Impact of a chemical perturbation on the functional traits of living neuronal networks in a model for Alzheimer's disease

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Abstract: Our understanding of the mechanisms underlying the process of network degeneration in Alzheimer's disease (AD) is still limited. However, a key agent associated to the disease is the protein Tau, whose malfunction is considered to cause neurodegeneration and death. In the present work we investigated for evidences of this malicious role of Tau using *in vitro* neuronal cultures. We analyzed recordings of spontaneous activity with and without Tau along different days *in vitro* to compare a healthy culture and one in which Tau protein was injected. The comparison was made through a set of complex networks descriptors. The results indicate that the Tau cultures shape a highly integrated network with a lot of neuronal activity, i.e., abnormally too active as compared to controls. The behaviour of Tau cultures leads to incorrect coding of information, as occurs in an Alzheimer's-affected brains, evidencing the involvement of the Tau protein in this disease.

I. INTRODUCTION

Alzheimer's disease (AD) is one of the major neurodegenerative disorders and is characterized by the progressive decline and ultimately affectation of multiple cognitive functions, such as memory impairment, loss of learning ability, and gradual decline in behavioural tasks [1]. AD accounts for approximately 70% of all cases of dementia [2], and its prevalence increases exponentially with age, rising from 3% between the ages of 65-74 years to 50% between the ages of 85 or older. In addition, it affects 25 million people worldwide [3].

The most specific pathological feature of AD is the loss of synapses. The protein Tau, when working correctly, is a central protein that organizes and stabilizes the structure of microtubules along neuronal axons. However, it has been observed in AD patients that Tau changes its function when becomes hyperphosphorylated, causing a degradation of axons and impairing axonal transport. This leads to a decrease in synaptic inhibition, which is important for proper flow of information in the brain [4]. Indeed, living neuronal circuits rely on a balance between excitation (tendency to activate neurons) and inhibition (tendency to inactivate them) to correctly transport and process information. This balance can be viewed as the correct timing of green and red traffic lights in a city. Thus, an imbalance between excitation and inhibition leads to a hyper-excitable network and, as a result, there is an incorrect dynamic functionality of the brain and information cannot be encoded correctly.

Since the human brain is highly complex, neuronal cultures are used to analyse the behaviour of neuronal circuits affected by AD in a control manner. This simplifies the system, which can be perturbed by injecting hyperphosphorylated Tau proteins, allowing us to observe differences in neuronal activity and connectivity between a healthy control culture and one affected by Tau. Therefore, these culture systems offer the opportunity to decipher mechanisms involved in neuropathogenesis.

II. MATERIALS & METHODS

Neuronal cultures.— Currently, neural cultures are particularly relevant for research into early markers of neurodegenerative diseases. They simplify the system and allow them to be disrupted, a task that cannot be carried out in the brain, as it is a highly sensitive system. In this way, *in vitro* neural cultures allow us to study the development of connections in living neural networks, and the interaction between connectivity, activity and function [5].

These *in vitro* preparations, for the specific case of our study, were obtained from embryonic rat cortical tissues, dissociating the neurons and culturing them on a 4 mm diameter glass substrate (Fig. 1A). In order to analyse the possible differences between healthy neurons and those affected by Alzheimer's disease, Tau proteins were injected into one of the two cultures to obtain a pathological neuronal network. These cultures were prepared by Dr. Soriano's group and were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Barcelona.

The self–organising potential of the neurons is such that within a few days after culturing them on the glass substrate, they reestablished a network with a rich spontaneous activity, which is recorded by calcium fluorescence imaging [5].

Calcium imaging.— Calcium fluorescence imaging is a technique for measuring the spontaneous activity of the neuronal network using a protein that is susceptible to fluorescent emission after binding Ca^{2+} ions. In other words, when the neuron is activated, a calcium intake occurs, modifying its intracellular concentration and causing a flux, which is detected by the fluorescent marker. Therefore, during neuronal activity, active neurons can be visualised as bright objects with single–cell resolution, as can be seen in Fig. 1A [5, 6].

