

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

# **Advanced Analysis of Extracellular Potentials: Exploring the Generation of Local Field Potentials**

**João Almeida Santiago**



Master in Bioengineering - Biomedical Engineering

Supervisor: Prof. Joost le Feber

Co-Supervisor: Prof. João Paulo Cunha

October 4, 2022



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Approved in oral examination by the committee:

Chair: Prof. Miguel Velhote Correia

External Examiner: Prof. Ivan Cohen

Supervisor: Prof. Joost le Feber

Co-Supervisor: Prof. João Paulo Cunha

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# Abstract

Local Field Potentials (LFPs) are extracellular signals believed to represent the activity of neuronal populations. Although the mechanisms behind the signal are consensual, their interpretation remains a challenge, with information contained in the signal waiting to be unraveled.

Using Microelectrode Arrays to perform electrophysiological recordings on *in vitro* cortical networks has proven to be a valuable research tool over the last years. With advances in signal acquisition techniques and data processing methodologies, the vast world of neuronal activity has started to be disentangled. Yet, much regarding these signals is still unknown or under debate, especially regarding LFPs and their relation with spiking activity.

Here, we used 60-electrode MEAs to record activity from dissociated cortical neuronal cultures and analyzed two separate frequency components: we detected Action Potentials (APs) in the high-frequency signal (above 300Hz) while recording LFPs in the low frequencies (below 35Hz). The developed project has three major lines of experimentation: firstly, we used transfected Channelrhodopsin-2 cultures with an H134R mutation, targeting only excitatory neurons, in an optogenetic stimulation protocol; secondly, we tested different combinations of neurotransmitter receptor blockers on cultures, being the used antagonists: PTX (50 $\mu$ M) for GABA<sub>A</sub>, and CNQX (10 $\mu$ M) and APV (100 $\mu$ M) for AMPA and NMDA, respectively; lastly, we simulated signals in Matlab to mimic the ones observed *in vitro*. Apart from simulations, the experiments were conducted while performing electrophysiological recordings.

Optogenetic stimulation results show a detectable negative deflection in the low-frequency component when cultures with suppressed activity are hit with a blue light pulse. This deflection increases in amplitude and peak latency with the duration of the light. This suggests that negative deflections in LFP signals represent an influx of cations to the cells (excitatory depolarization) with a possible gradual recruitment of voltage-gated ion channels.

Different recording conditions gave rise to distinct signals. In general, under control conditions, only a negative deflection is visible in the LFP signal, whereas blocking inhibition synchronizes the firing of the neurons and adds a measurable positive peak to the low frequency. We hypothesize that excitatory synaptic currents are the main contributors to the signal and that spiking activity influence can also be visualized when synchronicity in firing is very strong. The proposed hypothesis explains the observations in this study and is biophysically valid.

By simulating signals, we identify some artifacts and give some insight into potential effects of the filters applied to the signal. For example, an overshoot effect is generated by the filter which does not carry any relevant information.

All things considered, the developed project explored some concepts regarding *in vitro* cortical LFPs which may be relevant to understand the origin of such signals.

**Keywords:** Local Field Potentials (LFPs), Optogenetic Stimulation, Cortical *in vitro* cultures, Neuronal Excitation and Inhibition, Electrophysiology



# Resumo

Os Potenciais de Campo Local (LFPs) são sinais extracelulares que se acredita representar a atividade das populações neuronais. Embora os mecanismos por detrás do sinal sejam consensuais, a sua interpretação ainda é um desafio, com informação contida no sinal à espera de ser desvendada.

A utilização de microelétrodos para registos electrofisiológicos de redes corticais *in vitro* provou ser uma ferramenta de investigação valiosa nos últimos anos. Com os avanços nas técnicas de aquisição e metodologias de processamento de sinais, o vasto mundo da actividade neuronal começou a ser descoberto. No entanto, muito acerca destes sinais ainda é desconhecido ou está em debate, especialmente no que diz respeito aos LFPs e à sua relação com a actividade neuronal.

Aqui, utilizamos 60 microelétrodos para registar a atividade das culturas corticais, sendo analisadas duas componentes de frequência: deteção de potenciais de acção (APs) no sinal de alta frequência (acima de 300Hz) e registo de LFPs nas frequências baixas (abaixo de 35Hz). O projeto tem três experiências principais: primeiramente, utilizamos culturas transfectadas com uma mutação H134R de Channelrodopsina-2, afetando apenas neurónios excitatórios, num protocolo de estimulação optogenética; em segundo lugar, testamos diferentes combinações de bloqueadores de receptores neurotransmissores, sendo os antagonistas utilizados: PTX (50 $\mu$ M) para GABA<sub>A</sub>, e CNQX (10 $\mu$ M) e APV (100 $\mu$ M) para AMPA e NMDA, respectivamente; por fim, simulamos sinais em Matlab para mimetizar os observados *in vitro*. Exceptuando as simulações, as experiências foram acompanhadas da realização de gravações electrofisiológicas.

Os resultados da estimulação optogenética mostram que uma deflexão negativa na componente de baixa frequência é sentida quando culturas com actividade suprimida são atingidas com luz azul. Esta deflexão aumenta em amplitude e latência de pico com a duração da luz. Isto sugere que as deflexões negativas nos sinais LFP representam influxo de catiões (despolarização excitatória) com um possível recrutamento gradual de canais iónicos dependentes da voltagem.

As diferentes condições testadas deram origem a sinais distintos. Geralmente, no controlo, apenas uma deflexão negativa é visível no sinal do LFP, enquanto que o bloqueio da inibição sincroniza o disparo dos neurónios e adiciona um pico positivo mensurável à baixa frequência. Hipotetizamos que as correntes sinápticas excitatórias são as principais contribuintes para o sinal e que a influência da actividade neuronal também podem ser visualizada quando a sincronidade no disparo é muito forte. Esta hipótese explica os resultados observados e é biofisicamente válida.

Ao simular os sinais, identificamos alguns artefactos e temos alguma percepção dos potenciais efeitos dos filtros aplicados ao sinal. Por exemplo, um efeito de *overshoot* é gerado pelo filtro, o qual não transporta qualquer informação relevante.

Em suma, o projecto desenvolvido explorou alguns conceitos relativos *in vitro* aos LFP corticais que podem ser relevantes para compreender a origem de tais sinais.

**Keywords:** Potenciais de Campo Locais (LFP), Estimulação Optogénica, Culturas corticais *in vitro*, Excitação e Inibição Neuronal, Electrofisiologia



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João Almeida Santiago



*“Be not astonished at new ideas; for it is well known to you that a thing does not therefore cease to be true because it is not accepted by many.”*

Baruch Spinoza,  
Dutch Philosopher of Portuguese Origin



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# Abbreviations

|        |                                                           |
|--------|-----------------------------------------------------------|
| AMPA   | Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic Acid |
| AP     | Action Potential                                          |
| APV    | 2-amino-5-phosphonovaleric Acid                           |
| ChR-2  | Channelrhodopsin-2                                        |
| CNQX   | Cyanquixaline                                             |
| GABA   | Gamma-aminobutyric Acid                                   |
| LED    | Light-Emitting Diode                                      |
| LFP    | Local Field Potential                                     |
| MCS    | MultiChannel Systems                                      |
| MEA    | Microelectrode Array                                      |
| NBQX   | 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline         |
| NMDA   | N-methyl-D-aspartate                                      |
| PTX    | Picrotoxin                                                |
| RMS    | Root Mean Square                                          |
| st-LFP | Spike-triggered Average of the LFP                        |



# Chapter 1

## Introduction

"Sitting on your shoulders is the most complicated object in the known universe" -  
Michio Kaku

The brain is probably one of the most complex and fascinating structures in the human organism. As research in this field grows in interest among the scientific community, more and more is discovered about this complex organ that controls the human body and most of its conscious and unconscious actions. Technological developments and advances in investigation techniques broaden the horizons for discoveries, thus enlarging the knowledge we have of how processes take place in one of the most captivating systems present in human physiology: the nervous system. This knowledge is then prone to open doors for new approaches to the diagnosis and treatment of diseases as well as new paradigms on neuronal functions.

In fact, experimental work in this area has been growing exponentially, with a growing number of teams dedicating their efforts to solving the many mysteries of the brain. Despite a large number of recent discoveries, much is still unknown or under debate as neural functioning is not as straightforward as science would like it to be. Simply by keeping in mind that the brain has billions of neurons, each one connected to thousands of others, one can start grasping the complexity of interactions that take place every second, and understand the difficulty in coming up with accurate models for its mechanisms.

This is particularly interesting when one takes into account a large amount of yet-to-be-effectively treated diseases that affect the functioning of the nervous system, most of which verify a high incidence rate worldwide, hampering day-to-day life of numerous patients. As so, comprehending and describing the pathological pathways behind these conditions would be the vital first step in possibly coming up with new strategies for dealing with them and recovering quality of life for people who suffer from them. Naturally, one can only recognize when something is atypical after being familiar with the usual behaviors of the affected system. Hence, the perspective of one day being able to deal with situations that seem complicated in the present motivates scientists to keep unraveling the mechanisms that allow for the normal neural functioning of a human brain.

## 1.1 Motivation

Any research should start by questioning some phenomenon, being this the motivation of the work to be developed. In this particular case, the major focus is electrophysiological recordings and the valuable information that can be obtained from them. As in any investigation, the first step towards a plausible conclusion is disentangling complicated problems into smaller and simpler ones. In the case of neural functioning, cultures plated on Microelectrode Arrays (MEAs) are perhaps one of the most valuable tools for research. The freedom of experimentation that they offer, together with the ability to capture in real time the signals transmitted between different cells has opened doors to crucial findings and is very likely to keep doing so in the future. After all, it may well be in these signals that information can be contained, waiting to be translated into usable knowledge.

With this in mind, a lot of effort has been applied in decoding the neuronal potentials, in particular Action Potentials (APs) and, more recently, Local Field Potentials (LFPs). Understanding where such signals come from can be the fundamental step that needs to be taken in order to start retrieving physiologically relevant information from these. This, in turn, can be the key to deal with neurological diseases and paint a clearer picture on how exactly neurons respond to stimuli as well as why and when is this responsiveness affected.

## 1.2 Research Goals

The proposed project aims, in its essence, to expand the knowledge on LFPs, dwelling on what information is contained in these signals as well as what is their relationship with APs, which are already pretty extensively studied and characterized. In order to do so, several important questions are at the center of the conducted research:

1. **Is there a physiological relevance in local field potentials?** Some debate still exists amongst the scientific community on whether LFP signals are mainly noise from the recording setups or even a simple consequence of the respective high frequency component, with no extra information.
2. **What information is contained in the Local Field Potential?** Having the confirmation that the LFPs is a signal with biological content, one goal of the project is to elaborate on how this signal is generated and what phenomena can be described and therefore studied based on the new information in them contained.
3. **Is there a direct relationship between LFPs and APs?** Even though Action Potentials (and the information in them contained) are widely studied, their correlation with LFPs is still not fully characterized and could lead to new insights about neuronal functioning and the definition of neuronal excitability.

As so, the presented thesis aims to validate the role of LFP signals, to investigate whether they contain valuable information, not obtainable through the analysis of APs, to elaborate on this new content, and to characterize the dynamic relationship between the two signals.

## 1.3 Document Structure

The developed work is presented in this document, organized in principal 7 chapters, as follows:

- Chapter 1 presents some brief introductory notes on the motivation behind the project;
- Chapter 2 addresses some fundamental concepts important to understand the work to be presented, mainly focusing on the biophysics of neurons, on the way they interact with each other, and on how their activity can be captured for further analysis;
- Chapter 3 presents a review of what has been investigated so far in terms of LFPs, highlighting what is consensual among scientists and what remains under debate, motivating the work developed to fulfill such gaps;
- Chapter 4 contains the followed methodology regarding cell culturing, electrophysiological recordings acquisition, data treatment, signal processing, simulations in Matlab, and statistical analysis;
- Chapter 5 contains an extensive presentation of the results from this research across seven sections that contain information about the included cultures, the filtering results, the proof that there are differences between the two frequency components, the optogenetic stimulation outcome, the relation between APs and LFPs, the shapes observed in distinct recording conditions, and the simulated signals in Matlab;
- Chapter 6 discusses the results, looking for the common patterns between experiments and drawing conclusions from such, with the postulation of what information is contained in LFPs and the proposition of a new hypothesis for their generation;
- Chapter 7 offers a brief overview of the entire work comprising the principal findings that translate into contributions for the field, together with some prospects for future work to be developed;

Besides this, some additional material is also available for consultation:

- Appendix A gives an overview of the included cultures in the different conditions that were tested. Some characterization of each culture is made along with the description of the analysed electrophysiological recordings (with some important values presented). Rasterplots of the main conditions evaluated are also presented;
- Appendix B gives an overview of the included cultures in the optogenetic stimulation experiments. Some characterization of each culture is made along with the description of the analysed electrophysiological recordings (with some important values presented). Rasterplots of the main conditions evaluated are also presented;
- Appendix C addresses the representativeness of the average signals used in the analysis of results. This subject is first mentioned in Section 5.4.4, but a deeper description is provided in this appendix;



## Chapter 2

# Fundamental Concepts

This chapter aims to give a brief overview on some concepts that are essential for the understanding of this work, thus providing some background necessary to correctly interpret the results and follow their consequent discussion. To start with, neuronal functioning is briefly explained, with some notes on brain anatomy, biophysics of neurons, synaptic transmission, and membrane potentials, with a more detailed description of action potentials. Then, some important aspects regarding excitation, inhibition and their balance are addressed, mainly describing the principal mechanisms behind each and their importance in neuronal activity. Finally, regarding electrophysiological recordings and their advances, the use of *in vitro* models is explored, along with some intrinsic filtering properties that are important to account for.

### 2.1 Neuronal Functioning

#### 2.1.1 Mammalian Brains and the Cerebral Cortex

Recent scientific evidence suggests that all surviving mammals are descendants of the same ancestors, belonging to one of six major evolutive ramifications [1]. Given this fact, it is understandable that all mammals share some characteristics in terms of brain anatomy and neuronal functioning, which makes it possible to use animal models and then translate findings to the human condition, as will be approached in Section 2.3.1. A relevant common feature found in all mammalian brains, constituting a hallmark of evolution, is the dorsal cap of the neocortex with six cortical layers and a variety of cortical areas [1,2].

The brain is generally divided into six main structures: the medulla oblongata, pons, cerebellum, midbrain, diencephalon, and cerebrum [3], visible in the anatomical illustrations presented in Figure 2.1. We will focus on the cerebrum, also known as telencephalon, as it is in this structure that the cortex is located, being its biggest subdivision [1,4,5]. This structure is of utter importance in the mammalian brain, being responsible for complex cognitive behaviors [6].

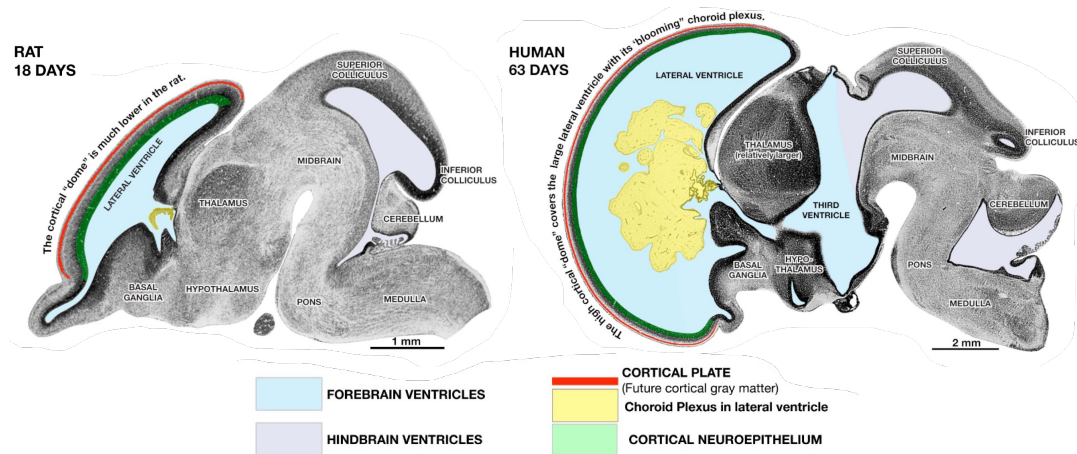


Figure 2.1: Human and rat brains at the same developmental stage: illustration showing the anatomical similarities between two mammalian brains. Adapted from [7].

The cerebral cortex can be divided into further smaller regions considering histological criteria, development timelines or evolutionary criteria [2]. Using the latter, three sections are considered: the archicortex (hippocampal region), the paleocortex (olfactory bulbs, limbic structures, piriform cortex, and secondary olfactory cortex), and the neocortex (youngest cortical areas with five cell-dense layers and one layer with sparse Cajal-Retzius neurons) [2, 4]. The different cortical layers are developed during the fetal stages and stay mature throughout adulthood [2]. The embryonic cortex is usually divided into three components: a cell-dense ependymal zone with proliferating precursors, an intermediate zone with migrating cells, and a cell-dense cortical plate [8].

Let's now consider two reference mammals: the human and the rat. In spite of the differences verified between them (for instance, different thickness of cortical layers [4] and distinct cortical folding [6]), the developmental stages of the structures are quite comparable [4]. Thus, rat cortical cultures are able to represent *in vivo* cell diversity, forming networks that demonstrate synchronous and spontaneous activity [9] (Section 2.3.1).

### 2.1.2 Biophysics of Neurons

Neurons are unique cells both due to their peculiar anatomical features and the remarkable way they interact with each other [10]. These cellular units of the nervous system can be described as electrically excitable cells that transmit signals through the body [11] and, in the case of cortical neurons, integrate thousands of inputs [12]. In their essence, neurons consist of a cell body with processes that are afferent (dendrites) or efferent (axons) [11], as shown in Figure 2.2. Despite their common anatomic features, neurons are the most polymorphic cells in the human body [10]. These cells can have various configurations depending on the arrangements of the three parts that constitute them [11], which are densely entangled, thus forming networks with other neurons and with other organs or cells [13]. Let's now analyze each neuronal constituent in more detail.

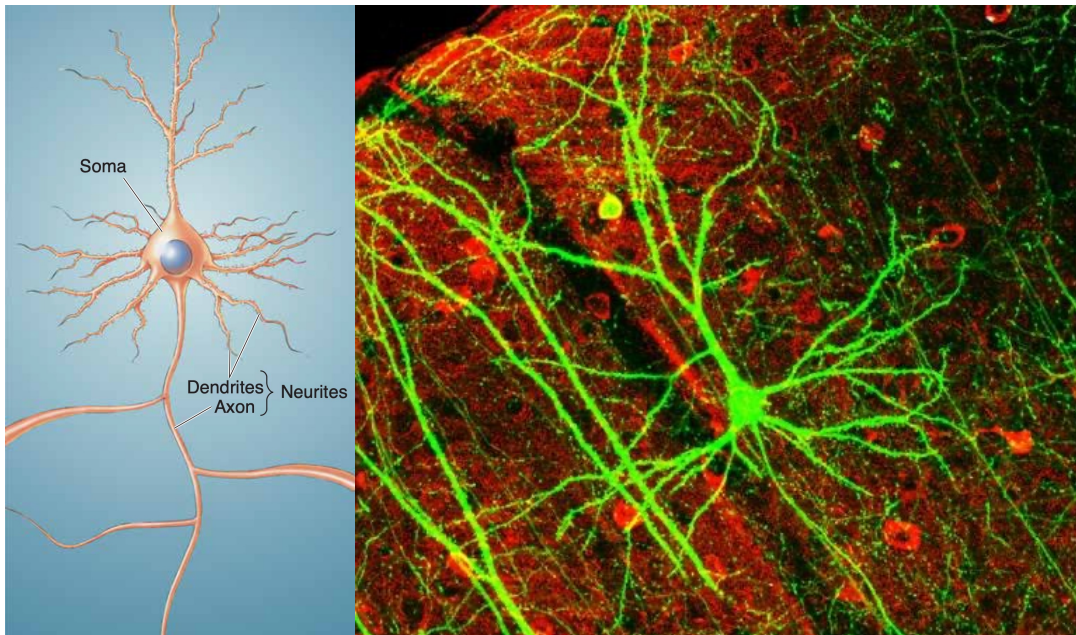


Figure 2.2: Microscopic view of a neuron: common simplified representation of a neuron (left) and microscopic image of a true neuron (right) where it is possible to distinguish dendrites, cell body and axons. Adapted from [14, 15].

The cell body (or soma) is the "central" part of neurons [16], containing all organelles necessary for cell functioning and survival [11]. Neurons possess all the typical cellular components and some specific ones: large and commonly spherical nucleus, Nissl substance (which distribution can be used to identify some types of neurons), endoplasmic reticulum (produce vital proteins), neurotubules for structure (random orientation in soma, aligned in axons), among others [10].

Dendrites tend to be thick and short protuberances with the same organelles as the cell body, participating in protein synthesis and lipid metabolism [17]. They are highly branched and elongated, located around the soma to generate a good surface for receiving stimuli [18]. Additionally, they have important synaptic regions along the main stem and in its spines (or thorns) [10].

Axons, oppositely to dendrites, are usually long and thin with few to no organelles. They are divided into different zones: the hillock (still contains some organelles), the initial segment (with the lowest threshold for AP generation [19]), the axon proper, and the terminal (divided into synaptic regions like the active zone) [10, 16]. Axons can be myelinated, having high concentrations of  $\text{Ca}^{2+}$  channels in nodal points, properties that confer their conducting efficiency and transmission speed [10]. Axons play a major role in the transport of substances from (anterograde) and to (retrograde) the cell body and in limiting the presynaptic synthesis of proteins and lipids [16–18].

Neurons can be classified as inhibitory, excitatory, or modulatory in terms of effect (see Section 2.2); or as motor, sensory or secretory (interneurones) in function [10, 20]. Morphological differences between neurons help making them specialized to their particular task [18]. Due to this, cortical circuits also activate different types of neurons based on the received stimuli [5, 21]

There are hundreds of different types of neurons in the mammalian brain [22], with several aspects differentiating them, including biophysical properties and firing patterns [21]. After reaching maturity, neurons are unable to divide themselves (their number in the organism is finite). Like so, as they get destroyed either naturally (in humans there is an estimated 20000 neurons daily dropout rate [10]) or erroneously (for example due to neurodegenerative diseases), an unrecoverable deficit starts to be noticed [11]. This, in turn, can lead to some conditions discussed in Section 2.2.

Let's now discuss how to model neurons and neuronal circuits. The cellular units of the nervous system present an extensive variety of morphologies, a factor that contributes to the generation of extracellular electric signals due to transmembrane processes [23]. In a very simplistic representation, the thin insulating layer created by the lipid bi-layer of the membrane can be compared to an RC circuit, having a certain capacitance in parallel with certain resistance and, in some cases, a certain inductance (being an RLC circuit in these situations) [23, 24]. Accounting for the spatial extension of neurons and considering the longitudinal extent much larger than the diameter of dendrites and axons, the dominant fraction of charge can be described by one-dimensional cable models. Thus, neuronal tissue can be represented as a series of circuits [23].

### 2.1.3 Synaptic Transmission

Neurons communicate with each other (as well as with other cells and organs) via a chemical process called synapse in which a particular message is not only transmitted but also interpreted simultaneously [11, 22, 25, 26]. This makes synaptic transmission the foundation of behavior and cognition [25], as they connect neurons, turning them into dynamic circuits that change over time [17]. In a very simple view, a synapse is formed by three major elements: a presynaptic active zone, a synaptic cleft, and a postsynaptic density [25], as seen in Figure 2.3.

Between neurons, synapses are established between the axon of a presynaptic unit and the dendrites of a postsynaptic one [10, 26]. While the postsynaptic mechanisms are usually more specialized, the presynaptic mechanisms are quite global, making it possible for one presynaptic neuron to communicate with various postsynaptic ones [17].

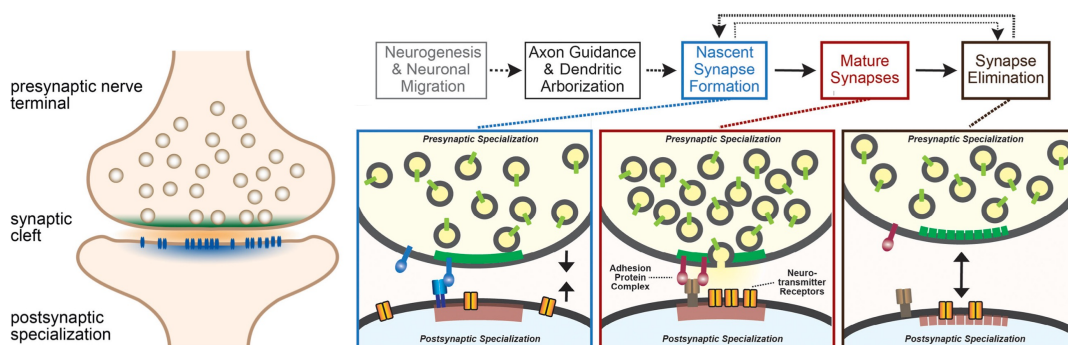


Figure 2.3: Synaptic transmission dynamics: overview of the elements that compose the synaptic connection between two neurons (left) and schematization of the synaptic life cycle with the three main stages highlighted (right). Adapted from [17, 25].

The presynaptic active zone consists of a highly patterned protein complex and lipids that regulates neurotransmitter release triggered by calcium intake [10, 17, 22, 25, 26]. When an action potential reaches this area,  $Ca^{2+}$  channels open and ions bind to vesicles full of neurotransmitters, activating them [22, 27, 28]. These vesicles, which change from synapse to synapse (along with their content), fuse to the membrane and promote exocytosis of neurotransmitters within time windows of milliseconds [27, 28]. There are over one hundred known molecules, being the most important (and most commonly segregated by neurons) Gamma-aminobutyric acid (GABA) and glutamate. Most neurons produce only one of these two being either inhibitory or excitatory, respectively (differences between these two types will be further analyzed in Section 2.2).

The synaptic cleft is a thin gap (15nm to 20nm [10]) rich in proteins that have important regulatory roles in synaptic maturation and transmission [25]. There may be even distinct subdomains of the cleft to regulate different functions [25]. It is through this space that neurotransmitters diffuse from the presynaptic release sites to the postsynaptic receptors present in the neuronal membrane, specifically in the postsynaptic density.

The postsynaptic density is also an aggregate of proteins, namely receptors [25]. The most abundant receptors are tetrameric and pentameric (>95%) and, due to their affinity to particular molecules, these elements are also responsible for the high specificity of the whole synaptic process [17]. Receptors have a tunable spatial distribution, forming nanoclusters (also called nanodomains) that regulate their activation [25]. On top of this, specific receptor recruitment may also be a reason behind the differences between the synaptic activity of separate neuronal populations [5, 29]. Kinetics of postsynaptic receptor binding is thought to be responsible for giving rise to slow synaptic currents [27].

All things considered, since there are various combinations possible between the synaptic components, synaptic transmission is asymmetrical (polarized) and tends to be very specific given its purpose and location [10, 17]. As to synaptic strength, multiple factors are considered key influences, such as the number of receptors, the concentration of neurotransmitters, the location of synaptic release and the trans-synaptic alignment between cells [5, 25].

Even though neurons are not renewable, synapses are constantly changing (Fig. 2.3) with old connections being eliminated by glia and new ones being formed by astrocytes [17]. It is thought that problems in synaptic genesis or functioning may be the origin of several diseases [17]. As so, strong regulatory machinery for synaptic assembly is of vital importance [26]. Although there is no definite description of how synapses are formed, it is believed that synaptic adhesion molecules (SAMs) play a vital role in the process [17, 26, 30] both by making and shaping them. Calcium channels have also been suggested to aid synaptic attachment and alignment [26].

So far, synapses between neurons have been presented in this Section. However, there is also synaptic transmission between neurons and other cells and tissues [11]. This communication takes place in neuromuscular junctions, where some relevant anatomical features are verified. On the one hand, neurons present an enlarged unmyelinated axon terminal that forms synaptic boutons covered with Schwann cells [10, 31]. On the other hand, the muscle membrane has a specialized region (the endplate) that is less dense and infolded, forming junctional folds [10, 31].

As to the modeling of synaptic processes, assuming that the ionic concentration of  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , among others, have a minimal effect on current flow, the voltage at the membrane at each point is dependent solely on the intracellular and extracellular voltages at that point [23]. This allowed for the application of the cable equation to a neuronal scenario, describing the neuron's current spread across axons and dendrites as well as the response elicited by current injection, taking into consideration the membrane's resting state [23]. The limitation of this approach is its incapability of modeling complicated 3D neuronal structures, making it necessary to reduce them to a set of 1D cable equations. Nonetheless, this has been a crucial step to describe the electrical behavior of neurons with active, linearized membranes [23].

#### 2.1.4 Membrane Potentials

The bilayered membrane that involves neurons works as a nearly perfect insulator with some proteins that work as ion gates. During a synapse, the release of neurotransmitters elicits the activation of specific receptors, which, in turn, promote the opening and closing of ionic channels, giving rise to membrane potentials due to the diffusion of charged ions [32–34].

In 1952, Hodgkin and Huxley were able to measure synaptic currents that result from ion exchanges between the intracellular and extracellular mediums. Today, 50 years later, the model that they proposed based on their experiments (Fig. 2.4), which granted them a Nobel Prize, still holds and can be used as a representation of the cell membrane [27]. They proposed that the cell membrane works as a capacitor in which input currents can further charge the capacitor or leak through one of three channels (with characteristic resistance and battery) [35]. They attributed one channel to sodium ( $\text{Na}^+$ ), one to potassium ( $\text{K}^+$ ), and one to other unspecific elements. Even though more ionic channels are now known, it is accepted that chloride ( $\text{Cl}^-$ ) is the third main contributor [11, 24, 32, 36]. Hodgkin and Huxley also proposed mathematical descriptions for their observations, with equations for the probability of the channels being opened or closed given the conditions [35]. Their work remains of utter importance when modeling membrane potentials.

Ion channels are selective pores that recognize specific ions based on size and polarity, having a protein that responds to voltage changes (the gate), responsible for the opening and closing of the channels [32]. Different channels have different biochemical properties (with specific subunits to confer a proper function) and can be distinguished and isolated using different blocking agents [32]. The dynamics of the channels also model the electric message that is being transmitted [32]. The flow of ions through these channels creates dipoles which, based on their alignment, can sum (if aligned), cancel (if opposing), or give no signal at all (if randomly disposed) [33].

All things considered, it is known that, in its resting state, the cell membrane potential is around  $[-75 : -40]\text{mV}$  and that the movement of ions through channels disrupts this equilibrium. The restoration of the equilibrium is aided by ionic pumps, even though these are not essential for the mechanisms responsible for the opening and closing of channels [32]. Calcium ( $\text{Ca}^{2+}$ ) channels have also been described as important for neurotransmission and disruption of extracellular potentials [17, 32]. The most important extracellular membrane potentials are Action Potentials (presented below in section 2.1.5) and Local Field Potentials (analysed in chapter 3).

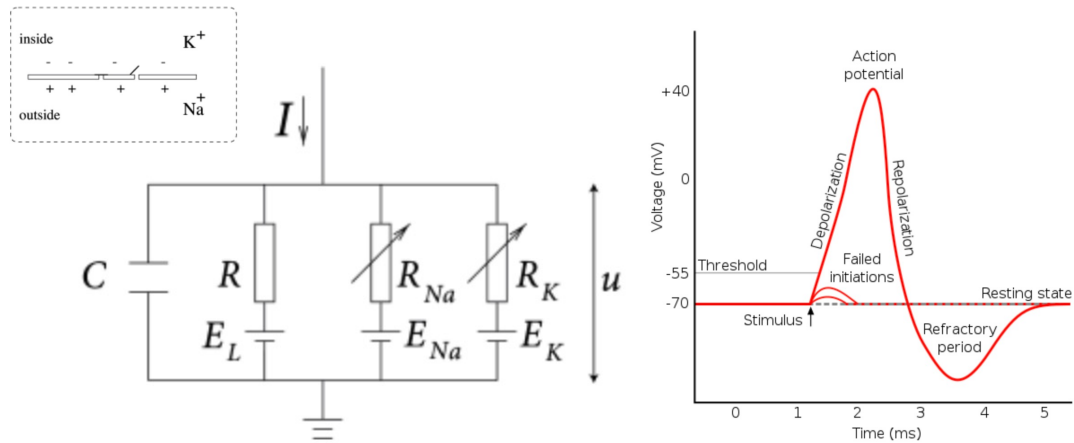


Figure 2.4: The Hodgkin-Huxley model and Action Potentials: basic schematization of the proposed model (left) and general shape of an action potential, with indication of main stages (right). Adapted from [35, 37].

