DISSOCIATED NEURONAL NETWORKS AND MICRO ELECTRODE ARRAYS FOR INVESTIGATING BRAIN FUNCTIONAL EVOLUTION AND PLASTICITY

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ABSTRACT

DISSOCIATED NEURONAL NETWORKS AND MICRO ELECTRODE ARRAYS FOR INVESTIGATING BRAIN FUNCTIONAL EVOLUTION AND PLASTICITY

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Doctor of Philosophy

Temple University, May, 2014

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For almost a century, the electrical properties of the brain and the nervous system have been investigated to gain a better understanding of their mechanisms and to find cures for pathological conditions. Despite the fact that today's advancements in surgical techniques, research, and medical imaging have improved our ability to treat brain disorders, our knowledge of the brain and its functions is still limited. Culturing dissociated cortical neurons on Micro-Electrode Array dishes is a powerful experimental tool for investigating functional and structural characteristics of in-vitro neuronal networks, such as the cellular basis of brain learning, memory and synaptic developmental plasticity.

This dissertation focuses on combining MEAs with novel electrophysiology experimental paradigms and statistical data analysis to investigate the mechanisms that regulate brain development at the level of synaptic formation and growth cones. The goal is to use a mathematical approach and specifically designed experiments to investigate whether dissociated neuronal networks can dependably display long and short-term plasticity, which are thought to be the building blocks of memory formation in the brain.

Quantifying the functional evolution of dissociated neuronal networks during in-vitro development, using a statistical analysis tool was the first aim of this work. The results of the False Discovery Rate analysis show an evolution in network activity with changes in both the number of statistically significant stimulus/recording pairs as well as the average length of connections and the number of connections per active node. It is therefore proposed that the FDR analysis combined with two metrics, the average connection length and the number of highly connected "supernodes" is a valuable technique for describing neuronal connectivity in MEA dishes. Furthermore, the statistical analysis indicates that cultures dissociated from the same brain tissue display trends in their temporal evolution that are more similar than those obtained with respect to different batches.

The second aim of this dissertation was to investigate long and short-term plasticity responsible for memory formation in dissociated neuronal networks. In order to address this issue, a set of experiments was designed and implemented in which the MEA electrode grid was divided into four quadrants, two of which were chronically stimulated, every two days for one hour with a stimulation paradigm that varied over time. Overall network and quadrant responses were then analyzed to quantify what level of plasticity

took place in the network and how this was due to the stimulation interruption. The results demonstrate that here were no spatial differences in the stimulus-evoked activity within quadrants. Furthermore, the implemented stimulation protocol induced depression effects in the neuronal networks as demonstrated by the consistently lower network activity following stimulation sessions. Finally, the analysis demonstrated that the inhibitory effects of the stimulation decreased over time, thus suggesting a habituation phenomenon.

These findings are sufficient to conclude that electrical stimulation is an important tool to interact with dissociated neuronal cultures, but localized stimuli are not enough to drive spatial synaptic potentiation or depression. On the contrary, the ability to modulate synaptic temporal plasticity was a feasible task to achieve by chronic network stimulation.

To my family,

Cristina, Anna & Salvatore

I would not be here without their unconditional love and support. More importantly, without them I would not be who I am today, for which I am so thankful.

To my friends,

The ones who have always been there for me and those I met more recently. In the hope that they will always be there.

To a very special person,

She is one of a kind. She pulled me through some of the toughest times of my life, by staying next to me, as only she can do and by helping me carry on. She gave me the strength not to give up and she gave me hope when everything was dark. Most importantly, thanks for what has yet to come and for being perfect to me.

…di piu'…

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CHAPTER 1

INTRODUCTION

1.1 Electrical Properties of the Nervous System: Early Studies

The idea of recording electrical activity from cells of the nervous system dates back to the 1660s, when a Dutch scientist, Jan Swammerdam, developed a system capable of electrically stimulating the thigh nerve of a frog and the connected thigh muscle contractions [1]. Although Swammerdam was the first to observe muscle contraction due to electrical stimulus delivery, it was Isaac Newton who was the first to hypothesize the electrical nature of signal propagation between nerves and muscles. It was 80 years later though, in 1791, that Luigi Galvani for the first time produced experimental support proving the electric nature of nerve impulse. Galvani also introduced the concept of ion channels and proved the relation between electrical impulse and muscle contraction strength.

Over the next few centuries, several scientists set off to investigate this phenomenon that was referred to as “the animal electricity”. Their studies led to the characterization of most phenomena that regulate action potential propagation, nerve signal conduction speed and the fact that nerve conduction was related to ion propagation. By the mid-1930s, the structure of the cell membrane was finally understood and the presence of ion channels was suggested, even though direct physiological evidence was still missing.

Such evidence arrived in 1936 when John Z. Young for the first time introduced into the scientific world the use of the squid axon to carry out electrophysiology experiments. But it was in 1949, when the voltage-clamp technique was designed by Kenneth Cole [2] and George Marmont [3] that modern electrophysiology was born. A few years later such a technique was employed by Alan Hodgkin and Andrew Huxley to propose the ionic theory of membrane excitation. Most importantly, Hodgkin and Huxley proposed the first mathematical model to describe the action potential formation and propagation in 1952 [4]. Their model is still used today and represents one of the best examples of how electrophysiology phenomena can be elegantly and accurately described in an efficient mathematical model even before they can be directly (experimentally) observed.

In this work we will focus mainly on a particular set of in-vitro neural experiments, referred to as Micro Electrode Array (MEA) recordings, in which live neurons can be cultured for weeks to months in special Petri dishes whose substrate embeds recording and stimulating electrodes. Since the pioneering work of Jerome Pine, who first proposed the use of MEAs to carry out electrophysiology stimulations and recordings of dissociated neuron networks [5], this type of technology has rapidly become a valuable tool to investigate neural functions and synaptic evolution at a network level.

1.2 Electrophysiology Experiments

For almost a century, scientists have been investigating the electrical characteristics of the brain and the nervous system in order to achieve a better understanding of their mechanisms and to find cures for pathological conditions. Despite the fact that today's

advancements in surgical techniques, research, and medical imaging have improved our ability to treat brain disorders, our knowledge of the brain and its functions is still limited. The reasons are manifold, and they are all related to the structural and functional brain complexity that is the result of millennia of evolution. The difficulty in understanding the brain derives from the fact that its properties and the way it processes information depend on how billions of neurons are arranged, connected and how electrical signals are transferred. Furthermore, the fact that functional and physical connectivity of different brains are rarely the same, even within the same species, makes investigating brain mechanisms even more challenging. One main reason why it is so arduous to understand brain mechanisms is our inability to record from large numbers of neurons simultaneously to investigate how the network functions.

For many years, researchers have designed electrophysiology experiments to investigate the electrical and functional behavior of brain regions and large neuronal populations. Their objective was to shed some light on the intricacy of neuronal connectivity and information propagation in the nervous system. Such experiments can be generally divided into two categories: 1) in-vivo experiments, in which the recordings are performed on live subjects; 2) in-vitro experiments, in which the electrical recordings are carried out on brain tissue, neurons or other tissue preparations grown in special cell culture incubators.

Choosing between these two experimental categories is a difficult task and it is a trade-off between benefits and drawbacks. For instance, in-vivo techniques are usually more complex and expensive to implement. They usually require sophisticated acquisition systems and the experiments are challenging to run because they involve

recording from live and behaving subjects. Moreover data quality and experimental results may be heavily influenced by experimental conditions or parameters not under the direct control of the investigators. Despite these limitations, these experiments are more complete and allow for better insights into the properties of fully functional and entire nervous systems.

In contrast, in-vitro experiments are easier to manage, simpler to run and to reproduce given the reduced number of variables that investigators have to deal with. Conversely, in-vitro models are often an over-simplified version of the nervous system regions under investigation and therefore inferences derived from such work are not always applicable to intact nervous system functions. One of the main advantages of in-vitro experiments is that they allow investigators to limit the number of variables to keep under control and to carry out experiments in more controlled environments. Given the numerous variables that play an important role in biological systems' functions, reducing the number of experimental variables becomes of vital importance when dealing with biological samples.

In this view, MEA technology represents a valuable compromise. It allows investigators to run experiments and test experimental conditions that would not be feasible to implement using traditional in-vitro or in-vivo techniques.

1.3 New Frontiers in In-Vitro Electrophysiology

Technology has advanced to where it is possible to design and grow living networks of neurons in culture dishes with predefined geometry and remarkably good reliability

[6]–[12]. Cortical neural cells in culture preserve many of the properties found in their in-vivo context, but important differences also exist. Therefore, the development of neuronal cultures in-vitro must be carefully investigated and documented if we want to generalize the results obtained from such cultures to entire nervous systems or clinical applications. In this respect, culturing neurons in-vitro gives the investigators the opportunity to derive parameters and mathematical models that could then be used to build new and more realistic computer simulations of neuron networks, which would be based on biologically derived neural patterns.

Furthermore, this technology has offered researchers the opportunity of studying neurons and their interactions in confined and highly controlled experimental conditions. This has improved our ability to control the numerous variables involved in such experiments and design experimental paradigms aimed at emphasizing specific aspects of neurons' functionality and connectivity. The idea behind this technology is that stimulation and recording of large networks of neurons, glial cells and astrocytes with a large number of electrodes can lead to a better understanding of the mechanisms behind the brain functions of learning, memory, neural signal coding and modulation.

1.4 Dissertation Organization

After having briefly described the basic research challenges that are faced when investigating the brain, the remainder of this document will proceed as follows:

- Chapter 2 will address MEA technology in detail, along with its applications, advantages and limitations. It will conclude by identifying the two basic MEA

recording problems being addressed in this dissertation, namely, (1) inadequate statistical tools available and (2) the question of whether external stimulation can generate neuronal plasticity.

- Chapter 3 will deal with the first of those problems, the inadequacy of the available statistical tools. The goal is to demonstrate an adequate statistical tool that can be applied to MEA recordings to study their functional evolution without making restrictive assumptions on the data.
- Chapter 4 will deal with the second problem, whether external stimulation can generate neural plasticity. The objective is to study the short and long-term plasticity effects induced by a select stimulation protocol.
- In Chapter 5 will discuss the implications and present the conclusion.

1.5 Goals and Objectives

This dissertation focuses on combining MEAs with novel electrophysiology experimental paradigms and statistical data analysis to investigate the mechanisms that regulate brain development at the level of synaptic formation and growth cones. The goal is to use a mathematical approach and ad-hoc designed experiments to investigate whether dissociated neuronal networks can dependably display long and short-term plasticity. Such phenomena are thought to be the building blocks of memory formation in the brain.

Even though several studies have investigated spontaneous electrical activity and stimulus-evoked activity in dissociated neuronal cultures, many questions still need to be

answered before these neuronal networks can be fully understood and integrated into larger and more complex systems [10], [13]–[17]. For example: (1) how a culture reacts if stimulated at various stages of development; (2) what the mechanisms that allow such cultures to consistently respond to stimulation are; and (3) what the real effects of stimulation on cultures stimulated repeatedly over time are as compared to "never-stimulated" control cultures. There have been no quantitative studies that assess how the development of dissociated cortical neurons can be affected by chronic external stimulation [18].

In this work, the objective is to investigate two specific questions:

1) Can a mathematical technique capable of reliably quantifying and emphasizing the physiological evolution of dissociated neuronal networks be developed? Such a technique would need to be able to account for the high variability and background noise that are characteristic of MEA recordings.

2) Are dissociated neurons capable of displaying memory formation phenomena? In this context, memory is defined at the neuronal level as synaptic plasticity, namely the ability of such cells to form, modify and delete connections based on the delivered electrical stimuli. It is known that plasticity effects are responsible for driving neuronal processing such as memory formation and "intelligence". The goal here is to test whether these preparations of dissociated neurons adapt to stimuli and respond to the external inputs through plasticity and synaptic modification in a stable way.

To address these two important MEA recording questions that are still unanswered, a new experimental paradigm and a new statistical analysis were designed to investigate: 1) the functional evolution of dissociated neuronal networks during in-vitro development,

using a statistical analysis tool to quantify network activity; 2) whether and how long-term and short-term memory mechanisms take place in dissociated cortical neurons when presented with external voltage stimuli.

1.6 Dissertation Contribution

This dissertation contribution to the research literature is two-fold. Firstly, it demonstrates the importance of statistical analysis techniques in MEA research to quantify the temporal evolution of dissociated neuronal networks. Precisely, the proposed statistical technique does not make use of any specific a-priori assumptions on the data distribution and combined with physiological metrics it has been proven to be suited to track and quantify the functional changes that take place during early neuronal development in in-vitro neuronal networks. The analysis results confirm the expected temporal evolution of synaptic connectivity as previously described in other work [19]. Furthermore, this dissertation results have demonstrated that neurons harvested from the same brain slice preparations display similar developmental behaviors, proving the importance of condition and genetic factors in MEA synaptic development. Another contribution is the importance of time window length selection when analyzing the stimulus-evoked responses in MEA experiments [20]–[23].

Secondly, in this dissertation it has been demonstrated that adopting a localized stimulation paradigm is not sufficient to induce network responses that are local to specific areas of the MEA dish. Moreover, using a specifically designed stimulation paradigm, this work has shown that the effects of electrical stimulation tend to decrease

over time, due to habitation phenomena that take place in dissociated rat neurons when chronically subjected to external stimulation. Furthermore, varying the stimulus delivery over time has highlighted the presence of short and long-term plasticity in these neuronal preparations, as measured both in terms of spike count and number of bursts in recordings.

CHAPTER 2

MICRO ELECTRODE ARRAY (MEA) RECORDINGS

2.1 MEA History

In the past, the electrical properties of neurons in cultures were studied using glass micropipette electrodes. Experiments carried out with this technique were often difficult to set up and difficult to reproduce because each electrode had to be manually positioned using some mechanical manipulator. Also, when using micropipette electrodes, it is difficult to record from more than a couple of neurons at a time. Therefore with such technology it is not feasible to study the behavior of neuron networks. Despite these limitations, micropipette electrodes were, for years, the main tools that neurophysiologists had to investigate single neuron characteristics, ion channels, pharmacology and synaptic plasticity in-vitro.

In order to overcome these limitations, a new experimental tool capable of monitoring activity of electrically excitable cells was introduced in 1972, when Thomas et al., introduced the use of Micro Electrode Arrays [24]. They were able to record field potentials from cultured sheets of cardiac tissue harvested from 6-10 day old embryonic chickens. After these first experiences with MEA technology, in 1979, Gross and Pine independently developed arrays for chronic multi-unit neuron recording and stimulation [5], [25]. For a few years, custom-made experimental tools were used and each lab

involved in this research field utilized their own proprietary hardware and software [5], [26]–[33].