Spontaneous activity was recorded with a fluorescence

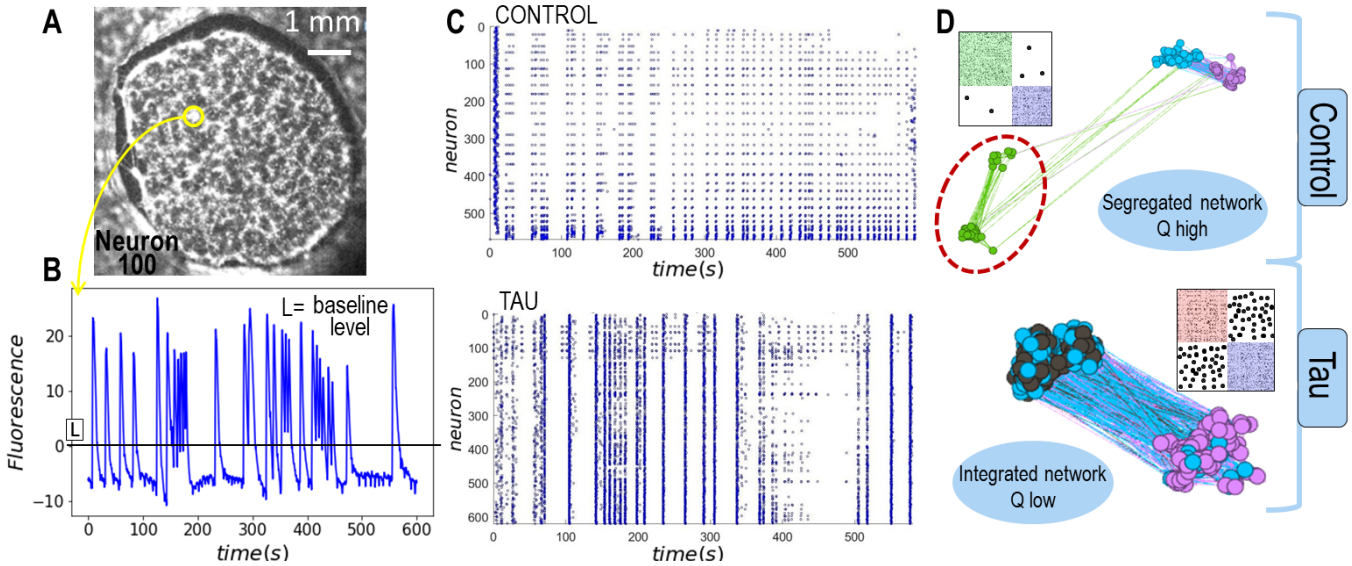

Fig. 1: **A:**In vitro culture visualised with fluorescence, where active neurons appear as bright dots. Belongs to the second 79. **B:** Fluorescence traces of the Tau culture of neuron 100, obtained from image A. **C:** Raster plot representing spontaneous activity (spike events), over time for each neuron in the network. The presence of activity is only represented by a dot if there is a relative change from a baseline level. **D:** Sketch and correlation matrices of functional differences between an integrated network (lower Fig.) and a segregated one (upper Fig.). Surrounding one of the communities of the segregated network, it is completely isolated, performing a specific task. This does not happen in the case of the integrated network.

camera and the calcium images obtained were analysed using the Netcal software, which corrects basal fluorescence, light fluctuations and drifts. In this way, the data were processed, obtaining peaks which indicate a joint activation of neurons, called a network burst, as can be seen in Fig. 1B. The data were then discretised into a table in which each element represents regions of interest (ROI), and from which, average fluorescence levels were extracted. These were transformed into a binary signal (spike train), being '1' if there is a relative change in neuronal activity from baseline levels of each ROI at a certain time t, or '0' if there is no increase in the relevant signal. From there, raster plots (Fig. 1C) were obtained.

This procedure, carried out using Dr. Soriano's software, made possible to obtain the data used in this study, which allowed us to compare the spontaneous activity between healthy neurons and those affected by Tau protein. The data provided are values of the fluorescence traces of about $700 - 750$ neurons every 0.03 s for 10 minutes.