### 2.1.5 Action Potentials

Known as the all-or-nothing signals based on a threshold value, Action Potentials consist of a spike-like depolarization of the membrane, generated by the opening and closing of ionic channels [32, 33, 38]. It is the electrical phenomenon through which neurons carry information to their axons, where it is translated into a neurotransmitter release. This information is enhanced by firing patterns that depend on network oscillations [39].

The disruption of the resting potential occurs in a sequence of stages (Fig. 2.4). When a given stimulus is received by the neuron (the binding of an excitatory neurotransmitter, for example) an influx of positively charged  $\text{Na}^+$  ions begins and the resting potential starts to increase [38]. If this is not high enough to induce a response, the membrane potential is quickly restored to equilibrium.

In cases in which the stimulus is strong enough to ensure that the threshold is surpassed, depolarization begins, a phase in which the potential of the membrane increases and even becomes positively charged as the action potential is generated [38]. After reaching a peak of around 40mV, repolarization begins, and the membrane's potential decreases. As channels remain open for a while after the resting potential has been achieved, there is some hyperpolarization as the membrane's potential goes below the resting one [38]. The resting state is restored by  $\text{Na}^+/\text{K}^+$  pumps during refractory periods that can be absolute ( $\text{Na}^+$  channels enter an inactive state during which they cannot be reopened) or relative (as  $\text{Na}^+$  channels gradually become active again, stronger stimuli may be capable of causing another discharge) [36].

After the synapse, and taking into consideration the models discussed in sections 2.1.2 and 2.1.4, it is possible to assume that the signal is transmitted through the postsynaptic neuron by a passive process (cable spread) in which excited regions have an excitatory effect on the following one, but not on the previous one, avoiding back-propagation [32]. In some cases, axons are coated with myelin, making this propagation much faster [32].

## 2.2 Inhibitory and Excitatory Activity

### 2.2.1 Neuronal Inhibition

Inhibitory neurons usually represent [10 : 20]% of a neural population and are essential to regulate the response to stimuli and general behavior of the culture, organizing network activity both temporally and spatially [40–42]. Different types of inhibitory neurons have been identified in terms of morphology and function [42]. The most common type of inhibitory neurons in the brain are (smooth dendritic) interneurons [5, 12]. These neurons release, in dense local boutons [42], neurotransmitters that promote the hyperpolarization of the postsynaptic membrane potential. GABA is the most important one related to mood and emotions [27, 32, 42–47]. This is done by promoting the opening of  $\text{Cl}^-$  channels, which lower the membrane potential, making it further away from the spiking threshold [19, 40]. Connectivity and recruitment are essential for the good functioning of inhibitory populations which are responsible for, among other tasks, regulating excitatory activity across the cortex as well as cognition [40].

Let's now focus some attention on the GABA family of neurotransmitters and their respective receptors. In early cortical networks, GABA has been shown to promote network bursts (periods of high activity in which most, if not all, neurons fire synchronously, followed by intervals of silence), instead of suppressing them [41, 48]. In fact, the transition from a depolarizing to a hyperpolarizing effect has been suggested to be a hallmark of GABA maturation [41, 48]. Nevertheless, even in mature cortical cultures, GABA seems to have the possibility of exerting an excitatory effect, showing the enormous complexity of neuronal circuits [19, 49]. The response elicited by GABAergic activity appears to be biphasic (Fig. 2.5), with a first fast component mediated by  $\text{GABA}_A$  receptors and a slower one mediated by  $\text{GABA}_B$  [27, 43, 45, 46, 50–52].

As to differences between the two types, while  $\text{GABA}_A$  is mostly responsible for reducing firing patterns,  $\text{GABA}_B$  tends to be more involved in dampening long active onsets [41, 51]. As so, blocking  $\text{GABA}_A$  transforms neuronal activity into an epileptic-like bursting behavior, and blocking  $\text{GABA}_B$  generates longer, but still finite, bursts [51].

Moreover,  $\text{GABA}_B$  is thought to have a very important role in mediating GABAergic activity, meaning that it can also reduce neurotransmitter release by hampering calcium intake [42, 45, 46, 53]. This could explain why, in some cortical regions, a single stimulus promotes an inhibitory response, while a train of stimuli promotes excitation [21].

$\text{GABA}_B$  receptors have been growing in interest not only due to their unique properties (they are non-ionotropic G-protein coupled and require two subunits to be functional [45, 47, 50]) but also because of their promising applications (analgesic response to acute pain or treatment for depression, alcohol addiction, and seizures [50]). Despite this, in *in vitro* conditions  $\text{GABA}_B$  receptors may be less present or even nonexistent [27, 51].

Inhibitory neurons receive inputs from various brain regions and give distinct responses to each in processes that may require different types of neurons, especially when dealing with complex tasks in which activity of cell assemblies may be crucial [40].

In order to be able to cope with different stimuli and give the most adequate response, different types of inhibitory mechanisms can be activated: feedforward (FF) in which a population of excitatory neurons activates an inhibitory population which, in turn, suppresses the activity of a different excitatory population [42,44,54]; feedback (FB) which is similar to the previous one but in the end the excitatory population being affected is the same that activated the inhibitory neurons [54]; and backward inhibition (BI) in which neural populations need to overcome reminiscent inhibition from previously performed tasks [55]. All these mechanisms can also be subject to neuromodulation, a characteristic that becomes important when studying them, looking for beneficial effects of altering inhibition as a way of treating some diseases [17,40].

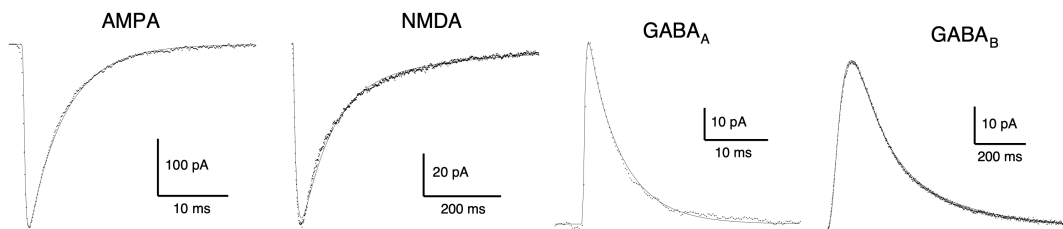


Figure 2.5: Synaptic currents generated by the principal neurotransmitter receptors: excitatory receptors AMPA and NMDA generate currents with bigger amplitudes than GABA receptors; NMDA and GABA<sub>B</sub> currents are slower than AMPA and GABA<sub>A</sub>. Adapted from [27].

### 2.2.2 Neuronal Excitation

Constituting around [80 : 90]% of neuronal cultures, excitatory neurons are responsible for the regulation of the general excitability of the nervous system [12]. The most common type of excitatory neurons in the cortex are pyramidal cells [5, 12]. Glutamate is one of the most important excitatory neurotransmitters, being implicated in processes related to memory [25, 27, 56]. When received by a postsynaptic neuron, excitatory neurotransmitters can promote the generation of an action potential by activating Na<sup>+</sup> and Ca<sup>2+</sup> channels. As positive ions flow into the cell, the membrane potential rises, becoming closer to the threshold value.

As seen in Figure 2.5, two major glutamate receptors are responsible for different components of the response to stimuli [25, 57, 58]. First, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are responsible for a fast reaction to the stimulus and then N-methyl-D-aspartate (NMDA) receptors mediate a slower part of the generated activity [27, 47]. It can be stated that AMPA is responsible for generating network bursts and NMDA for maintaining them [41]. For this reason, blocking AMPA receptors leads to the suppression of network activity while blocking NMDA receptors shortens the duration of active periods [41, 58].

Let's now focus on NMDA receptors. These have two different subunits that affect their functional properties (for example, the GluR-B receptor is only highly permeable to calcium if it is Q/R site-edited) [29]. NMDA receptors are of extreme importance in cortical activity and plasticity. They are thought to generate large slow postsynaptic potentials that may last for hundreds of milliseconds which become even larger if the dampening action of GABA is blocked [27,41].

Two other important processes are also thought to be provoked by NMDA receptors and are crucial in network development and functioning: long-term potentiation (LTP) and long-term depression (LDP) [59,60]. In particular, it has been hypothesized that there may be different threshold levels for calcium intake mediated by such receptors, inducing one of these processes (or none, in the "no man's land") [12,61]. On the one hand, LTP is a noticeable increase in synaptic transmission as a result of frequent stimulation and has been hypothesized to be involved in memory and learning mechanisms [60,62,63]. On the other hand, LDP refers to a state of synaptic transmission impairment that has been implicated in mnemonic processes [60,64].

When cultures are mature (after  $\approx 3$  weeks of growing) networks show (partially) synchronous activity that can be suppressed with AMPA and NMDA receptor antagonists CNQX and APV, respectively [48]. This supports the idea that network activity is largely influenced by excitation. One additional fact is that SAMs, introduced in section 2.1.3 are also thought to be involved in the regulation of excitatory activity [30].

### 2.2.3 The Excitation/Inhibition Balance

Since neurons receive inputs from both inhibitory and excitatory neurons, a proper balance between them is the key to the homeostasis of the nervous system and relies on the coregulation of inputs [12,20]. Altered excitability is thought to impair the proper processing of stimulus [12]. Recently, excitation/inhibition (E/I) imbalances, more specifically changes in the inhibitory system, have been shown to be the possible cause of multiple neurological disorders [12]. Here, we present three illustrative examples in which imbalances have been described. Needless to say that these are a small sample of the existing neurological conditions.

The first example is Autism Spectrum Disorders (ASD). Autistic brains have been shown to reveal a reduction in inhibitory neurotransmitters, mainly GABA, and their receptor units [65]. Moreover, an imbalance in excitation-inhibition, caused by GABAergic processes in the brain of people with ASD, is also believed to explain symptoms like atypical visual perception [66].

The second example is that of epilepsy. Two hallmarks related to this disease, more concretely to seizure generation, are hyperexcitability of individual neurons (lower threshold level for depolarization) and hypersynchronicity of neuronal networks (simultaneous discharging) [58,67]. Even though changes in the excitation-inhibition ratio may not be the only cause for this condition, it has been demonstrated that GABA and glutamate and their imbalance have been implicated in the pathogenesis of epilepsy [68].

The third example is Parkinson's disease. It has been defended that in the subthalamic nucleus (STN) of PD patients neuronal activity is composed of simultaneous and pulsing AP bursts caused by alterations in E/I balance leading to hyperactivity of neurons [69].

## 2.3 Electrophysiological Recordings

### 2.3.1 The Use of *in Vitro* Models

Animal models have been a vital research tool in some diseases, providing certain freedom of experimentation that has allowed numerous important discoveries [12, 41].

On the positive side, despite having different rhythms of growth, humans and other mammals share the same stages of neurological development, meaning that it is indeed possible to use animal models (like cultures obtained from rats) to study human diseases [4]. Moreover, monolayer cultures simplify the connections and, consequently, the observed signals.

On the negative side, the cortex has multiple organizational features which are very difficult to replicate *in vitro* [70, 71]. This, together with the incredible specificity that neuronal circuitry possesses *in vivo*, makes it quite challenging to translate results between models in some cases. However as discussed in Section 6.4.1, such models remain a good starting point for investigation.

### 2.3.2 Advances in Recording Techniques

Recent years have seen major innovation in recording techniques, especially with the appearance of multielectrodes that allow the recording of tens (or even thousands) of sites simultaneously with a growing capacity for streaming continuous data [34, 56, 72–74]. Cell culturing on multi-electrode arrays (MEAs) provides a non-invasive way of obtaining electrophysiological recordings from excitable cells [9, 57, 72]. As so, such *in vitro* cultures from dissociated neurons are good model systems to understand the dynamics of neuronal circuits (which can be translated to *in vivo* scenarios) based on precise and detailed analysis of neuronal data [9, 41, 56, 72].

As a matter of fact, more and more researchers use MEAs as a tool to study neuronal function [9, 56, 75, 76]. Simultaneous recording of high frequencies (information about spiking activity [34, 56]) and local field potentials over time makes it possible to study interactions of the cellular mechanisms behind pattern generation [75, 77, 78]. Furthermore, in present times, data collection allows information to be accessed in multiple spatio-temporal scales, consequently making it easier to find more complex patterns of neuronal activity [77].

### 2.3.3 Intrinsic Filtering Effects

Studies have demonstrated some filtering effect caused by the extracellular medium and ionic diffusion that may also contribute to ephaptic interactions [79, 80]. Moreover, chemical gradients and active transport cause significant ion movements in the brain, leading to the redistribution of charges due to ion buffering effects [81].

Research has shown the existence of an intrinsic dendritic filtering effect from the cable properties of dendrites [13, 74]. This effect depends on the number and thickness of dendrites [82], being difficult to define is not measurable. It may help explain the high variability in neural signals as different populations have different configurations and, consequently, different intrinsic filtering effects. Nevertheless, keeping recording setups equal, this effect should be negligible.

Lastly, there is the high-pass filter behavior of the electrodes of the MEAs (Fig. 2.6). Electrodes are commonly seen as capacitors. As so, from their impedance measurements as a function of the applied frequency, we can estimate their capacitance using equation 2.1:

$$Z_e = \frac{1}{j\omega C_e} \Leftrightarrow Z_e = \frac{1}{j2\pi f C_e} \Leftrightarrow C_e = \frac{1}{2\pi Z_e f} \quad (2.1)$$

in which  $Z_e$  is the electrode's impedance and  $f$  its frequency. Given that the imaginary part (j) is related to the phase, it can be left out of the final equation. Using data from three different MEAs, an average value of  $0.87 \pm 0.39\text{pF}$  was calculated for the capacitance of the electrodes.

When frequency values are high, following equation 2.1, the impedance values for the electrode will be very low, but if frequency values drop, then the impedance of the electrode may be noticeable. In fact, the voltage value measured is obtained through the following equation:

$$V_{Measured} = \frac{Z_a}{(Z_a + Z_e)} * V_{Real} \quad (2.2)$$

in which  $Z_a$  is the impedance of the amplifier,  $Z_e$  is the impedance of the electrode, and  $V_{Real}$  and  $V_{Measured}$  the actual value of the potential and the value picked up by electrodes, respectively.

Assuming that electrodes start manifesting an high-pass filtering effect when their impedance is greater or equal to the one from the amplifier ( $Z_e \geq Z_a$ ). As indicated in the MEA2100 headstage manual, the value for the impedance of the amplifier is  $1\text{G}\Omega$ . Resolving Equation 2.1 in function of the frequency we get:

$$f = \frac{1}{2\pi Z_e C_e} \quad (2.3)$$

We can then use the previously calculated values for  $C_e$  and for the impedance value of  $Z_a$ , obtaining a cut-off frequency of  $0.21 \pm 0.06\text{Hz}$ . This value is actually higher than the  $0.1\text{Hz}$  cut-off frequency defined in the acquisition setup, so it is possible that signals are indeed filtered by the electrodes. This phenomenon changes from electrode to electrode and is important to consider especially when running simulations.

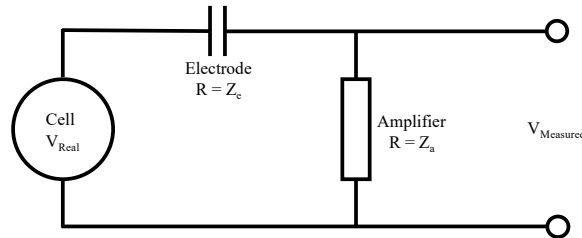


Figure 2.6: High-pass filtering effect of the electrodes: when electrodes are seen as capacitors they have a certain impedance ( $Z_e$ ) which significantly affects the voltage value measured ( $V_{Measured}$ ) when it becomes similar to the impedance of the amplifier ( $Z_a$ ).

## Chapter 3

# State of The Art

This project focuses on defining what neuronal mechanisms are behind Local Field Potentials, enabling associations between their shapes and the underlying phenomena that generate them. As so, in this chapter, an overview of what has been stated in the literature, including common assumptions about such signals, is presented. Then, a few research works are analyzed in more detail, given the fact that their focus was on finding the foundation of LFP signals, as is ours.

### 3.1 Local Field Potentials: an Introduction

Local Field Potentials (LFPs) are slow extracellular signals (lasting from hundreds of milliseconds to minutes [32]) which can be measured in the low-frequency component (below 300Hz) of electrophysiological recordings [72, 83]. These are highly non-stationary signals [39], with quite some variability depending on the used recording setup and the protocol applied [34, 74, 84].

Even though spiking activity is mostly encoded in the high-frequency component of recordings [73, 74, 85], studies suggest that spikes may also contribute to the generation of LFPs [33, 34, 79], but only if their energy surpasses noise levels [86]. Moreover, spiking patterns may be responsible for the oscillating behavior typically observed in the low frequencies of neuronal recordings [83]. If we consider signals acquired *in vivo*, the influence of spiking activity is even larger in the high-frequency band of LFPs (above 100Hz, the epsilon band) [87], serving as a signature of cell assembly activation [85].

On top of this, LFP signals are assumed to reflect dendritic (postsynaptic) currents caused by synaptic inputs [72–74, 85]. In other words, they are thought to result from the opening and closing of ionic channels as a response to the stimuli received [13]. Previous work shows that, as ions flow through specific channels, they generate inward synaptic currents (sinks) and outward recurrent currents (sources) separated in time (forming dipoles) [34, 88]. Such dipoles could provide a plausible explanation for the verified disruption of the extracellular potential [13, 79].

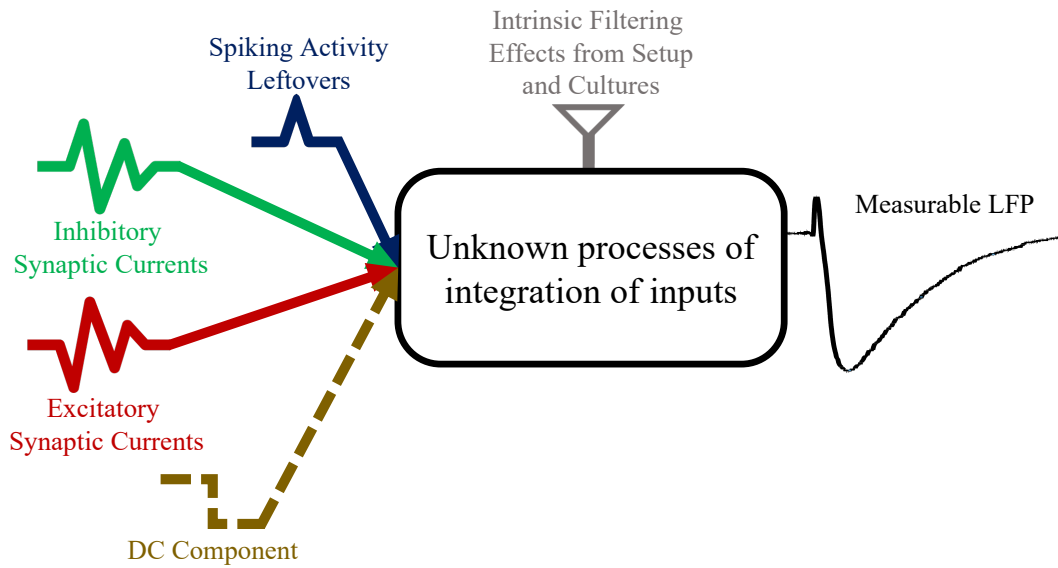


Figure 3.1: Black-box generation of LFPs: the exact mechanisms through which the commonly accepted contributors to the LFP signals and known intrinsic filtering effects are integrated into the final measurable signal is still unknown, despite its clinical relevance.

All things considered, one can think of LFPs as the final product of a black-box model (Fig. 3.1) in which the inputs can be: spiking activity, synaptic currents (both inhibitory and excitatory, pre and postsynaptic), and a possible DC component (which is filtered out in most methodologies). The precise way in which these inputs are incorporated into the measured signal is still not fully understood, making the interpretation of the generation of LFP signals a challenging task [34, 72–74, 77, 89].

In spite of their generation not being completely understood, LFP signals are quite used as they are easy to record and provide a unique window into integrative synaptic processes in cortical populations [34, 73, 74, 88–90]. Studies show that part of neuronal processing is done on a population basis, for which LFPs may be a measure of interest [88]. As a consequence, these signals are believed to differ from population to population [79]. One common view for LFP generation is that of "rings of neurons" around the electrode (Fig. 3.2), each one giving its contribution to the signal [73]. Signal strength for each ring is dependent on the balance between the decay of single-cell contribution and the increasing number of neurons per ring [13, 73].

Research has led to the conclusion that, besides the population that generates them, multiple spatial factors influence LFPs to a great extent such as neuronal morphology, distribution of inputs, population arrangement, synaptic connectivity, extracellular matrix conductivity, and input correlation [13, 34, 73, 77, 79, 83, 90]. A special focus has been given to this last factor, with various studies showing that correlated (and coordinated) sources tend to produce stronger signals that tend to be spread over larger distances [13, 73, 77, 83, 88, 90]. In addition, ephaptic coupling mechanisms also synchronize neuronal activity and affect extracellular signals [83].

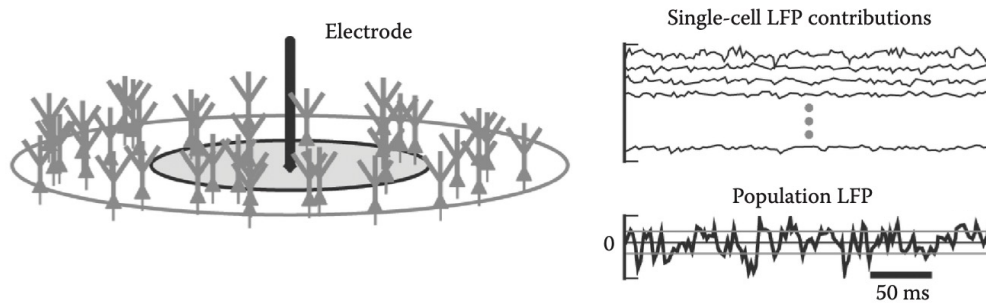


Figure 3.2: Contribution to the signal of neurons around the electrode: it is possible to imagine "rings of neurons" around the electrode, inside which each neuron generates a single cell contribution that is summed into the ring population LFP. Adapted from [13].

Curiously, some researchers find the term "local" misleading as it has been shown that LFPs may integrate thousands of inputs, having, therefore, multiple contributions from different sources [13, 34, 73, 77]. These can be either global or local, and the distinction between them is far from trivial [74]. Previous findings even suggest that signals recorded in one structure may represent the input in another structure [88].

All things considered, it is comprehensible to say that LFPs are difficult to generalize and interpret [13]. Nevertheless, their possible applications are well worth spending time studying such signals, since LFP signals are excellent candidates for prosthetic device control [13, 34, 90], with evidence showing that *in vivo* LFPs may even encode direction of movement [91]; characterization of the state of neuronal microcircuits [13]; evaluation of the pharmacological outcome of some treatments (for example, anti-epileptic drugs) [77]; and investigation of sensory processing, motor planning, and cognitive processes such as attention, memory, and perception) [34].

The currently most used techniques for the analysis of LFP signals tend to be Current Source Density (CSD) Analysis [13, 34, 72, 73] and the spike-triggered average of the LFP [34]. These focus more on using LFPs as tools than on comprehending their genesis. On that account, some modulation of LFP signals has been developed, but this approach remains not straightforward nor yet globally accepted [13, 34, 79]. All in all, as far as literature goes, LFPs remain a source of mystery among neuronal signals with proven physiological relevance.

## 3.2 Previous Research

Work in the field of LFPs is vast and pursues many different research goals. The principal objective of this study is to understand what information is contained in these signals regarding excitation and inhibition (as well as their balance). As so, examples of research projects focusing on the same questions are now highlighted. For each one, methodology, results and conclusions are addressed. Unfortunately, such literature on LFP experiments is notably sparse. In the end, a brief summary of the main contributions of the presented studies is presented (Section 3.3).

### 3.2.1 Research 1: Unitary Inhibitory Field Potentials

The first work addressed was developed in 2010 by Bazelot *et al.* [92]. Using thin rat hippocampal slices, this team measured intracellular signals with glass electrodes and extracellular signals with independent electrodes or arrays of electrodes (linear and curved). They then used excitatory receptor antagonists NBQX and APV along with inhibitory receptor antagonists picrotoxin and bicuculline to control neuronal activity in the preparations, evaluating the effect of changes in excitation and inhibition on the measured signals. They also manipulated the concentration of extracellular chloride to suppress postsynaptic inhibitory responses.

Regarding filtering of the signals, a bandpass of 500-3000Hz was used for the high-frequency component while for the low-frequency component, the chosen bandpass was of 1-300Hz (both of unspecified order). After filtering, they treated signals with an 'up-only' transform together with an amplitude threshold for event detection. The team used electrical parameters to distinguish between inhibitory and excitatory neurons.

As seen in Figure 3.3, two distinct signals were observed by this team: one fast (0.7-2.5ms) and frequent, and one slow (8-10ms) and sparser (putative LFPs). In some cases, the slow events were preceded by an extracellular action potential, but this was not a mandatory condition, with some slow events being measured without any APs in their vicinity.

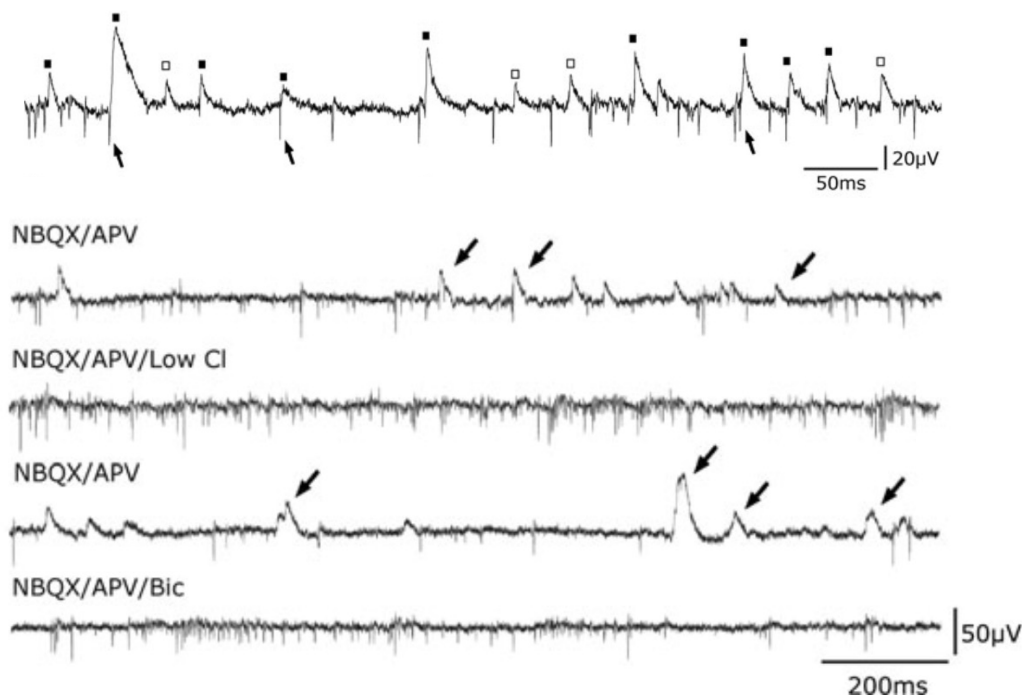


Figure 3.3: Unitary inhibitory field potentials: Extracellular recordings in control conditions (top) and in a series of combinations between inhibitory and excitatory blockers (bottom). On top, arrows represent Action Potentials and squares Field Potentials (filled when preceded by an AP). Below, arrows represent slow events. Adapted from [92].

This team suggested that pyramidal cells did initiate slow events in a few cases, but this was not a consistent phenomenon with differences being verified across recordings. The addition of excitatory receptor blockers did not suppress the events, although it reduced their frequency. Oppositely, lowering the extracellular  $\text{Cl}^-$  concentration and using inhibitory receptor antagonists did fully yet reversibly suppress the slow events, pointing towards an inhibitory origin (Fig. 3.3). On various occasions, extracellular field potentials were found to coincide with intracellular inhibitory postsynaptic potentials.

Moreover, their results show that single spike activity in the majority of interneurons did initiate a slow field potential, which could be recorded over distances of hundreds of micrometers, with a certain amplitude decay with increasing distance from the source. This spanning area is larger than that directly related to action potentials. As so, they propose that these are inhibitory local fields, partly shaped by the terminal distribution of the originating cell.

Differences in the slow events kinetics made it possible to separate events in terms of their origin (dendritic vs perisomatic), especially regarding frequency and time to peak/decay. The amplitude distribution of LFPs also gave some information on which was its originating interneurone.

The authors state that differences between this work and others that preceded it may be justified by variations in protocol and recording setup. In this work, experiments were conducted with the presence of NBQX and APV to prevent multiple interneurons from firing when innervated by a common pyramidal cell. Differences in the local density of terminals between pyramidal cells and interneurons (higher in the latter) may explain why only one of them generated measurable fields. They also suggested that excitatory neurons may generate extracellular events di-synaptically by activating an inhibitory interneurone.

### 3.2.2 Research 2: Myths and Misunderstandings

In 2016, Herreras conducted a review, highlighting the main misconceptions that have been aroused from previous research regarding the field of LFPs [93].

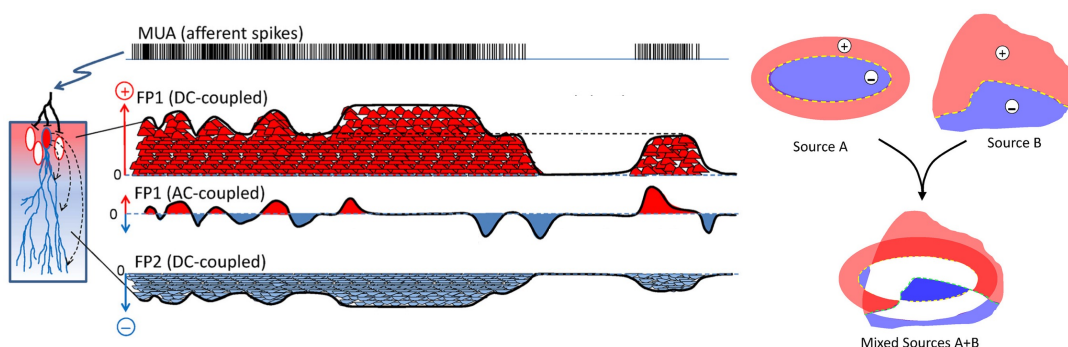


Figure 3.4: Some challenges regarding LFP acquisition: differences between AC coupled signals and the respective DC coupled ones (left) and spatial interaction of contributions between different sources (right). Adapted from [93].

He first stated that even though the biophysics behind field potentials is understood, the interpretation of the signals is not yet consensual as these seem to be a product of multiple structural and functional factors. Also, LFPs may significantly differ from structure to structure and reflect single or multiple cell activity, being most likely that cell aggregates gather the best conditions to generate measurable potentials.

Herreras discusses that not all types of neurons generate field potentials and that the ones that do may only be able to generate measurable potentials in some subgroups of co-activated synapses. Due to spatial arrangements, the contributions of different neurons will be integrated and, in that process, they may sum up or cancel each other out (Fig 3.4). The researcher indicates that this, along with the filtering properties of the tissue, varying spiking activity, and intrinsic currents, raises much debate around LFP interpretation.