For instance, in [26] the authors ran electrophysiology experiments on myocardial cells plated on custom-made MEA dishes. Instead of using traditional intracellular glass micropipettes, they were able to embed 25 recording and 6 stimulating electrodes onto the dish. Their experimental setup allowed them to successfully record extracellular potentials and to emphasize the importance of electrode array biocompatibility and longevity. In [27], Novak and Wheeler built a passive MEA and used it to record the abdominal ganglion of the marine mollusk *Aplysia californica*. Their MEA consisted of 32 gold electrodes laid on a glass substrate. The electrodes were arranged in a 4x8 grid with 200 μm spacing, and each electrode was 25 μm in diameter. Their main contribution to MEA fabrication was the fact that their dishes were durable, reusable and could be safely autoclaved. In [28], the authors set off to build their own MEA dishes, with the goal to develop biocompatible MEA recording devices that could combine extracellular recording capabilities with guidance of cells during growth, using surface topography techniques. This work represents one of the first attempts to influence cell development while simultaneously recording their electrical activity.

When the use of digital systems became more accessible, the first digital MEA recording systems were designed. For instance, in [30] Borroni et al., introduced one of the first integrated systems for recording and analyzing electrophysiological data from multiple channels. Their system was composed of an MS-DOS microcomputer, a 16-channel amplifier and electrode arrays with multiple tips that could be used both in intact and slice tissue preparations. Furthermore, their in-house developed acquisition software

allowed for data collection and on-line analysis of multiple-channel recordings. The real-time capabilities comprised averaging and current source density computation. In addition, their software had off-line capabilities, such as computing power spectra, peak amplitudes, area, latency and slope of user selected signal segments.

A further example of a PC-based system for acquisition and processing of MEA data was presented in [32], where Martinoia et al. introduced a simple and relatively inexpensive general-purpose acquisition system. Their system was able to acquire simultaneously up to 16 channels and store data directly on a computer disk. The main components of the complete MEA system were an instrumentation amplifier (AD521), followed by an inverting stage (implemented using an LF356 operational amplifier), for additional gain (see Figure 2.1) and an acquisition computer with in-house developed acquisition software (written in C) and a National Instruments 16-MIO-F5 A/D board to digitize the data. The system presented in this work is worth noting, because it represents the first complete digital MEA acquisition system.

Finally, it is important to mention the first complete closed flow chamber for long-

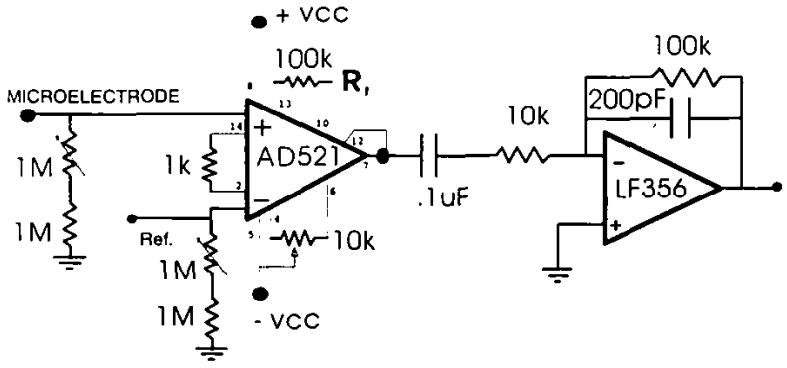

Figure 2.1: Amplifier first stage followed by a first-order band-pass filter. Reprinted from [32]

term multichannel recordings that was presented in [33]. This paved the way for a new class of more accurate experiments, in which the investigators could precisely and easily control the cultured cells' environmental conditions. In greater detail, Gross et al. built an autoclavable chamber with an associated medium circulation system. The chamber was specifically designed for multichannel electrophysiological recording from monolayer networks with multi-electrode matrices, for microscope observations of networks and cell manipulation.

At present, MEA is a commercially available technology comprised of various plug-and-play components and capable of recording from at least 60 electrodes. For instance, commercially available systems include: MEA1060-INV by Multichannel Systems (Germany) [34], Med64 by Alpha Med Scientific, Panasonic (Japan) [35], Center for Network Neuroscience (University of North Texas) (USA) [36], and OmniPlex Neural Data Acquisition System by Plexon Inc. (Texas, USA) [37].

2.2 MEA Experiments

Culturing dissociated cortical neurons on Micro-Electrode Array (MEA) dishes is a powerful experimental tool for investigating functional and structural characteristics of in-vitro neuronal networks. Over the few past decades, MEAs have been frequently used to investigate the mechanisms that take place at the network level among cultured neurons and to answer fundamental questions regarding the cellular basis of brain learning, memory and synaptic developmental plasticity. MEAs allow researchers to carry out long-term (up to a few months), non-invasive neural recordings using

experimental setups that are easier to control and less complex than similar in-vivo systems. Being able to observe in-vitro neuronal cultures for longer periods of time allows for better investigation of the mechanisms that take place during synaptic growth and development. In general, MEA research falls in one of two categories: hybrid systems, in which artificial and natural intelligence are merged to exploit the benefits deriving from their combined use; and realistic brain models that can be used to investigate how the brain works and how it forms structural and functional connections.

2.2.1 Closed-Loop Experiments and Hybrid Systems

MEA technology is commonly used to carry out real-time electrophysiology experiments both on in-vitro dissociated neurons and brain tissue slice preparations. The main characteristic of these experiments is that they are closed-loop experiments in which a feedback loop is implemented by delivering electrical stimuli to the electrodes while simultaneously recording from them. Electrical stimulation allows researchers to modulate neural activity in real-time in order to induce network plasticity or to simulate the effects of sensory input [38]–[41]. In [40], [42]–[45] the authors investigated how to use electrical stimulation to evoke and modulate neural responses. For instance, in [44] the authors demonstrated selective learning in a network of real cortical neurons by implementing a closed-loop stimulation protocol that allowed them to map evoked neuronal responses to known stimuli. These responses were used to generate learning curves that described how the repeated stimulation protocols were inducing changes in the functional connections of the neuronal network. The goal of hybrid systems is to integrate dissociated neural networks with an advanced system in which the network is used as a black box whose output controls the behavior of the next component of the

system. Feedback loops are generally used to select which neural network inputs have to be used to achieve a predetermined experimental task and to increase the system performance.

Seminal work on the efficacy of closed-loop systems is presented in [42], in which Bakkum et al. developed an adaptive training algorithm to investigate whether in-vitro neocortical networks could learn how to modulate their responses to achieve pre-determined activity states. They exploited the advantages of working with a feedback loop to continuously improve the system performance by effectively selecting stimulus sequences that led to system performance improvement. Furthermore, they emphasized that the use of a closed-loop system allowed them to improve over time the ability of the MEA networks to respond with the desired responses, without interfering with the network's functional connectivity. In other words, they showed that cultured neuronal networks could be used as a black box, if combined with a system that is capable of correctly mapping all their electrical responses to a select set of stimuli.

Other examples of MEA recordings integrated in a closed-loop system are presented in [40], [45], [46]. In these studies the investigators used external stimulation and feedback loops to build complex hybrid systems in which cortical neuronal cultures were used to control computer simulations through artificial sensory-motor loops. Specifically, in [43], Potter et al., have created a computer animation of a freely moving animal and aimed to control its movements using neuronal responses recorded in real-time from a network of dissociated rat cortical neurons. In Figure 2.2 the hybrid system is shown, in which an acquisition computer uses real-time data from living neurons to drive a software application, while controlling a stimulus generator that provides the feedback loop. Their

long-term goal was to use this closed-loop system to study learning in such in-vitro preparations. Despite the importance of their findings and the good performance they were able to achieve with their system, they could not quantify in detail how the complex network activity patterns were affected by stimulation and thus they could not precisely assess the effects of stimulation on network behavior. They were able to find that their hybrid system, thanks to the presence of a feedback loop, could successfully map the range of electrical responses that the networks generated. This allowed the system performance to be improved and demonstrated that MEA experiments are a promising tool for integrating natural and artificial "intelligence".

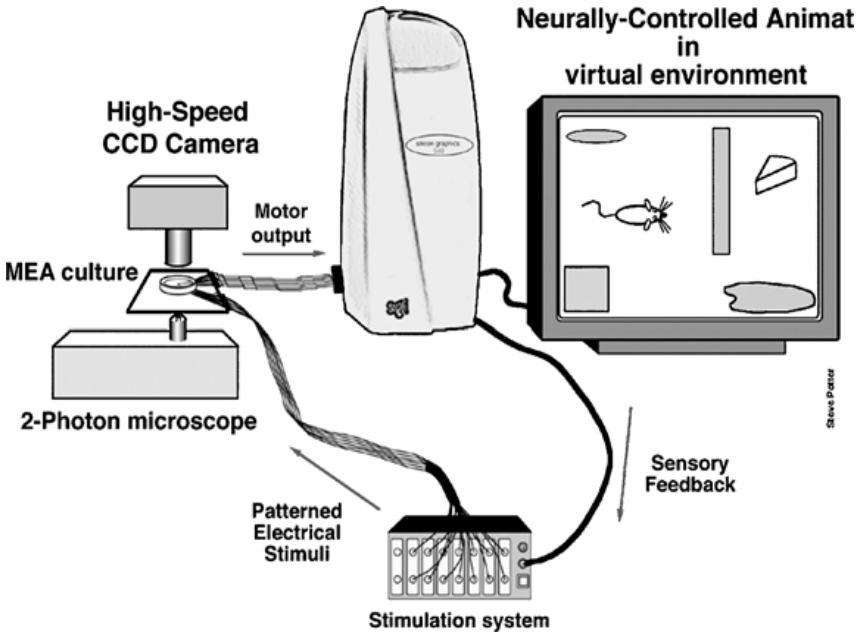

Figure 2.2: Scheme of a hybrid system. The goal of this system is to control computer simulation of the movement of a "virtual" rat. Reprinted from [43]

2.2.2 Realistic Brain Models

Since the first studies of Santiago Ramón y Cajal [47] dating back over a century ago, scientists have been interested in investigating how neurons are physically connected in our nervous system and what these connections mean from a functional neuronal network perspective. To date, we know that functional connectivity modulates cognitive and behavioral states in the brain, but very little is known about functional networks and complex neuronal ensembles involving overlapping or multiple anatomical structures [48], [49]. One limiting factor of this line of research is that most studies require the use of expensive and ad-hoc imaging tools. For instance, special MRI systems combined with tightly controlled experiments and powerful image processing techniques are required [49]. In contrast, MEA recordings represent an innovative tool to build a simplified and yet realistic neuronal model able to simulate the functions and properties of brain layers [45]. Starting from these simple brain layer models, the long-term goal is to increase the system complexity, trying to combine multiple layers and eventually building 3-D neuronal structures.

Neural network models have long been used to provide quantitative characterizations of neural processes. Combining MEA recordings with appropriate statistical analysis techniques represents an even more valuable tool to link electrophysiology experiments and computational modeling, by using the information from experiments to better identify parameters for more complex models. Alternatively, computational models can lead to the discovery of new statistical methods that may enhance our ability to extract features from experimental data.

Although the objective of most current MEA experiments is to connect external stimuli to induced network activity, the importance of developing appropriate statistical techniques to analyze neuron activity should be emphasized. This requires innovative mathematical approaches to integrate information derived from artificial and natural neural models. The advantages can be manifold; more reliable data analyses provide more accurate constraints and parameter values for dynamical models of neural systems. Finally, the improved ability to interpret neural activity, i.e. spike detection and sorting, can significantly expand the ability to design and build brain computer interface systems [50],[51].

Another relevant research study, in which the authors compared the electrical responses recorded from in-vitro dissociated cultured neurons to those obtained from a simulated neural network was presented in [52]. The goal of this research work was to investigate the network mechanisms of learning and memory using standard firing rate statistics. Specifically, they used a well-known neural model, called integrate-and-fire neural network and data recorded from live neurons to evaluate the performance of six statistical methods in detecting lasting functional changes in functional network connectivity. It is worth noting that their statistical method yielded comparable results when applied to both simulated network responses and MEA recordings. This process proves that MEA experiments can be used to improve our ability to build more realistic neural network models.

2.3 MEA Acquisition System

A complete MEA acquisition system is composed of five main components:

1. An MEA dish with live neurons cultured on it;
2. A specific MEA amplifier, to amplify signals coming from the MEA electrodes, see Figure 2.3;
3. A stimulus generator, to generate the voltage or current pulses necessary to deliver external stimulation to the MEA electrodes;
4. An acquisition computer, where the acquisition software runs and the necessary acquisition hardware is installed, such as A/D card, I/O ports and amplifier power supply.
5. A cell culture incubator, to preserve the best environmental conditions for the cells during long experiments;

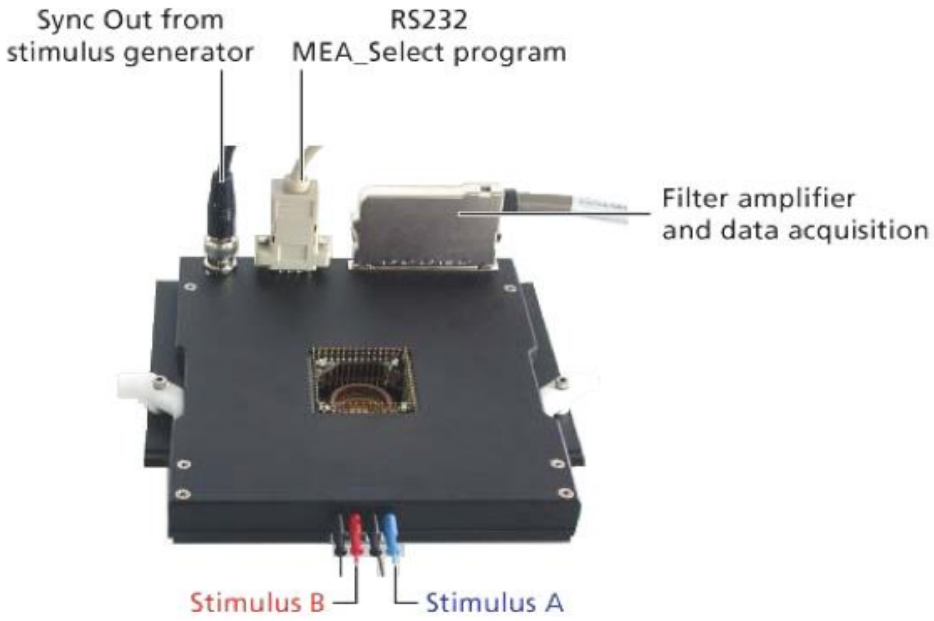

Figure 2.3: MEA 1060-Inv Amplifier. Reprinted from [34]

In our experiments we used a MEA1060-INV-BC acquisition system produced by Multichannel Systems (MCS, Reutlingen, Germany) [34]. We combined this system with 60-MEA-200/30-Ti MEA dishes produced by the same manufacturer. The characteristics of the MEA dishes and acquisition system that we present in the following are representative of other systems that are present on the market as well.