III. PROCEDURE & DATA ANALYSIS

A. Spontaneous activity

Spontaneous activity in cultures is characterised by episodes of intense, almost synchronous activity events in the form of collective neuronal features, called bursts, combined with a quiet interval of sporadic activity. Therefore, the peaks of the fluorescence signal identify burst episodes and are entirely related to spontaneous activity. The latter is involved in the development and interconnectivity of forming circuits. In the adult brain, it plays a key role in sensory stimulus selection, information processing and memory consolidation, among many other aspects. Accordingly, many neurodegenerative diseases have been shown associated with significant alterations in spontaneous activity patterns [5].

From the theoretical foundations mentioned in the section I, it is expected that, due to the relative decrease in synaptic inhibition, and consequently hyper–excitability in the network, the spontaneous activity for the Tau cultures to get altered as compared to the control ones.

B. Effective connectivity

Connectivity is the engine of a neural system and the distribution of connections outlines the circuits of the complex network, whose regions are functionally and structurally interconnected. Structural connections correspond to the physical wiring (synapses) that links and communicates neurons with each other; whereas functional connections represent the pathway along which information flows between active neurons. In the latter case, functional links between two neurons are understood in a general sense as statistical correlations between their dynamic patterns [5]. Mathematically, the functional connectivity between all ROI pairs can be obtained by considering Pearson's correlation [7]:

$$R_{ij} = \frac{\sum_{k=1}^{N} (a_{ik} - \bar{a}_i)(a_{jk} - \bar{a}_j)}{\sqrt{\sum_{k=1}^{N} (a_{ik} - \bar{a}_i)^2} \sqrt{\sum_{k=1}^{N} (a_{jk} - \bar{a}_j)^2}}, \quad (1)$$

where a_{ik} and a_{jk} are the spike trains of ROIs i and j, and $\bar{a}_i$, $\bar{a}_j$ their respective mean values. The coefficients

R_{ij} range from 0 to 1. If a pair of ROIs are perfectly functionally equivalent (have the same behavior), then the result will be 1. In general, neurons that have similar behavior tend to shape functional communities, which are groups of neurons that tend to connect with each other more strongly than with the rest of the network. Therefore, depending on the number of functional communities, the network will be more segregated or more integrated. Integration is associated with the ability of a neural network to function as a whole and exchange information efficiently, while segregation refers to the ability to distribute information to localised communities that perform specialised tasks [8]. This behaviour can be represented in Fig. 1D, in which correlation matrices are also shown, constructed from the values of the coefficient R_{ij} for each pair of ROIs.

To analyse the functional connectivity and the values of global efficiency and modularity, magnitudes explained in section III C, we have used a Matlab program called `Main_GTE.m`. Likewise, the neural networks have been represented using Gephi software, and the functional distance and the time evolution of the magnitudes mentioned above, with Python.

C. Global efficiency, Modularity & n. communities

Global efficiency, G_{eff}, quantifies the traffic capacity of a network, *i.e.*, the capacity to exchange and propagate information. It is defined as follows [7]:

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

where N refers to the total number of neurons, and d_{ij} indicates the minimum topological distance between neurons i and j. Therefore, a higher value of G_{eff} is associated with a more interconnected network.

On the other hand, **modularity**, Q, is related to the structure of the network and measures the degree of correlation between the number of links within communities and the links between communities. In other words, it quantifies the strength of division into communities, indicating how isolated they are from the rest of the network. It is obtained by complex mathematical algorithms and its value is between 0 and 1. When $Q = 0$, the network is highly integrated, being itself the only community. In contrast, when $Q = 1$, there are as many neurons as communities, indicating a strong community structure and leading to a segregated network.

Both magnitudes mentioned are related to the **number of communities**, N_{comm}. If there are many communities, then Q will be higher and G_{eff} will be lower, and the neurons will be more disconnected from each other.