According to this work, there are several conflicts with LFP signals. Firstly, the terminology local is itself admittedly confusing as local field potentials may have various contributions. In fact, the proportion between local and global contributions is not trivial to understand or calculate. Secondly, most research on LFP is done with AC-coupled amplifiers, which remove the DC component, depriving the signal of a true baseline value. Moreover, this can also have a big impact on the observed polarity of the signal (Fig 3.4). Thirdly, different recording strategies, data analyses, and protocols may have a huge impact on the heterogeneity of existing interpretations.

Herreras proposes that although many believe that LFP represents temporal spiking patterns around the electrode, it is more plausible that signals represent synaptic inputs (local or remote), based on available literature. Actually, he defends that a very weak temporal relationship between LFPs and spikes recorded from that same electrode should be expected, and, given some spatial arrangements, there is a strong possibility that LFPs measured in one structure reflect activity in another one. In fact, it is even possible that, due to spatial factors, the largest peak may not be in the source of the event.

In the opinion of this researcher, relating signal polarity with inhibition or excitation is a task that requires some attention and thought. Even though it seems intuitive that negative potentials should represent depolarization, the use of chemical tools like blockers may be of interest to validate such an assumption. This relation between the signal and inhibitory/excitatory activity is an interesting aspect to study since LFPs may indeed give some notes on E/I balance.

From the conducted analysis of the literature, it is understandable that LFP signals are quite varied and show some oscillations that may be of interest, especially the slow oscillation in the cortex, the generation of which remains unclear. Apart from that, LFPs can be defined as the extracellular electrical remnants of neuronal activity, meaning the net charge that remains after the summation of in and out currents. They originate from neuronal populations whose dynamics depend on input spikes and are heterogeneous in current densities over their own space.

Finally, a mention of the capacitive properties of the extracellular space is made as it may promote a low-pass filtering effect on currents. The conducted review paints a picture of how entangled LFP signals are with the space in which they are generated. Hence, when explaining the origin of signals in complex preparations, spatial discrimination techniques may be useful.

### 3.2.3 Research 3: Inhibitory Activity Primarily Reflected

In 2017, another work in the LFP field was carried out by Teleńczuk *et al.* [94]. Using human and monkey *in vivo* recordings captured using Utah arrays, this team calculated the spike-triggered average of the LFP (st-LFP) and a whitened version of this signal (wst-LFP). Prior to this step, signals were band-passed at 15-300Hz (unspecified order).

It is generally accepted that the nature of the LFP signal is dependent several factors such as neuronal morphology, synaptic receptors, ion channel kinetics, and electric properties of tissue. All of these contribute to the challenging interpretation of low-frequency signals. This team proposes that each spiking neuron establishes synapses with multiple other neurons, giving rise to many synaptic currents that interact with each other and originate the unitary LFP of that neuron. Multiple unitary LFPs are then summed into a measurable population signal in which contributions from single neurons are masked.

The st-LFP calculated by the team aims to recover the unitary contributions of single spiking neurons, but this signal is still affected by recurrent and ongoing LFP activity in the network. Both inhibitory and excitatory neurons displayed st-LFPs with broad components and averaging such signals over distances to the source showed spike-related components spanning over 1mm distances, with an exponential amplitude decrease with distance (Fig. 3.5). They state that such non-local and non-causal components are unsuitable for LFP interpretation.

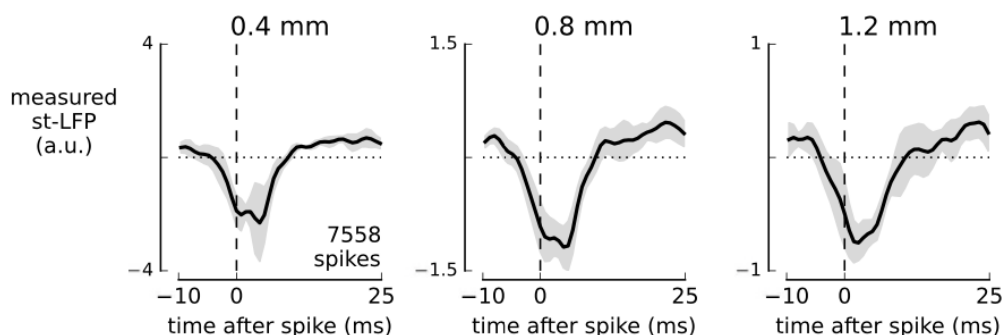


Figure 3.5: LFP spatial proliferation: measured st-LFP signals, averaged over distance to the "trigger" neuron. Adapted from [94].

In theory, interneurons are fast-spiking cells and pyramidal cells are regular spiking. Thus spike waveshape was used by the team to distinguish inhibitory and excitatory neurons. Despite this method not being consensual nor 100% efficient, accounting for up to 10% misclassified excitatory cells they defend that this should not hamper significantly the interpretation of results such as the observation that fast-spiking neurons correlate with LFPs at shorter latencies.

According to Teleńczuk *et al.*, volume conduction or synchronous activity of other neurons may result in a broader st-LFP signal. To solve this problem, they applied spatial filters, obtaining a signal that may originate from synaptic currents of the "trigger" neuron.

The results obtained by the team show that both excitatory and inhibitory neurons generate negative-going wst-LFPs of similar amplitudes but different latencies. This fact could suggest a di-synaptic contribution of excitatory neurons, through the activation of inhibitory ones. Moreover, they propose that the amplitude of the observed signal may be distorted by a series of factors like the low-pass filtering effect of the medium or the dendritic structure. Spatial filters remove the effects of passive conductivity, making signals slower in accordance to their putative synaptically-transmitted origin. Interestingly, the propagating speed, in this case, is consistent with the action potential propagation in unmyelinated fibers.

### 3.2.4 Research 4: Correlations with Neuronal Activity

In 2020 van de Weg developed some work on the relationship between LFPs and APs [33]. In this work, cortical networks of dissociated cells from newborn Wistar rats were grown and plated on top of 60-electrode MEAs. The author performed electrophysiological recordings and used a 1-35Hz band-pass and a 300Hz high-pass filters to separate the low and high-frequency components, respectively. AP detection was then performed and spikes were analyzed for artifact detection. Network bursts were detected using inter-spike intervals to cluster intra-burst and inter-burst spikes (active non-bursting cultures were also identified).

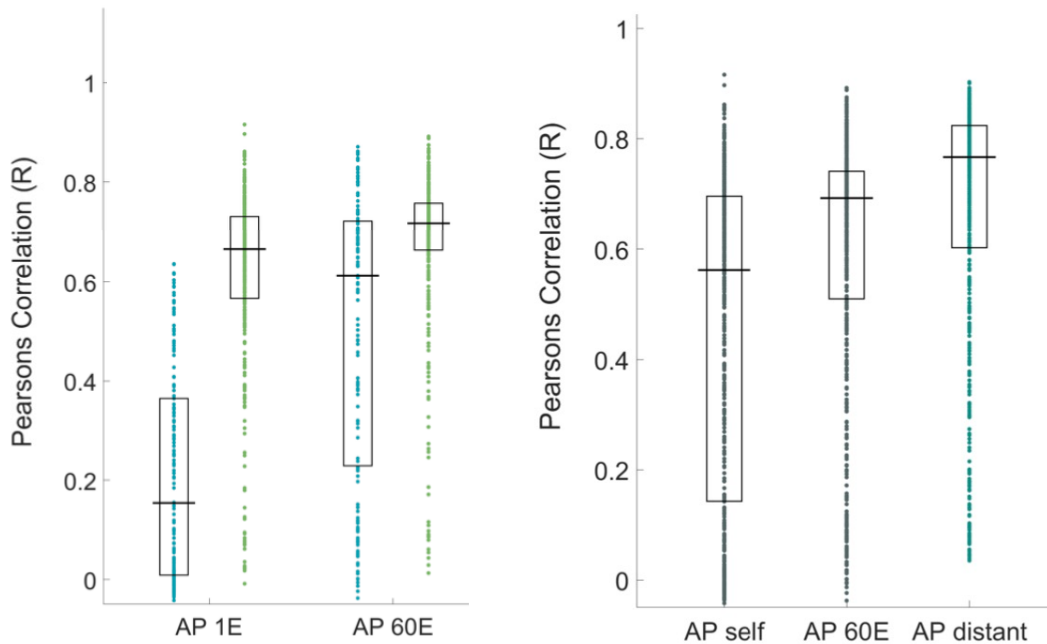


Figure 3.6: Correlation between LFPs and spiking activity: on the left, correlations using summed network activity (60E) are consistently higher than those of single electrodes (1E), and bursting activity (green) also correlates better than non-bursting (blue); on the right, LFP recorded on one electrode correlates better with distantly recorded APs (dist) than with network APs (60E) or from the same electrode (self). Adapted from [33].

In this research, Several parameters of the bio-signals were considered, like the area under the curve of the LFP and its absolute value, as well as the maximum peak potential. She calculated these using a sliding window of 0.5s (step size of. 0.05s), which was also applied in the high-frequency component for AP count. The work focused on correlations between LFPs and APs both with single electrode count or summed count over 9, 25, and 60 electrodes. AP-triggered LFPs were also obtained using AP timestamps to extract 2-second epochs from the LFP signal, but results were not extensively quantified.

The author states that maximum peak potential and absolute area under the curve both correlated nicely with AP activity (the latter was used for further analysis). As shown in Figure 3.6, correlations with summed network activity were, in general, superior to the single electrode approach. Moreover, electrodes that detected bursts tended to demonstrate a more noticeable link between spiking activity and LFP signals. Interestingly, signals seemed to have a strong remote contribution, as the correlation with AP count from a distant electrode was bigger than those of the network and the same electrode. In the majority of non-bursting cultures, no measurable LFP signals were recorded.

Other statements of relevance in this work are as follows: There is a possibility of electrodes picking up both frequency components (if covered by neurons and culture medium), only picking up one frequency component (if electrically isolated from the medium or not covered by any neurons), or picking up nothing apart from noise (electrically isolated); LFP signals correlate better with input (presynaptic) than with output (postsynaptic) spikes; output spikes are likely to generate further LFP signals when they arrive at their presynaptic active zones and are transmitted through synapses.

Van de Weg also suggests that, although only absolute AUC of LFP signals was analysed, other parameters like the maximum peak potential could be of interest and provide equally interesting results. Besides, LFP signals may contain information about the excitation/inhibition balance in cortical networks which remains unexplored.

### **3.3 Work (to be) Developed in the LFP Field**

This Section focused its attention on research conducted so far in the area of LFPs, both regarding their properties and their generation. As to the work already developed in the field, there seems to be a consensus about the biophysical sources behind the signals, but not about the exact way in which the signals are generated nor, consequently, on how they should be correctly interpreted. As these signals are very easy-to-record and show great potential for clinical applications, research in this field is of utter importance.

Some of the mentioned studies have proposed that information regarding excitation and inhibition (along with the balance between them) may be contained in LFP signals, but this is a possibility that remains unexplored. Retrieving such information from these easy to record signals would be of extreme clinical relevance, as it may provide a tool for the evaluation of neuronal network excitability.

One other apparent point of accordance between researchers is that changes in the recording setups and methodologies employed in this field have a major impact both on observed results and their following interpretation. It is not trivial to generalize and compare results across studies or even cortical areas. This is quite a challenge when working with LFP signals.

Despite recently developed valuable research, some important questions regarding LFP signals remain unanswered. Firstly, the generation of the signal is still a mystery, with few to no theories existing on how inputs are integrated into the measured potential. Secondly, the prominently inhibitory nature of such signals, as defended in previous work, needs to be validated and further studied, to evaluate if problems with inhibition in cortical networks can be observed in the signal (and how are they observed). Lastly, the putative correlation between APs and LFPs should also be confirmed and further explored as it may contain important information regarding the relation between neuronal input and output.

All things considered, from the research discussed here, it seems that LFPs are mostly generated by synaptic currents of inhibitory origin. The signal also seems to be influenced by spiking activity, demonstrating a strong correlation between the two frequency components. Nonetheless, working with LFPs is not straightforward, so these findings should be validated, and, if possible, expanded. Studies conducted so far on the origin of the signal tend to stimulate a single cell and evaluate the network response. However, evaluating signals generated upon the regular activity of cultures may be of interest. Finally, it is clear that LFP signals may have various clinical applications and that there is still a gap in understanding the information they encode which, in turn, hampers their usage.

## Chapter 4

# Materials and Methodology

The present chapter gives some insights into the materials used and methods followed during the project. First, we present the protocols for cell culture, followed by some elaboration on the acquisition of neuronal signals in the different experiments conducted. Then, we describe the data analysis performed on those signals and give an overview of the stimulation applied to some signals of interest. Lastly, we explain how the statistical significance of the results was evaluated.

### 4.1 Cell Culturing

#### 4.1.1 Normal Cortical Cultures

We grew cortical cultures following a slightly adapted cortex isolation protocol from previous experimental work developed in the Clinical Neurophysiology Group from the University of Twente [95, 96]. Briefly explained, we treated cells obtained from brain cortices of male and female newborn Wistar rats with trypsin and dissociated them by titration. We created a 1000  $\mu\text{L}$  well by attaching a silicone-made culture chamber on top of a multielectrode array (Multichannel Systems, Germany), precoated with polyethylene imine (PEI) to seed cultures. For cell nutrition and growth, the well contained R12H culture medium [97], half of which was refreshed twice a week. We only used cultures in experiments after growing them for at least two weeks in an incubator under standard conditions of 37°C, high humidity, and 5%  $\text{CO}_2$ . This period is essential for networks to be developed and start showing normal patterns of burstiness, a fundamental aspect of the conducted research.

#### 4.1.2 Viral Cortical Cultures

The ChannelRhodopsin-2 (ChR-2) is a valuable tool for the optogenetic stimulation of neurons. These non-selective cation channels are light-gated (a unique property), enabling the induction of depolarization and consequent firing of neurons using blue light pulses ( $\approx 470\text{ nm}$ ) [98].

Since their discovery, new variations of the ChR-2 have been engineered to overcome limitations and guarantee a successful stimulation of cell cultures. One particular example is the ChR-2 H134R mutation which has reduced desensitization (less intense response upon repeated exposure to the stimuli) and enhanced light sensitivity compared to ChR-2 [99]. As to their kinetics, it has been shown that when hit with blue light pulses, these channels present an opening rate much faster than their closing rate (ten to twenty times faster rates have been reported) [100].

Following one other protocol of a work developed in the abovementioned group, we transfected cultures with Adeno Associated Virus (AAV) containing the ChR-2 gene with an H134R mutation [101]. We used a promotor found only in excitatory neurons ( $\text{CaMKII}\alpha$ ) to ensure that only this cell type was mutated. The transfection was performed on Day *in Vitro* 1 (DIV1) as this was shown to produce the most effective results.

### 4.1.3 Neuronal Blockers

During experiments, we blocked inhibitory and excitatory activity (separately) to evaluate their contribution to the LFP signal. We used different blockers, one for inhibition (picrotoxin) and two others for excitation (Cyanquixaline and APV).

Picrotoxin (PTX) is one of the most traditionally used  $\text{GABA}_A$  antagonists, and its suppressing effect seems to be quite independent of the subunits present [102]. PTX consists of a combination of picrotin and picrotoxinin (the active component) in equimolar quantities. Its half maximal inhibitory concentration ( $\text{IC}_{50}$ ) has reported values of  $4\mu\text{M}$  [103]. It appears to be a rapidly metabolized substance, with an half-life of around 15 minutes [102, 103].

Cyanquixaline (CNQX) is a neuronal blocker that competes with AMPA and kainate (KA) receptors as well as with the glycine modulatory site on the NMDA receptor [58, 104]. However, its effect is most noticeable on AMPA receptors [105], which is also verified by the values of  $\text{IC}_{50}$ :  $0.3\mu\text{M}$  for AMPA,  $1.5\mu\text{M}$  for KA, and  $25\mu\text{M}$  for the glycine modulatory site [106]. With high concentrations of CNQX (such as  $10\mu\text{M}$ ) it is possible to have a disconnected network [107].

2-amino-5-phosphonovaleric acid (APV) is one of the compounds that are selective N-methyl-D-aspartate (NMDA) receptor antagonists [108]. It can be used to suppress network burstiness as a result of hampering excitatory synaptic transmission [109] but does not affect the spontaneous activity of neurons [110]. Values in the range of  $0.46\text{--}0.71\mu\text{M}$  have been reported for such antagonist when used with recombinant NMDA receptors [111]. However, it is most commonly used in the range of  $50\text{--}100\mu\text{M}$  to promote the blockade of excitation in neuronal cultures [57, 112, 113].

In order to block  $\text{GABA}_A$  receptors, thus blocking inhibitory activity in the cultures, we added a solution of picrotoxin (PTX) to the medium at a concentration of  $50\mu\text{M}$ . This was done by dilution of a  $40\text{mM}$  solution in ethanol. The dilution was performed in two steps: first, we added  $5\mu\text{L}$  to  $195\mu\text{L}$  of PBS (dilution factor of 40), bringing the concentration to  $1\text{mM}$ ; then, we transferred  $50\mu\text{L}$  of this new solution to  $\approx 1000\mu\text{L}$  of medium (dilution factor of 20), bringing the final concentration to the desired  $50\mu\text{M}$ . Even though the initial PTX solution is in ethanol, which is toxic for cells, being diluted by a factor of 800 makes its effect negligible. Thus, the final solution is safe to use with cell cultures without harming them.

Similarly, to block AMPA and NMDA receptors, we used solutions of CNQX and APV at final concentrations of 10 $\mu$ M and 100 $\mu$ M, respectively. For the CNQX, we prepared a first stock solution of 20mM by diluting  $\approx$ 464mg of powder in 100 $\mu$ L of PBS, and then diluted it twice: firstly, we added 10 $\mu$ L of this solution to 190mL of PBS (dilution factor of 20); after this, we pipetted 10 $\mu$ L of this solution to the  $\approx$ 1000 $\mu$ L of culture medium (dilution factor of 100). For the APV, from a previously existing stock solution of 20mM, we added 50 $\mu$ L to the culture medium (dilution factor of 200).

As so, the final concentrations of receptor antagonists used were: 50 $\mu$ M of PTX, 10 $\mu$ M of CNQX, and 100 $\mu$ M of APV, chosen based on the effects reported in the abovementioned literature.

## 4.2 Data Acquisition

### 4.2.1 Electrophysiological Recording Setup

We placed cultures on MEAs<sup>1</sup> inside a MEA 2100 headstage (Multichannel Systems, Germany)<sup>2</sup> for electrophysiological recordings. This was connected to MCS-Lite-IFB-C (an interface board multiboot), allowing for real-time signal detection, processing, and feedback. For data acquisition and storage, we used the MC\_Rack software (Multichannel Systems, Germany). Even though the program allows for real-time processing of signals, we stored raw data files and performed offline processing. Before running experiments, we used the MCS Filter Config software (Multichannel Systems, Germany) to set the system's cut-off frequencies of the second-order low-pass and first-order high-pass filters to 3000 Hz and 0.1 Hz, respectively. We acquired signals at a sampling frequency of 25 kHz in recordings of 3-15 minutes, depending on the stability of the recorded signal. Using the Multi Channel DataManager (Multichannel Systems, Germany), we saved files in .mcd format and later converted them to HDF5 files (to be processed in Matlab).

During recordings, we placed cultures inside a small chamber, supplied with a constant gas flow of 95% compressed air with 5% CO<sub>2</sub> to ensure cell viability during several hours of experimentation. We used a metal mesh to serve as a Faraday cage, reducing noise and enhancing the quality of recordings.

### 4.2.2 Optogenetic Stimulation Setup

In order to stimulate the cultures transfected with ChR-2, we placed Blue (470 nm)–74 lm@700 mA power LEDs (LuxeonStarLEDs, Canada) on a SinkPAD-II 20 mm Star Base approximately 7 cm above the top of the MEA with the Faraday Cage between them to mitigate the effect of electrical artifacts produced by the power cables. We used an in-house made LabVIEW program to control the tunable stimulation parameters: duration of the stimulus (time during which the LED is on) as well as different intervals of stimulation (time between two consecutive light pulses).

<sup>1</sup><https://www.multichannelsystems.com/products/60mea20030ir-ti-wo>

<sup>2</sup><https://www.multichannelsystems.com/products/mea2100-systems>

### 4.2.3 Experimental Protocol

We traced an experimental protocol for the project that consists of several steps with different conditions, each recorded for 5-11min (not very long because of the blockers' relatively short half-life duration). It is important to note that this protocol was inspired by works developed by other teams in an attempt to reproduce and expand their results. As the project advanced, the protocol suffered some slight changes in order to produce results more suitable for interpretation and discussion. As so, the experimental scenarios evaluated were as follows:

**Condition 1: Control.** We placed cell cultures inside the recording system with a conventional recording lid, and evaluated normal cell activity for some minutes.

**Condition 1.5: Control with stimulation (CStim).** We tested four of the five included cultures' responsiveness to light stimulation. To do so, we flashed light pulses of 0.1s each 10s on top of the cultures, maintaining the setup from the previous step.

**Condition 2: Inhibitory activity blocked (PTX).** After adding the PTX solution, excitatory only cell activity can be recorded. This step allows for a better understanding of the contribution of excitatory activity to LFP signals.

**Condition 2.5: PTX with stimulation (PTXStim).** Similarly to condition CStim, we stimulated the cultures with light pulses of 0.1s each 10s to evaluate whether there was a big influence of stimulation on the shape of the signals.

**Condition 3: All activity blocked (Blocked).** We added excitatory blockers (APV and CNQX), recording activity with both inhibition and excitation suppressed. This step is essential to understand if the signal is indeed related to neuronal activity in its genesis or not.

**Condition 4: Optogenetic Stimulation (Stim).** We applied different stimulation settings to evaluate how the signals changed with differences in pulse width and separation. 4 different values for stimulus duration were used (0.1s, 0.5s, 1s, 2s) in combination with three different intervals between stimuli (5s, 10s, 20s).

## 4.3 Data Analysis

The following section focuses mainly on the signal processing made in Matlab. For this, we wrote several scripts based on the work developed by van de Weg [\[33\]](#). For advanced analysis, we altered these scripts, evaluating aspects not approached in the previously carried out work.

### 4.3.1 Signal Filtering and Processing

We imported raw signals from HDF5 files and loaded them into a matrix of dimensions 60 x N (one line per electrode). We analyzed signals from all conditions in order to exclude electrodes that systematically presented a low signal-to-noise ratio throughout all experiments, making them not unsuitable for drawing proper conclusions. Electrode 15 (which worked as a "far away" reference) was excluded from analysis in all experiments.

Before analysis, we also trimmed the signals to a clean section, meaning that we disregarded very noisy initial moments. Considering a practical example, in every "PTX" and "Blocked" conditions, within the first 60-100s of recording, noticeable artifacts were induced by opening the recording cage to allow the addition of blockers. Such periods were not considered for analysis as they represent noisy events of known origin.

We used a threshold of 7 times the root mean square (RMS) value of the signal for AP detection in all experiments. We tested such value before analysis to ensure a good performance of the AP detection algorithm. In fact, neither setting a very high threshold nor a very low one is desirable. While the first will miss a lot of APs, the second will lead to noise being considered activity. After excluding all unsuitable electrodes, the remaining ones were considered valid and used for analysis in recordings of the experimental conditions as well as the optogenetic stimulation. This pre-processing of data gave rise to the dataset described in Section 5.1.

In a first moment, we filtered the signals to separate their two components. We applied a high-pass filter with a cut-off frequency of 300Hz to isolate the high-frequency component, and a low-pass filter with a cut-off frequency of 35Hz for the low-frequency component (both were second-order Butterworth filters). From this point, we analyzed each frequency component differently to obtain their relevant information.

On the one hand, we scanned the high-frequency content for AP detection with the abovementioned threshold value. The AP detection algorithm sets the threshold locally and not globally, meaning that it calculates the RMS in time windows of 60s with a step size of 30s in order to account for the inherent changing nature of the electrophysiological signals (which change temporally in mean and standard deviation). We saved a timestamp for the detected APs, together with a 6ms sample of the signal (from 2ms before until 4ms after the event). We then validated these putative APs using four different criteria (by adaptation of the work of Wagenaar et al. [114]):

1. In a 1ms vicinity, there should be no bigger peaks than the detected one;
2. APs cannot be wider than 0.16ms at 50% peak amplitude nor narrower than 0.24ms at 90%.
3. A maximum of 3 APs (per electrode) can share the same timestamp
4. Electrodes detecting as many positive as negative APs were discarded from the analysis

We registered the percentage of APs labeled as artifacts due to each one of these reasons (an overview per culture, per experimental step, can be found in Appendix A for the tested conditions, and in Appendix B for the optogenetic stimulation settings). Considering only the events that successfully fulfill all criteria, we counted APs per electrode in bins of 0.005s, with each bin corresponding to its central value (for example, we plot the 0s to 0.005s bin as the AP count value at 0.0025s). We also quantified the number of electrodes not detecting any AP throughout the recordings. We calculated firing rates per minute per electrode and plotted the mean shape of all the detected APs in each electrode to check for validity. To set the threshold for AP detection, we checked the RMS value of the signals (considered suitable if it was in the range of 3-5 $\mu$ V).

On the other hand, we down-sampled the low-frequency component of the signals, from 25kHz to 1kHz, avoiding problems with space for data storage without losing valuable information. We cut the signals from the optogenetic stimulation experiments to make the first stimulation begin at 0.5s (since the stimulus is given at a constant rate, it is possible to isolate the different periods of interest). We saved all of the variables in .mat files for further analysis.

### 4.3.2 Optogenetic Stimulation Evaluation

For each culture and each stimulation setting, we divided the signals collected from optogenetically stimulated cultures into epochs whose length was equal to the time between stimuli. As so, in all of them, the onset of stimulation was temporally aligned. The low-frequency signals are affected by very slow oscillations which are not physiologically relevant to the present study. In fact, their period is not compatible with any cell-related event, and they are thought to be related to other more complex processes like cognition. To serve as a correction, we subtracted the average value of the first 0.4s of signal (baseline) per stimulus. After this, we averaged all epochs of each electrode, allowing for the visualization of one representative waveshape per electrode per stimulation setting. We evaluated the validity of the average waveshape by visual analysis of maximum positive and negative deviations from it.

We then analyzed some main readouts from such average waveshapes to allow for comparison between different experiments and settings (Fig. 4.1). Firstly, the amplitude of the "negative" peak ( $A_N$ ) which represented the difference between the baseline and the minimum measured; secondly, the latency of the "negative" peak ( $\delta t_N$ ) corresponding to the time separation between the stimulus and the previously explained point; thirdly, the time constants for activation and deactivation of the ChR-2 ( $\tau_A$  and  $\tau_D$ , respectively) which correspond to the time necessary for each of these processes to reach 63% of the total variation of the potential.

One other parameter we evaluated in all experiments was the effect of the blockers (spiking activity must be suppressed in these experiments). To do this, we also split the signal containing the AP count at each moment into epochs with the same duration as the interval between stimuli. We obtained two readouts for comparison between responses given to various stimuli. Firstly, the total number of APs detected during those periods networkwide (averaged across all epochs). Secondly, we quantitatively evaluated burstiness.

For network burstiness evaluation, we first labeled the electrodes with 0 if no APs were detected and 1 if spiking activity was sensed in that electrode. After this step, each electrode was represented by an array of zeros and ones (as long as the number of timestamps in the signal). Then we summed all electrodes, creating a linear vector with the number of active electrodes per timepoint. A network burst was detected if the maximum number of synchronously active electrodes per epoch (considered to represent the peak of the putative network burst) was above five. Finally, if in more than 10% of all registered epochs there was a burst detected, then the culture would be classified as bursting upon stimulation.

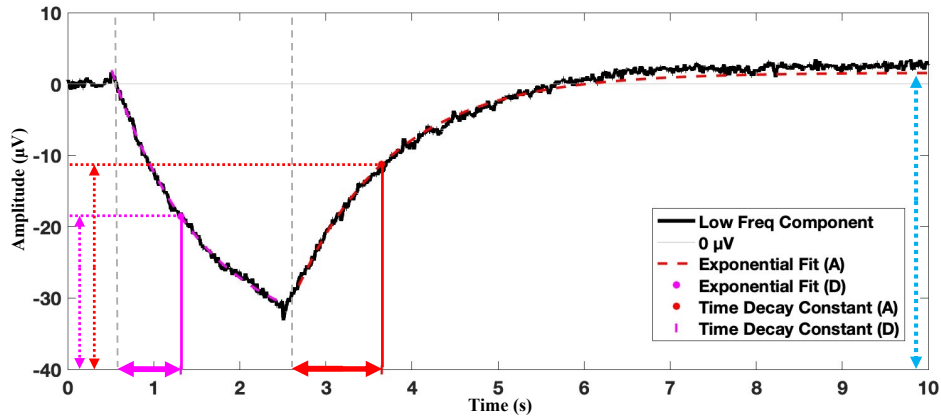


Figure 4.1: Time constant calculation for the optogenetic stimulation experiment: measured signal (black), exponential fits for activation and deactivation (pink and orange trace lines), total amplitude variation (blue dotted double arrow) and 63% of total variation in each direction (pink and orange dotted lines and dotted double arrows). The x values of the intersections (pink and orange lines) are the values necessary to obtain the activation and deactivation time constants (pink and orange double arrows, respectively).

### 4.3.3 AP and LFP Visualization and Quantification

We scanned pre-processed signals for features that may describe systematic behaviors. Regarding APs, the first focus of the analysis was the presence or absence of network bursts (by visual inspection and evaluation of the networkwide AP count). In the cultures with network bursts present, we selected 11 network bursts and used the moment in which they reached their peak as the timepoint of that given burst. The chosen number of selected moments in each experimental step has to do with the quality of control recordings in which, most times, it is already difficult to isolate 11 clearly separated bursts. If there were no measurable network bursts, we selected random time points for analysis. We calculated the burst height (maximum value of AP count reached) for each of the selected portions of the signal.

To analyze the low-frequency component of the signals, we cut windows of  $[-3 : 7]$ s around the burst peak for each electrode. Once more, to attenuate the sinusoidal fluctuations that affect the signals, we subtracted the baseline (mean value of the first two seconds of the considered recording portion) of each waveform. After performing this in each one of the conditions referred to in Section 4.2.3, we obtained readouts for comparison between conditions (Fig. 4.2). First, the amplitude of "1<sup>st</sup> positive" peak ( $A_{P1}$ ) is the difference between the baseline and the maximum measured right after the network burst. Second, the amplitude of the "negative" peak ( $A_N$ ) corresponds to the difference between the baseline and the minimum value measured after the network burst. Third, the amplitude of "2<sup>nd</sup> positive" peak ( $A_{P2}$ ) is the difference between the baseline and the maximum measured after the negative peak. Fourth, the time constant for restoration of the potential ( $\tau_R$ ) corresponds to the time it takes to reach 63% of the total potential variation.

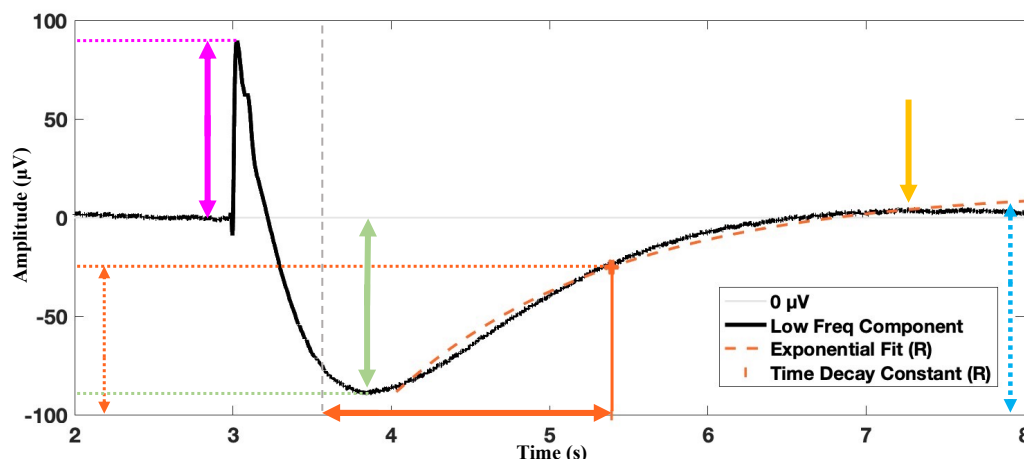


Figure 4.2: Main readouts for comparison across conditions and cultures: measured signal (black), exponential fit for restoration (orange trace line), total amplitude variation (blue dotted double arrow) and 63% of total variation (orange dotted line and dotted double arrow). The x value of the intersections (orange line) is the value necessary to obtain the restoration time constant (orange double arrow). Also, 1<sup>st</sup> positive peak amplitude (pink dotted line and double arrow), negative peak amplitude (green dotted line and double arrow), and 2<sup>nd</sup> positive peak amplitude (small difference between the grey line and the signal at the moment indicated by the yellow arrow).