In the MEA1060-INV-BC acquisition system, raw signals typically comprising as many as 60 voltage channels, are amplified by a 60-channel pre-amplifier and band-pass filtered. The pre-amplifier gain is generally set to 1200 V/V while its bandwidth ranges between 10 Hz and 3 KHz. The MEA dish is placed directly into the MEA preamplifier with a blanking circuit (MEA1060-BC-PA). Contact pins are embedded in the amplifier lid and they are directly in contact with the MEA contact pads. Positioning the pre-amplifier close to the recording sites (electrodes) is important to keep the signal-to-noise-

ratio of the system as high as possible. The amplifier is connected to the data acquisition computer via a single standard 68-pin MCS scable. The analog output signals of the MEA amplifier are then acquired and digitized by a dedicated A/D card, referred to as MC_Card, as shown in Figure 2.4. Alternatively, a USB based data acquisition device (USB-MEA-System) or a custom data acquisition system can be used. The MC_Card and the acquisition software are also responsible for controlling the MEA stimulus generator (STG-1002), which is a configurable 2-channel pulse generator. A block diagram of the complete system is shown in Figure 2.4.

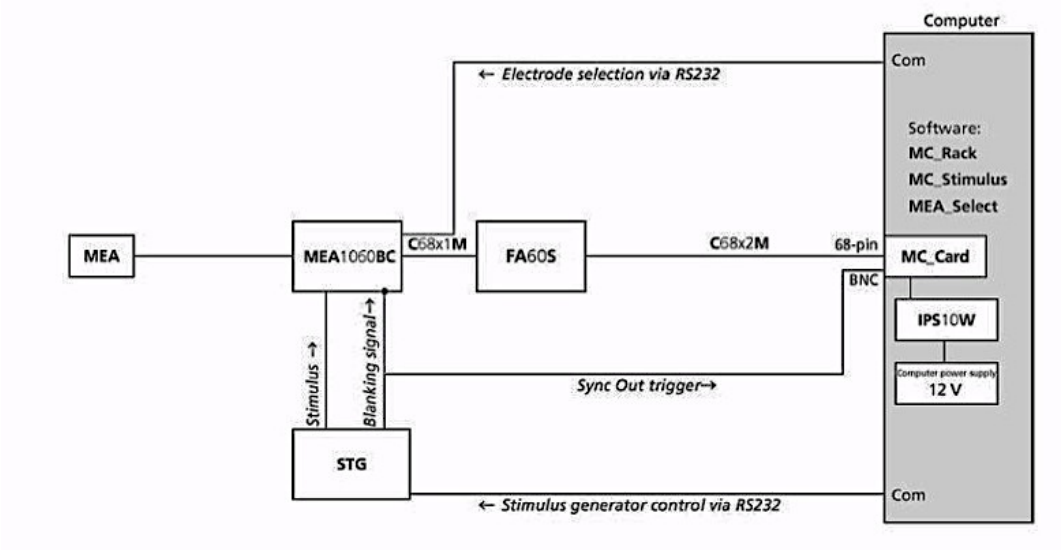

Figure 2.4: Block Diagram of a complete MEA Acquisition System. Reprinted from [34]

2.4 MEA Applications

Current MEA acquisition techniques show superior accessibility and flexibility compared to other electrophysiology models in terms of electrical recording and stimulation, pharmacological manipulation and imaging [49]. The long-term goal of investigators in this area is to use MEA technology to build a “brain-on-a-chip” that

would allow investigators to recreate various nervous system structures. These in-vitro systems would then be able to accurately mimic the behavior and characteristics of real brain structures. Possible applications include experimentation and assessment of novel treatments for brain disorders, evaluation of the effects of new drugs, and better understanding the connectivity, structure, and function of the brain [12],[53], [54].

From this perspective, MEA technology can be used to investigate how living neurons could interact with artificial systems with the goal of building hybrid systems where artificial and natural intelligence coexist. These hybrid systems could also be used to simulate and study different pathological situations or neurological disorders, such as epilepsy and stroke [12]. For instance, MEA technology can be used to investigate brain structures at the network level and to study the causes for most brain disorders such as Parkinson's disease, Alzheimer's and neuropathic pain. Furthermore, MEA technology can also represent a test bed for screening the effects of drugs during neuronal development. In this sense, MEA technology provides an electrophysiological platform that researchers can use to study the structural, biochemical or electrical events that take place in nervous system disorders.

This dissertation focuses in particular on combining MEAs with novel electrophysiology experimental paradigms and statistical data analysis to investigate the mechanisms that regulate brain development at the level of synaptic formation and growth cones. The goal is to use a mathematical approach and ad-hoc designed experiments to investigate whether dissociated neuronal networks can dependably display long and short-term plasticity. Such phenomena are thought to be the building blocks of memory formation in the brain.

2.5 MEA Recordings: Neural Spikes, Local Field Potentials and Spike Bursts

Neurons are electrical excitable cells that are the basic functional units of the nervous system. They are responsible for modulating and transferring information through electrical and chemical signals. From an electrophysiology perspective, the behavior of most types of neurons is defined as all-or-nothing, meaning that the electrical signal they use to communicate with each other only has two possible states, it is either on or off. These electrical signals are known as action potentials and they represent voltage variations generated by changes in ion concentrations between the extracellular and intracellular environment see Figure 2.5. Neural information is transferred every time a neuron receives an input that is above the threshold. More interestingly, neurons can

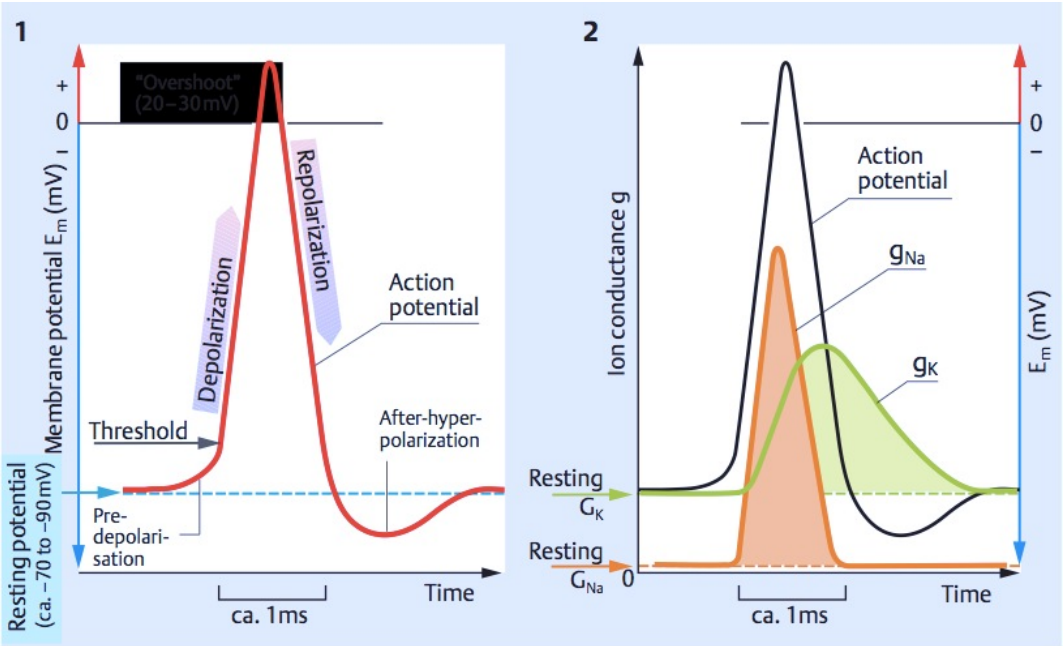

Figure 2.5: Action Potential Mechanisms. Reprinted from [87]

generate action potentials even if the single inputs they receive are not above the depolarization threshold. It is well known that neurons modulate and process information by temporal and spatial summation of stimuli. In other words, if the incoming stimuli are not able to generate an action potential individually, a neuron can sum all the incoming inputs spatially and temporally and if their summed depolarization is above the threshold then an action potential is generated. Figure 2.6 and Figure 2.7 respectively show the mechanisms of spatial and temporal summation. Specifically, the net influx of positive ions generates depolarization of the cell membrane. This event is also known as Excitatory Postsynaptic Potential (EPSP). A single EPSP is usually not enough to generate a postsynaptic action potential. In order to generate an action potential the simultaneous arrival of a large number of local depolarizations (temporal summation) in the *dendrites* is normally necessary. Furthermore, these multiple depolarizations are summed on the axon hillock, this phenomenon is known as spatial summation.

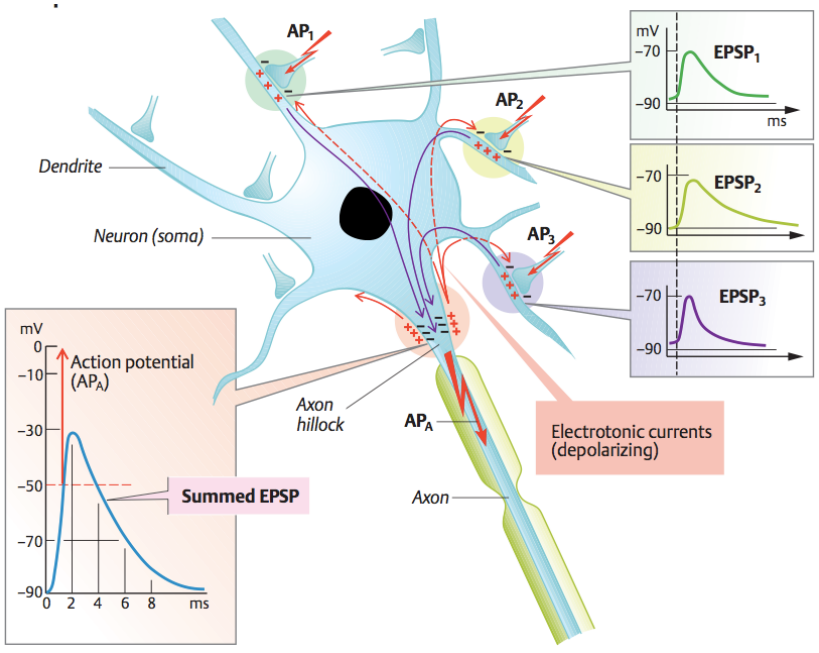

Figure 2.6: Spatial summation of neural inputs. Reprinted from [87]

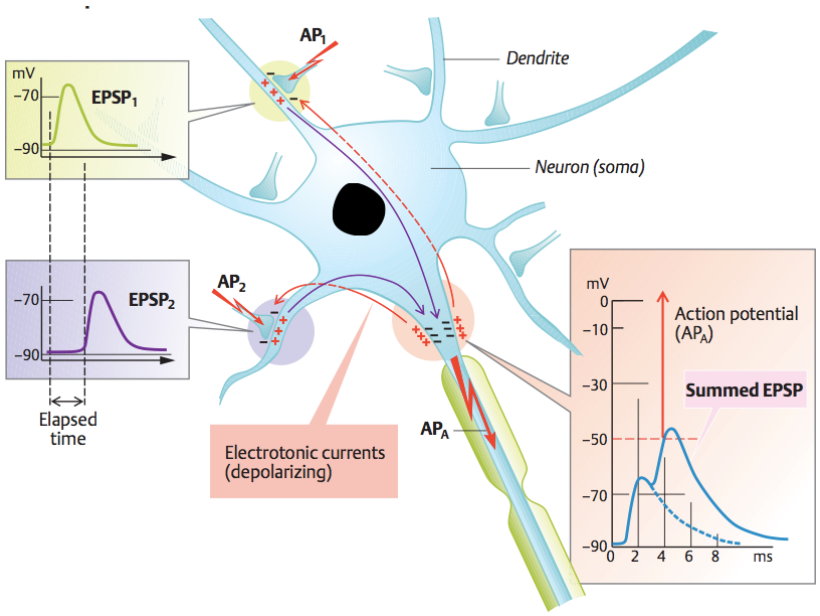

Figure 2.7: Temporal Summation of neural inputs. Reprinted from [87]

Over the past few decades several recording and stimulating techniques have been devised to investigate neuron electrical activity. These techniques range from single-cell intracellular recordings, such as patch-clamp approaches (see Figure 2.9), to multi-unit recording techniques, in which tiny electrodes are used to record the extracellular voltage variations. MEAs fall into the second category and utilize an electrode array to carry out simultaneous multi-unit extracellular recordings. When recording neural activity from MEA dishes, each electrode records the average extracellular field potential, namely the total electrical activity generated by the various (ranging from tens to hundreds) neural processes taking place in the extracellular environment that surrounds the electrode. An example of MEA device with dissociated neurons plated on is shown in Figure 2.10.

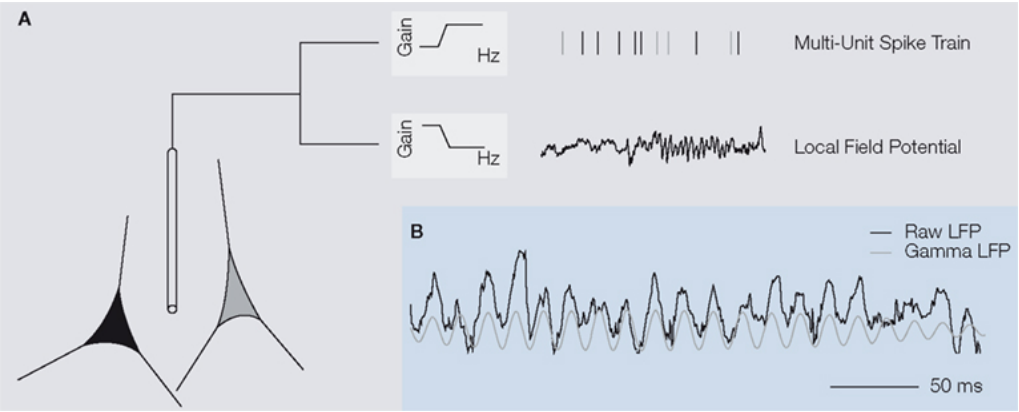

Figure 2.8: Example of Multi-Unit Spike Trains. Reprinted from [88]

Figure 2.8 shows an example of how multi-unit spike trains and Local Field Potentials (LFPs) can be extracted from continuous voltage recordings carried out from multiple neurons.

