elastic modulus of a PDMS polymer increases linearly with the amount of curing agent. Table I presents the elastic modulus for the studied curing agent percentages. Therefore, this mechanical property clearly reflects the increase in PDMS rigidity that comes with an increase of the percentage of curing agent.

C. Fluorescence imaging and pharmacology

We used fluorescence calcium imaging to record spontaneous neuronal activity. This technique is based on the visualization of the rapid increase of intracellular levels of Ca^{2+} during cellular communication. In order to do so, it relies on fluorescence sensors activated by calcium.

Our calcium-sensitive compound, GCaMP6s, is loaded into neurons via viral infection. Said compound undergoes a conformational change when it binds to Ca^{2+}, resulting in fluorescence emission. Therefore, when the bound GCaMP6s–Ca^{2+} complex is excited with blue light, the corresponding green emission can be captured by a fluorescence camera [2], thus allowing the monitoring of neuronal activity. Fig. 1B illustrates the increase of fluorescence that comes with a firing event that encompasses the entire network.

Culture connectivity can be modified with the use of drugs that block excitatory or inhibitory receptors, therefore preventing these neuronal subgroups to pass information, either to stimulate activity on other neurons (excitation) or to restrain it (inhibition). Our study included the use of *bicuculline* (BIC), an inhibition blocker that effectively makes the network more excitable on average.

D. Data analysis

The data was analyzed with a MATLAB-built software named NETCAL, short for 'Network Calcium analysis', developed by Javier G. Orlandi in 2018. NETCAL was designed to analyze high-speed high-resolution calcium imaging experiments [7].

Once the recording is loaded onto the software and preprocessed, the regions of interest (ROIs) must be defined. Our data was discretized using a 30×30 circular grid yielding a total of 732 ROIs per experiment, as seen in Fig. 1C. Subsequently, the software extracts the so-called traces, which illustrate the average fluorescence of each ROI as a function of time. Using the Schmitt inference, if activity is detected the ROI is assigned with the value '1' at time t, or '0' otherwise. Lastly, the data is represented in a raster plot, where each dot shows a ROI firing (a '1' in the time series), Fig. 1D. Apparent vertical lines in the raster indicate collective activity events that propagate very rapidly (few ms) across the network.

Afterwards, front velocity propagation was extracted using another of Jordi Soriano's softwares named *Visualize Front*. Assuming a circular propagation of the fronts, the software analyzes global firing events, which correspond to the vertical lines on the raster plot, and extracts the propagation velocity for each one of them.

E. Network measures

In order to further understand neuronal behaviour, we studied it using network theory. Complex theory refers to networks as an ensemble of nodes and their interactions through links [2]. In our study, the nodes correspond to the ROIs and the links to the physical connections, i.e., axons. We can determine how integrated a network is by computing its cross correlation. Essentially, when two ROIs have a similar train of spikes they are said to have a strong correlation or, in neuroscience terms, a strong *functional connectivity*. Thus, Pearson's correlation was computed for all pairs of ROIs as:

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

where $x_i(t)$ and $x_j(t)$ are the spike trains for neurons i and j, respectively, with mean values $\overline{x}_i$ and $\overline{x}_j$. Only the top 5% strongest connections were considered for further analysis, since they yield a reduced yet representative network structure. If r_{ij} belonged to the top 5% it was assigned a '1' and represented as a yellow dot. Oppositely, the correlation was considered weak and was set to '0'. Thus, the result is a 732×732 matrix containing the top 5% cross correlations, r_{ij}.

Once connectivity was defined, we computed a series of 'network measures' which give us some insight into the statistical properties of said network. Firstly, *Global efficiency*, G_{eff}, represents the network's ability to integrate information. It can be mathematically expressed as a function of the geodesic path [8]:

$$G_{\text{eff}} = \frac{1}{N(N-1)} \sum_{i \neq j}^{N} \frac{1}{d_{ij}}, \qquad (2)$$

where N is the number of nodes and d_{ij} is the topological distance between two nodes. G_{eff} ranges between 0 (the network is completely disconnected) and 1 (fully interconnected or 'complete graph').

The second descriptor is *Modularity*, Q. As its name suggests, it reflects the existence of communities of neurons, which are defined as neurons more interlinked within their group than with neurons outside of the community. Therefore, high modularity Q belongs to segregated networks and lower Q to highly integrated ones. The modularity is computed using recursive algorithms, and in the process, the cross correlation matrix is sorted into communities along the diagonal that maintain the original cross correlation of the network.

III. RESULTS AND DISCUSSION

A. Propagation velocity along development

In our experiments we analyzed 6 different rigidities that progressively increase with the percentage of curing agent, namely 1% (softest), 3%, 10%, 15%, 30% and 100% (stiffest), for DIV 6 and DIV 7. Results are shown in Fig. 2.