Two important observations: these readouts, along with the results obtained from the methods explained in Sections 4.5 and 4.4, will also be used to understand the effect of filtering on signals; also, due to considerable differences between conditions, activation was not characterized here.

Even though the majority of the electrodes displayed similar patterns, some electrodes had to be separately analyzed. One clear example of such a situation can be viewed in Figure 4.3 in which different shapes are highlighted in different colors. We identified electrodes of interest qualitatively (by visual inspection) and quantitatively (very different readouts). Their analysis focused on whether the distinct waveshapes were physiologically relevant.

To evaluate changes between experimental conditions, we evaluated statistical differences between the main readouts (see Section 4.6). We also calculated the correlation between the average waveshapes for each condition. Finally, we performed a visual analysis of the waveshapes, aiming towards finding a hypothesis that coherently explained the observed divergences between signals.

## 4.4 Additional Tests

We used some extra MEAs to test the influence of the blue light stimulation pulses in the obtained signal. As so, we placed two MEAs under the LED (one completely empty and the other filled with PBS) and performed electrophysiological recordings. Additionally, using one MEA with a cortical culture, we performed two recordings with the same stimulation settings. However, in one of the instances, we placed an opaque object between the LED and the culture.

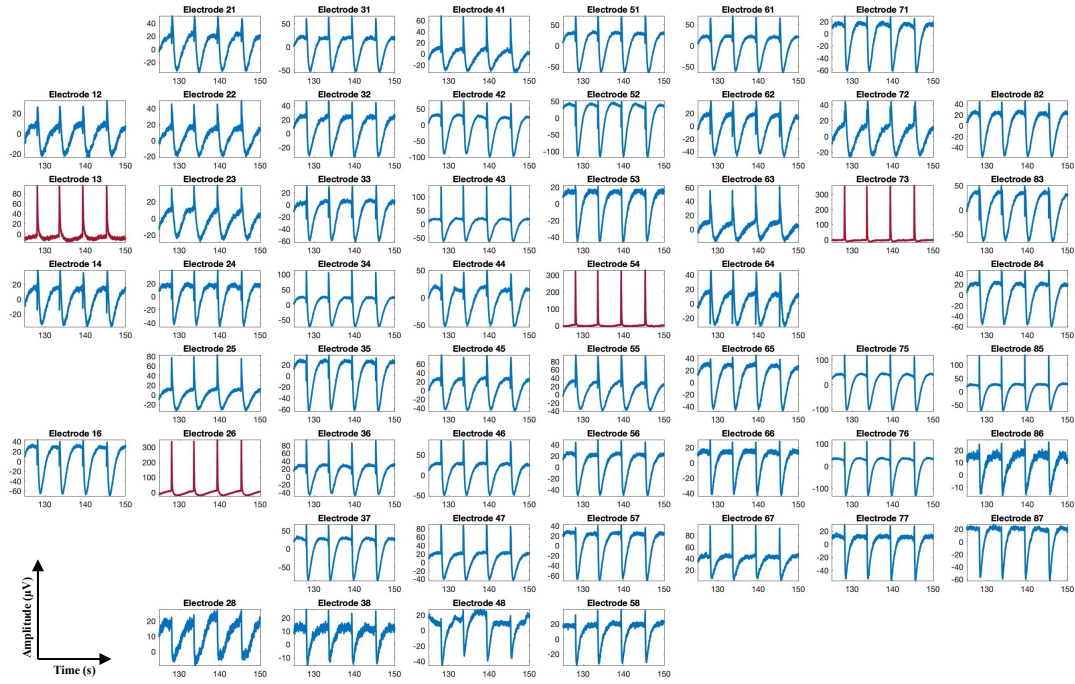


Figure 4.3: Example of LFP shape divergence within a culture: two shapes were detected in culture C1 (blue and red) which made in necessary to perform some extra analysis on the signals.

## 4.5 Signal Simulations

We simulated signals in Matlab in order to evaluate the effect of the filtering settings applied and try to retrieve the unfiltered signal. This step, although important to understand which features are intrinsic to the biosignals and which are a mere consequence of the applied filtering, is not crucial to hypothesize about the origin of the LFP signals. In fact, filters only respond to perturbations in the signal, and, as so, if the recording did not change in time, no shape would be generated only by the applied filters. Nonetheless, we ran simulations regarding both the stimulus-induced and the raw LFP shapes.

To simulate the stimulus, we generated a square pulse (positive amplitude of  $100\mu\text{V}$  in a 2s on and 8s off cycle). For the raw signal simulation, we only simulated the low-frequency component (we did not consider the high-frequency content). Each period of the simulated signal consisted of a sinusoidal drop for the negative deflection and an exponential function for the recovery. We added a sinusoidal wave of very low frequency to the complete signal (mimicking fluctuations verified *in vitro*), in addition to some white noise.

To see the effect of synchronicity in the sensed signals, we generated 100 positive sinusoidal waves of unitary amplitude and 1ms period. We then summed them all across a window of 5ms. We tested three scenarios: full synchronicity (all peaked at the same time), 50% synchronicity (half of them peaked at random times), and no synchronicity (each one peaked at random times).

## **4.6 Statistical Analysis**

For statistical analysis, we tested normality using the Shapiro-Wilk test with a significance level of 0.05. In the case of normally distributed samples, we applied a paired t-test to evaluate significant differences. Otherwise, we performed a Wilcoxon test with the same goal. In both cases, significance was of  $\alpha = 0.05$ . When comparisons between different cultures had to be made, given the fact that the number of considered electrodes was different for each one of the five cultures, we performed a Welch two-sample t-test to assess statistical significance ( $\alpha = 0.05$ ).

## Chapter 5

# Results

In this Chapter, the results obtained throughout the developed project are presented, starting with a brief description of the included cultures and electrodes, followed by the analysis of signals in a sequence of ascending complexity. In the end, some main results are summarized, reflecting the main contributions of this work.

### 5.1 Included Cultures Overview

Neuronal activity in five different cultures was recorded with the filter settings described in Section 4.2.1, resulting in five different sets of signals (C1 to C5) with multiple conditions (see Section 4.2.3). Cultures C1 to C3 were used after 21 days of growth while cultures C4 and C5 were only 15 days old. In all of them, more than 50% of the total number of electrodes were considered valid for analysis. The number of electrodes that fulfilled the inclusion criterion were: 54 in C1 (90%), 56 in C2 (93%), 37 in C3 (62%), 35 in C4 (58%) and 34 in C5 (57%). Considering the five experiments, 217 out of 300 electrodes were included in the analysis (72%).

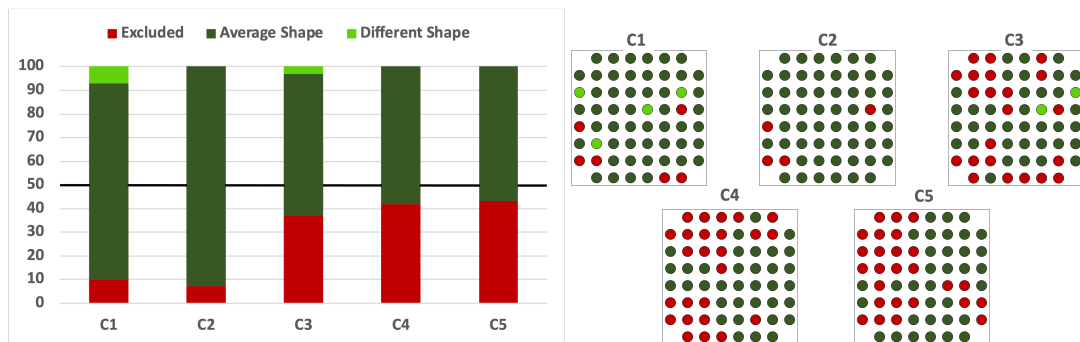


Figure 5.1: [Cultures and Electrodes Included in Analysis: quantification of excluded and included electrodes (left), and distribution of such electrodes on the MEAs (right). Different waveshapes detected in the LFP signals, separately analyzed are shown in different shades of green.

Moreover, regarding the identification of different shapes (see Section 4.3.3) it is possible to find, within the included electrodes, only 1 single waveshape in most cultures (C2 to C5) and 2 different waveshapes in cultures C1 (see Section 5.6.5). The distribution and quantification of electrodes capturing each one of these waveshapes, along with the percentage of excluded electrodes per culture, can be visualized in Figure 5.1. The selected electrodes have a RMS value in the range of 3-5 $\mu$ V with cell activity and in the range of 2.5-3 $\mu$ V when all activity is blocked. In some conditions, additional electrodes were excluded during AP validation for detecting as many positive APs as negative (see Section 4.3.1). When performing statistical significance analysis, only valid electrodes in all conditions were considered. A deeper characterization of the included cultures in recordings and stimulation can be found in Appendices A and B, respectively.

## 5.2 Signal Acquisition and Filtering

Figure 5.2 illustrates the main results obtained in a step-by-step view from the raw signal to the filtered components. We obtained two different signals after separating the high and low-frequency components. We performed spike detection and quantification per electrode (AP Count) using the high-frequency content. Then we summed such values, obtaining the networkwide AP Count in which we visually verified the presence of bursting behavior in examples like Figure 5.2. In the low-frequency component, after testing higher cut-off frequencies, results remained extremely similar, indicating that we were not losing information with the lower cut-off value.

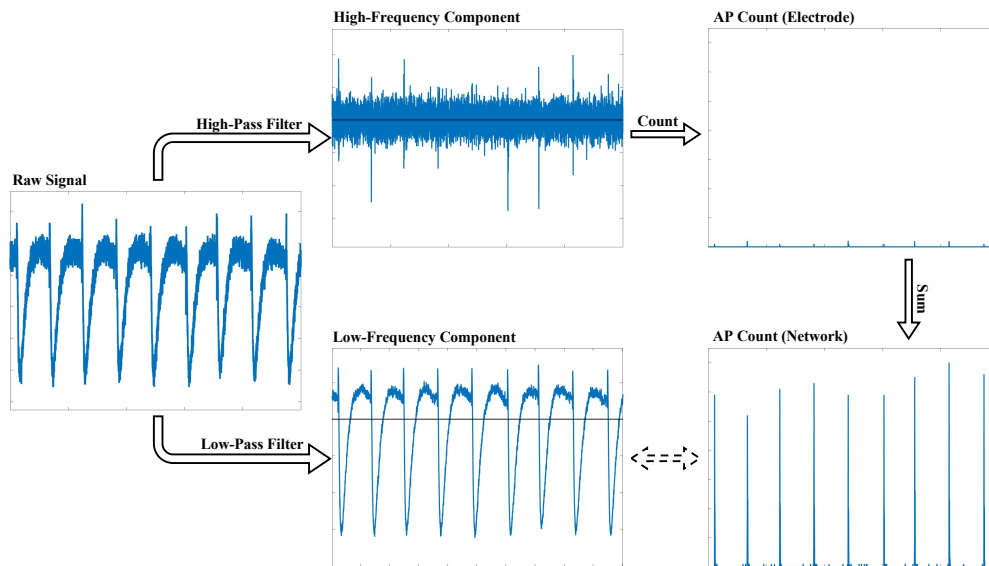


Figure 5.2: Filtering and processing applied to the raw signals: the raw signal (left) was filtered with a 300Hz high-pass filter (top middle) and a 35Hz low-pass filter (bottom middle) separately. The high-frequency component was used to perform AP detection on an electrode basis (top right) which was summed to obtain the networkwide count (bottom right). The dashed double arrow highlights the possible interdependence between network activity and LFPs.

### 5.3 Differences in Frequency Components

Comparison between Control, Blocked, and Stim (2s/10s) conditions allowed the visualization of three distinct scenarios in the two different frequency components. Figure 5.3 shows examples, with the AP detection resulting from the high-frequency component analysis in the top row and the low-frequency signals in the bottom row. In Control condition, with the cultures presenting their normal behavior, it is possible to measure local field potentials as well as to detect APs across the whole network. In Blocked condition, since excitation and inhibition are both blocked, none of the components show activity, meaning no APs detected nor measurable LFPs. In Stim condition, it is possible to measure fluctuations in the low-frequency component, but very few shapes in the high-frequency component are considered to represent true action potentials.

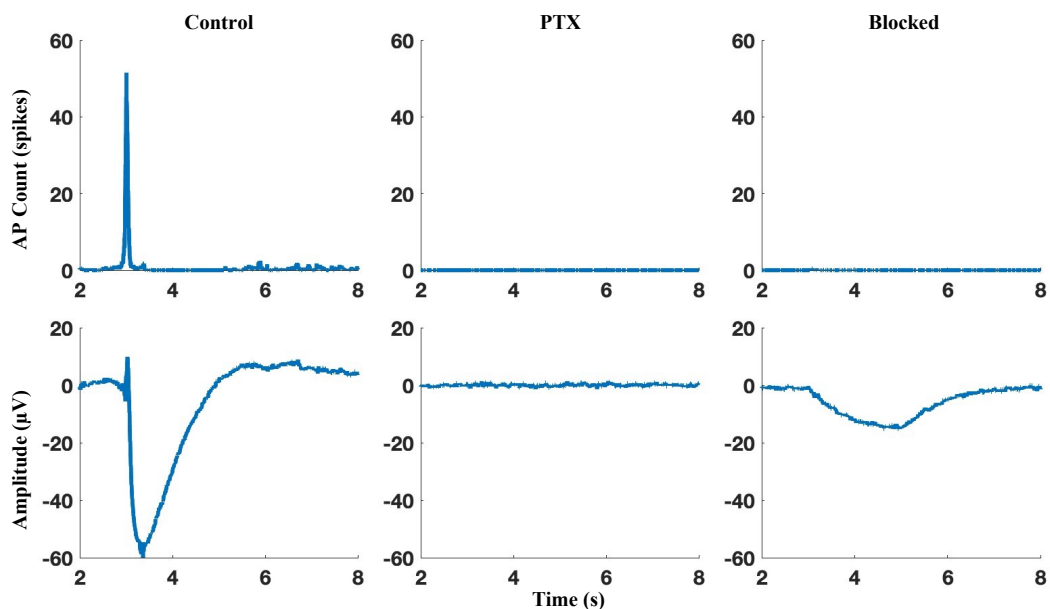


Figure 5.3: APs and LFPs obtained in different recording conditions: Action Potentials detected in Control, Blocked and 2s/10s Stim conditions, summed across all electrodes (top). Low-frequency component for each of the conditions (bottom).

### 5.4 Optogenetic Stimulation

#### 5.4.1 Effect of Blockers

The first step was to evaluate the neuronal blockers' efficiency in suppressing signals. Qualitative evaluation is possible by visualization of the raster plots (Fig. 5.4). When inhibition is suppressed the culture should acquire a typical rhythmic bursting behavior. Then, when excitation is also blocked, most of the spiking activity ought to be successfully silenced. Having visually confirmed activity suppression, the main quantitative evaluation parameters are presented in Table 5.1.

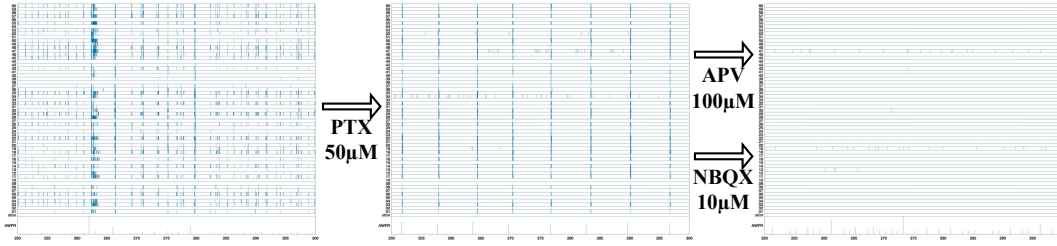


Figure 5.4: Raster plots of normal activity (left), blocked inhibition with PTX (middle) and blocked activity with PTX, APV and CNQX (right).

By analyzing Table 5.1, in all cultures, right upon addition of the suppressors, with no stimulation being applied, neural activity was very low and no culture was bursting. After this, the number of APs detected tends to increase as time passes by, making the background color scale also accompany these increments in spiking activity.

Upon stimulation, however, results regarding burstiness evaluation were not so consistent. In three of the cultures (C1, C4, and C5) the number of APs detected during the considered epochs remained substantially low and no network bursts were detected. In culture C2, the network started bursting with optogenetic stimulation in all settings and the number of detected APs was considerably high. Lastly, in culture C3, blockers were still exerting their effect in the first stimulation settings applied (0.5s/10s) but, after that, started fading out, hence the increase in APs detected and the reappearance of measurable network bursts in the remaining tested conditions.

Considering the abovementioned results, cultures C1, C4 and C5 will be the main ones used for the analysis of results regarding the signals obtained with optogenetic stimulation in the following sections. Some analysis of one of the remaining cultures is also presented in Section 5.5, although this will focus on more specific behaviors observed in that culture, which may be general.

Table 5.1: AP quantification and burstiness evaluation during stimulation

| Stimulation Settings | APcount Networkwide |          |           |         |        |
|----------------------|---------------------|----------|-----------|---------|--------|
|                      | C1                  | C2       | C3        | C4      | C5     |
| No Stim/10s          | 9 ± 4               | 36 ± 13  | 56 ± 22   | 1 ± 2   | 1 ± 2  |
| 0.1s/5s              | 17 ± 4              |          |           |         |        |
| 0.1s/10s             |                     | 158 ± 23 |           | 4 ± 2   | 10 ± 4 |
| 0.5s/5s              | 26 ± 3              |          |           |         |        |
| 0.5s/10s             |                     | 360 ± 32 | 59 ± 10   | 8 ± 2   | 1 ± 1  |
| 1s/10s               | 47 ± 55             | 125 ± 19 | 1019 ± 27 | 8 ± 2   | 5 ± 2  |
| 1s/20s               |                     |          | 472 ± 320 |         |        |
| 2s/10s               |                     | 164 ± 22 | 1062 ± 31 | 14 ± 11 | 1 ± 1  |
| 2s/20s               | 57 ± 6              |          |           |         |        |

Numbers in green indicate absence of network bursts, while numbers in green indicate bursting behavior; Background color scale indicates the order of application of the stimulation settings (red → orange → dark yellow → light yellow → white, from first to last).

### 5.4.2 Effectiveness of the Stimulation

When all activity was blocked no measurable signal was detectable in the low-frequency component in the successfully suppressed cultures (green signals in Fig. 5.5). Upon stimulation with the blue LED, signals started to reappear in the low-frequency component, even though no considerable APs were detected in the high-frequency component (Blocked condition in Fig. 5.3).

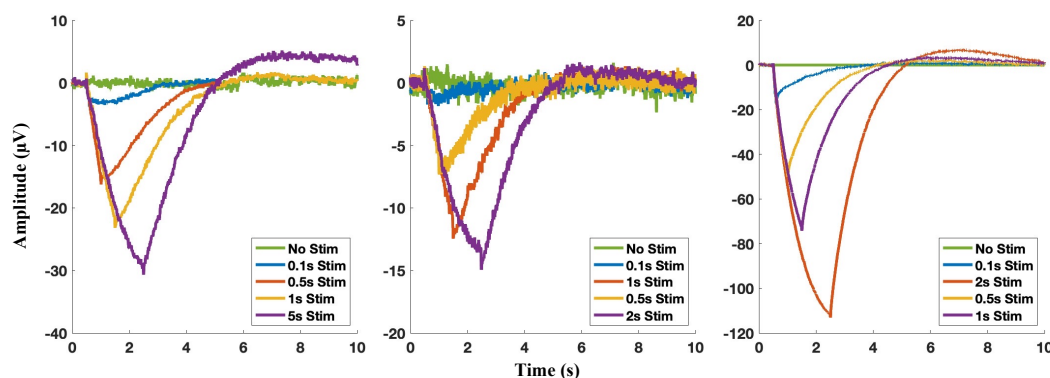


Figure 5.5: Example of signals obtained by stimulating suppressed cultures: the order of stimulation settings in the label of each plot indicates the order in which such settings were applied to that culture, from top to bottom

### 5.4.3 Signals Captured by Different Electrodes

In Figure 5.5, the observed shapes correspond to one electrode per culture. In fact, after analyzing the signals captured by each one of the electrodes, one electrode per culture appeared to be representative of all (see Section 5.4.4). Looking at the set of signals obtained when we stimulated culture C1 for 2s each 20s (visible in Fig. 5.6), it is possible to clearly perceive the similarities and differences between the signals captured by the different electrodes of the MEA. These results were very comparable across cultures.

Given certain stimulation settings, signals captured from all valid electrodes in the MEA showed a very high correlation with R values in the range of 0.85-1.00 (for all cultures). All signals were aligned temporally using the timepoint of the network burst so that a separate biased alignment of the various channels did not influence results. Nonetheless, signals differ in amplitude with electrodes spread across the MEA measuring values that go from -20 $\mu$ V all the way down to -70 $\mu$ V as illustrated in Figure 5.6 (middle portion) employing a color map.

Interestingly, it is possible to see that, independently of the stimulation settings, it is visible that the same set of electrodes tends to pick up the signals with the highest amplitudes or the ones with the lowest amplitudes. Taking Figure 5.6 as an example, once more, it is possible to see that in the grids presented in the middle portion, the lower left corner tends to be redder, indicating signals with larger amplitudes, while the upper left corner appears to capture smaller signals.

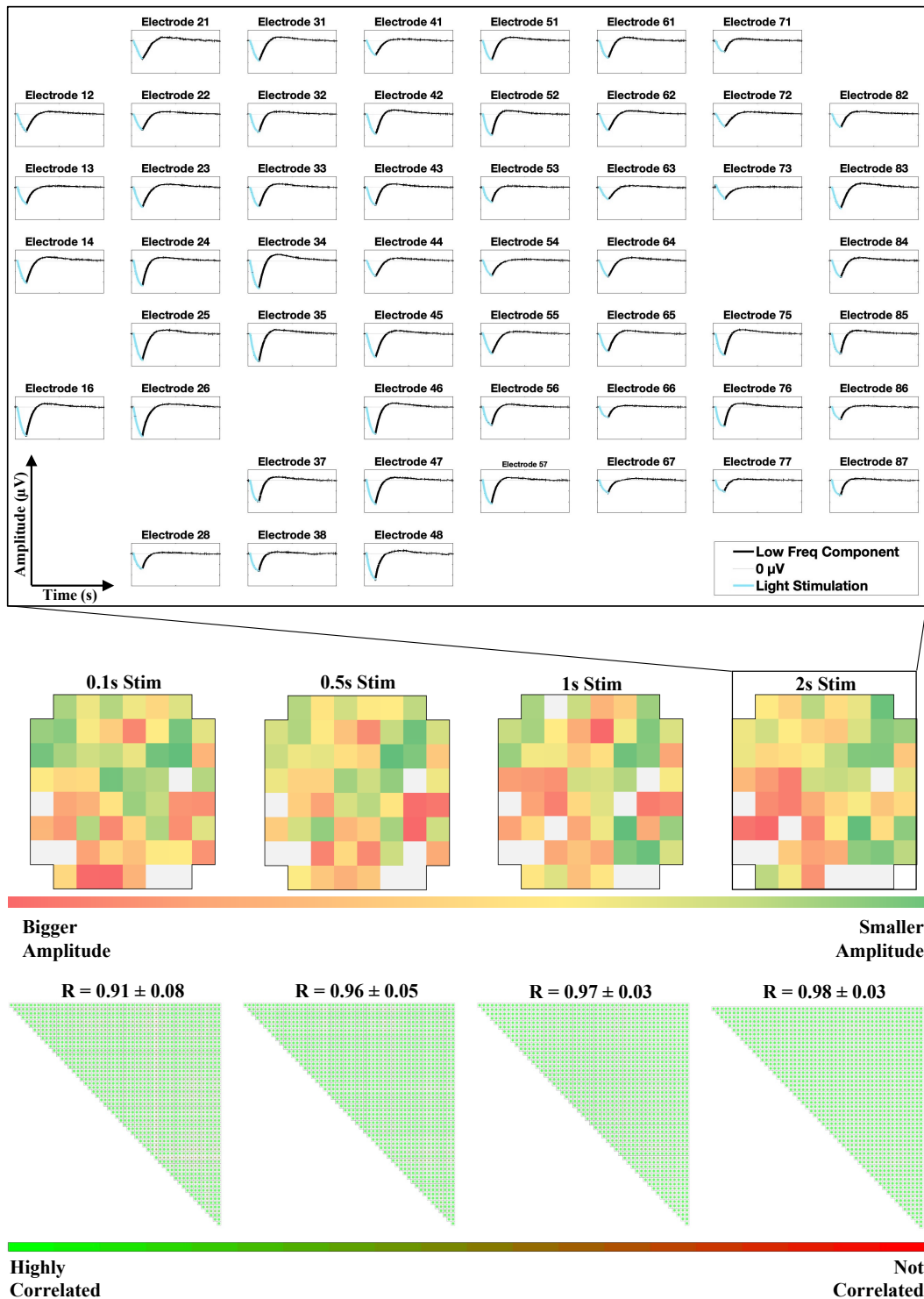


Figure 5.6: Similarities between signals captured by the different included electrodes with the low-frequency signals (top) displayed in their recording positions in the MEA (AP Count in top right corner); Color map of the amplitudes of the signals per electrode, in each of the stimulation conditions (red represents a bigger variation in potential); Correlation matrices (bottom) between all valid electrodes per stimulation setting (green represents a very high correlation).

#### 5.4.4 Representativeness of the Average Waveshape

Another key aspect of the analysis is that, as explained in Section 4.3.2, the considered waveshape is, in fact, the average waveshape of the response to a series of stimuli during a certain recording period. As so, the maximum deviations from such waveshape must be small. Only then can we consider it representative of the response. In general, this variation is not high, and average signals are a good representation of the signal's response to the stimulus, as seen in Figure 5.7.

It is important to note that the maximum deviation from the average signal is inversely proportional to the duration of the stimulus. This implies that longer stimuli elicit responses that do not vary as much (as shown in Fig. 5.7 in which the duration was of 2s), in comparison with shorter stimuli in which deviations from the average signal tend to be larger. For a deeper analysis of the such phenomenon, see Appendix C. Nonetheless, the average signal was considered representative enough to be used in the interpretation and generalization of the results.



Figure 5.7: Representativeness of the average waveshape of signal's response to stimuli (traced in black), displayed in position of recording, with the maximum deviations (blue areas). Note: subplots are presented with different ranges in y-axis to enhance the visualization of the variation.

#### 5.4.5 Signal's Amplitude vs Stimulation Settings

The first evaluated readout between stimulation settings was the difference in amplitude of the negative deflection observed (referred to as negative peak from now on). Since we subtract baseline values from the signals, comparison between different conditions and cultures is possible. The main results of this evaluation can be visualized in Figure 5.8.

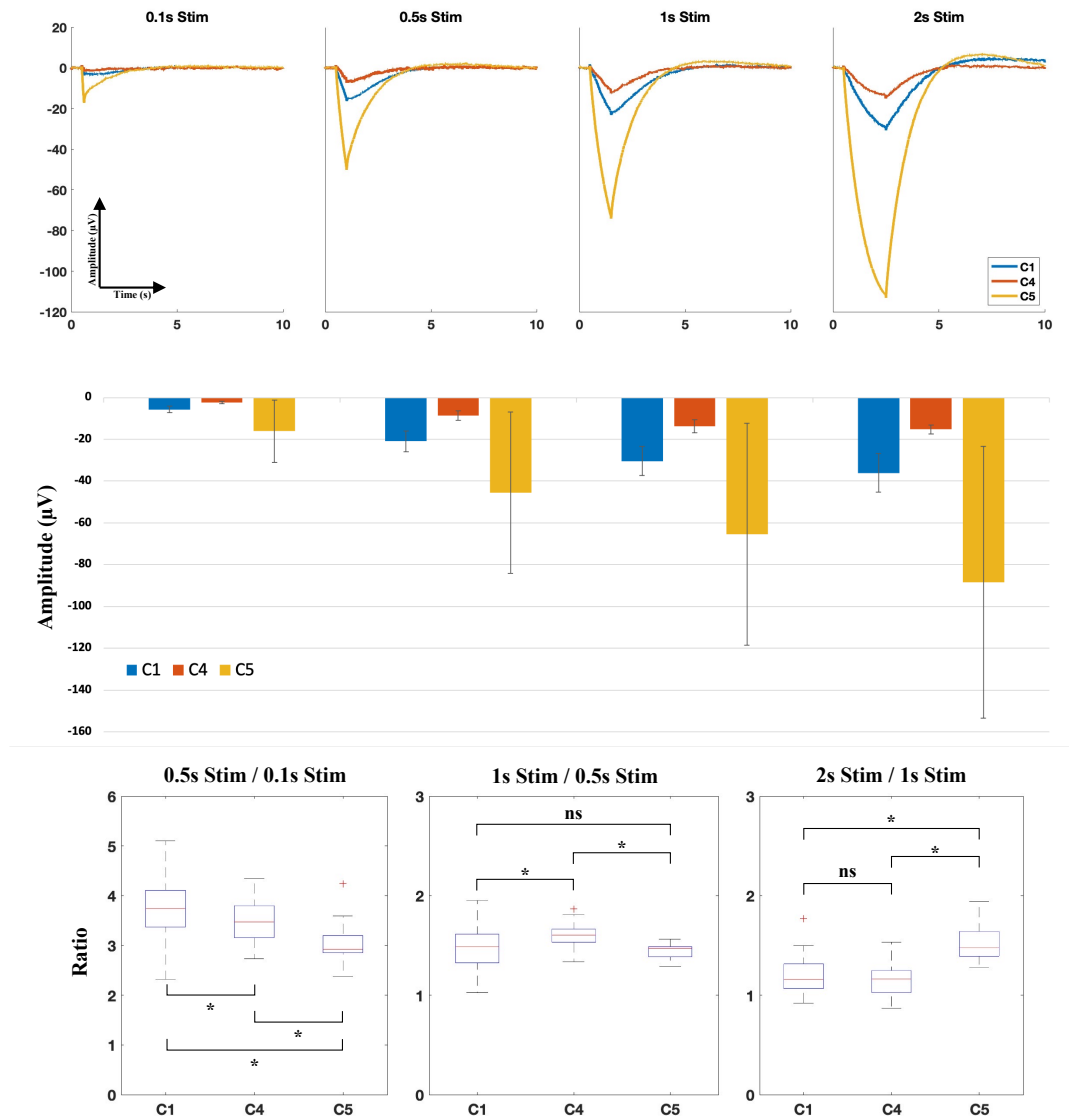


Figure 5.8: Variation of the amplitude of the signals with the stimulation settings: Plot of a representative waveshape per culture, per stimulation setting, evidencing the changes in amplitude between cultures and settings (top); Bar plot of the average amplitudes measured (with the respective standard deviation) per stimulation setting, per culture (middle); Comparison of ratios between amplitudes measured with different stimulus duration (bottom), with \* $p < 0.05$  and non significant changes indicated with ns.