From the information mentioned in Sections I and III A, it is expected that the Tau culture will have a smaller number of communities, due to the high integrability of the network associated with the decrease of inhibitory synapses. This leads to an excess of communication between all neurons and a global and non-functional exchange of information.

IV. RESULTS & DISCUSSION

A. Spontaneous activity

Fig. 2A represents the fluorescence amplitude as a function of time for a pair of neurons (control and Tau) at day *in vitro* DIV 12. However, in practice, representations have been made of not just 1 but a random 20 neurons to see if they were all behaving similarly. In this way, comparing both cultures, it was found that in most cases there was a significant increase in the spontaneous activity of the Tau culture, as expected. To generalise this behaviour to the whole network, we calculated the averages of activity for different DIVs, as can be seen in Fig. 2B. It can be noted that in the first DIVs, the activity of both cultures is quite similar and does not follow a clear trend, as it belongs to the beginning of the maturation process [9]. Over time, the Tau culture shows an increase in spontaneous activity, leading to over–excitation. However, from DIV 14 onwards, the two culture types are getting closer again. We hypothesize that the Tau culture activates plasticity mechanisms and self–regulates. Then, it starts to decrease activity to approach a moderate rate and correct this deficiency. This behaviour cannot be extrapolated to the brain, as the disease is constantly evolving in it, and unlike the culture, it is not isolated.

Following the analysis of the spontaneous activity, colour maps of the local maxima of the fluorescence traces were made to observe the homogeneity of the cultures. For all the DIVs, more heterogeneous maps were obtained for the Tau culture, which shows specific areas of high activity. In contrast, the colour map of the Control culture is more homogeneous, as expected, as it presents more periodic bursts with similar intensities. This behaviour can be verified in Fig. 2C. In general, the Tau culture shows higher peaks, of different intensities, and larger areas, resulting in a higher interburst interval (IBI).

B. Functional connectivity

From Fig. 2D, it can be seen that both cultures are organised in functional communities, although in different ways. The control culture has a strong modular organization. The Tau one has less modular organisation and a more integrated network, as the number of coloured boxes is lower. It also has a higher overall connectivity, since there is a high density of black dots. Fig. 2E shows the location of the neurons in the culture and the extension of the modules. For the control condition, modules are compact in space (similar colors are nearby) while for Tau they are mixed. Thus, the Tau network is more integrated, since neurons from different communities are more spread out, exchanging information more effectively with each other. In contrast, the network of the Control culture is more segregated, i.e. each community is concentrated in a specific region and performs specialised tasks.