DIV 6 represents earlier stages of development of the culture. Front propagation velocities appear to decrease as rigidity grows. This behaviour seems to be reflected in all rigidities except for 10%, which has the highest velocities out of all.

On the other hand, cultures at DIV 7 reached a superior state of development. Front propagation velocity appears to have a more stable behaviour among the softest substrates, from 1% up to 15%. However, it must be noted that 10% concentration still presents the highest velocities. Once 30% rigidity is reached, there is a drastic drop of the propagation velocity, which intensifies even further for the stiffest substrate.

Front propagation velocities depend on a series of factors, namely average axonal length, connection strength, and the balance between excitation and inhibition [2]. In our study we assumed equal connection strength for all connections, and leave discussion of inhibitory drugs for later on. Average axonal length defines the average connectivity of the neuron, which in turn, characterizes velocity of propagation. As we previously mentioned, velocities tend to decrease with rigidity. Thus, this leads us to believe that mean axonal length also decreases with rigidity.

However, when comparing these results with literature, we found conflicting results. In 2009, Teixeira *et al.* [9]

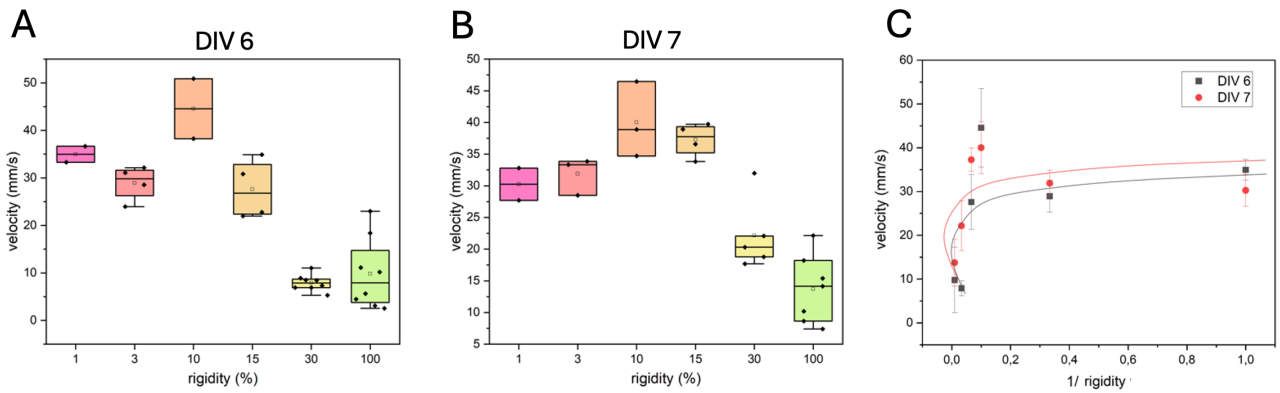

FIG. 2: Impact of rigidity on velocity. (**A**) Box plot of the front propagation velocities for increasing substrate rigidity, expressed as percentage of curing agent, in cortical rat cultures at DIV 6. (**B**) Corresponding results for DIV 7. (**C**) Front velocity propagation as a function of the inverse of substrate rigidity. Curves represent qualitatively the behaviour of Ref. [4].

showed that soft substrates promote maturation of stem-cell derived neurons. This study showed that neurons differentiated on soft substrates had long neurites proving substrate elasticity as an essential parameter in neuronal maturation. Whereas, two years later, Chou *et al.* [3] found the opposite. When researching the effect of different rigidities on the development of neuroblasts, they found that neurite projection was 35% shorter on PDMS than on glass.

Moreover, we tried to fit our results with the expected theoretical curve for velocities found by Jacobi *et al.* [4], as seen in Fig. 2C. The model predicts propagation velocities in a one-dimensional neuronal network as a function of the fraction of unblocked receptors. In our case, propagation velocities were expressed as a function of the inverse of rigidity. Even though a similar behaviour was obtained, all velocities were slower, typically by a factor of 3 to the original model. Since that model is a one-dimensional idealization, and our study is two-dimensional, we have to consider that in the latter, average neuronal connectivity is inferior, thus resulting in lower velocities. Conceptually, in two dimensions the area that axons need to cover is much greater than in one dimension and, consequently, the propagation of information is slower.

Neuronal cultures change very rapidly during the developmental stages. Fig. 2C reflects larger velocities for DIV 7 when compared to DIV 6. Our proposed conceptual model is shown in Fig. 3. For early stages, neurons only connect to a first neighbour approximation. Since the path information has to take to be propagated is longer, it results in lower propagation velocities. On the other hand, when development reaches the young stage at DIV 7, the connections between neurons are longer. Neurons at larger distances are now interacting with each other, as a result of increased mean axonal length. Therefore, front propagation velocities for DIV 7 are higher on average.