From both qualitative and quantitative evaluations, it was possible to see that the longer the stimulus' duration, the bigger the amplitude of the measured negative peaks. There does not seem to be one common range for amplitude values dependent on stimulation time between cultures, with culture C5 measuring systematically larger signals than the remaining cultures and C4 the smallest ones. However, when comparing the ratios between 0.5s and 0.1s stimulation, 1s and 0.5s stimulation, and 2s and 1s stimulation, it becomes evident that these values are not so distinct and that common ratios of 3-4 times bigger, 1.3-1.7 times bigger, and 1-1.5 times bigger, respectively, can be verified across cultures, even though being statistically different in some instances.

#### 5.4.6 Latency of the Negative Peak vs Stimulation Settings

Another parameter that we evaluated in the stimulated cultures was the latency of the negative peak. Regarding this, we verified that the latency was mostly coincident with the duration of the stimulus, meaning that the negative deflection accumulated while the LED was on (Fig. 5.9).

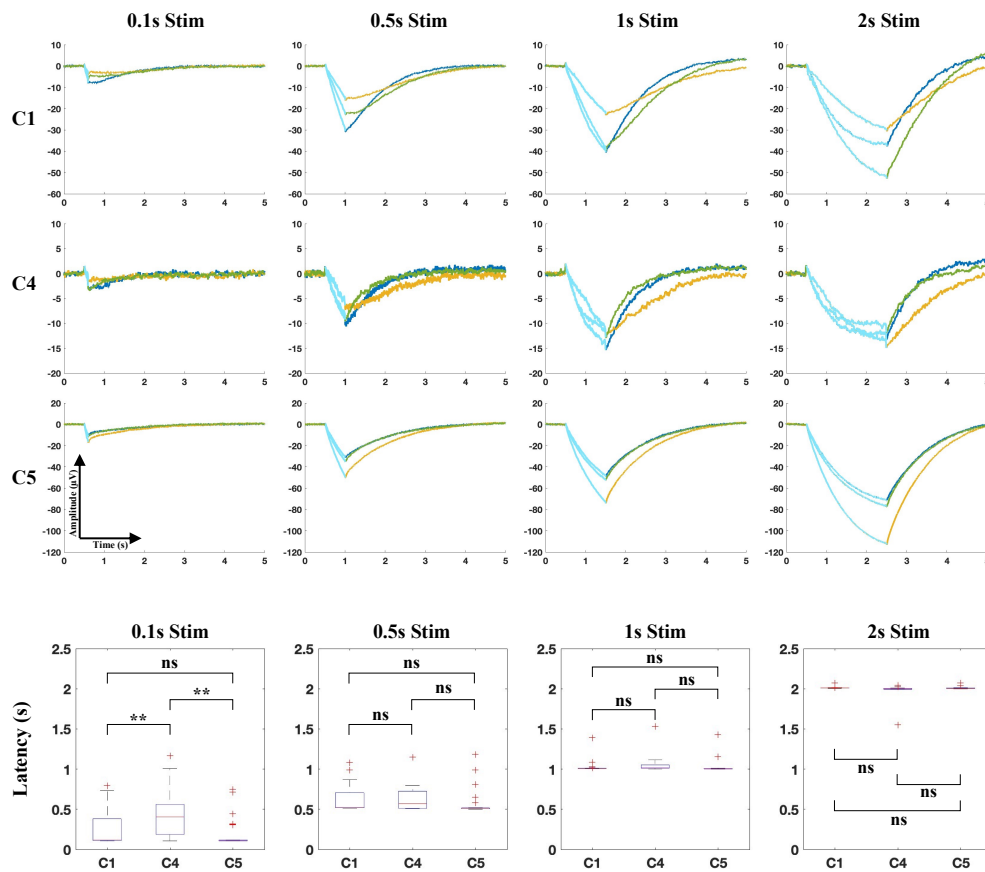


Figure 5.9: Variation of the latency of the negative peak with the stimulation settings: plot of three random representative electrodes per culture, per stimulation setting (top), highlighting the time during which the blue LED was on (light blue traces); Latency times between different cultures and stimulus duration (bottom), with \* $p < 0.05$  and non significant changes indicated with ns.

By simply looking at the top panel of Figure 5.9, it is possible to see that the latency times between cultures are very close to each other (temporal alignment along the columns of the grid of plots). However, the amplitudes measured in each culture are in completely different ranges with  $C1 = [-60 : 10]\mu V$ ,  $C4 = [-20 : 5]\mu V$  and  $C5 = [-120 : 20]\mu V$ . This difference in amplitudes is in accordance with what we exposed in the previous section.

### 5.4.7 Activation and Deactivation Time Constants vs Stimulation Settings

Specific decay time constants characterize diffusion processes. For this reason, we calculated the time constants for the activation (settlement of the negative deflection) and deactivation (restorations of the potential) of the low-frequency signals using the methods explained in section 4.3.2. Results are presented in Figure 5.10. Regarding activation, results were, in most cases, significantly different both between cultures to which the same stimulation was applied and between different settings in the same culture. Just like the latency and the amplitude of the verified deflection, the time constant for the descending part of the signals seems to increase consistently with pulse duration.

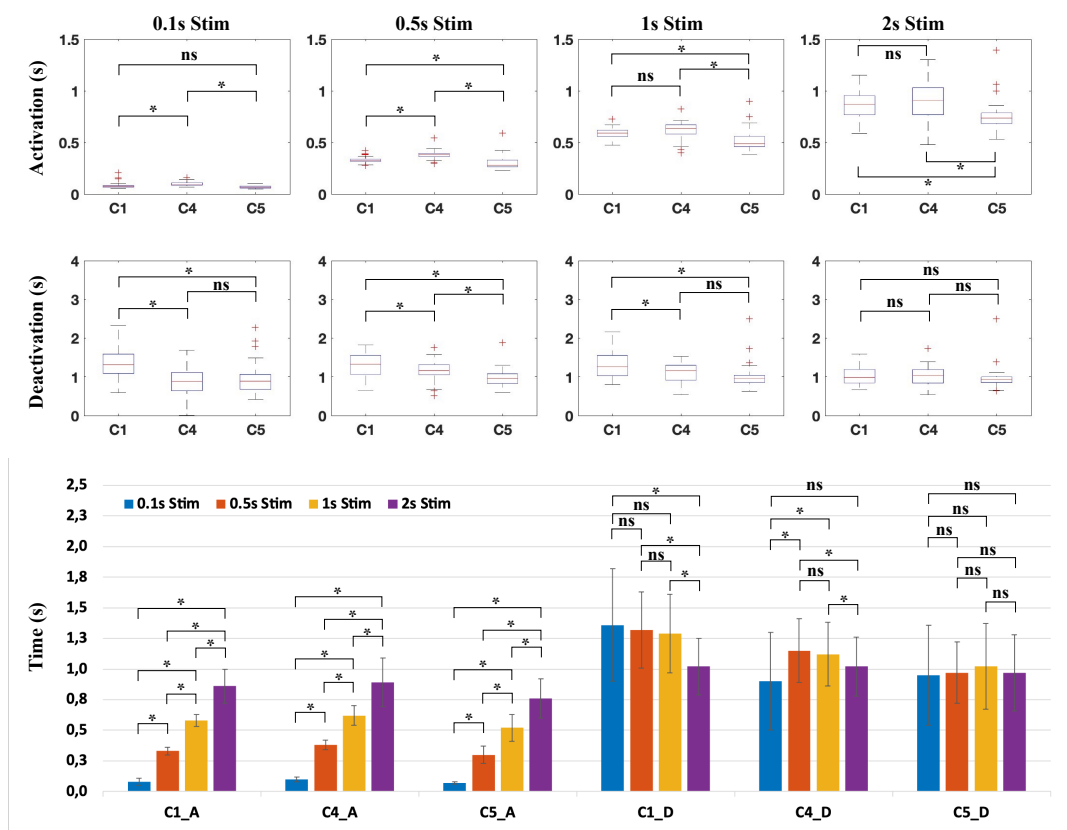


Figure 5.10: Activation and deactivation time constants for the different stimulation settings: Comparison between different cultures with the same settings (top) and between different settings applied to the same culture (bottom), with  $*p < 0.05$  and non significant changes indicated with ns.

Moreover, the value of the activation time itself increased with longer stimulus going from values around  $[0.07 : 0.1]$ s for 0.1s pulses up to  $[0.76 : 0.86]$ s for 2s ones. Such results go along with the finding that the negative deflection accumulates as long as the stimulus is applied, as shown in Section 5.4.6, particularly in Figure 5.9.

Regarding deactivation, results were more consistent between cultures and within the same culture. For the 2s stimulus, all cultures in all stimulation settings had a deactivation time of around 0.9-1.1s. Deactivation times at shorter stimulus duration were often significantly different between cultures, although differences were small. All in all, there seems to be a relatively constant value per culture for deactivation (and a common range across all cultures).

#### 5.4.8 Overshoot and Baseline vs Stimulation Settings

The last evaluated readouts were the overshoot after the negative peak and the baseline of the recording. Firstly, as can be seen in Figure 5.5, in some cases, after the negative peak, a small positive overshoot can be visualized. Hence, to verify whether this represented a systematic phenomenon or not, the overshoot was quantified. Secondly, as mentioned in Section 4.3.2, since slow oscillations are not under evaluation in the present study, we subtracted baseline values from the signals. These values were still registered and checked for systematic patterns.

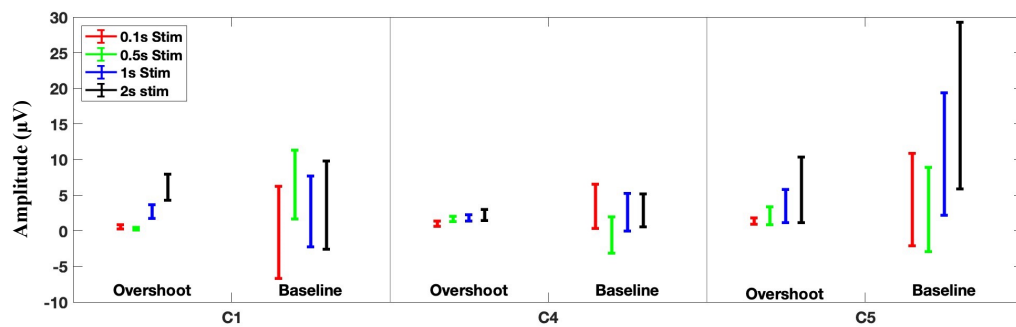


Figure 5.11: Overshoot and baseline values for the different stimulation settings. For each of the cultures two sets of ranges of values are traced: overshoot values on the left and baseline values on the right.

Regarding overshoot, by analysis of the plots in Figure 5.11, it was possible to state that there was a tendency in values to increase with the duration of the stimulus with 0.1s stimuli always registering the smallest range for overshoot values ( $[0.48 : 1.38]\mu\text{V}$ ) and 2s stimulation the highest one ( $[2.21 : 6.12]\mu\text{V}$ ). The overshoot values increase  $[0.9 : 1.53]$  times from 0.1s to 0.5s stimulation and  $[1.23 : 2.46]$  times from 1s to 2s settings.

We found no systematic patterns between the different tested cultures regarding baseline values. Besides, the differences in stimulation settings did not seem to have any proportional effect on baseline values, with all settings registering similar ranges for baseline values.

### 5.4.9 Relevance of the Measured Signals' Content

In order to understand if the measured signals could be mere electrical artifacts of the recording setup, we conducted three extra experiments, as explained in Section 4.3.2. As shown in Figure 5.12, these waveshapes were very different from the ones obtained when the cultures were optogenetically stimulated (Fig. 5.5).

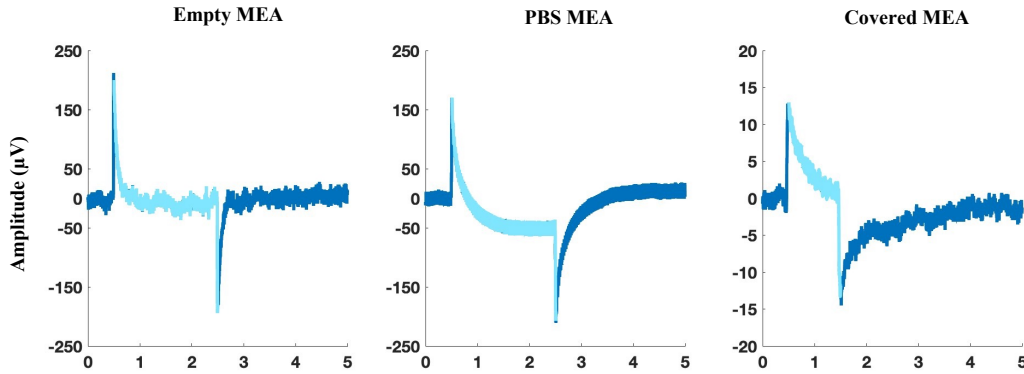


Figure 5.12: Electrical artifacts picked up by the electrodes: Average waveshape of the signal captured using an empty MEA under the LED (left), a MEA filled with PBS under the LED (middle), and a MEA with cells in culture medium but in which the LED was covered using an opaque material (right). Light blue traces indicate the time during which the LED was on.

Firstly, with the empty and PBS MEAs, amplitude values were in a  $[-200 : 200]\mu\text{V}$  range which is much larger than the amplitude values for the signals obtained with the cells. Secondly, the signals' shape in these cases accurately resembles a square pulse to which a band-pass filter was applied (see Section 5.6.5). When the LED was covered, preventing cultures from receiving the light stimulus, the shape of the low-frequency signal remained similar to the ones observed with the other two MEAs, but the amplitude of the deflections was much smaller ( $[-15 : 15]\mu\text{V}$ ).

## 5.5 AP Count vs Low-Frequency Component Waveshapes

In culture C3, as the blockers began to fade out, the culture started regaining network activity, and bursts became more noticeable. This, in turn, had an effect on the low-frequency signal, in which a strong positive deflection (referred to as positive peak) started to be more prominent prior to the negative peak, which also increased in amplitude as the bursts got more intense. In fact, all electrodes seemed to pick up signals that showed a similar response to the changes in network bursts, with high correlations between the values of the amplitudes of both the positive and negative peaks, as well as with the intensity of the network bursts (measured as the number of spikes in each burst). Figure 5.13 visually summarizes the abovementioned results.

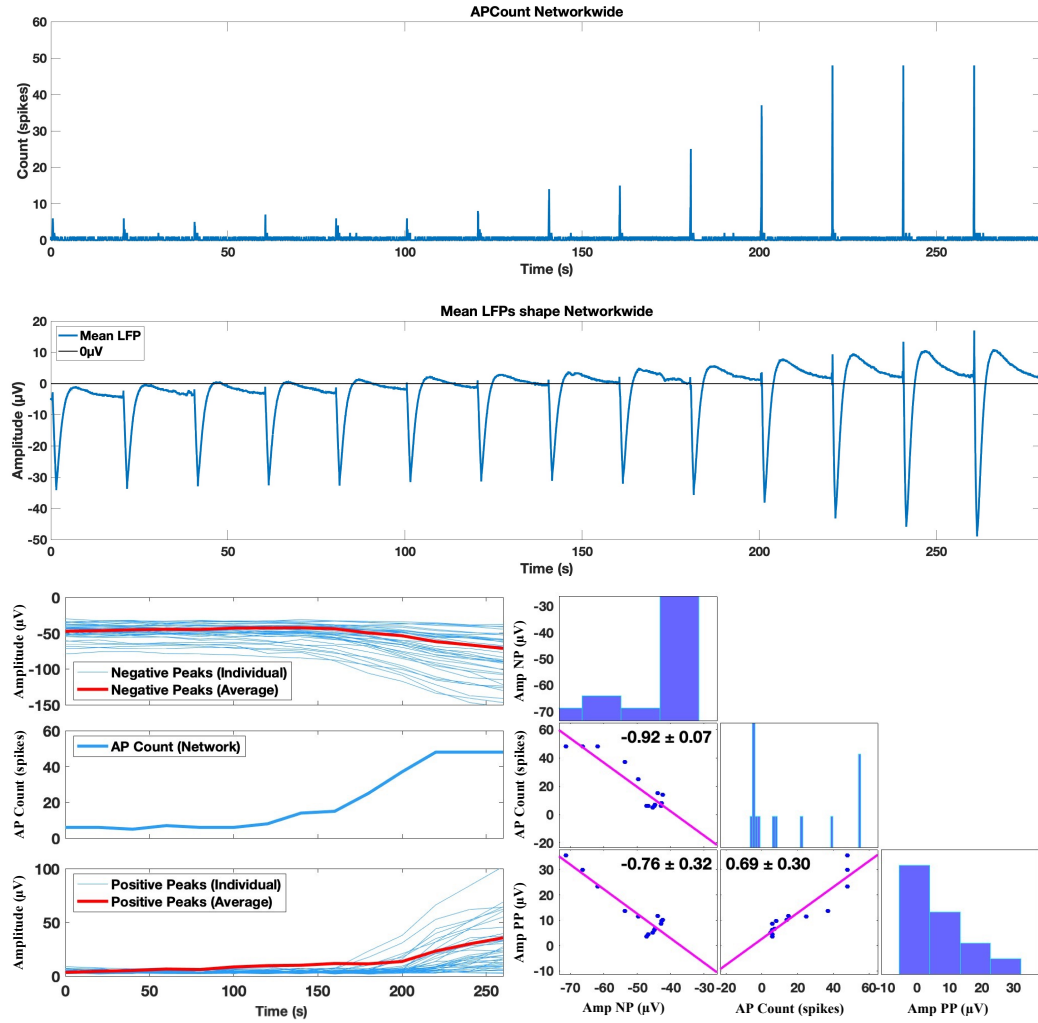


Figure 5.13: Network bursts' effect in the low-frequency component: Plots of the AP count (networkwide) and average waveshape of the low-frequency component (top); Traces of the variation of the amplitude of the positive and negative peaks per electrode (bottom left); Correlation matrix between negative peaks, AP count, and positive peaks (bottom right).

## 5.6 Disentangling the "Black-Box" Generation of LFPs

So far, we have shown that the signals measured in the low-frequency component are not only a leftover of the spiking activity nor electrical artifacts of the recording systems. It is now time to focus on exploring the processes behind the generation of the signal and how the putative major contributors are combined in the final waveshape. For this, it is crucial to distinguish general patterns of the LFPs from punctual deviations (also checking whether these different signals are of relevance or not).

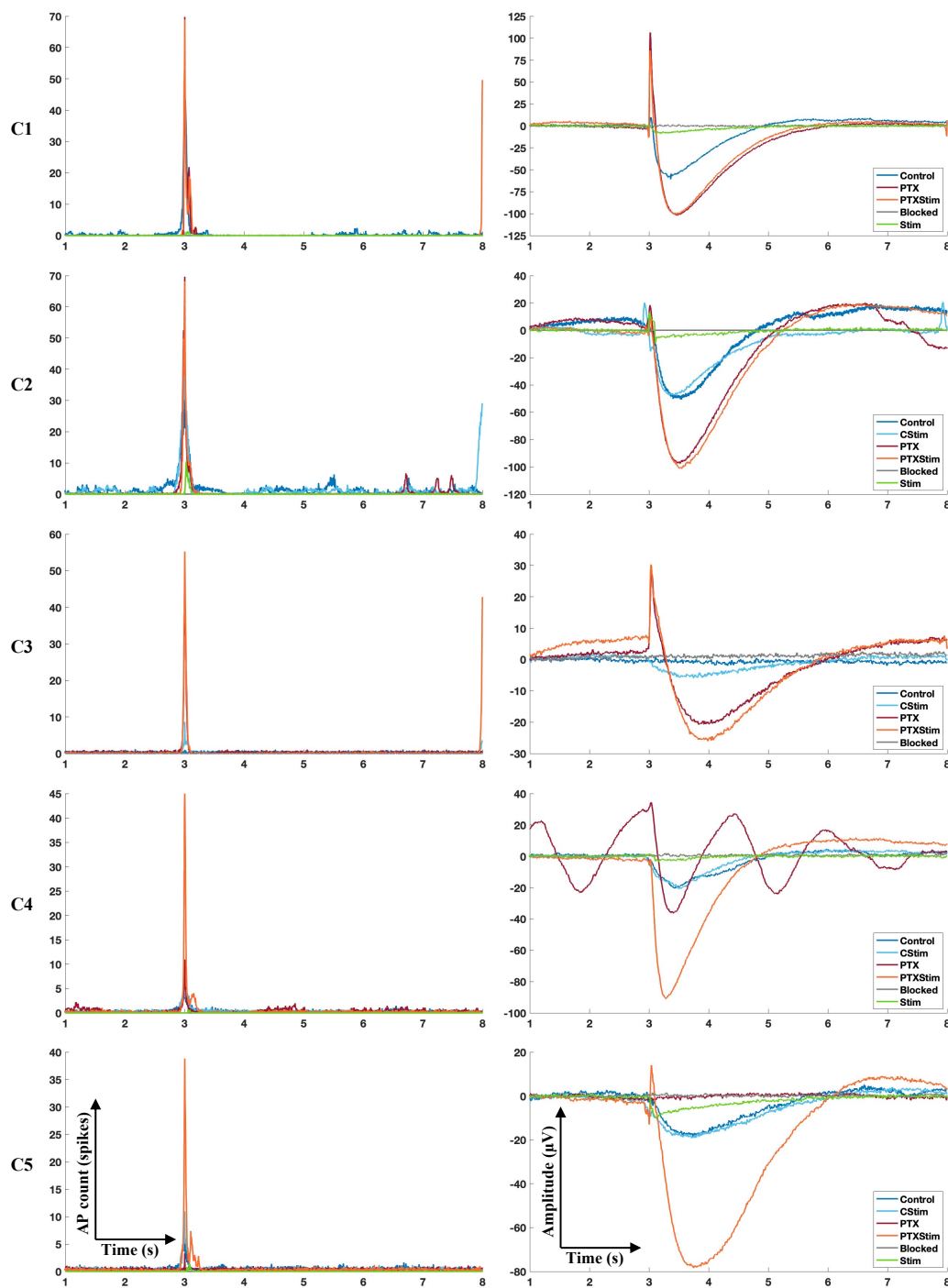


Figure 5.14: Observed signals for all cultures in the different conditions: Plots of the AP count (networkwide) per culture per condition (top); Representative waveshape per culture per condition of the low-frequency component recorded (bottom).

### 5.6.1 Culture by Culture Overview of the Signals

The same tested conditions in different cultures produced slightly different signals that translated different behaviors in both frequency components (Fig. 5.14). Regarding the low-frequency component, the major observations per culture were as follows:

1. Culture C1: commonly observed shape with a short positive peak before a very noticeable negative deflection in Control; PTX with and without stimulation produced similar signals, in which the amplitudes of both the positive and negative peaks increased; blocking all activity suppressed the signals, and optogenetic stimulation promoted their reappearance.
2. Culture C2: Control with and without stimulation gave rise to a comparable negative deflection being the only difference between these conditions slightly larger positive peaks when stimulated; PTX and PTXStim were very similar in shape and amplitude values; we detected no deflections in Blocked condition; Upon stimulation, a signal could be observed.
3. Culture C3: in Control condition, no deflections were clearly visible unless the culture was hit with the blue LED; the addition of PTX with and without stimulation produced a wave-shape similar to the ones observed in the previous cultures; we did not identify significant variations in potential when activity was blocked; no stimulation results are reported.
4. Culture C4: we recorded a consistent waveshape in Control and CStim, with a prominent negative deflection; in PTX without stimulation, there was overlap and summation of signals resulting in an oscillatory-looking waveshape which was "corrected" when we stimulated the culture; once more, we did not detect deflections in Blocked condition, but did capture small ones upon stimulation.
5. Culture C5: we observed the standard waveshape in Control with and without stimulation; the addition of PTX did not produce the expected response in the culture, with no detectable signals being picked up in the low-frequency component without stimulation; PTXStim is characterized by a very prepotent negative deflection; signals were, again, suppressed upon the addition of CNQX and APV; small consistent deflections were verified upon stimulation.

Table 5.2: Network burstiness registered in the different recording conditions

|         | C1      | C2      | C3     | C4      | C5      |
|---------|---------|---------|--------|---------|---------|
| Control | 51 ± 19 | 36 ± 17 | 0 ± 1  | 7 ± 2   | 9 ± 7   |
| CStim   | —       | 50 ± 12 | 9 ± 4  | 10 ± 3  | 11 ± 3  |
| PTX     | 68 ± 5  | 71 ± 4  | 48 ± 8 | 11 ± 3  | 3 ± 2   |
| PTXStim | 68 ± 12 | 68 ± 7  | 55 ± 3 | 44 ± 10 | 39 ± 15 |
| Blocked | 0 ± 0   | 0 ± 0   | 0 ± 0  | 0 ± 0   | 0 ± 0   |
| Stim    | 0 ± 0   | 0 ± 0   | —      | 0 ± 0   | 0 ± 0   |

Values highlighted in red are cases in which the behaviour of the culture differed from the majority; Traced cells indicate that no recording for that condition is available.

As to differences in spiking activity (Table 5.2), concisely, it is possible to see that, in cultures C1 and C2, there was considerably high activity in control conditions, which increased even more upon the addition of PTX, and was completely suppressed with CNQX and APV. In culture C3, Control condition showed asynchronous activity, with very few or no APs being coincident, which improved with stimulation and the addition of PTX. When all activity was blocked we did not record measurable spiking activity in this culture. In cultures C4 and C5, in Control condition, with and without stimulation, there were some network bursts (though they were not strong). The addition of PTX had almost no effect in culture C4 and even reduced activity in C5, but, once stimulated, bursts started to reappear. Finally, these last two cultures were also effectively blocked by the AMPA and NMDA antagonists.

### 5.6.2 Differences Between Electrodes

Before discussing the exact fluctuations verified in the high and low-frequency components in each recording condition, it is vital to see if the waveshapes observed across the different electrodes are consistent. Moreover, checking if any electrodes picked up on distinct signals yet accordant may be of interest for analysis. In order to do this, the two principal readouts used were the amplitude of the positive and negative peaks (Fig. 5.15).

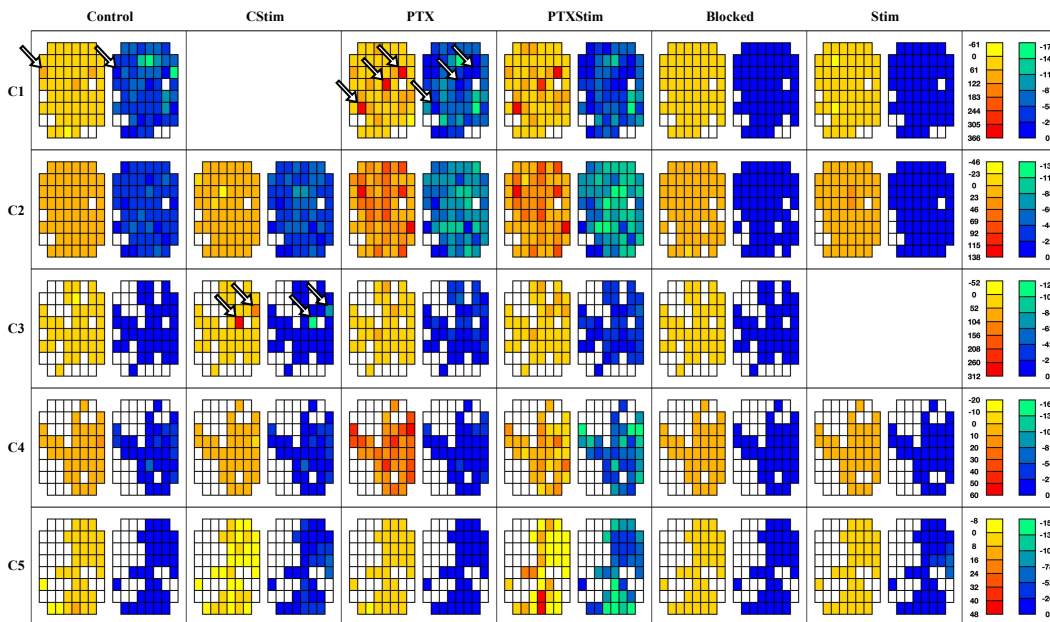


Figure 5.15: Distribution of peak amplitudes across cultures and conditions: for each culture and for each condition two coloured grids are presented, the left one representing the amplitudes of the positive peaks and the right one the amplitude of the negative (scales in  $\mu\text{V}$  are presented per culture on the right). White squares represent excluded electrodes. Arrows represent electrodes with different, yet consistent waveshapes, which will be analyzed separately

Firstly, as was previously mentioned, it is possible to see that the PTX condition, especially when stimulated, yields the highest measured values per culture, both for positive and negative peaks. Since we block inhibition in this condition, this may be of interest.

Secondly, in Blocked and Stimulation conditions, almost every electrode picks up signals with both positive and negative peaks very close to zero. Such results point towards an effective blockade of all neuronal activity and indicate that the deflections produced by optogenetic stimulation, although existent, are not very noticeable compared to the LFPs measured in other conditions (such results will be exposed ahead, in Section 5.6.3).

Thirdly, some relation between positive and negative peak amplitude seems to exist, with electrodes picking up the highest values for the positive peak and also showing the lowest ones for the negative deflection. This will be further explored ahead.

One last observation to be made is that some electrodes did pick up different yet consistent waveshapes, marked with arrows in Figure 5.15, which will be analyzed in Section 5.6.5.

### 5.6.3 Different Signals Reflect Distinct Conditions

The next step was to compare all cultures and try to find common patterns that could describe how the signals vary with the tested conditions. For this, it is important to look at Figures 5.14, 5.15, and 5.16 as well as to Table 5.2. Moreover, it is important to note that because these signals have high variability it is difficult to set the biological relevance of some phenomena.

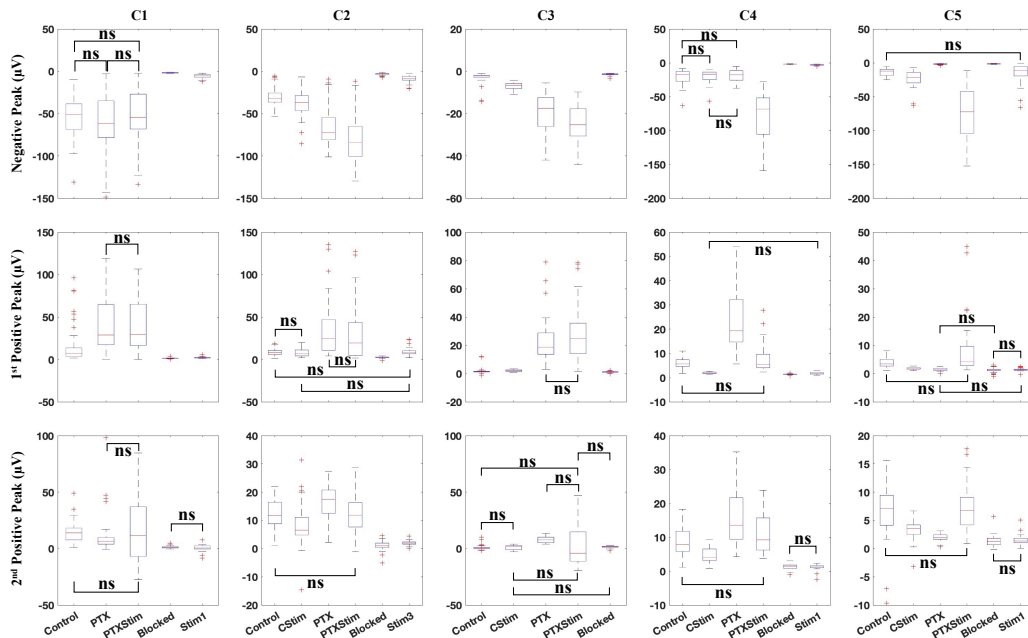


Figure 5.16: Quantitative analysis on the changes in measured signals: variation of the amplitudes of the negative peak (top) and the positive peaks that come before (middle) and after (bottom) with non significant changes indicated with ns.