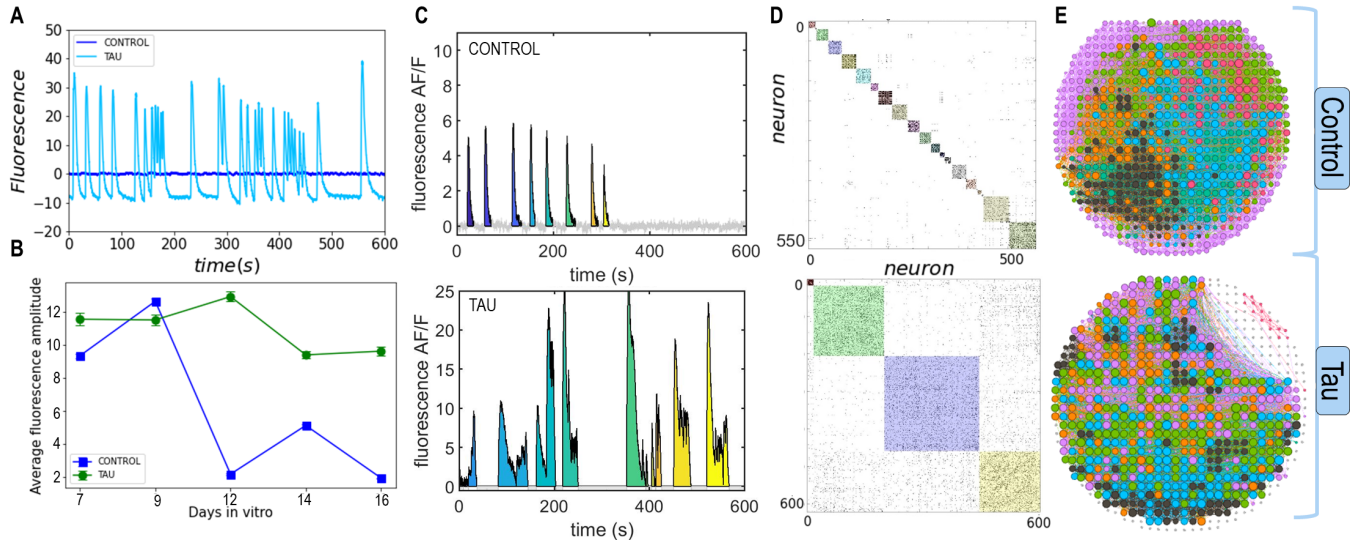

Fig. 2: **A:** Fluorescence traces representing the spontaneous activity of the Control and Tau cultures as a function of time at DIV 12 of neuron 620. **B:** Time evolution of the average local maxima of the fluorescence traces for each neuron in both cultures. The error bars represent the standard deviations across maxima in each culture. **C:** Area under the fluorescence signal of each burst, visualising the fluorescence traces of 2 randomly chosen neurons at DIV 12. Note that the vertical scale is different in control and Tau. **D:** Correlation matrices of both cultures at DIV 12, which show the segregated or integrated behaviour of each one of them. The black dots represent the functional connections and the coloured boxes represent the functional communities present in the network. **E:** Spatial maps of the effective networks for each culture. Neurons are colour–coded according to the functional community to which they belong. The diameter of each neuron is proportional to the number of functional connections.

Overall, the Tau system is more synchronous, with spread out communities that make neurons to connect across long distances. In addition, using Python, we computed the functional Euclidean distance from one neuron to another and, on average, functional connections extend a longer distance for Tau as compared to control. Indeed, at DIV 12, we observed that, for Tau, about 3300 connections connected at a distance of 0.20 mm. By contrast, for the control case, about 1100 connections connected at a distance of 0.15 mm.

Finally, we analyzed the number of connections that a neuron established. In the case of the Tau culture, many neurons have a single connection, and after that, most of them have an average of 40 connections. For control culture, the average was always below the Tau condition. This result makes sense, since the latter network is more integrated.

The evolution of the most important connectivity descriptors along DIV (G_{eff}, Q, N_{comm}) is shown in Fig 3A. Tau is always different from control, although the differences slightly change along time. Thus, in general, the functional organization follows the same trend, with Tau showing more connections, a higher G_{eff}, a lower Q, and N_{comm}.

C. Extension of the analysis to other data series

All the information presented above are results obtained with a single data series, *i.e.*, a single pair of control and Tau cultures. In summary (Fig. 3A), the Tau culture has a higher G_{eff} and a lower Q than control. This is an expected trend for the strongly synchronous Tau system.

To investigate whether the results hold in other experimental repetitions, and to have more statistics, the same analysis has been done with more series. Unfortunately, as shown in Fig. 3B, we immediately observed that there was a lot of variability and the averages followed an unclear trend. The G_{eff} value of the Tau culture is lower than the Control one, when in principle it should be the other way around. Moreover, the values of Q and N_{comm} are very similar in both cultures. Even so, at DIV 12, the results for a high synchronization in the Tau culture are fulfilled.

The variability in the averages of the 3 series may be explained by many factors, such as the fact that it is a living system and therefore is very fluctuating; the existence of errors when performing the experiment; and the difference between the brain and a neuronal culture, since the first one may have an integrated element that the culture does not have.