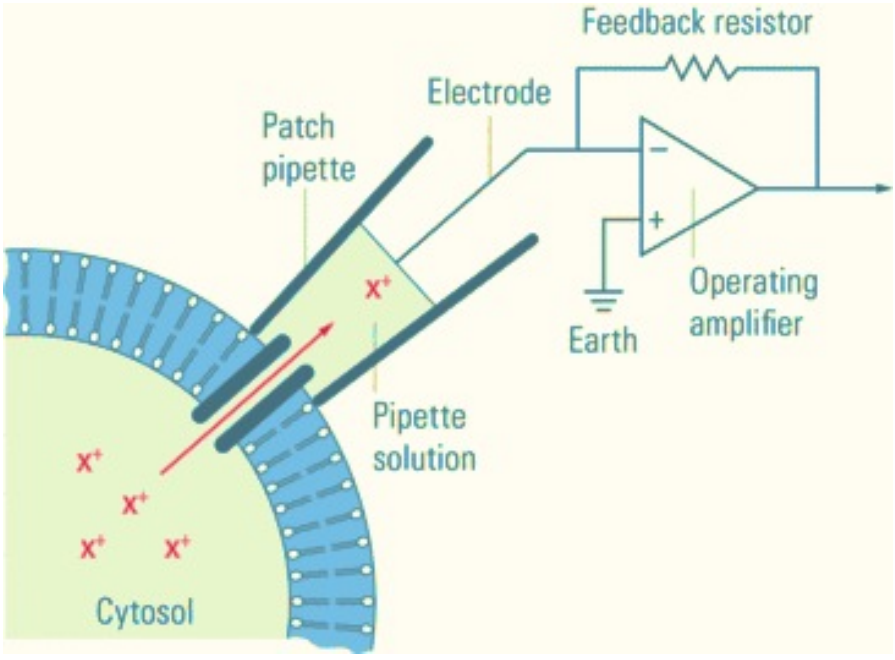

Figure 2.9: Patch-clamp technique. Reprinted from [89]

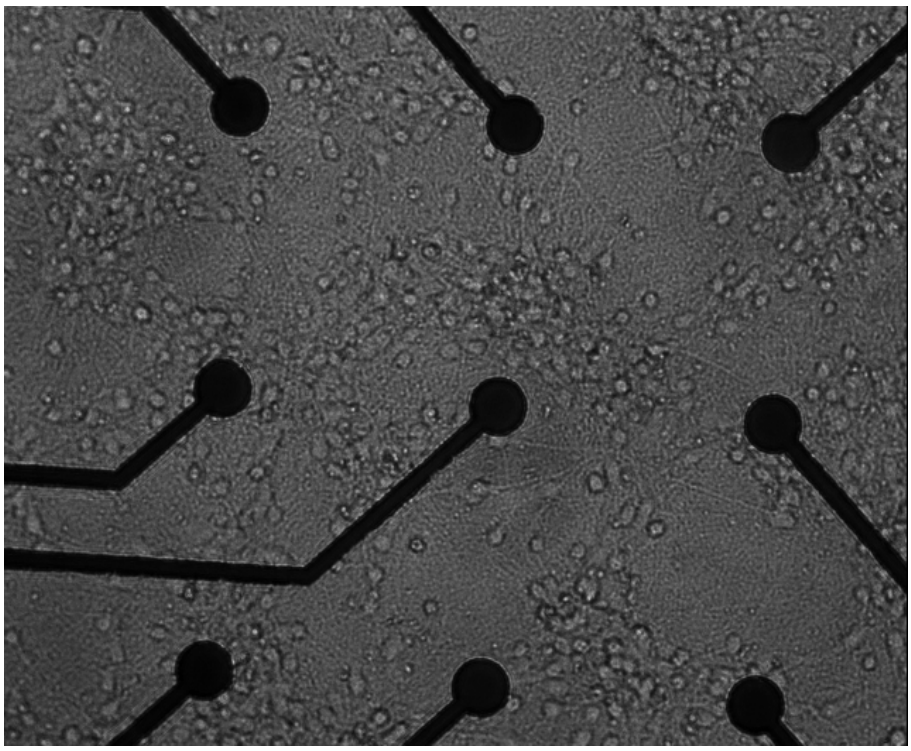

Figure 2.10: Example of extracellular multi-unit recording. MEA dish.

The neural extracellular potential is usually considered to be composed of two main components (Figure 2.8): 1) High frequency voltage fluctuations, ranging between 300 Hz and 3 kHz, usually called neural spikes; 2) Low frequency voltage fluctuations, less than 200 Hz, generally referred to as Local Field Potentials (LFPs). These two components have usually been considered related to different aspects of neural activity, but the full extent of the link between them and the underlying brain activity remains yet to be investigated. [55] Figure 2.11 shows an example of neural spikes and LFPs and how these can be derived from raw MEA recordings, simply by bandpass-filtering the raw data.

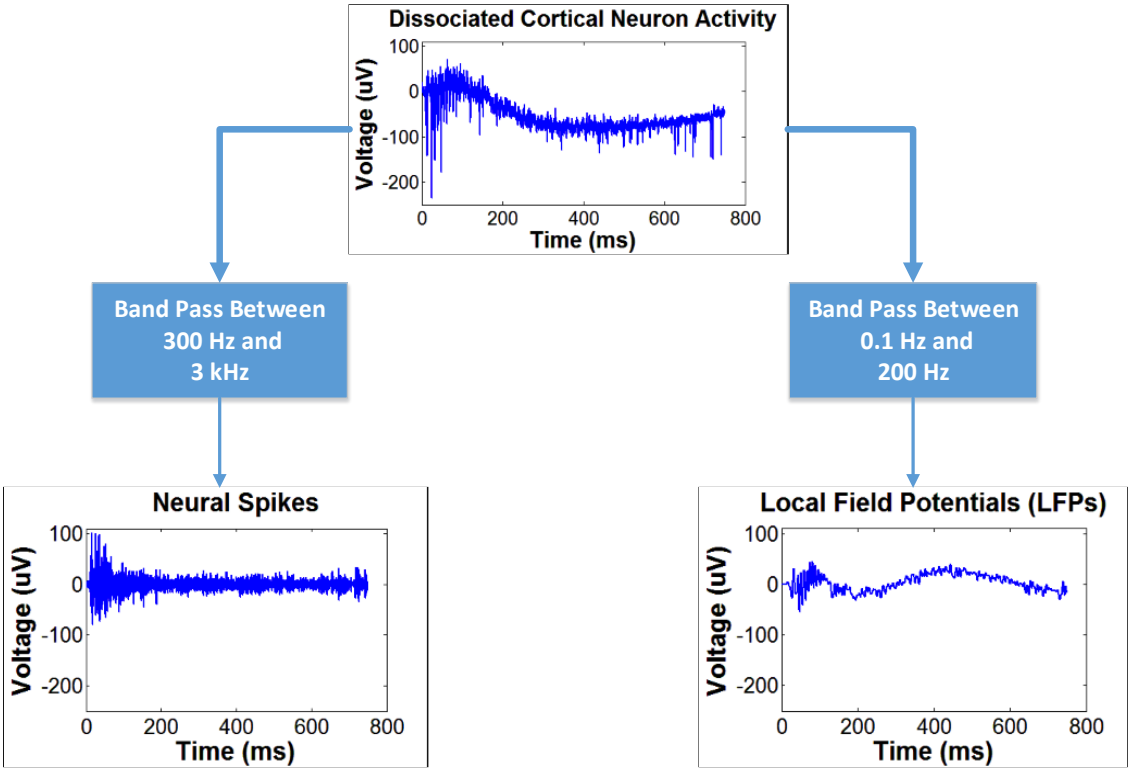

Figure 2.11: The two main components of MEA recordings: Neural Spikes (bottom left panel) and Local Field Potentials (bottom right panel)

Another important characteristic of MEA recordings is the bursting activity that dissociated cultured neurons display in-vitro. Although in the literature there are several definitions for such bursts, in this work we define an activity burst as an activity pattern consisting of a train of high frequency spikes, usually involving most active channels of a MEA dish. It is well-documented [14],[38] that these bursting activity patterns begin to appear a few days after plating the neurons and continue to develop over the course of in-vitro neuronal growth and are considered a natural (normal) characteristic of dissociated neuronal networks. Interestingly, during in-vitro development, the vast majority of the cultures respond to delivery of electrical stimuli by significantly increasing the bursting activity. A widespread stabilization of electrical activity is usually observed after the culture's third week of age.

2.5.1 Neural Spikes

Typically, a neuron consists of the soma or cell body and two types of processes: the axon and *dendrites*. The neuron receives afferent signals, which could be either excitatory or inhibitory, from up to a few thousand other neurons via its *dendrites* and sums the signals along the cell membrane of the soma. The axon stems from the axon hillock of the soma and it is along this process that the transmission of efferent neural signals occurs. *Axons* often have branches that further divide and terminate in multiple swellings called synaptic buttons. A synapse is the site where the axon of a neuron communicates with other neurons. With very few exceptions, synaptic transmission in mammals is mediated by chemicals (i.e. neurotransmitter release), not by electrical signals.

Neurons form large networks (ranging from thousands to millions of cells) in the nervous system and use action potentials, also called neural spikes to communicate with each other and process information. Recording individual spikes can be technical challenging, especially when trying to record activity from large neuron populations. In practice, in order to be able to record single spikes, it is typically necessary that the recording electrode size be comparable in size to the neuron being recorded. This implies that such electrodes will not be able to record activity from many other cells, meaning that the number of neurons that can simultaneously be observed is limited by the number of electrodes. On the contrary, if the goal is to record from a large population of neurons, the recording electrode size will usually be larger. This implies that a single electrode is capable of recording neural activity from tens or hundreds of cells simultaneously. Each MEA electrode is designed to record extracellular activity as generated by multiple neurons. In fact, MEA electrodes are typically laid out in a planar 8x8 grid (with missing

corners); further details and dimensions of standard MEA dishes are shown in Figure 2.13. Briefly, the electrode size is 30 µm in diameter and they are spaced by 200 µm. In comparison, the typical neuron soma is in the range of 4-100 µm in diameter. Therefore, given its size, the MEA electrode can record activity generated by tens or hundreds of cells, and hence these types of recordings are also called multi-unit recording (MU) and usually reflect the spatially averaged activity of local neural populations. Figure 2.12 shows a typical MEA experimental setup.

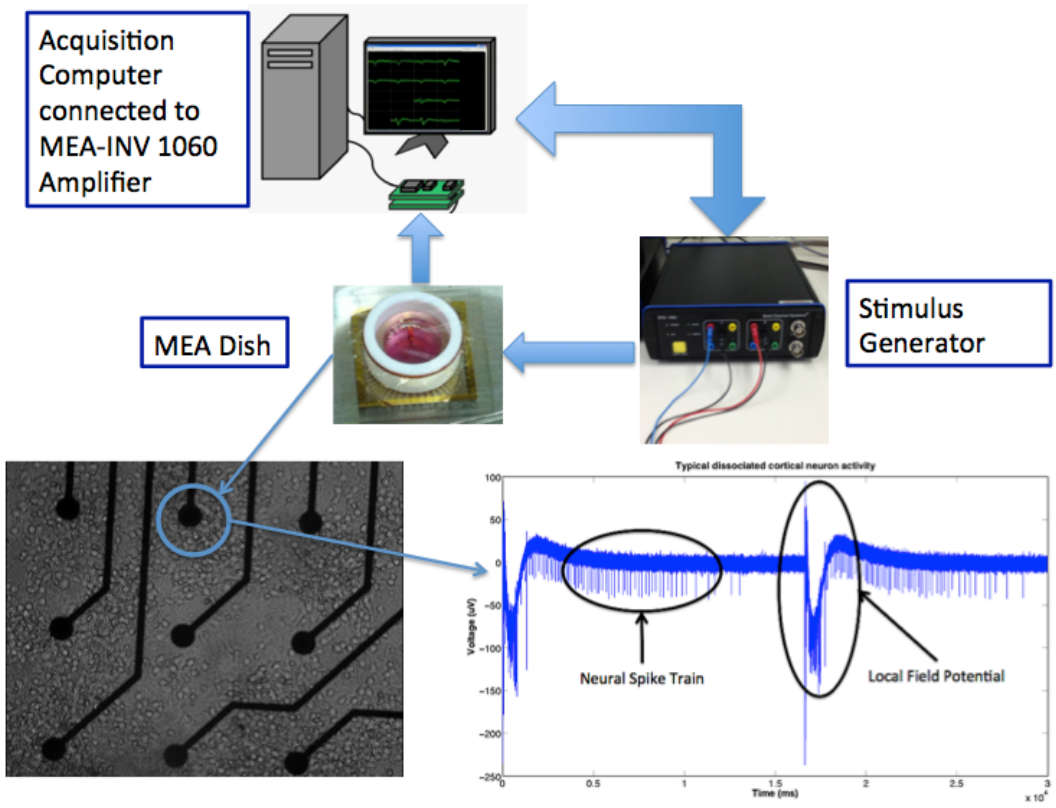

Figure 2.12: Example of MEA signal recording

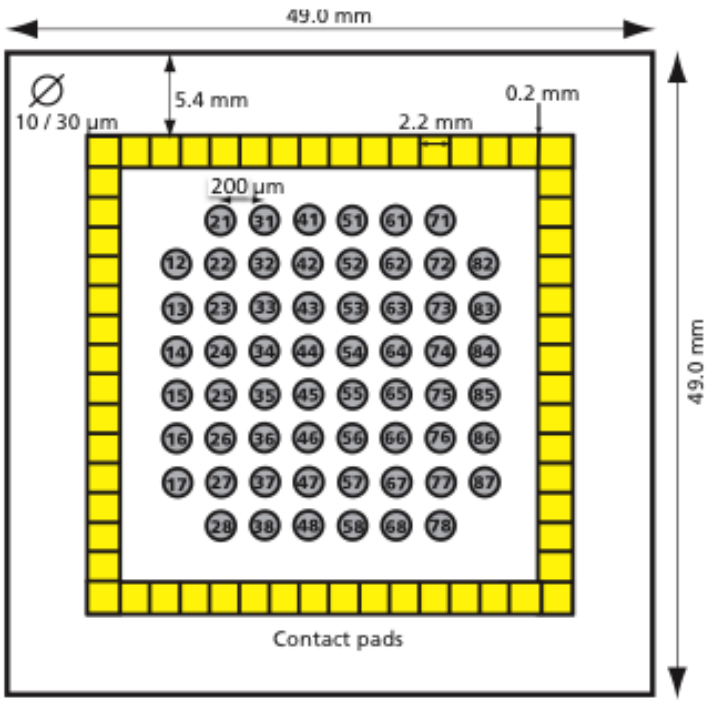

Figure 2.13: Standard Electrode Grid 8x8 and dimensions.
Reprinted from [34]

Over the years, several signal processing techniques have been used to detect spikes and isolate them from other neural signal components. These spike detection techniques can be based on simple voltage thresholds or they can be based on more complex processing based on template matching or adaptive signal processing techniques. Typically, spike detection algorithms use some kind of signal preprocessing to enhance spikes and reduce noise before using a threshold to detect spikes. The threshold can be static or dynamic, according to the specific application requirements. An alternative approach consists of detecting spikes using their specific waveforms to train template-matching algorithms. Examples of threshold spike detectors are presented in [56], [57], while examples of template-matching spike detectors are presented in [51], [58].