In culture C1, during Control, it is possible to see a signal which suffers a negative deflection and is then exponentially restored to its baseline value. Adding PTX, a very prominent positive peak before the negative deflection starts to be noticeable (accompanying a significant increase in burst intensity), and the amplitude of the negative deflection also increases, even though not significantly. Stimulation of the culture in this condition had no significant effects on the signal, with amplitudes of both the first positive and the negative peaks remaining statistically similar. Translated to values, the negative peak amplitude was  $-56.91 \pm 31.33\mu\text{V}$ ,  $-62.60 \pm 35.32\mu\text{V}$ , and  $-54.24 \pm 33.48\mu\text{V}$  for Control, PTX, and PTXStim, respectively. As to the positive peak amplitude, it changed from  $15.55 \pm 21.77\mu\text{V}$  in Control to  $54.39 \pm 76.95\mu\text{V}$  in PTX and remained at  $51.69 \pm 70.31$  in PTXStim. Adding excitatory blockers causes the disappearance of the peaks ( $-1.70 \pm 0.43\mu\text{V}$  for the negative and  $1.36 \pm 0.56\mu\text{V}$  for the positive, which are on the same level as random amplitudes intrinsic to the signal). Even though optogenetic stimulation generates some new deflections without significantly changing spiking activity, these are much smaller than the ones observed previously, being the increase verified in the positive peak (goes up to  $2.02 \pm 0.73\mu\text{V}$ ) smaller than the one in the negative values (decrease to  $-5.90 \pm 2.11\mu\text{V}$ ).

In culture C2, the shape observed in Control condition was in accordance with the one from the previous culture with a slight difference in amplitudes (peaks in the values of  $-30.72 \pm 10.82\mu\text{V}$  and  $8.63 \pm 4.04\mu\text{V}$ ). Stimulation of the culture in Control condition did not seem to highly affect the shape of the signal, with peak values shifting to  $-37.45 \pm 15.05\mu\text{V}$  and  $8.23 \pm 5.10\mu\text{V}$ , respectively. Such difference was significant for the negative peak amplitude, but not for the positive one. Once again, the addition of PTX led to an increase in peak amplitudes ( $-67.44 \pm 22.32\mu\text{V}$  and  $34.50 \pm 30.52\mu\text{V}$ ), as well as in burst intensity. Upon stimulation, the positive peaks remained consistent ( $29.06 \pm 30.50\mu\text{V}$ ), but there was a significant increase in negative peak amplitudes ( $-79.74 \pm 26.27\mu\text{V}$ ). Similar to the previous culture, blocking all activity suppressed the measured potentials ( $-3.19 \pm 1.13\mu\text{V}$  and  $2.44 \pm 0.98\mu\text{V}$  peaks) and network bursts. Optogenetic stimulation gave rise to significantly different signals with amplitudes of  $-8.72 \pm 4.02\mu\text{V}$  and  $8.94 \pm 4.18\mu\text{V}$  with no significant changes in spiking activity.

Control condition in culture C3 showed a different behavior from the already analyzed ones. Despite there being a lot of spiking activity, its synchronicity was very poor, and we detected no intense network bursts. Instead, neurons were firing independently, preventing the generation of a measurable LFP signal (negative peaks of  $-3.07 \pm 2.83\mu\text{V}$  and positive ones of  $1.87 \pm 2.49\mu\text{V}$ ). Stimulation of the culture with blue light pulses promoted synchronous firing of neurons, and this caused the low-frequency component to start showing some significantly different deflections of  $-11.84 \pm 21.86\mu\text{V}$  and  $14.36 \pm 55.57\mu\text{V}$ . Adding PTX promoted the appearance of intense bursts and a prominent positive peak ( $23.15 \pm 16.29\mu\text{V}$  without stimulation and  $28.39 \pm 20.30\mu\text{V}$ , no significant difference) before a noticeable negative one ( $-18.97 \pm 8.93\mu\text{V}$  without stimulation, and  $-24.83 \pm 8.64\mu\text{V}$ , marking significant increase when stimulated). We also verified ascending values of burst intensity for these four conditions, all significantly different from each other. The addition of APV and NCQX successfully suppressed activity, with no network bursts detected and peaks decreasing to values of  $-1.40 \pm 0.46\mu\text{V}$  and  $1.10 \pm 0.43\mu\text{V}$ .

In culture C4, there were fluctuations similar to the ones observed in cultures C1 and C2 in control conditions, although these were smaller ( $-21.16 \pm 12.18\mu\text{V}$  and  $6.17 \pm 2.43\mu\text{V}$  peaks), following network bursts which were also less intense. Stimulation of the culture provoked no significant differences in the negative peak ( $-20.51 \pm 10.13\mu\text{V}$ ) but significantly reduced the positive one ( $1.86 \pm 0.40\mu\text{V}$ ). When treated with picrotoxin, the culture started bursting repeatedly but not intensely. Such behavior generated very unusual waveshapes in all electrodes with very different changes in peak amplitudes from the ones observed in the previous cultures. Stimulating the culture maintained a constant frequency in bursting, shaping the low-frequency signals to a more consistent shape accordingly to the ones observed in other cultures (peaks of  $-78.17 \pm 34.04\mu\text{V}$  and  $7.90 \pm 5.96\mu\text{V}$ ). Blockade of all activity promoted the expected result of attenuating the deflections ( $-1.84 \pm 0.40\mu\text{V}$  and  $1.42 \pm 0.26\mu\text{V}$  peaks). Without spiking activity, stimulation of the culture using blue light pulses of 0.1s (the same as in all stimulation applied in other conditions) generated a small yet detectable signal in the low-frequency component with a positive peak of around  $1.77 \pm 0.51\mu\text{V}$  followed by a negative potential deflection of  $-3.06 \pm 0.65\mu\text{V}$ .

Finally, in culture C5, Control condition with and without stimulation generated significantly different signals (despite no significant changes in burst intensity) in which the waveshape was consistent with the ones previously observed (peaks of  $-13.95 \pm 5.73\mu\text{V}$  and  $3.85 \pm 1.89\mu\text{V}$  for Control without stimulation and peaks of  $-23.69 \pm 12.91\mu\text{V}$  and  $1.81 \pm 0.42\mu\text{V}$  when stimulated). The addition of PTX had the opposite effect of what would be expected, suppressing the signals in the low-frequency component ( $-1.89 \pm 0.61\mu\text{V}$  and  $1.49 \pm 0.53\mu\text{V}$  amplitudes). Stimulation of this culture generated measurable network bursts and signals with positive peaks of  $8.44 \pm 10.64\mu\text{V}$  and negative ones of  $-75.84 \pm 37.47\mu\text{V}$ . APV and NCQX silenced the network bursts and weakened the deflections (amplitudes dropped to  $-1.47 \pm 0.40\mu\text{V}$  and  $1.20 \pm 0.81\mu\text{V}$ ). As seen in previous cultures, despite no significant change in the network activity, signals with peak amplitudes of  $-16.31 \pm 14.92\mu\text{V}$  and  $1.41 \pm 0.56\mu\text{V}$  were observed upon optogenetic stimulation.

Although in Figure 5.16 the value for two positive peaks are presented, the analysis focused mainly on the positive peak that preceded the negative one. This is due to the fact that the second positive peak is largely affected by the filtering applied (if not completely created by it), a phenomenon which is further explored in Section 5.7.2. Nevertheless, we present such values as they also gave some insight into an interesting phenomenon exposed in Section 5.6.5.

Scanning all the observations made above, it is possible to highlight some patterns that are consistent across cultures under the same conditions:

1. In the majority of the cases, the signals from Control and CStim conditions were very similar with average correlation values in the range of [0.73 : 0.93] in cultures C2, C4, and C5 where shapes were consistent and 0.13 in culture C3 in which control condition did not show measurable LFPs;
2. From Control to PTX, there is an increase in bursting intensity, along with bigger amplitudes for both positive and negative peaks of the low-frequency signals, except for cultures C4 and C5 in which the picrotoxin did not have the expected effect;

3. PTX and PTXStim conditions were also very similar to each other with correlation values of [0.72 : 0.95] for cultures C1 to C3 and much lower values ([0.05 : 0.20]) for cultures C4 and C5 in which the blocker was not so effective;
4. In Blocked condition, with all inhibitory and excitatory activity blocked, we did not detect bursts, and the waveshapes observed were successfully suppressed in all tested cultures with no measurable peaks surpassing the [-4 : 4] $\mu$ V range;
5. Stimulation of the Blocked cultures did not induce significant changes in the spiking activity but allowed for the generation of weak yet consistent waveshapes in the low-frequency component, with the negative peak being more noticeable than the positive one (except in culture C2 in which peaks seem to be symmetric, something to be explored in Section 5.7.1);
6. In all cultures and all conditions, there were some fluctuations in the values of the peaks (Fig. 5.15), but even with those the shape tended to be consistent;

#### 5.6.4 Evaluation of the Restoration Time Constants

The last readout for the different evaluated conditions was the restoration time constant. Considering per culture only the conditions that produced measurable LFP signals, the average value for the restoration time constant was  $1.33 \pm 0.32$ s for culture C1,  $1.17 \pm 0.21$ s for culture C2,  $1.37 \pm 0.30$ s for culture C3,  $1.22 \pm 0.26$ s for culture C4, and  $1.35 \pm 0.29$ s for culture C5. These values are comparable with those obtained in the optogenetic stimulation experiments for cultures C1, C4, and C5 ( $1.25 \pm 0.33$ s,  $1.05 \pm 0.29$ s, and  $0.98 \pm 0.33$ s, respectively). Nevertheless, such differences in time constant values were significant in all three considered cultures.

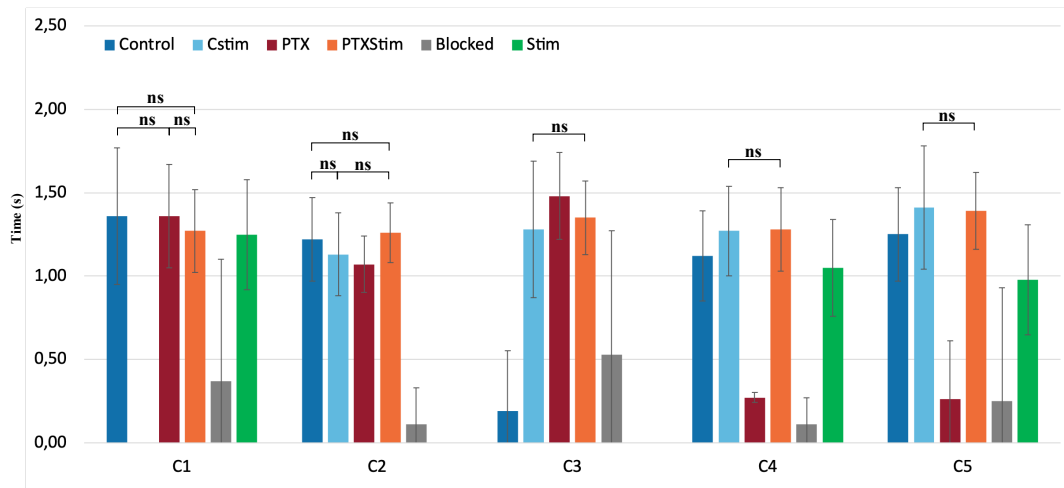


Figure 5.17: Comparison of restoration time constants: values for different conditions applied to each culture, with non significant changes indicated with ns. Green bars correspond to the average between deactivation time constant values for all stimulation settings applied to each culture included in the optogenetic experiment.

### 5.6.5 Different Waveshapes of Interest

As shown in Figure 5.15, there were some electrodes in which we could observe some distinct interesting waveshapes. The first case to be analyzed is a set of four electrodes in culture C1 that picked up a predominantly positive waveshape (referred to as distinct shape), as opposed to the rest of the electrodes in the MEA, as can be seen in Figure 5.18.

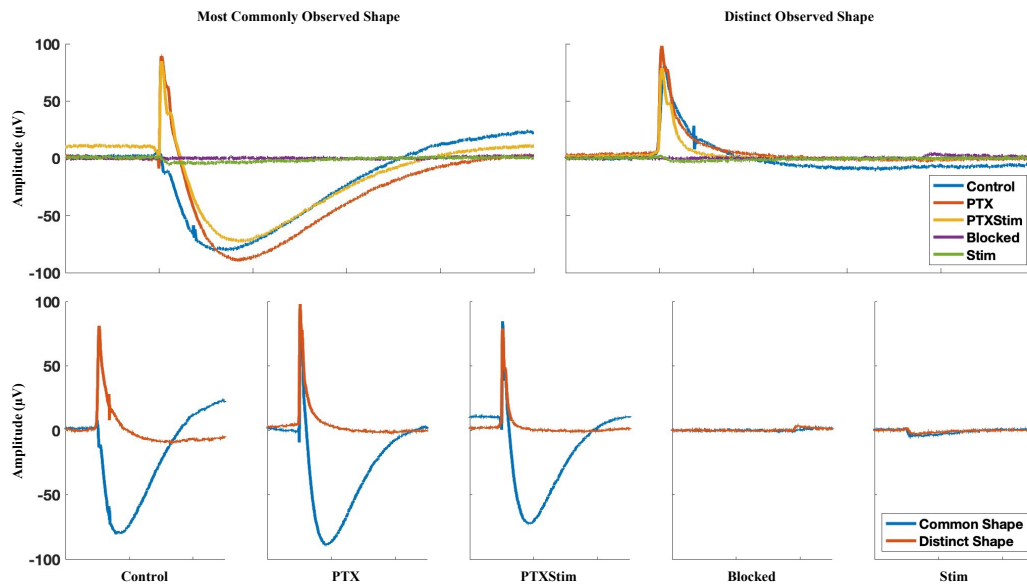


Figure 5.18: Distinct yet consistent waveshapes observed in C1: 5s traces for one selected electrode representative of each distinct waveshape observed with the set of different recording conditions (top) with a 5s plot of both signals for each condition (bottom).

The two signals observed are similar in their "positive content" but very distinct in the negative y-axis. First, both have the same onset timepoint, meaning that the observed perturbation initiates simultaneously in both electrodes. In Control, the blue shape does not have any noticeable positive peak, being completely different from the red one. In PTX and PTXStim conditions, however, the positive peaks of both red and blue shapes coincide in time. While the blue electrode suffers a negative deflection after reaching the maximum, the red one drops progressively to zero. Blocking all neuronal activity suppressed the signals in both electrodes. Interestingly, both electrodes pick up a similar negative waveshape when optogenetically stimulated.

Figure 5.19 shows one other case of a strong deviation from the most common waveshapes observed in culture C3. Here, a set of two electrodes picked up a particular waveshape in CStim. Still, in the remaining conditions, they recorded signals consistent with the rest of the electrodes. Even though this phenomenon was not as consistent as the previous one, the enormous discrepancy in amplitude values verified in CStim (amplitudes increase from tens to thousands of micro-volts) made it compelling to evaluate.

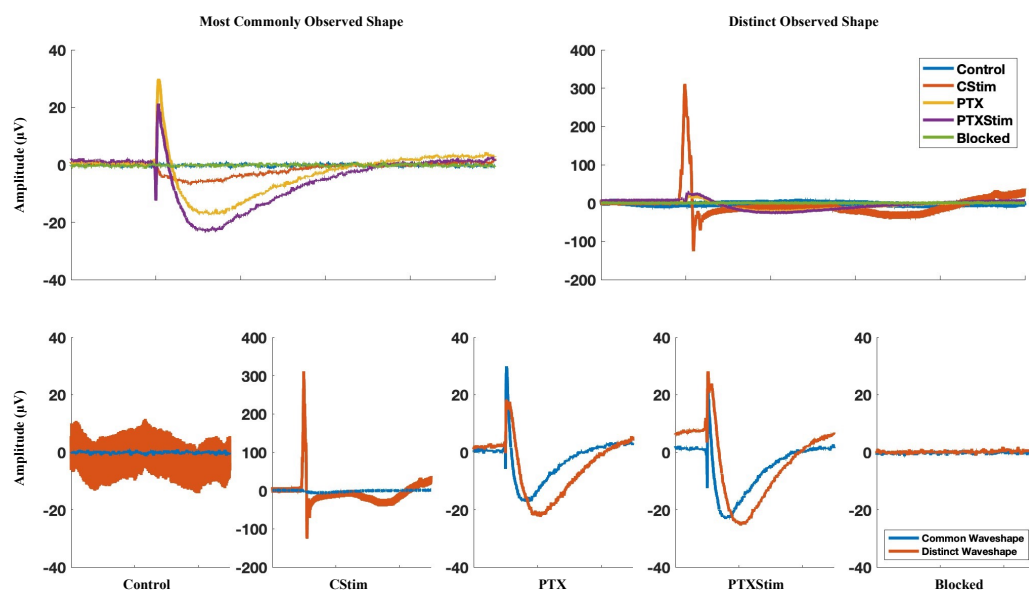


Figure 5.19: Distinct waveshapes observed in C3: 5s traces for one selected electrode representative of each distinct waveshape observed with the set of different recording conditions (top) with a 5s plot of both signals for each condition (bottom).

Finally, in cases like the example presented in Figure 5.20, a steep negative spike, randomly spread through the MEA, was visible prior to the commonly observed shape. Nonetheless, after the addition of PTX, the signals did not show this sharp negative peak but presented an oscillation between the positive and negative ones. We observed these waveshapes across cultures (with a few electrodes in each culture picking up such signals). In total, 14 of the 217 included electrodes (6%) registered these sharp spike-like events prior to any of the commonly seen deflections in the LFP signal.

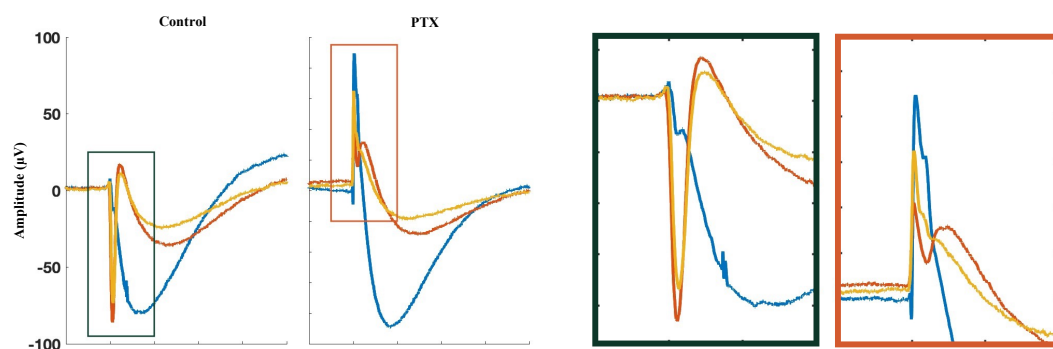


Figure 5.20: Common perturbation in signals observed across cultures with 5s plots of signals captured by three different electrodes for each condition

## 5.7 Looking for Answers in Signal Simulations

The last part of the project made use of simulations in Matlab to mimic the observed signals and understand what could be the exact effect of the applied filters and the explanation behind some of the observed waveshapes. Moreover, we also present a simulation on the impact of spiking activity leftovers.

### 5.7.1 The putative Shape of Measured Artifacts

In order to evaluate whether the shapes observed upon optogenetic stimulation of silenced cultures could be mere artifacts, we conducted three experiments that led to the observation of the wave-shapes visible in Section 5.4.9, Figure 5.12. Therefore, it is possible to compare such signals with the one obtained by stimulating a square-shaped wave, shown in Figure 5.21. Even though it is not shown here, by tuning the cut-off frequencies, shapes varied in settling times, becoming more similar to the covered MEA signal if we used lower cut-off frequency values for the high-pass filter. This may be useful when considering the possible intrinsic filtering effect of the electrodes (for further explanation, see Section 2.3.3).

The results of this simulation identified a very specific waveshape that gives rise to consistent fluctuations in the signals from stimulated cultures. This seems to have a non-physiologically relevant origin, a fact that opens doors for future work in which one fully removes the influence of such artifacts from the signal. This will be further explored in Sections 6.1.2 and 7.2.

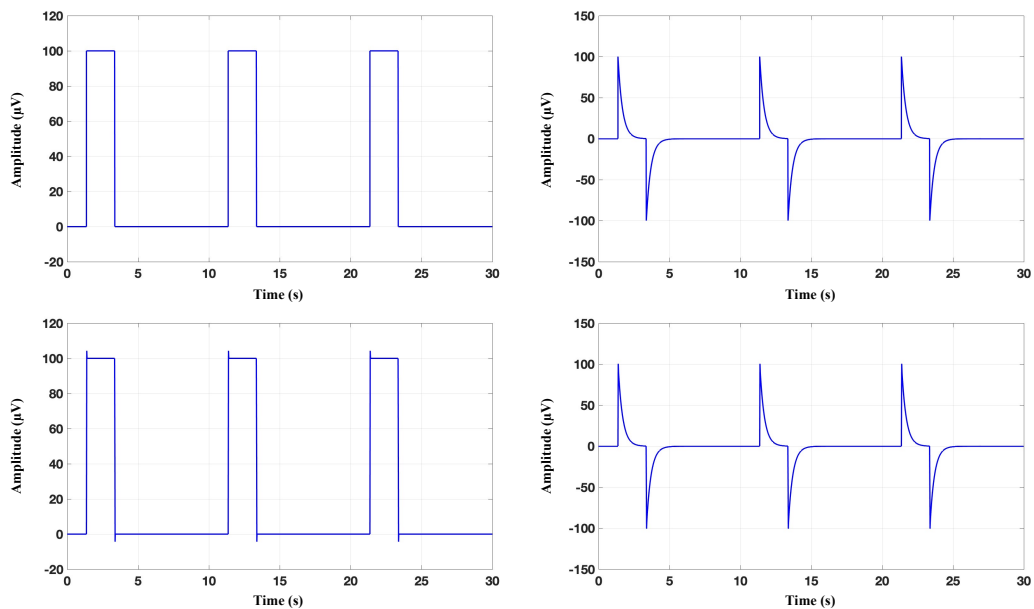


Figure 5.21: Simulation of an electrical artifact resulting from stimulation: raw simulated signal (top left), high pass filtered signal (top right), low pass filtered signal (bottom left), and band pass filtered signal (bottom right)

When stimulating cell cultures, in some cases, it is still possible to observe some effect of these artifacts at the beginning and end of the stimulation period (Fig. 5.22). As so, these peaks should not be considered for biophysical interpretations as they most probably do not derive from any cellular process but rather from the use of the LED to activate the ChR-2 channels. This could be a pertinent aspect to consider in future works, as is mentioned in Section 7.2.

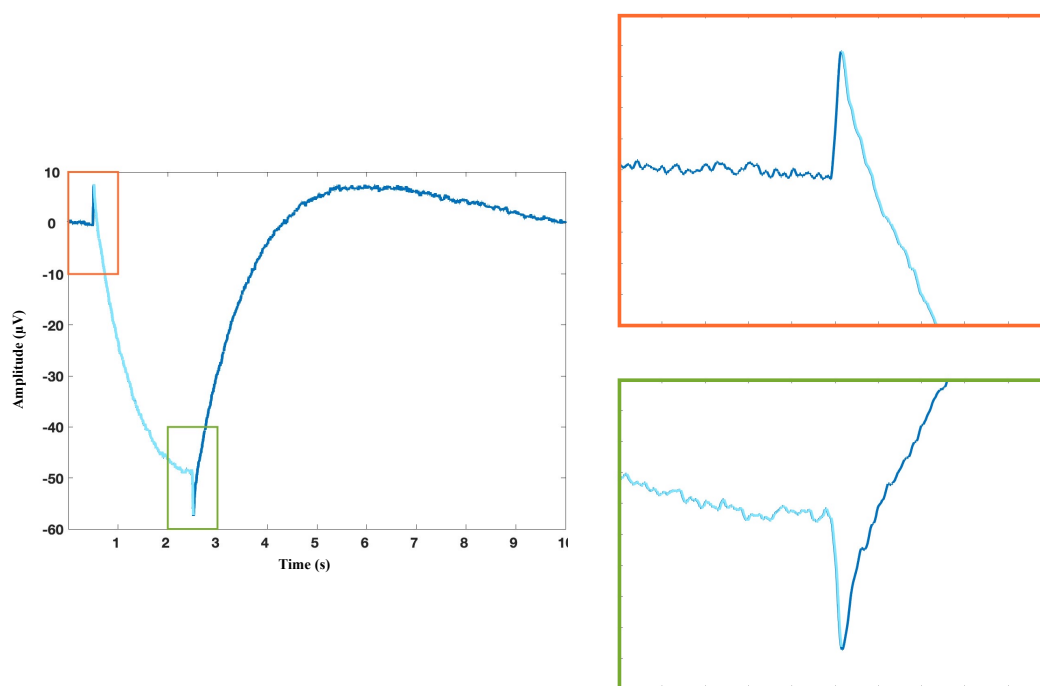


Figure 5.22: Measured artifacts in the LFP waveshape: average waveshape obtained by optogenetic stimulation of one of the cultures (left) with close ups on two segments of the signal in which it is possible to see electrical artifacts from the switching on (orange) and off (green) of the LED.

### 5.7.2 Overshoot and Width: Consequences of Filtering?

We aimed to understand the major effects of the applied filtering settings on a waveshape that could resemble the deflections visible throughout the project. As so, we created a simulated signal with repeated negative deflections over time and a basal sinusoidal fluctuation to mimic results found *in vitro*. After being band passed with the same cut-off frequencies used in the recordings, the signal suffered some alterations. Even though the negative deflection maintained its overall shape, its amplitude was considerably smaller (around  $-60\mu\text{V}$  compared to the  $-100\mu\text{V}$  in the simulated raw signal), and the deflection's width also slightly increased (visually evaluated). A small, yet detectable, overshoot after the negative peak was visible as well. The sinusoidal background fluctuation was soothed but not fully eliminated.

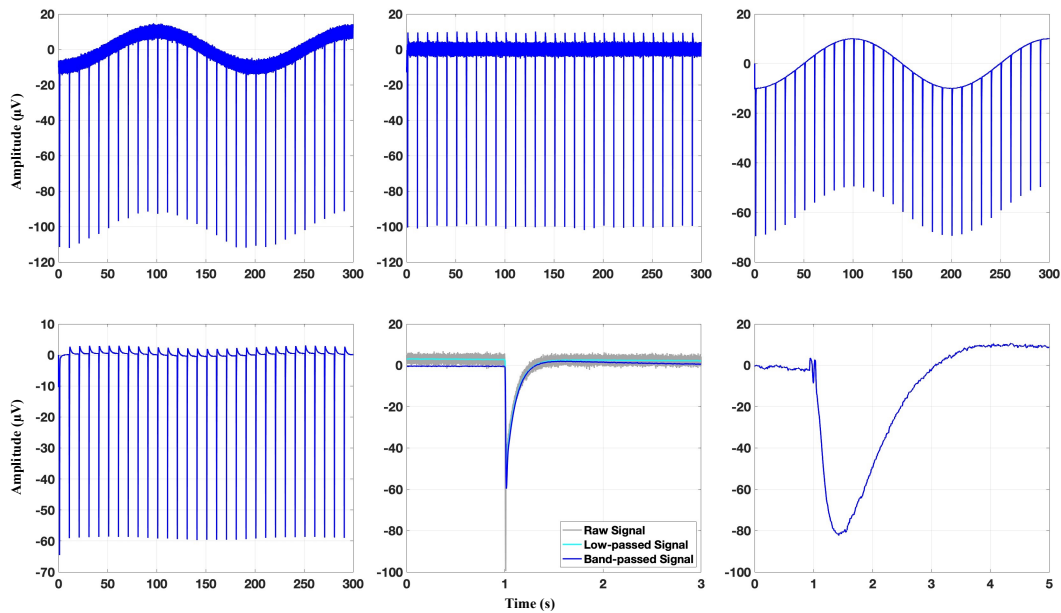


Figure 5.23: Simulation of the low-frequency component: raw simulated signal (top left), high pass filtered signal (top middle), low pass filtered signal (top right), band pass filtered signal (bottom left), 3s window close up of the band pass filtered signal (bottom middle), and 5s window close up of the measured signal in one of the cultures for comparison (bottom right).

It is relevant to refer that the present simulation does still not mimic all the dynamic components of the LFP signals and that, even though it already allows for paramount observations, it is vital to keep improving it in order to withdraw proper conclusions about the consequences of filtering. The differences between the signals are visible when comparing the last two plots of the bottom row in Figure 5.23. Hence, this part of the project does not allow for final statements but rather for a hypothesis that may be validated one day with extra work.

### 5.7.3 Spiking Activity Leftovers

In order to study whether spiking activity in the presynaptic neuron has any effect on the observed shape in low-frequency signals, a simulation was run on Matlab with 100 spikes. Figure 5.24 represents this simulation, in which each spike generates a small positive deflection (this assumption is further discussed in Section 6.4). As so, forcing the spikes to occur all at the same time generated a very noticeable spike. However, if half were at the same time and the other half at random points, a less prominent spike was produced. Lastly, completely random time points were masked in the white noise, generating oscillations in the measured signal.

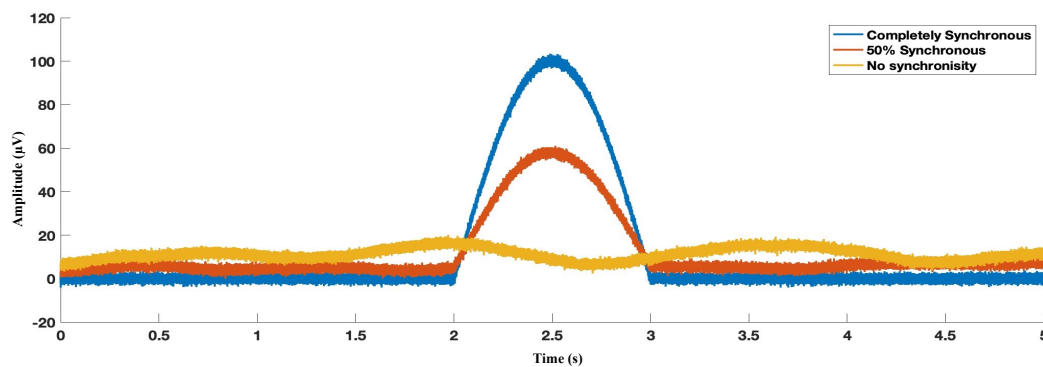


Figure 5.24: Simulation on the hypothesis about the burst leftover summation: summation of 100 signals assuming full, 50% or no specific synchronicity of such signals.

## 5.8 Summary of Results

The project aimed to answer a set of research questions presented in Section 1.2. As so, let's now sum up the most important results, relating them to the question they may help give an answer to:

### **Is there a physiological relevance in low-frequency signals?**

First of all, the results show that even when no bursts (or APs at all) are detected, it is possible to generate deflections in the low frequencies. Secondly, the shapes of signals observed in extra MEAs with no cultures or with the covered stimulus were quite distinct and resembled a band-passed square waveform.

### **What information do the Local Field Potentials contain?**

Optogenetic stimulation in silenced cultures generated a signal with a negative deflection whose amplitude and latency increased with the duration of the received stimulus. Blocking only inhibition or both inhibition and excitation led to the measurement of differently shaped signals, and the differences between conditions were consistent across cultures. Filtering settings may be responsible for altering the observed shape, namely making the negative deflection wider and adding the overshoot effect.

### **Is there a direct relationship between LFPs and APs?**

When the effect of blockers started to fade out in culture C3, it was possible to observe that changes in the intensity of network bursts were shaping the low-frequency signal measured. We found a significant correlation between the AP count and the amplitude of both the positive and negative peaks of the LFP, which is in line with previous work.

## Chapter 6

# Discussion

The results presented in the previous Chapter will now be discussed in this present Chapter. On the one hand, this discussion aims to hypothesize on the reasons and mechanisms behind the observer results and, on the other hand, the comparison between the developed work and the conclusions from previously conducted research (mainly the ones approached in Section 3.2) is of interest. In the end, the take-home messages are briefly summarized as well as some difficulties felt during the development of the project.

### 6.1 General Observations

#### 6.1.1 Frequency Components Contain Different Information

One of the revolving questions around LFPs is still how to interpret them and exactly what new information (if any) they contain. Although some researchers have already defended that high and low frequencies encode different aspects of neural activity [13, 34, 92, 94], there is still some lack of evidence that the two components contain different information.