Finally, it should be noted that panels A, B in Fig. 3 are computed by excluding the non–active neurons, which are the ones that are dying and changing the network. They were excluded since the network measures (G_{eff}, Q and N_{comm}) are very sensitive to the presence of non–connected nodes and become unreliable. For instance, each non–active neuron is counted as a single community (N_{comm} becomes too large) and G_{eff} quickly

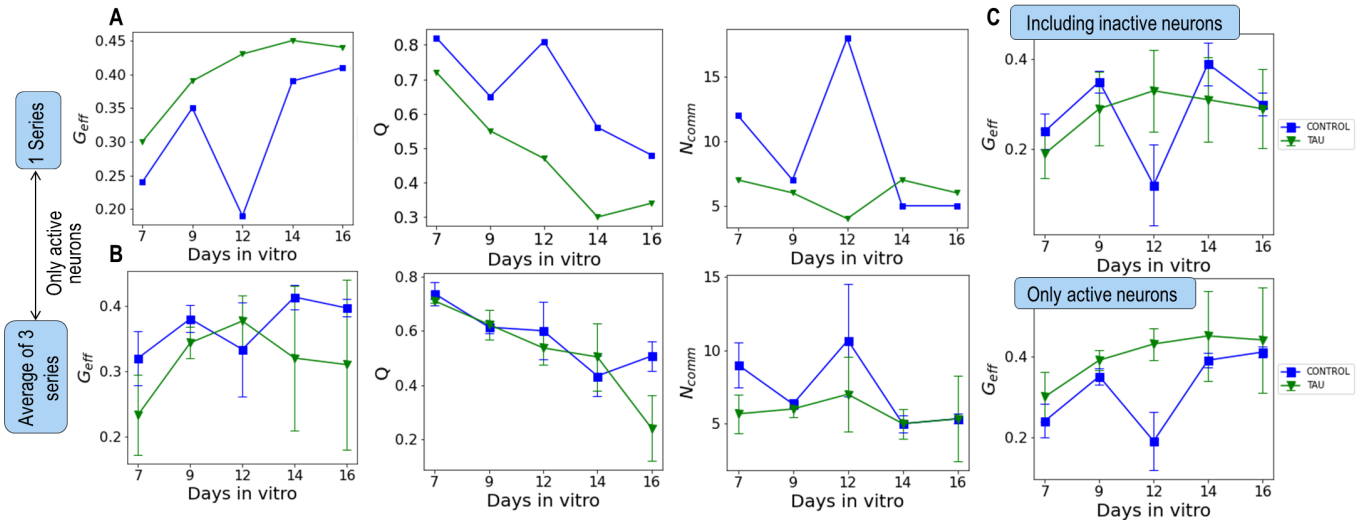

Fig. 3: Time evolution of different network parameters, obtained with an acceptance threshold $Z_{score} = 2$. **A:** Time evolution of global efficiency, modularity and number of communities obtained from the data of a single series. **B:** Time evolution of global efficiency, modularity and number of communities obtained from the average of 3 data series. The error bars shown are calculated from the standard error between the 3 series. **C:** Time evolution of the global efficiency, obtained from the data of a single series, showing the differences between including or not the non-active neurons.

drops to zero. Thus, the results are distorted. A comparison without and with non–active neurons is shown in Fig. 3C. When non–active neurons are included, only comparable trends are obtained at DIV 12.

V. CONCLUSIONS

With the first set of data, we have obtained results that show an excess of spontaneous activity, functional connectivity and synchrony in the Tau culture. In addition, based on $G_{\text{eff}}^{C}<G_{\text{eff}}^{Tau}$ and $Q^{C}>Q^{Tau}$, we can conclude that the neuronal network of the Tau culture is characterised by high integration and incorrect coding of information. This behaviour is similar to that of a brain affected by AD and therefore good results are obtained.

However, when more statistics are included, the expected trend does not hold, since there is some variability, especially in the values of G_{eff}, Q and N_{comm}. Therefore, these parameters by themselves may not be sufficient to completely pinpoint damage and other statistical approaches may be required. This fact shows an honest view of science, as there are many phenomenon and conditions that have to be taken into account and can lead to discrepancies in the results. Even so, in both cases there are differences between cultures, showing a certain trend, and at DIV 12 the expected results are obtained, whether non-active are included or excluded.

This study provides evidence for the involvement of the Tau protein in AD, although more research is still needed, since adding more statistics leads to more variability in the results.

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