2.5.2 Local Field Potentials (LFPs)

Local Field Potentials (LFPs) are commonly related to the inputs of a neural ensemble and its processes within the neuronal network. In this view, LFPs are primarily associated with synchronized synaptic signals, sub-threshold membrane oscillations and spike after-potentials. Understanding LFPs is important because there are some studies suggesting that LFPs could be responsible for some very specific aspects of neural signal modulation. LFPs have different characteristics depending on which brain region they are recorded from. In the hippocampus, for example, LFPs are usually characterized by strong theta oscillations, between 4 and 10 Hz. These oscillations are believed to be critical for temporal coding of information and synaptic plasticity in the hippocampus region. In most applications, as well as in clinical studies, LFPs are usually divided into different frequency components. These are the same oscillations that are associated with EEG recordings: delta (< 4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), gamma (30-80 Hz) and high gamma (> 80 Hz).

In common traces of quiescent networks, most of the signal power is found at low frequencies, indicating that rhythms like delta and theta are predominant. Studies have shown that when a network is stimulated, gamma band oscillations are enhanced and activity at lower frequencies is suppressed [59]. On the contrary, increased power in the gamma band means that the neural network is engaged in some kind of processing activity. At a given recording site, gamma rhythms are stronger for some stimuli than others, generally displaying selectivity and a preference similar to that of nearby neuronal spiking activity. For instance, in higher cortex, gamma power is prominent during working memory and learning. Interestingly, irregular gamma activity has been observed

in neurological disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia, and epilepsy [60].

The main motivation for measuring LFPs is that they provide a measure of local neuron population activity, which, despite being less direct than neural spikes, is nonetheless practical for studying the overall behavior of select brain region [61][62]. This information is complementary to that provided by action potentials since it relates to events that eventually might lead to the generation of action potentials, but may not clearly manifest in action potential patterns in cases where excitatory inputs are subthreshold or offset by concurrent inhibition.

It is worth noting that extracellular recordings are bipolar recordings, that is signals are measured as the voltage difference that occurs between two electrodes. One of the electrodes is usually called the active or recording electrode and it is placed in proximity of the neurons from which we want to measure action potentials from. The other electrode is commonly called the reference or ground electrode, and as its name suggests it is used as a voltage reference level. MEA dishes can have different ground electrode configurations, but the most common experimental choice is to use as reference and ground electrode a specific electrode on the dish that is hundreds of time larger than the recording electrodes. Such a setup has a relevant flaw when recording LFPs, because of the fact that using a distant reference electrode prevents the investigators from having accurate information on the activity source location. Attempts to provide a general solution to this problem have not been successful, because, as discussed above, the factors that impact LFP recordings, both physiological (e.g., strength, spatial extent and symmetry of activation in the neuronal substrate), and technical (e.g., electrode

characteristics and reference site) are difficult to assess. In this view, all that can be said with certainty for MEA recordings, is that the source of the LFP is generated somewhere in the conductive medium. This implies that identifying the sources of activity becomes even more challenging, because of volume conduction effects.

2.5.3 Bursting Activity

Over the past decade, one of the most investigated phenomena in MEA applications is the bursting activity of dissociated neuronal networks. These bursts are defined as brief periods during which the spike rate of many cells or electrodes exceeds the baseline rate several fold. Previous studies of neural development in-vitro all agreed that population bursts are a major component of cultures' activity patterns. Burst activity is a fundamental characteristic of the developing brain and plays an important role in establishing appropriate connections. This phenomenon represents the exploring behavior of in-vitro systems due to the lack of natural neural input [63].

Figure 2.15 shows an example of MEA recordings displaying synchronized activity on most electrodes. These activity bursts have been investigated in several studies [10], [14], [38], [39], [63]–[66], and they have been mainly associated with the lack of external inputs in MEA neuronal preparations. Given their regular presence in MEA experiments, they are often used to measure the network developmental stage and maturation. The reasons why this bursting activity is such a predominant feature of in-vitro brain preparations and not of in-vivo studies is not yet understood. Therefore, such activity patterns are important parameters to be investigated and analyzed. Indeed, these periodic bursts represent a relatively stable activity characteristic in networks of cortical neurons. They offer the opportunity to study basic mechanisms of network firing, such as how

synchronization is achieved and how connectivity determines patterns of activity in the neural population during different stages of in-vitro development.

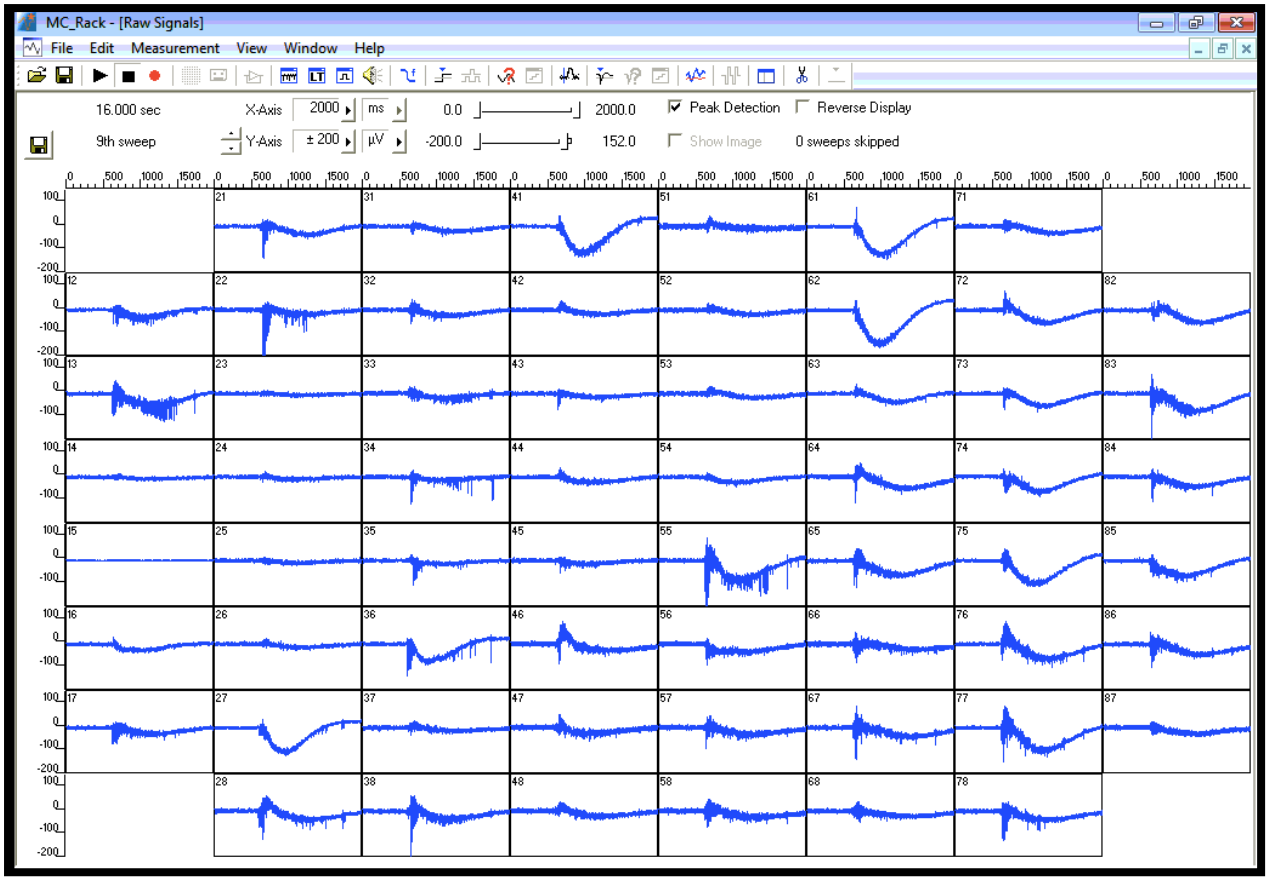

Figure 2.14: Example of MEA recordings showing synchronized activity on most channels.

CHAPTER 3

QUANTIFYING DISSOCIATED NEURONAL NETWORK TEMPORAL EVOLUTION

3.1 Introduction

Although hybrid neural-electrical circuits have been demonstrated, their functionality is inherently limited when the neuronal network is treated as a black box. An understanding of how dissociated neuronal networks evolve with respect to specific stimuli (or lack thereof) will lead to hybrid systems with greater functionality and robustness. Our long-term goal is to understand how these systems respond and evolve when presented with external stimulation. More in detail, we want to investigate how and why dissociated cortical neurons vary their electrical activity over time [17], [67], [68]. Such an investigation requires new and appropriate mathematical tool to provide us with better approaches to quantify functional activity evolution in dissociated cortical networks.

In the literature, there is a general lack of adequate statistical tools for processing and quantifying large spike-based data sets. [21] This deficit hinders investigators' ability to identify significant changes in network connectivity amid populations of weakly tuned

neurons with high spontaneous activity. As a result, the ultimate goal of exploring the relationship between neural circuit topology and behavior is compromised. Existing tools such as activity task neuroimaging are insufficiently sensitive both temporally and spatially [18]. Although various approaches have been presented for analyzing spike behavior in MEA recordings, these methods have tended to focus on raw statistical correlations without necessarily yielding meaningful insights into physiological network topology. For instance, in [69] the authors use Hidden Markov Models (HMMs) to estimate the number of states the neurons in the network can have. The authors assumed that neuronal networks only adopt three different firing patterns. This simplification was necessary to implement the HHM technique, but at the same time such an approach fails to capture the high variability and variety of neuronal network electrical responses.

Others [70] have proposed to use dynamic Bayesian networks to discover excitatory relationships in MEA recordings. In this work the authors tested a computer algorithm capable of emphasizing the excitatory statistical connections in discrete-time networks. Their main assumption is that in the network only excitatory connections are important, while inhibitory connections are neglected. With respect to neuronal networks, such an assumption cannot be considered valid, thus their mathematical approach cannot capture the full complexity of live neuron interactions. In both of these studies, the authors realize the importance of applying statistical techniques to identify sequences of firing neurons and find the functional network connectivity. However, despite the recognition of the relevance of statistical methods, there is a lack of literature investigating the physiological aspects of neuronal development [49], [50].

It is proposed here to use a well-known statistical technique that has been proven successful in separating the non-null from null cases in multiple hypothesis testing. For the first time, this work will statistically quantify the temporal dynamics of dissociated cultured neuronal networks, without simplifying the underlying biological model. This is achieved by applying the False Discovery Rate (FDR) statistical analysis technique to MEA recordings and using its results to quantify biological and electro-physiological properties of dissociated neuronal networks during their first five weeks in-vitro. FDR identifies significant stimulus-response pairs among the numerous spontaneous spikes from the cultured neurons. Moreover, the FDR technique has been proven to be a valuable tool to overcome the traditional issues in multiple hypotheses testing problems, namely controlling the probability of erroneously rejecting even one of the true null hypotheses, otherwise known as the family wise error-rate (FWE) [71]. This allowed an investigation of the temporal evolution of cultured neural networks as they are presented with electrical stimulation during early development.

3.2 Methods

3.2.1 Data Collection

The statistical analyses presented here were performed on neural spike data made available by Dr. Steve Potter in the Laboratory for Neuroengineering at Georgia Institute of Technology and Emory University School of Medicine. They comprise a series of MEA recordings from cultures of dissociated rat cortical neurons with bursting activity

patterns, recorded over the first five weeks of their in-vitro development. Details of the cell culture methodology and electrophysiology can be found in [14].

3.2.2 Signal Processing

To investigate network changes, we analyzed a large MEA data set composed of neuron spikes recorded from cultures of dissociated rat cortical neurons plated on MEA dishes with 59 recording electrodes each. There were 15 high-density high-volume ("dense") cultures, as well as 7 high-density small-volume ("small") and 6 low-density high-volume ("sparse") ones. The culture density was chosen when plating the dissociated cortical neurons onto the MEAs, as described in [14]. Further details on different plating densities can be found in Table 1. Some neuron cultures were dissociated from the same original brain tissue; such cultures were defined as belonging to the same "batch" of brain tissue. The number of neuronal cultures dissociated from each batch is shown in Table 1.

Table 1: Information on different densities of dissociated cortical neurons

	DENSE	SMALL	SPARSE
Plating Volume (μL)	20	5	20
Density of Suspension (cells/μL)	2500	2500	625
Nominal Number of Plated Cells	50000	12500	12500
Number of Studied Cultures	15	7	6
Number of Studied Batches	6	2	2

Neural network activity was recorded during the first five weeks of each culture's in-vitro development. During this period, stimulation sessions (typically occurring daily) were comprised of 50 electrical stimulus pulses delivered to each of the 59 electrodes.

These stimuli were delivered sequentially to every electrode on the MEA, once every 300 ms while neural responses were recorded from all other electrodes. Although it is well-known that neuronal network responses elicited during such stimulation sessions are complex and may last longer than 300 ms [72], [73], we focused on network responses that occurred within selected time window durations, namely 50 ms, 100 ms and 150 ms after stimulus onset. This allowed us to account for three specific components of network responses known as 1) the network "direct responses" to stimulation, that are those occurring between 0 and 20 ms after stimulus (50 ms windows); 2) the "early post-synaptic spikes", occurring 5–1000 ms after stimulus presentation (100 ms windows); 3) "Culture-wide barrages", occurring at latencies greater than 100 ms (150 ms windows). These responses are thought to be the most representative of the stimulation effects The stimulus-evoked spike count was normalized by subtracting the average spontaneous spike count averaged over the chosen time window. The spontaneous spikes were recorded on the same experimental day as the stimulus-evoked spikes, from the same neural network. This technique allowed us to account for the natural variability in neuron firing activity that occurs as a result of axonal growth and network changes over time. The same stimulation protocol was delivered to every culture. [14]

Each culture yielded a 59x59x50 data matrix (stimulated electrodes x recording electrodes x number of trials) of normalized spike counts on each day. We then averaged across trials to produce a 59x59 matrix of stimulus-response pairs (Z_{kj}) per day per culture. These matrices were then interpreted for statistical significance (see Section "Statistical Analysis"). Only those stimulus-response pairs determined to be statistically significant were used in the subsequent quantitative connectivity analysis.