The results obtained with the optogenetic stimulation suggest that distinct neuronal processes generate the two frequency components. Even though spike activity was completely suppressed, signals in the low-frequency component were measurable. The hypothesized mechanism behind the LFP waveshape is further explored in Section 6.4, but it seems plausible that while spiking activity does contribute to the generation of the signal, as stated by other authors [33, 34, 79], it is not the only contributor. In fact, as proposed by Einevoll *et al.*, synaptic currents seem to be overriding contributors to the signal [34]. Nonetheless, the way in which all these different inputs are integrated into the final LFP signal remains a question to be further discussed in this report.

All in all results strongly point towards the possibility of extracting novel and relevant information from LFP signals. For this reason, a deeper look into the similarities, differences, and interdependence of APs and LFPs may lead to new insights into neuronal excitability.

### 6.1.2 LFPs Are Distinct From Measured Artifacts

One other possibility to consider would be that the signals measured in the low frequencies are artifacts of the recording systems, having no biological or physiological information behind them. Even more, when adding optogenetic stimulation, it becomes possible that the observed wave-shapes correspond to electrical artifacts caused by switching on and off the LED. We performed three additional experiments to test this. The obtained waveshapes are different from the remaining recordings (Fig. 5.12). In fact, they derive from a square pulse waveshape which is most likely to be an artifact related to the recording protocol or system (or even both).

Interestingly, the amplitude of such artifacts was much larger in the empty and PBS MEAs than in the covered ones. Since TiN electrodes are not light-sensitive, the fact that the light is covered must not have a direct impact on the signal. Instead, a filtering effect of the culture medium and the cells, softening the amplitude of this artifact, is the most probable cause for the differences.

### 6.1.3 Selected Low Cut-off Frequency

Although it has been stated in the literature that the low-frequency component can correspond to potentials up to  $\approx 500\text{Hz}$  [34], in more recent studies, most protocols opt to filter the low-frequency components with low pass filters with 300Hz cut-off frequencies [92, 94]. However, following the filtering protocol established in a previous study [33], signals were filtered with a 35Hz low pass filter, since this is often the maximum frequency in EEG recordings. Several reasons justify the chosen value: 1) Since the signals are captured from monolayer cultures, their nature is not as complex as the ones captures with slices or even *in vivo*, thus not having so much information spread across different frequency bands; 2) after testing higher cut off frequencies for the same signals, results remained the same, indicating that no information was being lost with the lower cut off value; 3) narrowing the range of frequencies to which the filter is permissive generates cleaner signals, making it simpler to evaluate common patterns and withdraw conclusions.

### 6.1.4 Previous Work in the Field

Although LFPs are widely studied in the present, and their biophysical principle and validity are already commonly accepted by most researchers, there are few works in the field that set out to establish the relationship between the observed signals and ongoing excitatory and inhibitory activity [33, 92, 94]. In addition, the high variability of the low-frequency component (depending on intrinsic properties of the cultures), paired with the significant influence of the recording setup and protocol (which differ from study to study) makes comparisons between results from different teams quite challenging.

Regarding the optogenetic stimulation experiments, to the best of our knowledge, no similar works seem to have been conducted. This makes it necessary to clearly describe the observed patterns and look for an explanation that may one day be validated with similar experiments being conducted by other teams.

## 6.2 Optogenetic Stimulation

### 6.2.1 A Biophysical Origin to the Negative Deflections

Upon optogenetic stimulation of the cultures, signals in the low-frequency component started to show a small yet noticeable negative deflection. Given the slow kinetics of the measured signal, which may also influence the applied filters as is discussed in Section 6.3, the most plausible phenomenon to generate them are currents resulting from the movement of ions through the ChR-2 membrane channels.

Extracellular potentials like the ones observed in these experiments can be interpreted as being of either ionic or capacitive origin, as has already been stated in other works [44]. Given the fact that the used channel ChannelRhodopsin-2 had a mutation that made it selective to excitatory neurons, the ionic origin seems to be the most probable one for the observed negative deflections. This means that when stimulated with blue light, the  $\text{Ca}^{2+}$  channels of the ChR-2 open and cations start flowing from the extracellular medium to the inside of the neurons, creating current sinks throughout the culture medium. Such ionic exchange gives rise to a drop in the measured potential that is picked up by electrodes. To re-establish the cell's homeostasis cations are released to the culture medium via different channels in different locations, generating sources and contributing to the restoration of the equilibrium potential.

This would indicate that using the MEAs with one electrode serving as a reference, the negative deflections would be associated with depolarization of the membrane (typically related to excitatory activity). Some attention will be focused on this point, as this seems to corroborate the conclusions of Bazelot *et al.* [92] and contradict the findings of Teleńczuk *et al.* [94].

The first important factor to consider is that cell preparations were different from study to study (hippocampal slices, *in vivo* recordings, and cortical monolayer cultures), and this has been proven to be a crucial determinant of the dynamics of the signals. Moreover, the recording setups and electrodes were also different, which possibly implies different references for measurements, affecting the observed shapes. In the work of Teleńczuk *et al.* the distinction between inhibitory and excitatory neurons was done based on the shape of action potentials, which is not a trivial (nor consensual) method. Finally, in both studies, one neuron was being stimulated while in this experiment, ChR-2 channels were the target of stimulation. All things considered, it is arduous to attribute the verified differences to a single factor.

### 6.2.2 Effect of the Stimulation Settings

Signals observed in the low-frequency component showed some consistent changes depending on applied stimulation settings. Regarding the negative peak, both amplitude and latency were some readouts of interest due to their almost linear dependency on the duration of the stimulus. In fact, contrary to what would have been expected, signals did not seem to show clear signs of reaching a saturation level, the most likely behavior if ChR-2 were the only channels opening upon being stimulated with the blue LED, given their fast opening and closing rates [100].

The much slower signals observed may be a result of the recruitment of new voltage-gated cation channels as a response to the influx of  $\text{Ca}^{2+}$  ions. This gradual recruitment and opening of channels would explain both the fact that the amplitude keeps dropping while the LED is on and the slow dynamics as synaptic currents that last over a second have been measured as a result of the gating mechanisms by NMDA receptors after blocking the uptake of GABA [27].

In general, the shape of the stimulation response was consistent across the various electrodes of the MEA, with very high correlation values and considerably small standard deviation values for readouts such as the activation and deactivation constants. Deviations from the average waveshape were not very strong and one electrode could be used to generalize conclusions.

The amplitude of the signal, however, did vary from electrode to electrode. Interestingly, for the different stimulation settings applied to the same culture, the mapping of measured amplitudes across the MEA was consistent, meaning that some electrodes kept measuring the signals with the highest amplitude in all recordings. This phenomenon could be related to the generation of current sources and sinks spread spatially across the MEA, causing signal amplitudes to be dependent on the distance to these points. The generation of sources and sinks due to ionic flows has already been defended by other works in the field [93].

Baseline values did not appear to have any meaningful relationship with the stimulation settings applied with the same quite wide range of baseline values being registered in all stimulation settings in each culture. These very low frequencies may then be related to other more complex processes and be of interest in future works [92], but this may only be correctly explored after the basis of the signal generator is well established. It is also a possibility that these oscillations in monolayer cultures are a mere consequence of the recording setup used. Whichever the right explanation behind them is, for the ongoing project the main focus is not on them, thus normalization of all signals by their baseline.

All things considered, the results from this first part of the developed project seem to indicate that a negative deflection sensed in the low-frequency component in the used recording setup encodes a flux of positively charged ions through into the neurons, possibly being related to depolarization of the membrane and, consequently, excitatory activity.

### 6.3 True Signals vs Consequences of Filtering

We will now address whether the most commonly observed waveshape (or, at least, part of it) is a consequence of the filtering applied to the raw signal, focusing on some distinct aspects.

#### **The low frequency signal encodes information besides bursting activity**

Optogenetically stimulated neurons with no bursting activity still showed some negative deflections in the low-frequency component. This suggests that the low-frequency signal is not fully dependent on the high-frequency content and can be detected even without any spiking activity. Thus, it is possible to state that the two signals may have some interdependence but generate from different phenomena.

**The negative deflection is not just a consequence of the first positive peak**

Using simulated signals not shown in the report, we verified that the applied high-pass filter settings could never generate a negative deflection more prominent than the positive peak that preceded it, a phenomenon seen in multiple of the analyzed recordings. Moreover, in some cases, signals with the same amplitude for the positive peak had significantly different values for the negative one. It is, for these reasons, plausible to assume that the negative deflection's origin stands in other processes rather than simply in the filtering of the signal.

**There is some influence of the filtering methodology on the observed shapes**

Be that as it may, it is crucial to consider that filtering of the signal did have some effects on the observed waveshape, which cannot be forgotten during the interpretation of the results. For instance, it is very likely that the second positive peak (often referred to as "overshoot") is a consequence of the filtering, as simulations presented in Section 5.7.2 helped verify. In fact, by considering the results presented in Section 5.6.3, more negative values for the negative peak tended to be accompanied by a bigger second positive peak. This hampers the interpretation of the signal after reaching the negative peak, as the high-pass filter seems to manipulate its characteristics (width and overshoot).

## 6.4 Interpretation of the Generation of LFP Signals

It is now time to focus on one of the most challenging tasks of the project: finding a physiologically correct interpretation of the signals observed in the different recording conditions tested. Let's hypothesize that we have three principal contributors to the signal: the leftovers of spiking activity, inhibitory synaptic currents (mostly fast from GABA<sub>A</sub> receptors), and excitatory synaptic currents (fast from AMPA receptors and slow from NMDA receptors). The optogenetic stimulation experiment's results suggest that excitatory synaptic currents translate into negative deflections. Oppositely, inhibitory ones must be positive deflections.

When the nerve pulse reaches the axonal terminations, it promotes the opening of voltage-gated calcium channels through which ions flow along their concentration gradient [115]. As so, upon the arrival of the electrical impulse to the terminals, the concentration of positively charged ions in the extracellular medium becomes higher than that of the intracellular medium. This discrepancy could be measured as a positive peak which is then restored with the influx of Ca<sup>2+</sup>. The leftovers of spiking activity are, therefore, hypothesized to be viewed as positive spikes with a duration similar to their originating network burst.

Having defined the key players, let's discuss their incorporation into the final signal in each condition. The first important aspect of the proposed hypothesis is that synchronicity of the network would be an essential condition for observing spiking activity leftovers. As seen via simulation (see Section 5.7.3), the same amount of spikes can give rise to different signals depending on their firing alignment (asynchronous firing of neurons may be responsible for the observed smooth fluctuations that affect signals' baseline).

In Control condition, despite the fact that there is a lot of spiking activity and even network bursts, the fact that there are still neurons firing during inter-burst spikes may indicate that even though there are enough synapses to generate measurable currents, their synchronicity is not strong enough to induce a measurable 1<sup>st</sup> positive peak. Since APs are events that can be as fast as 1ms, it can happen that in a 5ms bin, a substantial quantity of events happen but produce a small peak (Fig. 5.24). This first peak has sensibly the same duration as the network burst. After this, inhibitory and excitatory synaptic currents interact with each other and give rise to a predominantly negative deflection. This implies that inhibitory currents (mostly slow ones from NMDA receptors) are completely surpassed by excitatory ones. The fact that the negative peak clearly outlasts burst duration may have to do with the gradual recruitment of ion channels after the synapse, mixed with some effects of filtering.

All things considered, apart from culture C3 in which activity was not synchronous enough to generate any measurable LFPs, the proposed hypothesis for the observed waveshape is that mildly synchronized spike leftovers generate a small positive peak and excitatory activity masks inhibitory currents and generates a negative deflection.

In CStim, there is not a response consistent enough for drawing conclusions. In general, waveshapes remain similar, but across cultures significance of changes in negative and positive peaks' amplitude varied. It is important to refer that in culture C3, the usage of the LED helped promote a more synchronized firing to generate measurable synaptic currents but, as for spiking leftovers, the stimulation did not seem to have a strong effect. The proposed hypothesis for this condition is, as so, similar to the one for Control condition.

Adding PTX to the cultures blocks GABAergic transmission, leading to an increase in excitatory activity. This causes neurons to fire less independently and, consequently, more synchronously, which could be the reason behind the significant growth in the first positive peak amplitude verified in all cultures, except for C4 and C5 (to be discussed below). The negative deflection also shows an increase in amplitude which could come from the combination of two factors: due to the high pass filtering, a bigger positive peak will be compensated with a bigger negative deflection; with inhibition blocked, excitatory synaptic currents are not partially canceled and fully contribute for the deflection.

The proposed hypothesis suggests that, with blocked inhibition, the first positive peak becomes more perceptible due to higher synchronicity in spiking activity. Moreover, the negative peak increases as expected both because there are no more positive synaptic currents, and it could be that the membrane recruits more NMDA receptors as a result of blocking inhibitory activity in cortical cultures.

Similarly to what happened between Control and CStim, variations between the conditions PTX and PTXStim showed some changes from culture to culture, being significant in some cases but not in others. Hence, no prime changes were sensed in the dynamics of local field potentials and, as so the proposed hypothesis for the PTXStim signals is the same as for PTX (a positive peak directly related to network activity and a slow negative deflection majorly reflecting NMDA receptor kinetics).

In cultures C4 and C5, picrotoxin had an unexpected effect. Considering that these two cultures were grown for less time than the others, the culture's maturity may have played a central part in this. One step of maturation of neuronal cultures is the conversion of GABA<sub>A</sub> from excitatory to inhibitory. It could be possible that these cultures were not mature enough, and blocking these receptors had a mixed effect on culture C4 and an opposite effect on culture C5 than expected. Upon stimulation, since these cultures still had the AMPA and NMDA receptors unblocked, signals started to be measurable. Stimulation alone was not strong enough to fully synchronize spiking activity and generate the pronounced positive peaks (in CStim, stimulation of the cultures also did not consistently generate this peak).

Blocking all neuronal activity naturally promotes the full suppression of the signals. Considering the proposed hypothesis, this is the expected outcome, as there are no synapses to generate synaptic currents and no synchronous spiking activity to generate leftovers for the signal. Upon stimulation, the opening of ionic channels induces currents that give rise to the observed negative deflections. In some cases, during stimulation, a positive peak seemed to be present. However, it is more likely that those cases are actually electrical artifacts caused by the usage of the LED rather than generated by neuronal activity.

In culture C3, the effect of the blockers started to fade out, and the shape of the signals changed in response to alteration in network dynamics. Here, we hypothesize that only APV and CNQX were not actively blocking excitatory receptors anymore, but PTX remained effective in the culture. This phenomenon can have two possible explanations: a longer half-life time for the inhibitory neuronal blocker (which does not seem to be the most likely cause) or a possible deterioration of the excitatory blockers solutions over time (in the case of culture C3 they were not freshly prepared before the experiment, but had been prepared before for culture C2). Regardless of the justification, the spiking behavior was very similar to the one observed in PTX conditions. As expected, the shapes observed in the low-frequency component were also similar. This means that a positive peak from synchronous bursts started to appear, and the negative deflection got progressively bigger as burst intensity increased.

On the whole, the proposed hypothesis successfully explains all the waveshapes observed in the various recording conditions. Moreover, our findings corroborate the assumption that spiking activity does contribute to the low-frequency signal, in accordance with previous work [33, 79]. Besides this, it points towards a predominantly excitatory contribution for the LFP signal, another conclusion that contradicts what has been stated by other teams working on the field [92, 94]. In Section 6.2, we already debated the polarity of the signals. However, both Bazelot *et al.* and Teleńczuk *et al.* suggest that inhibitory activity contributes the most to LFP signals.

For this, apart from the already approached differences in recording setups, it is essential to note that, in both studies, they only stimulated only one neuron and measured the respective network response. That is the biggest and probably most important difference between the previous work and the one presented here, in which cultures were either recorded without stimulation or evenly stimulated. The unblocked activity of the entire network in which excitatory neurons are more abundant may have a pivotal role in shaping the signal.

### 6.4.1 *In vitro* model is merely a starting point

Previous research widely defends that LFPs are influenced to a great extent by the spatial characteristics of their location of propagation [13, 79, 93] as well as by the dynamics of the cell populations that generate them. Monolayer cultures in MEAs provide freedom of experimentation, being easy to manipulate and relatively rapid to obtain (in a three-week period a functional network is established and ready for experimentation).

As a matter of fact, even though the environment verified *in vivo* is much more complex than the one simulated *in vitro*, the latter still provides a crucial model for studying neuronal functioning. Hence, the proposed hypothesis may be valuable for works with monolayer models of neurological diseases but also as a starting point to increase complexity in neuronal structures and see how the signals react and change. Research regarding neurological disorders has benefited a lot from such models on MEAs, and present works still use them as a tool for mimicking the pathological states of neuronal microcircuits. Thus, it is likely that *in vitro* models will keep being the starting point of many discoveries.

Keeping the enormous relevance of *in vitro* models in mind, the results observed in this work may give some indispensable insight into how LFP signals reflect neuronal excitability in monolayer cortical cultures. We can hypothesize that a frequent pattern of fluctuations with a striking positive peak followed by a clear negative deflection can be a sign of enhanced excitability of the cultures, which can be a relation of interest when modeling diseases.

## 6.5 Some Difficulties in Signal Interpretation

Even though the majority of the waveshapes measured by different electrodes were consistent and the proposed hypothesis is able to explain them, some distinct shapes were observed in some cultures, as presented in Section 5.6.5. The odd shape observed in a couple of electrodes in culture C3 appears to be an artifact in CStim, which masks the signal and has no physiological relevance. That is because only in this condition was the detected shape very different from the rest of the electrodes. Consequently, such instances may be overlooked.

The spiking shape measured by some electrodes in culture C1 throughout all tested conditions, shown in Figure 5.18, was a far more intriguing case to explain. In this work, we do not pay much attention to GABA<sub>B</sub> receptors due to the fact that, while GABA<sub>A</sub> receptors do not require potent stimuli to be activated, GABA<sub>B</sub> synaptic mechanisms are only activated upon the manifestation of high levels of input via pre-synaptic activity [27] and can be less powerful in *in vitro* preparations [51]. However, not using any antagonist for such receptors, paired with the fact that, in some conditions, pre-synaptic synchronicity may generate large inputs, could indicate that the membrane actually recruits some of these receptors. Still, only further experimentation could properly answer the remaining questions regarding such a possibility (it seems unlikely that inhibition kicks in before excitation and that it dominates excitation during the onset of network bursts).

Another hypothesis that could be considered and further explored is that these electrodes do not pick up activity from any neuron. This scenario also seems quite unlikely, given that these electrodes did pick up negative deflections during optogenetic stimulation experiments. All things considered, due to the consistency in the waveshape of these signals and lack of variability across conditions, along with the very low number of electrodes picking up such waveshape, it is also plausible that these do not encode any physiologically relevant data and are just artifacts.

The other phenomenon, exposed in Figure 5.20, is also challenging to explain. Due to its non-systematic nature (not commonly observed across cultures and conditions), it is difficult to state, without a doubt, whether it is just another punctual measurement artifact or a signal that may encode some yet-to-be-understood neuronal activity.

One last challenge when working with LFPs is the slow fluctuations that affect these signals. As to the spiking synchronicity simulation, asynchronous activity may actually produce such sinusoidal waves, which may also be thought-provoking for future research.

## 6.6 Importance of Signal Simulations

Given the available time for the conducted research, the final simulations were still not as close to the observed waveshapes as would be ideal. Continuing to work on such methodologies may be a helpful tool as it can lead to a deeper understanding of how the unfiltered signals may look and also provide a bridge between different datasets of collected signals. As a practical example, there are signals collected during previous research on the topic [33] that, due to the fact that they have distinct filtering settings applied, present a different waveshape from the one observed in the here presented results. However, if by applying the different filter settings to a common simulated signal, differences in shape correspond to the ones verified between the collected signals, it would be possible to use both datasets to draw conclusions.

## 6.7 Take-Home Messages

The results from the optogenetic stimulation experiments served as a starting point for the discussion of what may be the mechanisms behind LFP signals. The critical analysis conducted here focuses on understanding what novel information can be retrieved from LFP signals. Even with no measurable spiking activity, we could observe negative deflections in the low frequencies, which indicates that different neuronal processes are behind each component. That, in turn, highlights the relevance of understanding what novel information can be processed from the low-frequency component, as this shows there it may detect neuronal processes even when they maintain subthreshold neuronal activity. Furthermore, it was possible to clearly identify the influence of artifacts in the signal and understand that these do not have any biophysical origin. Finally, we defend that with this recording setup and methodology, such negative deflections are produced by cations flowing into the cells and consequent membrane depolarization (i.e. excitatory activity).

In general, LFP signals seem to have two measurable deflections, each generated by a different process, of variable amplitude across all tested conditions. Accompanying network bursts, a first positive deflection (the positive peak) seems to be directly linked to network spiking activity (its width accompanies the duration of the burst) and is highly dependent on the synchronicity of the input APs. Then, a slower negative deflection (the negative peak) is sensed in all conditions, apart from those in which blockers completely suppressed the signals. Here, the balance between inhibitory and excitatory synaptic currents, with a stronger contribution of the latter, seems to be the key elements to signal generation. Considering the results from optogenetic stimulation and the kinetics of receptor pathways, it seems most likely that slow NMDA receptors are the major contributors to the measured signal.

We observed some still not fully understood shapes in some cultures, the extent to which these represent physiologically relevant information or not is yet to be properly studied and defined. Other possibly appealing food for thought may include the actual influence of GABA<sub>B</sub> receptors on the observed signals could also be further exploited in future work.

Computational signal simulation proved to be a valuable tool and should be developed in future work to broaden the understanding of the generation of LFPs. Not only did simulations clearly identify the artifacts as band-pass filtered square pulse waves, but they also clarified that filtering LFP signals in some conditions generates an overshoot effect after the negative peak.

The results presented in this report are puzzling in the sense that they corroborate previous work on some aspects (spiking activity does shape the LFP signal) but seem to contradict it in others (excitatory activity seems to dominate inhibitory activity in the generation of LFP signals).

All things considered, the *in vitro* models on monolayer cultures are valuable scientific tools. This project took advantage of the freedom of experimentation they offer to hypothesize about LFP signal generation within a cortical neuronal population.

## Chapter 7

# Conclusions and Future Work

The last Chapter of this document focuses mainly on summing up the conducted research work, highlighting the main contributions that emerged from it. Moreover, we make some suggestions for future work which could further expand the obtained results and allow for an even deeper understanding of the subject under research.

### 7.1 Main Conclusions and Contributions

In this Section, we present the main conclusions of the work and highlight some contributions to the field of LFP processing and interpretation.

After reviewing previous research in this field, it became clear that, even though the main contributors to the signal are already accepted, there is still no consensus on the exact integration of inputs into the measurable signal. **As far as research in the field goes, the major influences seem to be spiking activity and currents resulting from ion exchanges through the membrane.**

Using H134R mutated Channelrhodopsins it was possible to measure local field potentials without having any spikes detected in the high-frequency component (neuronal blockers suppressed all activity). The opposite scenario in which we record a lot of spiking activity, but no LFP signals are visible, is also possible. **Ergo, the high and low-frequency components of the recorded signals encode different aspects of neuronal activity.**

We postulate that the influx of positive ions results in negative deflections measured in the low-frequency signal. We also suggest that activating ChR-2 channels promotes the gradual recruitment of voltage-gated channels, explaining the slow kinetics and the "triangular" shape of the observed signals which resulted from the influx of positively charged calcium ions. **In short, we show that negative deflections represent depolarization of the post-synaptic neuron (i.e. excitatory synaptic currents).**

The proposed hypothesis from the collected results is that spiking activity gives rise to a positive peak when synchronous enough, something that becomes very noticeable when we block inhibition and cultures acquires hyperexcited behavior with synchronous and intense network bursts. The integration of various synaptic currents generates a negative deflection in the signal, pointing towards a bigger contribution of excitatory activity to the signal. **As so, we propose that highly synchronous bursting activity, together with inhibitory and excitatory synaptic currents, generate LFPs.**

If this explanation holds, then LFP signals provide a measure of neuronal input. Paired with AP detection in high-frequency recordings, this signal can one day be used to define a measure of neuronal excitability. Besides, since spiking activity does influence the signal's shape, it may be possible to see some shapes that may represent enhanced excitability in cultures. **Hence, the proposed hypothesis may hold the key for the clinical use of LFPs in defining neuronal excitability in cortical cultures.**

Simulation of the stimulus and the signal offered a valuable tool for understanding the influence of artifacts on the observed shapes when applying stimulation to the cultures. Furthermore, they also give insight into the extent to which the used filters may shape the signal, especially by making the negative deflection a bit wider and adding an overshoot effect to compensate for such deflections. **For this reason, simulations in Matlab permit the distinction between true signals and artifacts as well as some of the effects that the applied filtering techniques have.**

There are still some phenomena that remain puzzling after the conducted research, but given the high variability of the signals, it is not surprising that some inconsistencies and deviations may arise from time to time. Some examples include (but are not limited to) the following questions: Why do negative deflections outlast network bursts for so long? What process generates the positive spike-like observed shapes? Which deviations are physiologically relevant and which are mere artifacts? **Further exploration of the subject and validation of the proposed hypothesis may give answers to these yet-to-be-explained details.**

The developed project did produce some valuable results that reinforced some previous statements from previous work in the field, contradicted some conclusions from previous research, and also gave rise to a new theory that aims to explain how these signals are generated. **All things considered, LFPs may bring new information regarding neuronal activity in *in vitro* monolayer cultures to the table, being an important signal when it comes to neuronal excitability.**

## 7.2 Future Work

Even though the developed project produced some interesting results, with a possible understanding of the neuronal processes behind the generation of Local Field Potentials, other hypotheses may still be tested and further explored. In this Section, we suggest future work to continue deciphering the origin of LFPs in monolayer neuronal cultures and their full potential regarding their application as a measure of excitability.

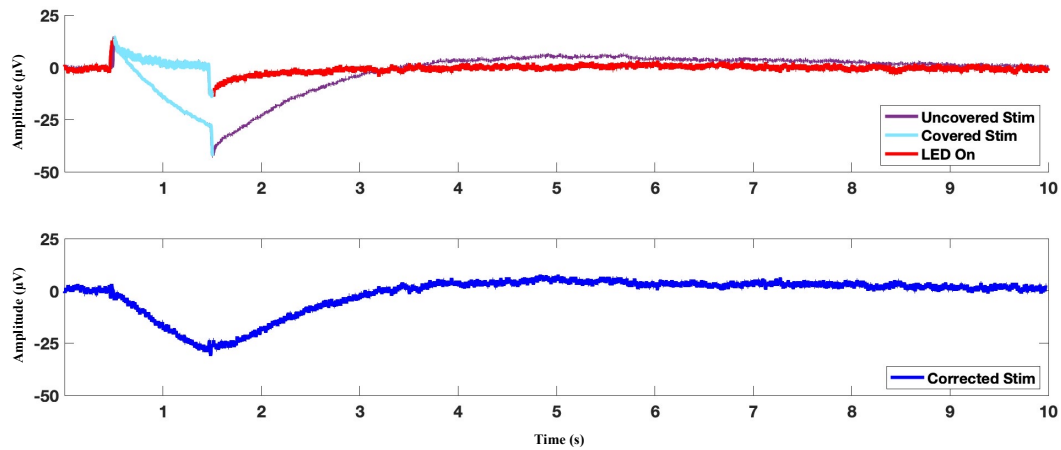


Figure 7.1: Motivation for future work with optogenetic stimulation: signals observed with the stimulus covered and exposed to the culture (top) can be subtracted to obtain the corrected signal without the influence of the measured artifacts (bottom).

The protocol of optogenetic stimulation can be readjusted to include two complementary conditions. On the one hand, it would be advantageous to record activity with the covered stimulus in each MEA before conducting the rest of the recordings, as it makes it possible, by registering the exact timestamps of stimulation, to subtract the artifacts from the measured signals (Fig. 7.1). Unfortunately, we only performed such recording in one extra MEA, but it proved to be a promising technique for the future. On the other hand, it could also be interesting to block free  $\text{Ca}^{2+}$  ions in the medium and stimulate the silenced cultures to see if the negative deflections were still visible. If that is the case, then the observed perturbations in potential are not a product of positive ions moving through the membrane, as hypothesized here.

As to the tested conditions, it would be interesting to have a scenario where we only block excitation, whereas inhibitory receptors are still active. In this case, should the proposed hypothesis be true, we expected neuronal activity in such conditions to generate positive deflections from anions entering the cells as opposed to what happens in the tested PTX and PTXStim conditions. Moreover, selectively blocking AMPA receptors, but not NMDA ones would be an interesting way of relating the negative deflection with the slow closure of such channels. Blocking  $\text{GABA}_B$  receptors may also be decisive in validating that their influence is not strong.

It is paramount to keep expanding the knowledge of LFPs, by studying progressively complex scenarios and evaluating the visualized signals. Only after fully understanding the phenomenon in its most uncomplicated representation can one start hypothesizing about the more complete structures. This process may be crucial for establishing the bridge between *in vitro* and *in vivo*.

Finally, we would like to inspire a clear description of the signal acquisition setups and processing methodologies. Such effort will facilitate the correct comparison between different works developed in the field.



## **Appendix A**

### **Included Cell Cultures in Recordings**

The first Appendix for consultation provides some characterization of the cortical cultures used in the developed project. For each one of the cultures, we present some values regarding recording settings and network activity. Additionally, we exhibit three raster plots, one for Control neuronal activity, one for only excitatory activity (PTX), and one for no activity (Blocked).

## A.1 Culture C1 Overview

Table A.1: Characterization of the included electrodes in culture C1

|                                     |                          |                                    |                                    |                                    |                                    |
|-------------------------------------|--------------------------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|
| <b>MEA Number</b>                   |                          | 40464 (Culture C1)                 |                                    |                                    |                                    |
| <b>Days <i>in vitro</i></b>         |                          | 21                                 |                                    |                                    |                                    |
| <b>Condition</b>                    |                          | <b>Control</b>                     | <b>PTX</b>                         | <b>PTXStim</b>                     | <b>Blocked</b>                     |
| <b>Duration (s)</b>                 |                          | 754                                | 627                                | 658                                | 337                                |
| <b>AP Thresh (x * RMS)</b>          |                          | 7                                  |                                    |                                    |                                    |
| <b>Excluded Electrodes</b>          | Reference                | 1                                  |                                    |                                    |                                    |
|                                     | Too Noisy                | 5                                  | 5                                  | 5                                  | 5                                  |
|                                     | Pos = Neg                | 0                                  | 0                                  | 0                                  | 1                                  |
| <b>Total Excluded Electrodes</b>    |                          | <b>6</b>                           | <b>5</b>                           | <b>5</b>                           | <b>6</b>                           |
| <b>Artifacts Detected (%)</b>       | Bigger Peaks in Vicinity | 14,3                               | 14,4                               | 15,0                               | 4,6                                |
|                                     | Tip not Sharp            | 12,7                               | 20,3                               | 20,4                               | 8,2                                |
|                                     | Stem not Sharp           | 0,0                                | 0,0                                | 0,0                                | 0,0                                |
|                                     | Too Sharp                | 0,0                                | 0,0                                | 0,0                                | 0,0                                |
|                                     | Too many Electrodes      | 2,7                                | 24,6                               | 22,0                               | 0,0                                |
| <b>Total Artifacts Detected (%)</b> |                          | <b>29,6</b>                        | <b>59,3</b>                        | <b>57,5</b>                        | <b>12,8</b>                        |
| <b>Electrodes with no APs</b>       |                          | <b>0</b>                           | <b>0</b>                           | <b>0</b>                           | <b>40</b>                          |
| <b>RMS (<math>\mu</math>V)</b>      |                          | <b><math>3,6 \pm 0,6</math></b>    | <b><math>3,4 \pm 0,4</math></b>    | <b><math>3,2 \pm 0,5</math></b>    | <b><math>2,8 \pm 0,2</math></b>    |
| <b>AP Peak(<math>\mu</math>V)</b>   |                          | <b><math>-30,5 \pm 29,8</math></b> | <b><math>-21,1 \pm 16,0</math></b> | <b><math>-21,3 \pm 17,0</math></b> | <b><math>-42,8 \pm 29,1</math></b> |
| <b>Firing Rate (spikes/min)</b>     |                          | <b><math>95 \pm 97</math></b>      | <b><math>102 \pm 64</math></b>     | <b><math>139 \pm 80</math></b>     | <b><math>1 \pm 4</math></b>        |

Culture C1 was very active in control conditions, with some noticeable network bursts in the raster plot. The addition of PTX had the expected effect, increasing firing rates and promoting an epileptic-like behavior with systematically intense bursts and sparse activity in inter-burst intervals. CNQX and APV successfully suppressed spiking activity (with 40 out of the 54 included electrodes picking up no APs at all).