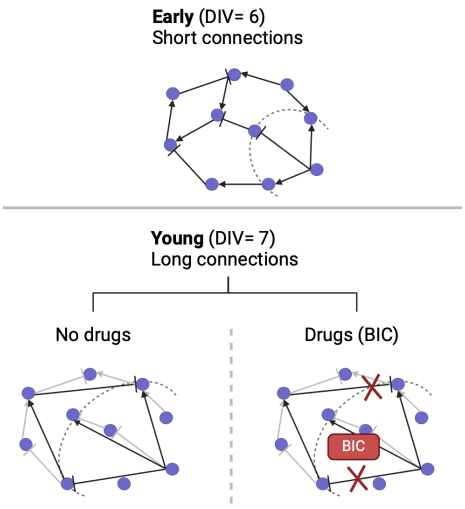

FIG. 3: Conceptual model for the development of cultures. Top: early cultures (DIV 6) present short connections with excitation and inhibition. Bottom: young cultures (DIV 7) present longer connections with inhibition and excitation in basal conditions, but in the presence of bicuculline (BIC) inhibition is blocked. Black arrows show majority connections and grey arrows remnants of short connections which may still be viable. Created with *BioRender.com*.

B. Functional connectivity

In basal conditions, there is a certain balance between excitation and inhibition which, as previously stated, has an effect on the velocity propagation of activity fronts. Fig. 4 shows the sorted cross correlation matrices before and after the application of bicuculline in a culture with a 15% curing agent PDMS substrate.

When no drugs are present, inhibitory and excitatory connections are activated. Consequently, certain regions in the culture are constantly slowing down the propa-

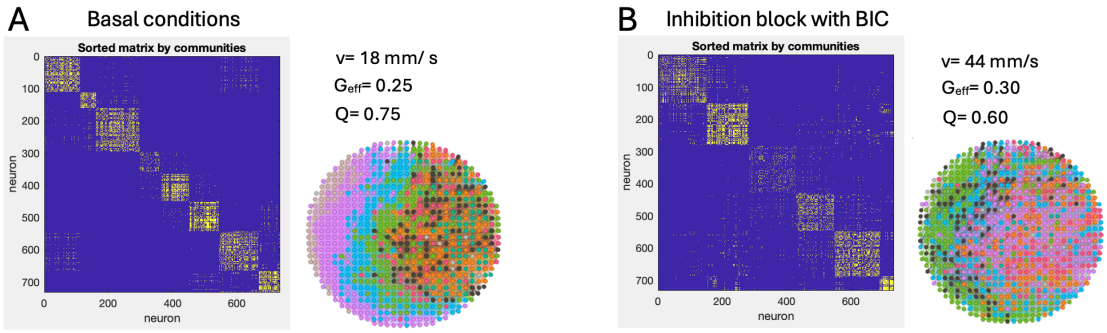

FIG. 4: Sorted cross correlation matrices and network maps for 15% concentration curing agent (moderate rigidity). (**A**) Data with no drugs. (**B**) Data after application of bicuculline. In the matrices, yellow dots indicate a functional connection. Corresponding network measures are shown in both cases. For the maps, circles are ROIs and colors indicate communities.

gation of information. On the contrary, in the presence of bicuculline, inhibitory connections are fully silenced. In turn, the network's ability to distribute information across all regions increases. This behaviour is portrayed in bottom section of Fig. 3.

Results are shown in Fig. 4. System dynamics reveal communication patterns between ROIs. As expected, in the presence of bicuculline, modularity decreases and global efficiency increases. When inhibition action is blocked, the network becomes more integrated. Networks are shown as *Gephi graphs* and provide a visual representation, where colors reflect the different communities present in the sorted cross correlation matrices.

Globally, the blocking effect of bicuculline translates into an increase of propagation velocities. More segregated networks (product of the presence of inhibition), Fig. 4A, present lower propagation velocities. Whereas, when networks are more integrated, Fig. 4B, the propagation velocities are higher.

IV. CONCLUSIONS

Our study shows that substrate rigidity does have an impact on local and global network parameters. Preliminary results have proven that rigidities above 6 MPa, those corresponding to 30%, substantially decrease front propagation velocity. Hence, we arrive to the conclusion that substrate rigidity shapes axonal length. We hypothesize that softer substrates would promote axonal elongation, whereas stiffer ones would have the opposite effect. Furthermore, the characterization of the early developmental stages of cultures has also proven to influence axonal length.

Overall, substrate rigidity and developmental stage are two essential factors that must be taken into account when studying *in vitro* culture dynamics. Nevertheless, our research has also confirmed modeling *in vitro* neuronal cultures is as challenging as it is significant.

Acknowledgments

I would like to extend my profoundest gratitude to my advisor Dr. Jordi Soriano, for guiding me throughout the project while also sharing his passion for research. In addition, I would also like to thank PhD student, Mireia Olives, and Belén Montenegro with whom I shared countless hours in the laboratory. Finally, I would like to extend my deepest appreciation to my parents and sister for their unconditional support.

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