In order to be able to quantify changes in the connectivity graphs with respect to time, we used two measures per experimental day: the average length of significant pairwise stimulus-response connections and the number of connections that every node displays. The former is a measure of how physically far the neurons can extend their connectivity pathways. The latter is a measure of how many significant connections every node can either generate or receive. In other words, this is a measure of how many significant hubs the network displays on any specific experimental day. We defined "supernodes" to be those nodes that display at least three significant connections, either incoming or outgoing. The existence of supernodes is consistent with the notion that biological networks tend to form 'small-world' networks, as previously showed in [74]. It is worth noting that the MEA electrode grid used in this work is directly connected to the underlying neuron network. However, given the limited number of electrodes and their size and spacing, this electrode grid cannot capture the full extent and complexity of the actual neuron connectivity. Consequently, every electrode (or node) is actually simultaneously recording from (and stimulating) multiple neurons (ranging from tens to hundreds). Considering the high neuronal connectivity, a single stimulus pulse is therefore potentially able to induce stimulus-evoked responses across the whole network either directly or through one or more synapses. As a result, when we identify connections and connectivity graphs, we are actually measuring connections between electrodes (nodes) and not single cells. Although it is not easy to quantify the exact number of neurons involved, it is reasonable to assume that each supernode connection comprises a number of main neurons ranging between 80 and 200. This can be seen in Figure 3.1, where a typical network of dissociated neurons plated on MEA is shown.

Every electrode is in direct contact with multiple neurons and how these neurons have multiple connections with several neurons forming large connectivity clusters.

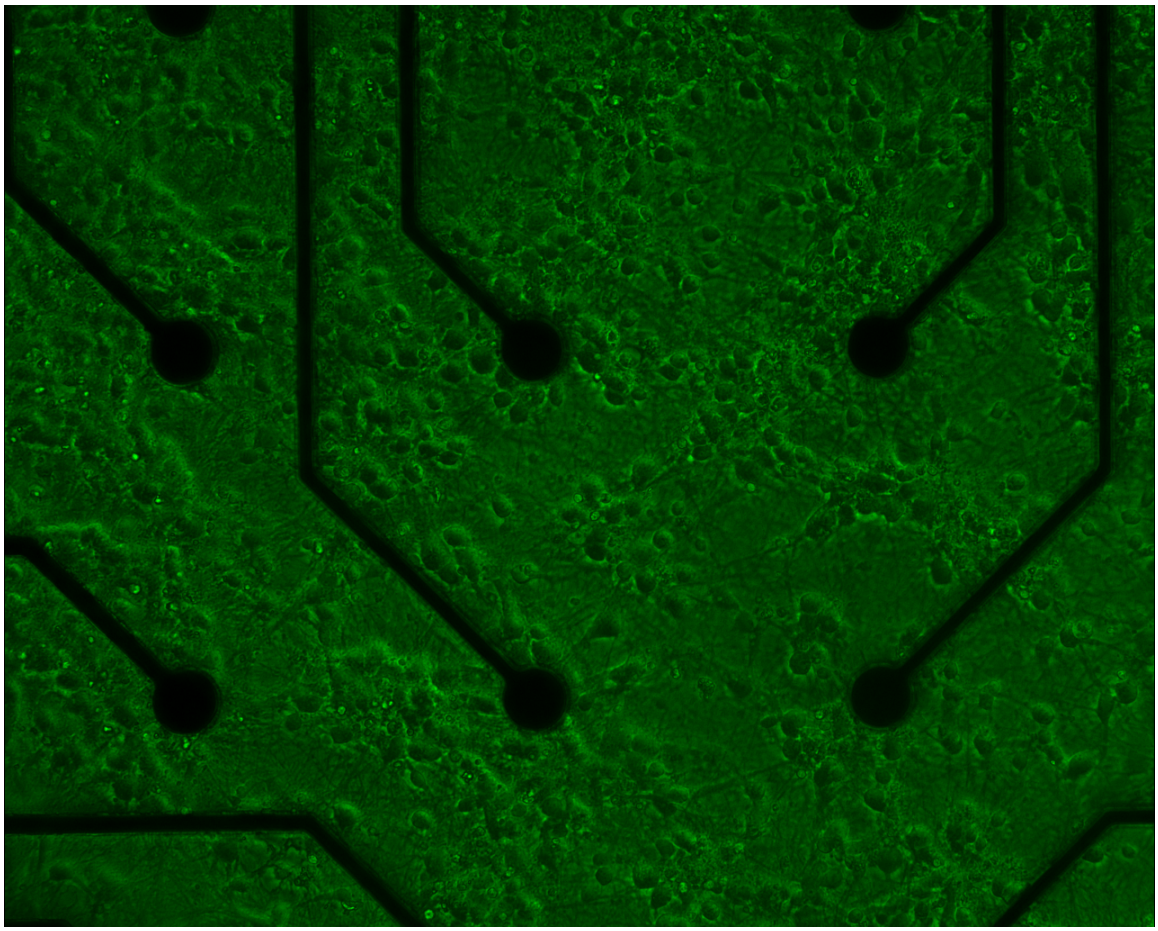

Figure 3.1: Multiple connections of dissociated cortical neurons plated on MEA.

3.2.3 Statistical Analysis

In order to identify statistically significant stimulus-response pairs, we implemented the False Discovery Rate (FDR) analysis technique. The FDR technique is a multiple hypothesis testing procedure whose objective is to control the expected proportion of incorrectly rejected null hypotheses, as shown by the following equations. We chose to use FDR because it has been proven to be effective when testing multiple hypotheses [71]

in high dimensionality data sets. In our case the null hypothesis is that a given stimulus-response pair is not statistically significant. We applied the FDR to the average number of evoked spikes relative to the average number of spikes recorded when no stimulation was delivered, (Z_{kj}) as shown in Figure 3.2.

3.2.4 Statistical Significance Test: FDR

The False Discovery Rate is defined as:

$$FDR = E\left[\frac{V}{R}\right]$$

where:

- V is the number of false discoveries
- R is the total number of discoveries

FDR procedures are designed to control the expected proportion of incorrectly rejected null hypotheses, also called false discoveries V. In this work we chose $\text{FDR} = 5\%$. The null hypothesis was defined as:

$H\{0,kj\}$: While stimulating electrode k, electrode j does not respond.

Therefore, the FDR guarantees that no more than 5% of the stimulus-response pairs identified as being significant will actually be insignificant. The FDR was applied to each of the 59x59 elements of the matrix Z_{kj} (stimulus-response activity pairs, normalized by the network spontaneous activity) recorded for every experimental session and for every culture. The implemented mathematical analysis is shown in Figure 3.2.

Mathematically, the FDR technique defines the two hypotheses as follows:

$H_{\{0,kj\}} : Z_{kj} \sim N(\mu_0=0, \sigma^2) = f_0(Z_{kj})$
$H_{\{1,kj\}} : Z_{kj} \sim f_1(Z_{kj})$

The statistic T_{kj}, referred to as "local FDR" is then defined as:

$$T_{kj} = \frac{(1-\epsilon)f_0(Z_{kj})}{\epsilon\, f_1(Z_{kj}) + (1-\epsilon)f_0(Z_{kj})} = \frac{(1-\epsilon)f_0(Z_{kj})}{f(Z_{kj})}$$

In other words, the local FDR function quantifies the relative likelihood of H0; values of T_{kj} close to 1 indicate a high likelihood of H0 whereas values closer to 0 indicate a low likelihood. In other words, the local FDR function is a measure of how similar the two distributions $f_0(Z_{kj})$ and $f(Z_{kj})$ are, where $f_0(Z_{kj})$ is the null distribution density function and $f(Z_{kj})$ is the alternative distribution function [75]. The parameter ϵ is called the non-

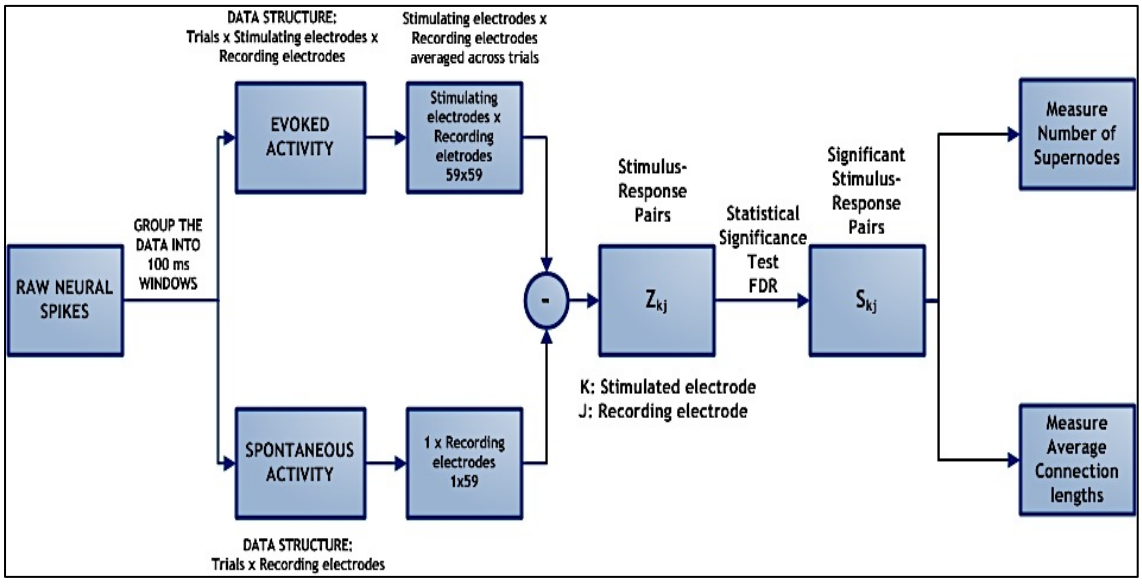

Figure 3.2: Block Diagram of the Implemented Neuronal Spike Statistical Analysis. The raw neural spikes are divided into two groups, evoked activity and spontaneous activity, respectively stimulated and non-stimulated experimental sessions. Then the raw spikes were divided into 100 ms time windows (100 ms after stimulus onset for evoked spikes) and averaged across repetitions (time windows). The average spontaneous activity was subtracted from the average evoked activity and fed into the False discovery Rate (FDR) statistical analysis technique. The output of the FDR is the significant stimulus-response pairs. Using these significant pairs we computed their average connection lengths and the number of supernodes.

null proportion [71], [75] and represents the number of expected significant stimulus-response pairs. In our analysis we expect ϵ to always assume small values because we expect the number of significant connections to be lower than the number of possible pairwise connections in the network. The value of the parameter ϵ is estimated from the data before computing the local FDR functions [71]. If T_{kj} is close to 1, then the two distributions defined in the hypotheses are similar and the null hypothesis is selected. This indicates no significant relation between the stimulus, delivered to electrode k, and the neural response recorded at electrode j.

Next, the 59x59 T_{kj}'s are ranked from the smallest to the largest. The ordered local FDR functions are called $T_1, \ldots, T_p$ where p = 59x59. Significant local FDR functions are therefore T_i, for $i \leq k$, such that:

$$k = \max\left(I: \frac{\sum_{I=1}^{k} T_I}{k}\right) \leq FDR$$

This technique therefore guarantees that the average false positive rate over all significant stimulus-response pairs will be less than 5%.

3.3 Results

Figure 3.3 shows typical connectivity graphs for two different cultures harvested from different brain tissues, on three separate days. Each red arrow indicates a statistically significant connection between a stimulated electrode and a recording one, as identified by the FDR analysis. Figure 3.3 suggests that neuronal connectivity tends to evolve over time, with increases in both the number of statistically significant stimulus/recording

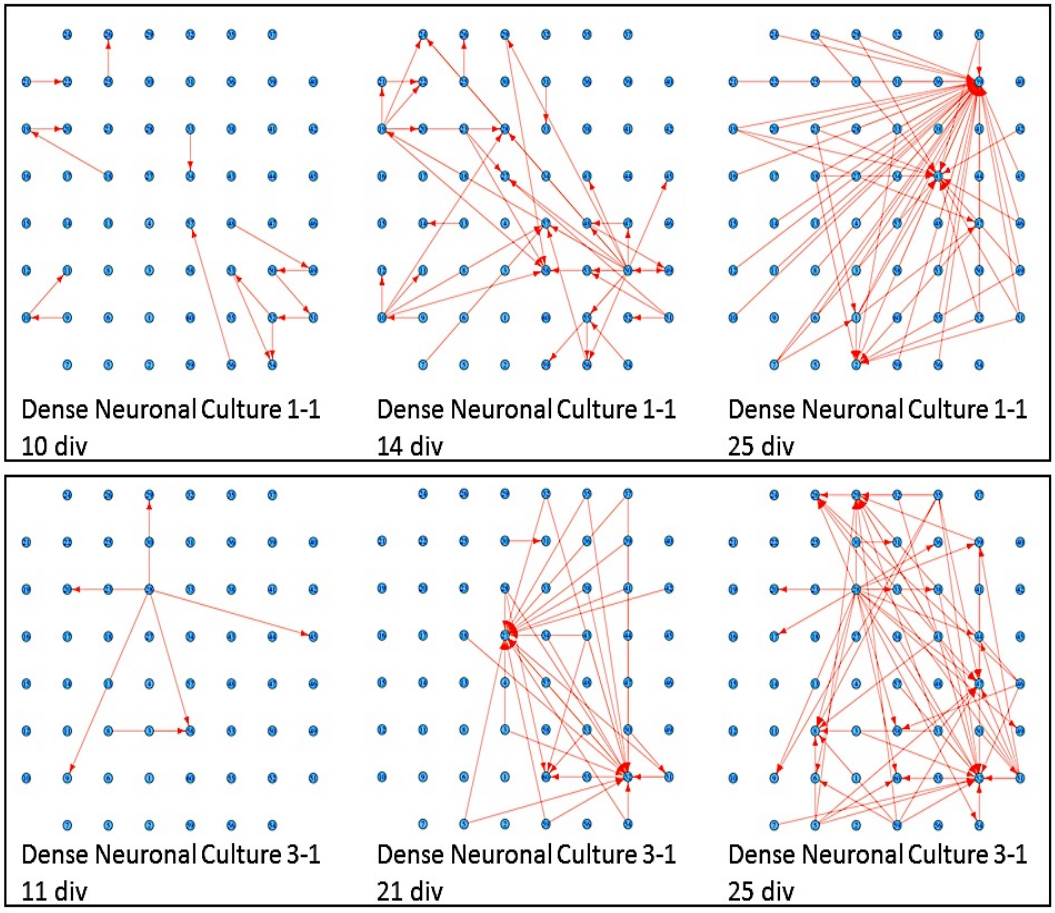

Figure 3.3: Connectivity Graphs for dense neuronal cultures on different days after plating. The top Panel shows results for culture 1-1, on days in-vitro (div) 10, 14 and 25. The bottom panel shows results for culture 3-1, on days in-vitro 11, 21 and 25.

pairs as well as the average length of connections and the number of connections per active node.