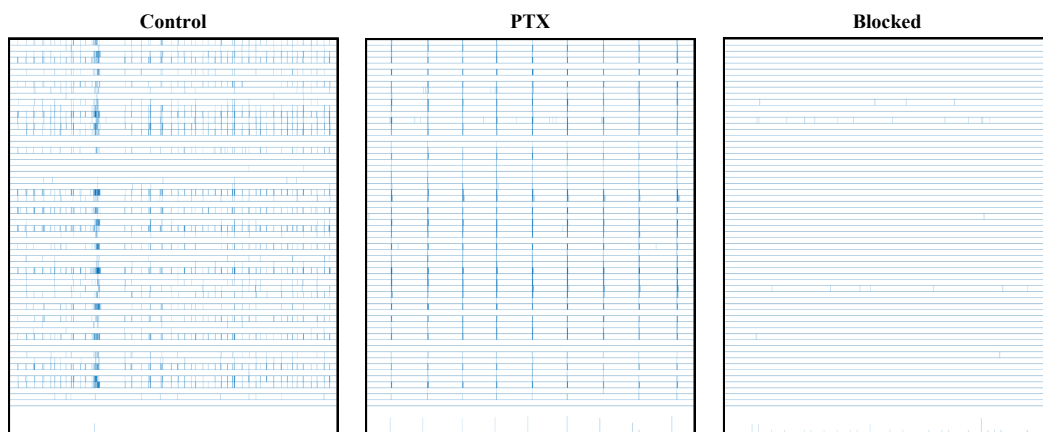


Figure A.1: Rasterplots from different conditions (culture C1)

## A.2 Culture C2 Overview

Table A.2: Characterization of the included electrodes in culture C2

| MEA Number                   |                          | 40315 (Culture C2) |                  |                  |                  |                  |
|------------------------------|--------------------------|--------------------|------------------|------------------|------------------|------------------|
| Days <i>in vitro</i>         |                          | 21                 |                  |                  |                  |                  |
| Condition                    |                          | Control            | Cstim            | PTX              | PTXStim          | Blocked          |
| Duration (s)                 |                          | 317                | 302              | 605              | 657              | 300              |
| AP Thresh (x * RMS)          |                          | 7                  |                  |                  |                  |                  |
| Excluded Electrodes          | Reference                | 1                  |                  |                  |                  |                  |
|                              | Too Noisy                | 7                  | 7                | 7                | 7                | 7                |
|                              | Pos = Neg                | 0                  | 0                | 0                | 0                | 4                |
| Total Excluded Electrodes    |                          | 8                  | 8                | 8                | 8                | 12               |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 10,2               | 13,0             | 13,8             | 12,1             | 20,2             |
|                              | Tip not Sharp            | 9,7                | 13,5             | 22,1             | 18,9             | 18,5             |
|                              | Stem not Sharp           | 0,2                | 0,1              | 0,1              | 0,0              | 0,1              |
|                              | Too Sharp                | 0,0                | 0,0              | 0,0              | 0,0              | 0,0              |
|                              | Too many Electrodes      | 1,2                | 3,5              | 23,6             | 29,1             | 0,0              |
| Total Artifacts Detected (%) |                          | 21,3               | 30,2             | 59,5             | 60,2             | 38,8             |
| Electrodes with no APs       |                          | 0                  | 0                | 0                | 0                | 4                |
| RMS ( $\mu$ V)               |                          | 3,3 $\pm$ 0,6      | 3,2 $\pm$ 0,4    | 3,2 $\pm$ 0,4    | 3,3 $\pm$ 0,5    | 2,6 $\pm$ 0,2    |
| AP Peak ( $\mu$ V)           |                          | -40,6 $\pm$ 18,8   | -34,4 $\pm$ 16,2 | -34,4 $\pm$ 16,2 | -34,8 $\pm$ 15,7 | -38,4 $\pm$ 29,0 |
| Firing Rate (spikes/min)     |                          | 258 $\pm$ 210      | 271 $\pm$ 167    | 142 $\pm$ 75     | 167 $\pm$ 84     | 4 $\pm$ 5        |

Culture C2 was very active in control conditions, with some noticeable network bursts in the raster plot. The addition of PTX had the expected effect, promoting an epileptic-like behavior with systematically intense bursts and sparse activity in inter-burst intervals. Since this culture had a lot of neurons firing between network bursts, blocking inhibition actually made firing rates drop, as neurons fired less frequently but more synchronously. CNQX and APV successfully suppressed spiking activity (even though only 4 of the 48 included electrodes did not pick up APs at all).

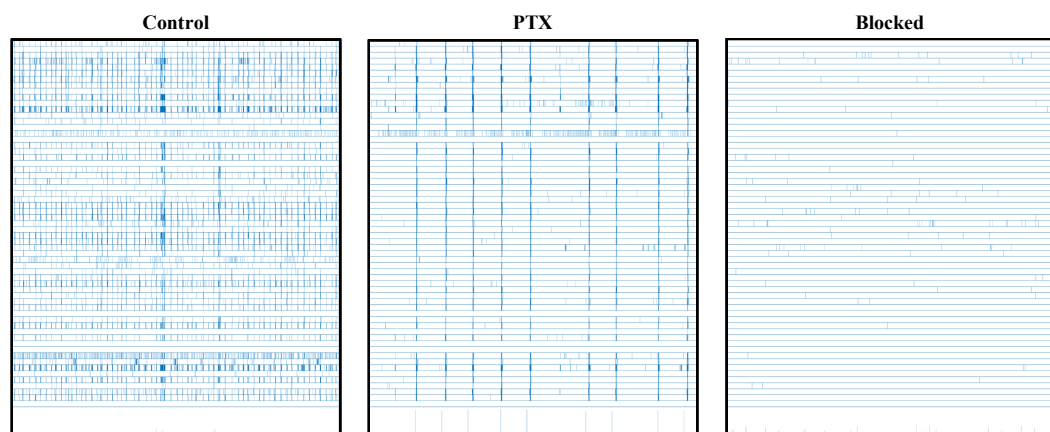


Figure A.2: Rasterplots from different conditions (culture C1)

### A.3 Culture C3 Overview

Table A.3: Characterization of the included electrodes in culture C3

| MEA Number                   | 40317 (Culture C3)       |                  |                  |                  |                    |
|------------------------------|--------------------------|------------------|------------------|------------------|--------------------|
| Days <i>in vitro</i>         | 21                       |                  |                  |                  |                    |
| Condition                    | Control                  | Cstim            | PTX              | PTXStim          | Blocked            |
| Duration (s)                 | 349                      | 304              | 427              | 616              | 229                |
| AP Thresh (x * RMS)          | 7                        |                  |                  |                  |                    |
| Excluded Electrodes          | Reference                | 1                |                  |                  |                    |
|                              | Too Noisy                | 22               | 22               | 22               | 22                 |
|                              | Pos = Neg                | 0                | 0                | 0                | 0                  |
| Total Excluded Electrodes    | 23                       | 23               | 23               | 23               | 23                 |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 1,9              | 5,7              | 9,1              | 17,9               |
|                              | Tip not Sharp            | 5,2              | 6,5              | 15,2             | 23,7               |
|                              | Stem not Sharp           | 0,0              | 0,0              | 0,0              | 0,1                |
|                              | Too Sharp                | 0,0              | 0,0              | 0,0              | 0,0                |
|                              | Too many Electrodes      | 0,0              | 0,0              | 2,0              | 3,9                |
| Total Artifacts Detected (%) | 7,2                      | 12,2             | 26,3             | 45,5             | 2,1                |
| Electrodes with no APs       | 3                        | 2                | 0                | 0                | 20                 |
| RMS ( $\mu$ V)               | $3,3 \pm 0,3$            | $3,3 \pm 0,7$    | $3,3 \pm 0,3$    | $3,6 \pm 0,4$    | $2,9 \pm 0,2$      |
| AP Peak( $\mu$ V)            | $-37,0 \pm 28,1$         | $-31,1 \pm 12,3$ | $-31,2 \pm 12,1$ | $-34,0 \pm 15,1$ | $-32,46 \pm 12,74$ |
| Firing Rate (spikes/min)     | $126 \pm 168$            | $72 \pm 87$      | $156 \pm 183$    | $198 \pm 133$    | $9 \pm 33$         |

Culture C3 was mildly active in Control condition but did not present network bursts (neurons tended to fire asynchronously). The addition of PTX had a softer effect than expected, still promoting intense network bursts but with a lot of asynchronous activity in inter-burst intervals. CNQX and APV successfully suppressed spiking activity (with 20 out of the 37 included electrodes picking up no APs).

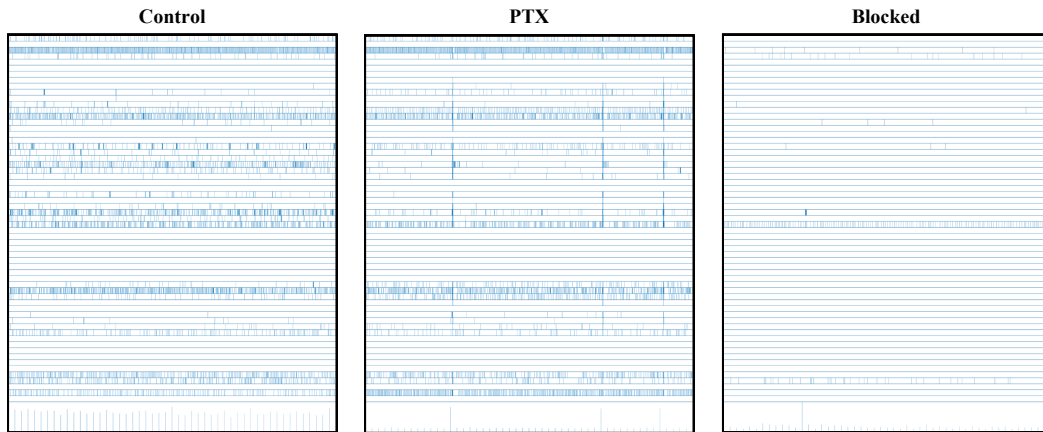


Figure A.3: Rasterplots from different conditions (culture C1)

## A.4 Culture C4 Overview

Table A.4: Characterization of the included electrodes in culture C4

| MEA Number                   |                          | 40409 (Culture C4) |                  |                  |                  |                 |
|------------------------------|--------------------------|--------------------|------------------|------------------|------------------|-----------------|
| Days <i>in vitro</i>         |                          | 15                 |                  |                  |                  |                 |
| Condition                    |                          | Control            | Cstim            | PTX              | PTXStim          | Blocked         |
| Duration (s)                 |                          | 352                | 369              | 140              | 350              | 294             |
| AP Thresh (x * RMS)          |                          | 7                  |                  |                  |                  |                 |
| Excluded Electrodes          | Reference                | 1                  |                  |                  |                  |                 |
|                              | Too Noisy                | 25                 | 25               | 25               | 25               | 25              |
|                              | Pos = Neg                | 0                  | 0                | 0                | 0                | 0               |
| Total Excluded Electrodes    |                          | 26                 | 26               | 26               | 26               | 26              |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 7,6                | 7,8              | 10,4             | 12,4             | 3,6             |
|                              | Tip not Sharp            | 3,5                | 2,8              | 5,4              | 11,4             | 9,1             |
|                              | Stem not Sharp           | 0,0                | 0,0              | 0,0              | 0,0              | 0,0             |
|                              | Too Sharp                | 0,0                | 0,0              | 0,0              | 0,0              | 0,0             |
|                              | Too many Electrodes      | 0,0                | 0,0              | 0,0              | 1,2              | 0,0             |
| Total Artifacts Detected (%) |                          | 11,1               | 10,6             | 15,8             | 25,0             | 12,7            |
| Electrodes with no APs       |                          | 0                  | 1                | 0                | 0                | 25              |
| RMS ( $\mu$ V)               |                          | $4,5 \pm 0,7$      | $4,3 \pm 0,7$    | $4,5 \pm 0,7$    | $3,9 \pm 0,6$    | $3,4 \pm 0,7$   |
| AP Peak ( $\mu$ V)           |                          | $-37,7 \pm 14,4$   | $-35,8 \pm 13,3$ | $-33,5 \pm 14,5$ | $-28,0 \pm 11,2$ | $-32,7 \pm 8,7$ |
| Firing Rate (spikes/min)     |                          | $117 \pm 146$      | $118 \pm 137$    | $149 \pm 166$    | $152 \pm 134$    | $0 \pm 1$       |

Culture C4 was mildly active in control conditions but did not present network bursts (neurons tended to fire asynchronously). That could be a consequence of the state of maturation of the culture. The addition of PTX had the expected effect, increasing firing rates and promoting an epileptic-like behavior with systematically intense bursts and sparse activity in inter-burst intervals. CNQX and APV successfully suppressed spiking activity (with 25 of the 34 included electrodes picking up no APs).

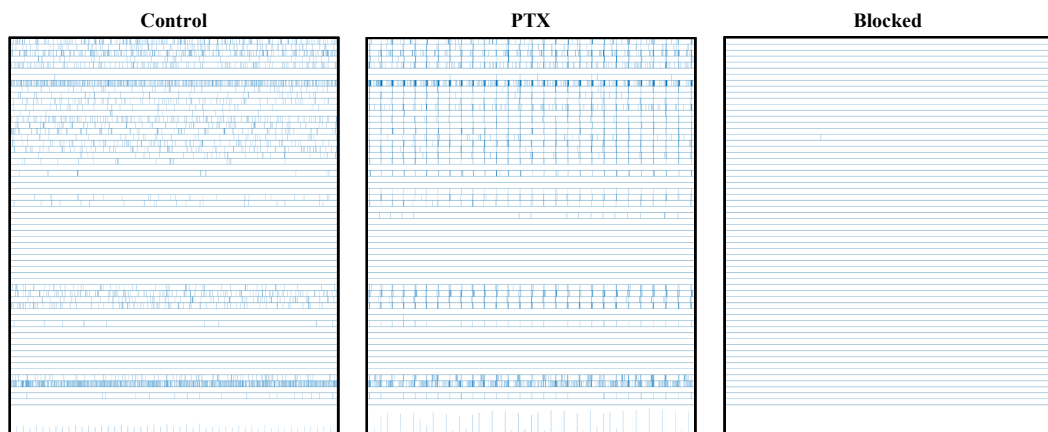


Figure A.4: Rasterplots from different conditions (culture C1)

## A.5 Culture C5 Overview

Table A.5: Characterization of the included electrodes in culture C5

| MEA Number                   |                          | 40417 (Culture C5) |                  |                  |                  |                 |
|------------------------------|--------------------------|--------------------|------------------|------------------|------------------|-----------------|
| Days <i>in vitro</i>         |                          | 15                 |                  |                  |                  |                 |
| Condition                    |                          | Control            | Cstim            | PTX              | PTXStim          | Blocked         |
| Duration (s)                 |                          | 319                | 335              | 80               | 312              | 279             |
| AP Thresh (x * RMS)          |                          | 7                  |                  |                  |                  |                 |
| Excluded Electrodes          | Reference                | 1                  |                  |                  |                  |                 |
|                              | Too Noisy                | 26                 | 26               | 26               | 26               | 26              |
|                              | Pos = Neg                | 0                  | 0                | 0                | 0                | 0               |
| Total Excluded Electrodes    |                          | 27                 | 27               | 27               | 27               | 27              |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 6,0                | 6,5              | 4,7              | 12,8             | 0,0             |
|                              | Tip not Sharp            | 5,0                | 6,5              | 3,5              | 16,2             | 4,0             |
|                              | Stem not Sharp           | 0,0                | 0,0              | 0,0              | 0,0              | 0,0             |
|                              | Too Sharp                | 0,0                | 0,0              | 0,0              | 0,0              | 0,0             |
|                              | Too many Electrodes      | 0,0                | 0,0              | 0,0              | 2,3              | 0,0             |
| Total Artifacts Detected (%) |                          | 11,1               | 13,0             | 8,3              | 31,3             | 4,0             |
| Electrodes with no APs       |                          | 0                  | 0                | 3                | 0                | 27              |
| RMS ( $\mu$ V)               |                          | $3,9 \pm 0,7$      | $3,7 \pm 0,7$    | $3,4 \pm 0,5$    | $3,4 \pm 0,6$    | $2,8 \pm 0,4$   |
| AP Peak ( $\mu$ V)           |                          | $-36,8 \pm 15,5$   | $-36,6 \pm 16,6$ | $-28,2 \pm 10,2$ | $-28,9 \pm 12,4$ | $-22,7 \pm 8,7$ |
| Firing Rate (spikes/min)     |                          | $165 \pm 204$      | $159 \pm 189$    | $146 \pm 182$    | $183 \pm 168$    | $0 \pm 1$       |

Culture C5 was mildly active in Control condition but did not present network bursts (neurons tended to fire asynchronously). That could be a consequence of the state of maturation of the culture. The addition of PTX did not have the expected effect, with a decrease in firing rates and no detected bursting activity. Instead, spikes remained abundant yet asynchronous. CNQX and APV successfully suppressed spiking activity (with 27 out of the 33 included electrodes picking up no APs at all).

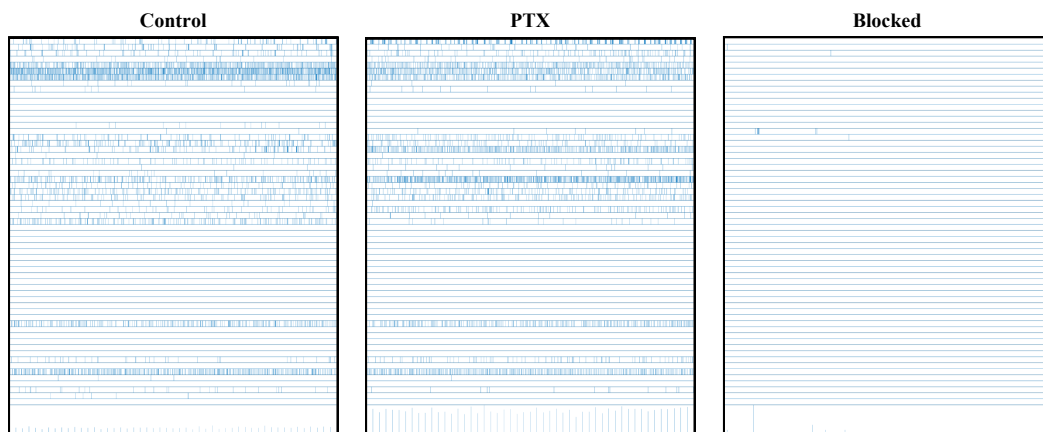


Figure A.5: Rasterplots from different conditions (culture C1)

## **Appendix B**

### **Included Cell Cultures in Stimulation**

The second Appendix for consultation provides some characterization of the cortical cultures used in the optogenetic stimulation experiment. For each one of the cultures, we present some parameters regarding recording settings and network activity. Additionally, we exhibit two raster plots showing network activity in two different stimulation conditions (the first and the last applied). Cultures C2 and C3 were omitted from this Appendix as we did not use them for analysis.

## B.1 Culture C1 Overview

Table B.1: Characterization of the included electrodes in culture C1

| MEA Number                   |                          | 40464 (Culture C1) |                  |                  |                  |
|------------------------------|--------------------------|--------------------|------------------|------------------|------------------|
| Days <i>in vitro</i>         |                          | 21                 |                  |                  |                  |
| Condition                    |                          | Stim1              | Stim2            | Stim3            | Stim4            |
| Stimulation Settings         |                          | 0,1s / 5s          | 0,5s / 5s        | 1s / 10s         | 2s / 20s         |
| Duration (s)                 |                          | 199                | 301              | 285              | 292              |
| AP Thresh (x * RMS)          |                          | 7                  |                  |                  |                  |
| Excluded Electrodes          | Reference                | 1                  |                  |                  |                  |
|                              | Too Noisy                | 5                  | 5                | 5                | 5                |
|                              | Pos = Neg                | 0                  | 1                | 3                | 2                |
| Total Excluded Electrodes    |                          | 6                  | 7                | 9                | 8                |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 15,3               | 14,8             | 13,9             | 10,5             |
|                              | Tip not Sharp            | 2,9                | 0,9              | 5,0              | 0,7              |
|                              | Stem not Sharp           | 0,0                | 0,0              | 0,1              | 0,0              |
|                              | Too Sharp                | 0,0                | 0,0              | 0,0              | 0,0              |
|                              | Too many Electrodes      | 0,0                | 0,0              | 0,0              | 0,0              |
| Total Artifacts Detected (%) |                          | 18,1               | 15,7             | 19,0             | 11,2             |
| Electrodes with no APs       |                          | 37                 | 37               | 36               | 33               |
| RMS ( $\mu$ V)               |                          | $2,8 \pm 0,2$      | $2,8 \pm 0,2$    | $2,8 \pm 0,2$    | $2,8 \pm 0,2$    |
| AP Peak( $\mu$ V)            |                          | $-26,0 \pm 10,8$   | $-43,4 \pm 26,3$ | $-30,9 \pm 21,0$ | $-28,0 \pm 16,2$ |
| Firing Rate (spikes/min)     |                          | $4 \pm 12$         | $6 \pm 21$       | $6 \pm 18$       | $3 \pm 14$       |

In culture C1, we see that the optogenetic stimulation promotes a few spikes. However, these are very sparse in quantity and do not compare to the spiking activity demonstrated by the culture in Control condition. From the first setting applied to the last, there was no increase in neuronal activity, with the effect of the blockers remaining stable.



Figure B.1: Rasterplots from different conditions (culture C1)

## B.2 Culture C4 Overview

Table B.2: Characterization of the included electrodes in culture C4

| MEA Number                   |                          | 40409 (Culture C4) |                 |                  |                 |
|------------------------------|--------------------------|--------------------|-----------------|------------------|-----------------|
| Days <i>in vitro</i>         |                          | 15                 |                 |                  |                 |
| Condition                    |                          | Stim1              | Stim2           | Stim3            | Stim4           |
| Stimulation Settings         |                          | 0,1s / 10s         | 1s / 10s        | 0,5s / 10s       | 2s / 10s        |
| Duration (s)                 |                          | 301                | 299             | 300              | 300             |
| AP Thresh (x * RMS)          |                          | 7                  |                 |                  |                 |
| Excluded Electrodes          | Reference                | 1                  |                 |                  |                 |
|                              | Too Noisy                | 25                 | 25              | 25               | 25              |
|                              | Pos = Neg                | 1                  | 1               | 1                | 1               |
| Total Excluded Electrodes    |                          | 27                 | 27              | 27               | 27              |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 3,1                | 4,7             | 3,0              | 8,5             |
|                              | Tip not Sharp            | 1,6                | 0,0             | 0,0              | 6,2             |
|                              | Stem not Sharp           | 1,6                | 0,0             | 0,0              | 0,0             |
|                              | Too Sharp                | 0,0                | 0,0             | 0,0              | 0,0             |
|                              | Too many Electrodes      | 0,0                | 0,0             | 0,0              | 0,0             |
| Total Artifacts Detected (%) |                          | 6,3                | 4,7             | 3,0              | 14,7            |
| Electrodes with no APs       |                          | 24                 | 28              | 25               | 24              |
| RMS ( $\mu$ V)               |                          | 3,1 $\pm$ 0,4      | 3,1 $\pm$ 0,4   | 3,0 $\pm$ 0,4    | 3,0 $\pm$ 0,4   |
| AP Peak( $\mu$ V)            |                          | -29,3 $\pm$ 5,3    | -27,9 $\pm$ 5,6 | -29,9 $\pm$ 13,9 | -27,7 $\pm$ 4,4 |
| Firing Rate (spikes/min)     |                          | 1 $\pm$ 3          | 1 $\pm$ 5       | 2 $\pm$ 5        | 2 $\pm$ 6       |

In culture C4, we see that the optogenetic stimulation promotes a few spikes. However, these are very sparse in quantity and do not compare to the spiking activity demonstrated by the culture in control condition. From the first setting applied to the last, there was no increase in neuronal activity, with the effect of the blockers remaining stable.



Figure B.2: Rasterplots from different conditions (culture C1)

### B.3 Culture C5 Overview

Table B.3: Characterization of the included electrodes in culture C5

| MEA Number                   |                          | 40417 (Culture C5) |                  |                  |                  |
|------------------------------|--------------------------|--------------------|------------------|------------------|------------------|
| Days <i>in vitro</i>         |                          | 15                 |                  |                  |                  |
| Condition                    |                          | Stim1              | Stim2            | Stim3            | Stim4            |
| Stimulation Settings         |                          | 0,1s / 10s         | 2s / 10s         | 0,5s / 10s       | 1s / 10s         |
| Duration (s)                 |                          | 300                | 301              | 326              | 302              |
| AP Thresh (x * RMS)          |                          | 7                  |                  |                  |                  |
| Excluded Electrodes          | Reference                | 1                  |                  |                  |                  |
|                              | Too Noisy                | 26                 | 26               | 26               | 26               |
|                              | Pos = Neg                | 0                  | 1                | 0                | 0                |
| Total Excluded Electrodes    |                          | 27                 | 28               | 27               | 27               |
| Artifacts Detected (%)       | Bigger Peaks in Vicinity | 0,6                | 0,0              | 2,1              | 0,0              |
|                              | Tip not Sharp            | 7,8                | 0,0              | 12,5             | 3,3              |
|                              | Stem not Sharp           | 0,0                | 0,0              | 0,0              | 0,0              |
|                              | Too Sharp                | 0,0                | 0,0              | 0,0              | 0,0              |
|                              | Too many Electrodes      | 0,0                | 0,0              | 0,0              | 0,0              |
| Total Artifacts Detected (%) |                          | 8,4                | 0,0              | 14,6             | 3,3              |
| Electrodes with no APs       |                          | 17                 | 27               | 26               | 19               |
| RMS ( $\mu$ V)               |                          | $2,8 \pm 0,4$      | $2,7 \pm 0,3$    | $2,7 \pm 0,3$    | $2,7 \pm 0,3$    |
| AP Peak ( $\mu$ V)           |                          | $-27,5 \pm 12,7$   | $-35,5 \pm 17,3$ | $-38,5 \pm 23,9$ | $-25,7 \pm 16,5$ |
| Firing Rate (spikes/min)     |                          | $2 \pm 3$          | $0 \pm 1$        | $0 \pm 1$        | $1 \pm 2$        |

In culture C5, we see that the optogenetic stimulation promotes a few spikes. However, these are very sparse in quantity and do not compare to the spiking activity demonstrated by the culture in control condition. From the first setting applied to the last, there was no increase in neuronal activity, with the effect of the blockers remaining stable.



Figure B.3: Rasterplots from different conditions (culture C1)

## **Appendix C**

# **Representativeness of the average signals**

The third Appendix for consultation briefly discusses the effect of stimulation settings on signal variability and, consequently, on the representativeness of the average signal. Across cultures, the influence of stimulation settings on the average waveshape of a given electrode seemed to follow one of two lines of evolution: on the one hand, as the duration of the stimulus increased, responses detected by one electrode got more consistent with less deviation from the average waveshape (electrode 1); on the other hand, with increasing duration of the stimulus, recovery of the signal increased in variability, even though the response to the stimulus itself (the negative peak) did not present many deviations from the average waveshape (electrode 2). In general, the average waveshape was a representative signal for the responses recorded by a given electrode. We did not find any cases in which the average shape and the highlighted ones did not agree.

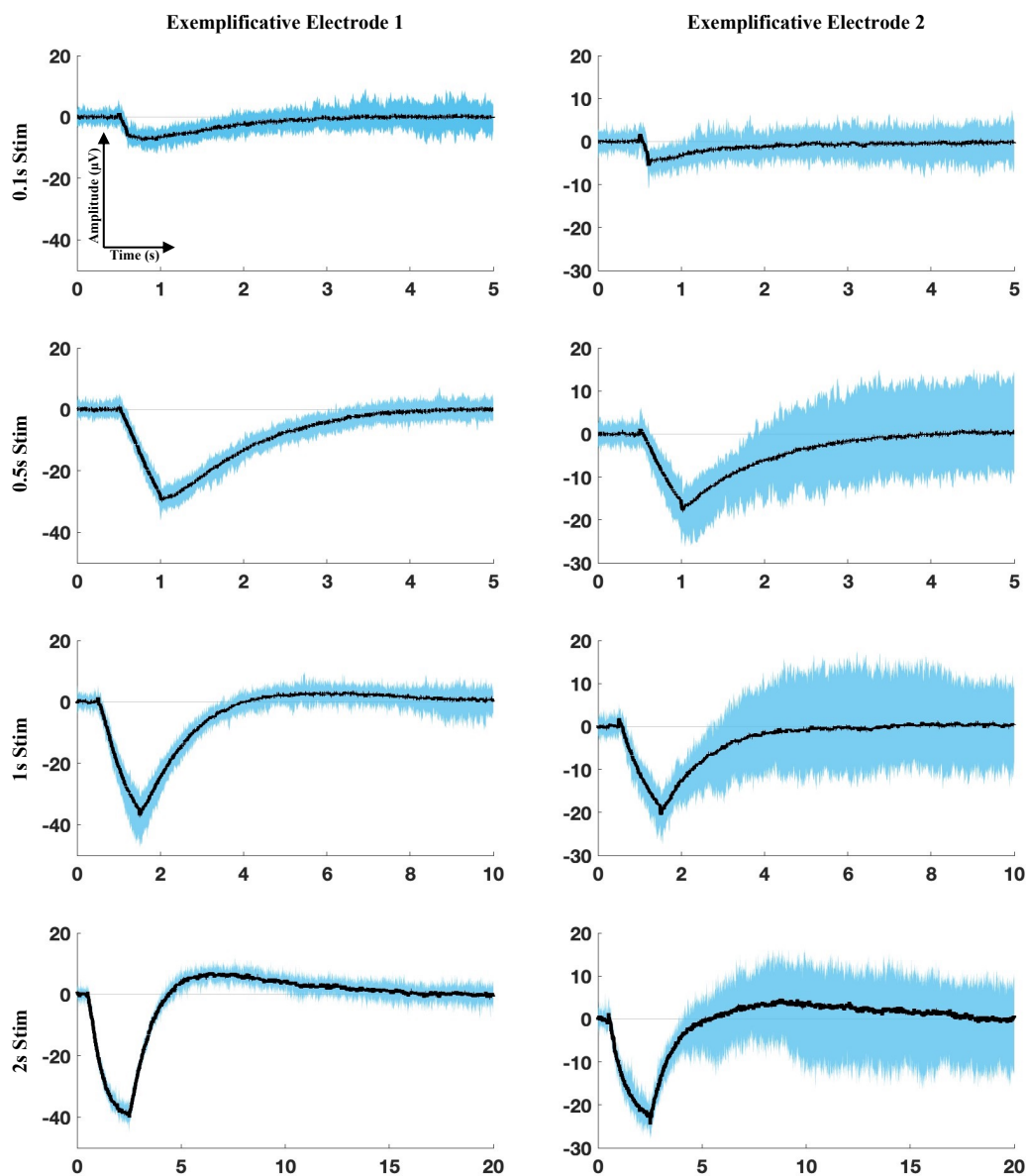


Figure C.1: Influence of stimulation settings on average waveshape: two exemplificative electrodes are presented with the most commonly observed evolution of the average waveshape per electrode for the different stimulation settings applied.

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