In order to better analyze the changes in electrical activity versus time (and among different plating densities and neuron batches), we averaged the connection lengths and the supernode counts across cultures harvested from the same batch. The resulting graphs are shown in Figure 3.4, Figure 3.6 and Figure 3.5 where average connection distances and average supernode counts are shown respectively for dense, small and sparse cultures when a 150 ms time windows was chosen to record network activity. For every cell density, the number of cultures that we used to compute the average within batches is

different and it is indicated in the figures with ***n***. Due to the fact that recordings were not performed every day, we used a dotted blue line to indicate missing experimental days while a solid black line was used to plot the actual data sample means. The red error bars indicate the standard errors obtained when averaging cultures derived from the same batch. From these graphs, it is noticeable how the average connectivity pair lengths increase over time and then reach a plateau, following the expected network temporal evolution. Moreover, this behavior is observed to be consistent across cultures of different densities and across different time window durations (data not shown). An increase in average connection length means that stimulus-evoked responses are recorded from electrodes that are physically further from the stimulated electrode; evoked electrical activity is propagated more easily in the dish and for longer distances. The functional evolution in the studied neuronal networks seems to perfectly reflect the natural temporal evolution of neural circuit formation. In fact, neural circuit formation occurs in three distinct stages: 1) Immature synapses form between *axons* and *dendrites*. 2) Synapses undergo maturation, which involves the conversion of silent synapses to active ones. 3) Excess synapses are eliminated or pruned to refine the neuronal connections within the circuit [19].

Figure 3.4, Figure 3.6 and Figure 3.5 reveal consistent neural development within batches, with more variable trends across batches. To quantify this, we computed the statistical significance of changes for both connection lengths and number of supernodes within and across batches. To test the statistical significance of the average changes we performed a one-way ANOVA test within batches and across batches.

Our results with respect to 50 ms and 150 ms windows suggest that the connection length variability within batches is not statistically significant ($p \geq 0.05$). On the contrary, cross-batch variability was statistically significant for connection lengths (*p* values for 50 ms and 150 ms windows were *respectively p50 = 0.0207* and *p150 = 0.0107*). No significant variations were observed in both incoming (*p50 = 0.357* and *p150 = 0.204*) and outgoing (*p50 = 0.295* and *p150 = 0.992*) supernode counts. In contrast, variations observed within and across batches for a 100 ms observation window tested non-significant with p values for mean connection lengths, mean incoming and outgoing supernodes respectively equal to $p_{length} = 0.673$, $p_{incoming} = 0.357$ and $p_{outgoing} = 0.295$. We hypothesize that the observed variability in the analysis results is mainly due to the fact that when using different time window durations, one might or might not include in the analysis some of the phases of the network stimulus-evoked responses. In this view, our statistical analysis emphasizes the importance of the time window length selection and that common values that have been commonly utilized in previous works, such as 100 ms windows, might not yield optimal results.

Furthermore, we tested the effects that different time windows had on the analysis results and whether the time window related changes were statistically significant. So we performed a one-way ANOVA test on the average connection lengths computed for the six dense batches in each time window. Then we performed an ANOVA multiple comparison test to identify which group means were statistically different from each other. The results of the multiple comparison test are shown in Figure 3.7, in which, the three group means and their 95% confidence intervals for every dense batch are shown. In batch 1 and batch 3, there are group means significantly different from the others. In

batch 1 the second group mean (100 ms window) is significantly different from the other two group means, while in batch 3 is the third group mean to be significantly different from the others. It can be seen how differences between results generated using different time windows are significant in batch 1, where the 100 ms group mean is significantly different from the other two ($p = 7.051 \times 10^{-4}$) and in batch 3 where 150 ms group mean is significantly different ($p = 8.126 \times 10^{-6}$). This implies that different time windows generate significantly different results in two batches out of six. On the contrary, differences between the three group means are non-significant for the remaining batches.

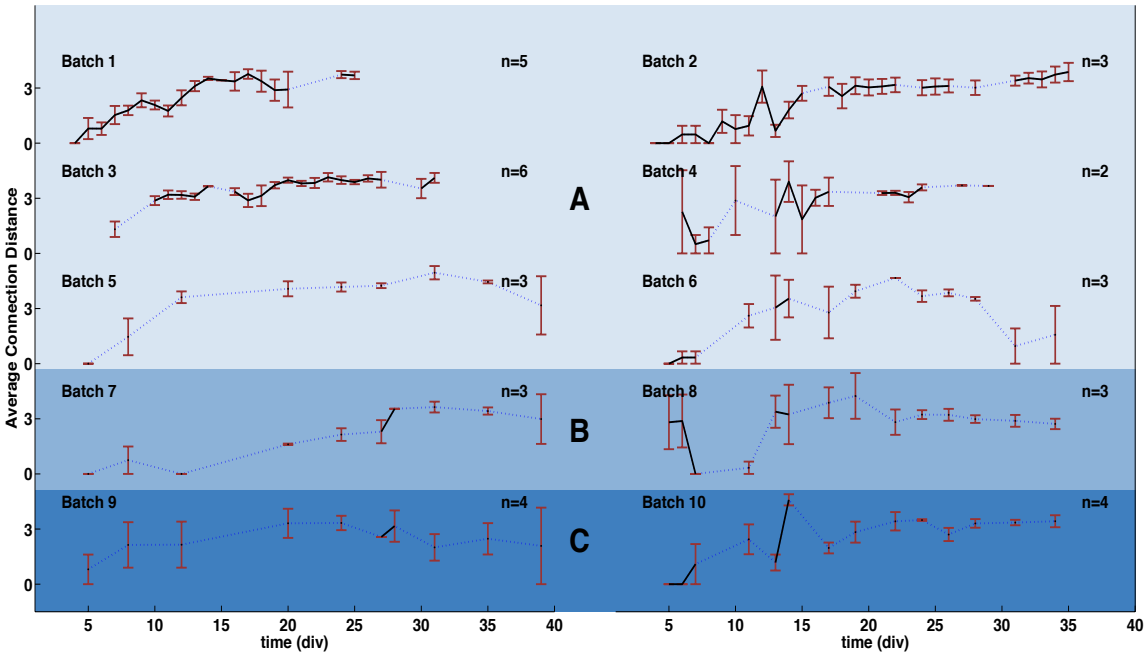

Figure 3.4: Connection Lengths averaged across cultures harvested from the same neuron batch when using a 150 ms time window. A) shows results derived from 6 dense culture batches. B) Results derived from 2 sparse density batches and C) displays results for 2 small density batches. Each panel shows results with respect to a different batch. The blue solid line represents the average connection length within batches, while red vertical lines are the corresponding standard errors. n represents the population size for each batch.

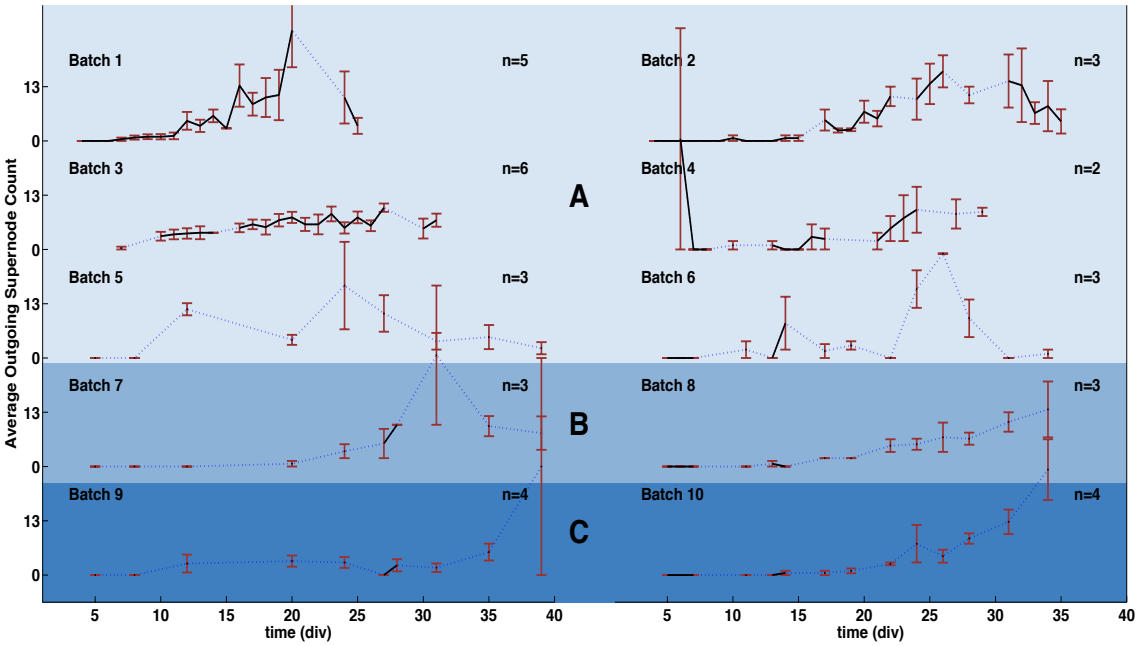

Figure 3.5: Outgoing supernode number averaged across different cultures harvested from the same neuron batch when using a 150 ms time window. A) shows results for 6 dense culture batches. B) shows results for 2 sparse density culture batches and C) for 2 small density cultures. Blue solid lines represent the average supernode number within batches, while red vertical lines are the corresponding standard errors. n represents the population size for each batch.

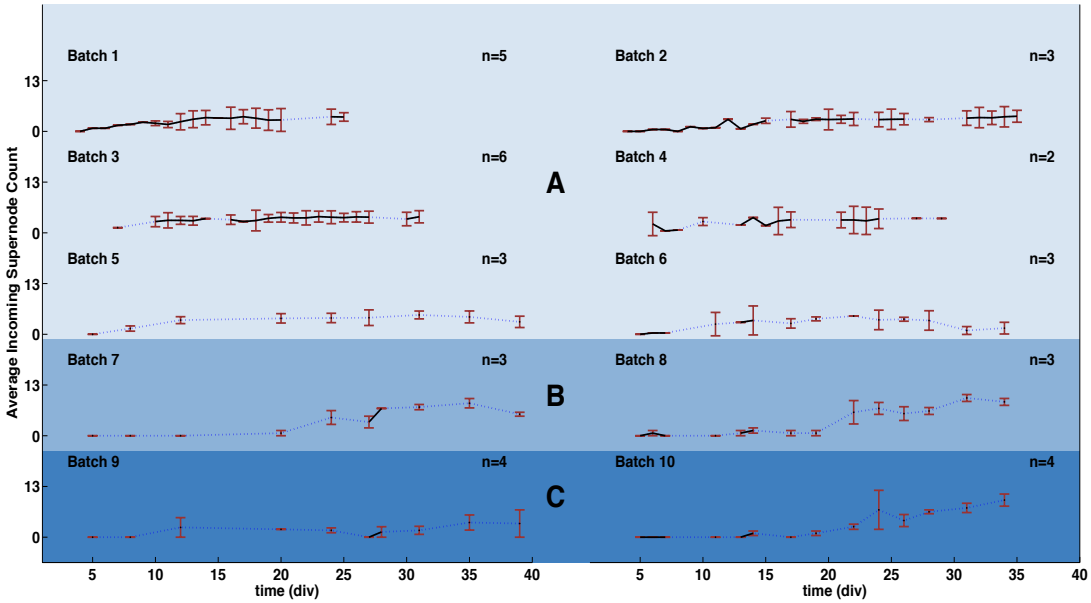

Figure 3.6: Incoming supernode number averaged across different cultures harvested from the same neuron batch when using a 150 ms time window. A) shows results for 6 dense culture batches. B) shows results for 2 sparse density culture batches and C) for 2 small density cultures. Blue solid lines represent the average supernode number within batches, while red vertical lines are the corresponding standard errors. n represents the population size for each batch.

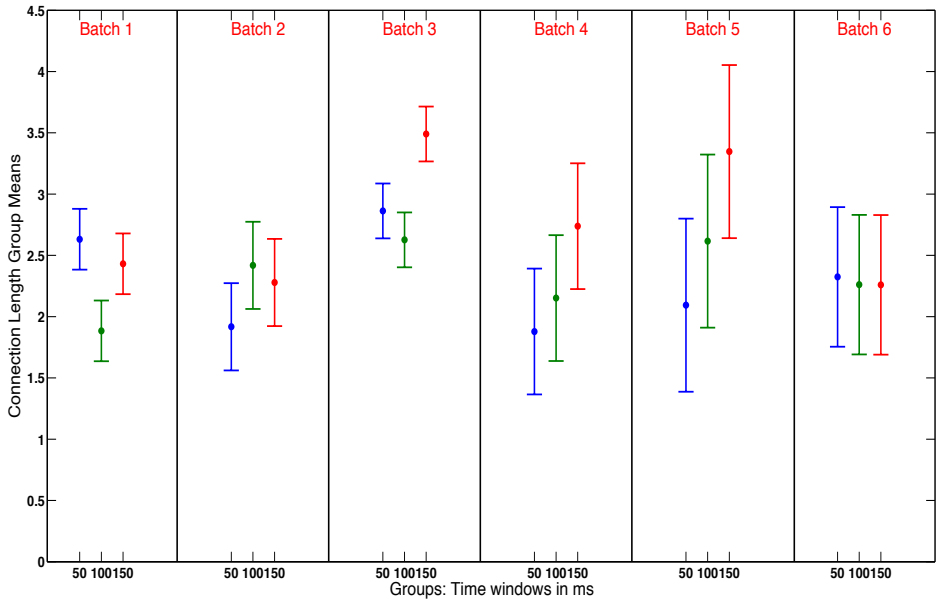

Figure 3.7: Results of an ANOVA multiple comparison tests between the three time windows' group means for each batch. The six panels show the three time window means (dot in the center of the lines) and 95% confidence intervals around them. When confidence intervals computed for different groups overlap, it means that such groups are not significantly different.

3.4 Discussion

This work has adapted a statistical technique for identifying significant neuronal connectivity between pairs of electrodes in a micro-electrode array dish. This work has furthermore developed two metrics for describing connectivity in the MEA dish: (1) the average distance between stimulus and recording electrodes, and (2) the existence of "supernode" electrodes, which form functional hubs connecting to a large number of other electrodes. Finally, this work has used these metrics to quantify connectivity trends in MEA cultures of dissociated rat cortical neurons, including culture preparations characterized as "Dense", "Sparse" and "Small". In all cases, the MEA dishes showed two phases of development with respect to neuronal connectivity over a period of about 40 days. The first phase was characterized by relatively little significant neuronal

connectivity within the MEA dish, this phase typically lasted five days. The second phase, lasting 10-15 days, is characterized by a rapid growth in the sophistication of network connectivity, both in terms of average connection length and number of supernodes. At the end of the second phase, network growth tends to plateau. It is interesting to notice that towards the end of the experiments (35 div) our statistical results show that the number of significant connections begins to decrease in some batches. This might be caused for several reasons including changes in neuron density, glial cell proliferation or the fact that the networks might become less sensitive to stimulation over time. We observed that after 35 div the spontaneous firing rate can start to decrease and stimulus evoked responses decrease accordingly. This is in agreement with what found in [14], where the authors found that after one month in vitro, the network's overall firing rate was lower while its bursting activity increased.

Furthermore, our findings for 50 ms and 150 ms time windows, suggest that the observed neuronal networks display similar behavioral trends within neuron cultures derived from the same brain tissue with non-significant variations in their connection lengths. On the contrary, temporal evolution seems to display statistically significant differences when analyzing cultures harvested from different brain tissues, as quantified by the ANOVA test results (p value for 50 ms windows is $p50 = 0.0207$, and p value for 100 ms windows is $p150 = 0.0107$).

Two plausible explanations can justify the observed behaviors: 1) Cultures derived from the same brain tissues were grown, fed and recorded from at the same time and exposed to the same experimental conditions. On the contrary, experimental conditions might have been slightly different for cultures derived from different batches because

they were grown during different periods of time. In this view the different experimental conditions could explain the high variability across batches. 2) Despite these neurons having been dissociated before plating, they could still retain some innate characteristics and properties originating from the brain tissue they were derived from. While the former explanation is more plausible, considering neuron sensitivity to experimental conditions. The latter is intriguing because it suggests that dissociated neurons retain essential properties of the original brain cortical tissue they were harvested from. If so, then electrical activity may be determined by genetic factors to a much larger extent than previously thought. Further investigation is warranted.

In previous studies, the gold standard to quantify the electrical activity of neuronal networks cultured on MEA dishes was to measure the overall network activity by summing the number of spikes detected per unit time over all electrodes [14]. Although this metric has proven beneficial when assessing the total network activity or network bursting activity, it is not specific or accurate enough to quantify the networks' temporal evolution. Furthermore, given the randomness and variability associated with the spontaneous activity of such networks, it also lacks the statistical features that are valuable to minimize the effects of randomness in MEA recording results. Our findings suggest that FDR analysis is a valuable technique to investigate and quantify dissociated cortical networks' temporal evolution when combined with more physiological metrics that can track changes in network activity.

One last consideration regarding the statistically significant connectivity graphs that are the results of the FDR analysis. It is important to notice that the identified connections are not necessarily direct connections between two nodes, but they can hide intermediate

hops and more complex activity patterns. This issue gets even more complex if we keep in mind that the electrode connections are an overall and over-simplified representation of the neuron network connectivity. Unfortunately, with this kind of MEA dishes it is arduous to track the real neuronal connections that underlie electrode activation.

Despite the results presented in this work, further studies will be necessary to understand the role of chronic external stimulation in dissociated cortical neuron development. Specifically, while this work identifies characteristic phases of MEA network development, it is not known whether those changes are occurring spontaneously or in response to the daily stimulation protocol. Further investigation is needed in which the neuronal connectivity of unstimulated MEA arrays is compared to that of chronically stimulated ones. Preliminary evidence [17] suggests that electrical activity may shape network functional properties.

Our findings are consistent with previous results in the literature. For instance in [76], the authors have investigated the presence and the importance of "brain hubs" in functional brain organization. These brain hubs play a key role in global information integration between different parts of the brain connections.

In the future, we will develop this work by investigating the specific role of electrical stimulation in regulating neuronal development. Specifically, we will implement associative learning protocols in MEA dishes such as those described in [77]. Protocols will use two different sets of external electrical stimuli. The Unconditional Stimulus (US) will be chosen from those stimuli that do not produce any evoked network response, whereas the Conditional Stimulus (CS) will be chosen from among those stimuli that produce a distinctive network activity. By comparing the network responses to the

different stimuli and characterizing their temporal evolution, we will be able to study in greater detail the learning processes that take place in dissociated cortical neurons. Furthermore, in order to improve the significance of our analytical approach, the methods introduced in this work could be applied to synthetic data following the approach presented in [78] and this will be the subject of a future study and publication.

3.5 Conclusions

In this chapter we investigated how dissociated cortical neurons respond to chronic electrical stimulation. In particular we have investigated the temporal evolution of neuronal activity in response to a constant electrical stimulation protocol over the first 5 weeks of neuronal development. Our goal was to quantify changes in neuronal network connectivity, in dissociated cortical neurons using statistical analysis. We hypothesized that both external stimuli and network functional evolution were fundamental in neuronal development as previously shown in the literature. In fact, our results show an evolution in network activity in two ways. Neuronal connectivity tends to evolve over time, with changes in both the number of statistically significant stimulus/recording pairs as well as the average length of connections and the number of connections per active node. We therefore propose that the FDR analysis combined with two metrics, the average connection length and the number of highly connected "supernodes" are meaningful techniques for describing neuronal connectivity in MEA dishes. Furthermore, our results indicate that when analyzing stimulus-evoked responses recorded within 50 ms and 150 ms time windows from stimulus onset, cultures dissociated from the same brain tissue

display trends in their temporal evolution that are more similar than those obtained with respect to different batches, as quantified by the statistical tests within and across batches. We suggest two hypotheses that could help explain the observed phenomena: 1) Cultures derived from the same brain tissues were cultured and exposed to experiments in the same time periods and under very similar experimental conditions, this could have induced the similarities in the observed results. 2) Our findings could indicate that even after dissociation, these neurons pre- served some of the properties and characteristics of the original brain tissue they were harvested from. This would indicate that genetic information and genetic programs control neural development and neural firing more than previously hypothesized [79].

CHAPTER 4

INVESTIGATING LONG AND SHORT-TERM MEMORY FORMATION IN DISSOCIATED NEURONAL NETWORKS

It is a well-known phenomenon that external electrical stimulation can affect the functional activity of dissociated neuron networks [10]. The ability to drive the network to respond in a desired way provides a valuable approach to study changes in functional connectivity induced by external stimuli. For instance, in [16] and [80] Jimbo et al. demonstrated that local tetanic stimulation induces long-lasting (longer than 30 min) changes in network responses. They used the number of spikes recorded across all the electrodes to measure network responses. The most relevant aspect of this work is that for a given tetanic stimulation, activated neurons showed similar changes in activity level, as quantified by the overall network activity. In other words, all the activated neurons either increase their responsiveness or decrease to the stimulus. The result is interesting because it proves that despite the large number of neurons and synapses involved in the network response phenomena, potentiation or depression are pathway-specific and not neuron-specific. In the literature, several studies have shown that these networks exhibit a variety of recurring activity patterns that can be modified by electrical stimulation [81] [14]. Therefore, many research labs have designed experiments whose goal is to utilize stimulation to modify the behavior of such neuronal preparations and interact with them. The most common issues with such experimental setups are the high variability in the

network responses and the randomness in background activity, thus making the neuronal activity difficult to interpret. The observed variability has often been attributed to the natural plasticity of the nervous system, but there are no studies that have specifically investigated to what extent this plasticity can be associated to in-vitro memory formation.

The aim of this chapter is to investigate whether dissociated neuron networks can exhibit memory phenomena, defined as the ability to learn and remember the applied stimulus paradigm. In [82], [83], it has been shown that low-frequency stimulation can weaken synaptic transmission and such an effect is known as long-term depression (LTD). LTD might serve as a learning mechanism in its own right or might be a means of ensuring homeostatic stability by preventing an increase in overall activity in potentiated networks.

In order to answer this question, a set of experiments has been designed and implemented, in which the MEA electrode grid is divided into four quadrants, two of which are chronically stimulated (Q1 and Q3, see Figure 4.3), every two days for 1 hour. This stimulation paradigm is applied for ten days, while the remaining two quadrants (Q2 and Q4, see Figure 4.3) are never stimulated and can then be used as an on-dish control. After this period of chronic stimulation, the stimulus delivery is suspended in Q3 for ten days; Q3 is therefore the "experimental" quadrant. During this period only Q1, defined as the "test" quadrant, is stimulated. Ten days later, stimulation delivery is resumed into both quadrants (Q1 and Q3) and their responses are compared to quantify what level of plasticity has taken place in the network and how this relates to the stimulation interruption.

One of three possible outcomes is expected:

1. The experimental quadrant responds to the resumed stimulation with a lower activity compared to the test quadrant to which chronic stimulation was regularly applied.

2. The experimental quadrant responds with higher activity compared to the test quadrant.

3. The experimental quadrant shows no significant difference from the always-stimulated quadrant.

Moreover, the experimental paradigm made it possible to study differences in the test quadrant's stimulus-evoked activity, once the chronic stimulation was resumed, and find out if the activity in the experimental and test quadrants eventually went back to the activation levels they displayed before suspending the stimulation.

Even though the electrode grid was divided in different areas, such a division does not apply to the underlying neuronal networks. In fact, the cultured neurons are capable of generating connections that spread out across any area of the MEA dish. This implies that stimulation, even if localized to a specific site, could evoke responses and modifications in different locations of the dish. As a result, the observed spatial changes in the neuron activity might not be exclusively contained within a given quadrant. Nonetheless, it was anticipated that the effects of the applied stimulation should be stronger or more lasting in proximity of the delivered stimulus pulses. In order to identify spatial changes in network activity evoked by local stimuli, both the total activity (entire electrode grid) and local activity (in each quadrant) were quantified. This allowed for detecting statistically significant local changes (across quadrants) with respect to the induced spatial activity changes in the networks.

4.1 Methods

4.1.1 Cell Culture Preparation

Primary neuronal cultures were obtained from BrainBits, LLC (IL, USA). Briefly, cortical tissue from embryonic Sprague–Dawley rats at day 18 of gestation is harvested by caesarian section from anesthetized pregnant dams. MEA dish preparation and neuron dissociation has been proceed as previously described in [14]. Namely, rat embryonic cerebral cortices are dissected from the brain and dissociated first by enzymatic digestion in papain solution (20 min at 37 °C) and subsequently by mechanical dissociation with a fine tipped Pasteur pipette. The resulting tissue is re-suspended in Neurobasal medium supplemented with 2% B-27 and 1% Glutamax-I (NbActiv4, BrainBits, IL, USA) at the final concentration of 4000 cells/ul. The dissociated neurons are then plated onto MEAs previously coated with poly-D-lysine and laminin to promote cell adhesion. Cultures are kept in an incubator at 5% CO2 at 37°C and transferred to a dedicated acquisition incubator with the same environmental conditions during the experimental sessions. To reduce thermal stress of the cells, MEAs are kept at 37°C by means of a controlled thermostat (MCS, Reutlingen, Germany). Half of the culture medium is changed twice per week, immediately after the recording sessions.

4.1.2 Data Collection

Microelectrode arrays (Multichannel systems, MCS) consist of 60 TiN/SiN planar round electrodes (30 um diameter; 200 um center-to-center inter-electrode intervals) arranged in a square grid (without corners; see Figure 4.3), with the only exception of a larger ground electrode replacing one of the recording electrodes. All dish chambers are

sealed with a gas permeable Teflon membrane to prevent contamination and evaporation [8]. The activity of all cultures is recorded using a MEA1060-INV System, from Multi Channel Systems (MCS, Runtgen, Germany). Voltage signals are amplified 1200x, sampled at 20 kHz and acquired through the data acquisition card and MCRack software (MCS). During stimulation sessions, electrical stimuli were delivered through a two-channel stimulator MCS STG1004. Data processing is performed off-line in a two-step process: first spike detection and parameter extraction are carried out using MC_Rack software, (MCS); then data analysis and statistical testing are performed with MATLAB© (The Mathworks, Natick, MA, USA).

4.2 Experimental Paradigm

The stimulation protocol implemented in this work was designed to investigate two phenomena: 1) Whether localized electrode stimulation affects the stimulus-evoked responses in the two stimulated quadrants (Q1 and Q3) differently from what happens in the control quadrants. Dividing the electrode grid into quadrants allows for a comparison of network responses between regions and to quantify how localized evoked-responses are (see Figure 4.3). In other words, it was investigated whether chronic stimulation affects the responses of the local neurons (either by potentiation or depression), and whether such phenomena are mostly seen in the experimental quadrants given the higher levels of stimulation they are exposed to. 2) Whether varying the external stimulation protocol every ten days, during the 30-day long experiments, generates short-term or long-term potentiation and/or depression in dissociated cultured neurons.

In this view, the experiments were designed to elucidate both the temporal and spatial aspects of network activity and to reveal variations in synaptic plasticity. The goal was to use localized stimulation to investigate local and global changes within a single neuronal network with respect to the electrode grid location while tracking its temporal response to chronic stimulation. The choice to divide the stimulation protocol into four phases will determine whether synaptic plasticity mechanisms are evoked, either in the short or long-term and how permanent any such effects are.

In summary, the experimental paradigm consisted of dividing the MEA electrode grid into four quadrants (two test quadrants and two control quadrants). Then voltage stimulus pulses were delivered using eight preselected electrodes in each of the two test quadrants. The experiments were divided into four ten-day long phases in which different stimulation protocols were implemented. No stimulation was delivered in the first experimental phase, while during the last three experimental phases, chronic external stimulation was delivered for one hour every other day. Every recording session started 20 minutes after placing the dish onto the amplifier, thereby giving the neurons enough time to recover from mechanical stress due to movement. Every complete experiment consisted of the following experimental phases:

Phase 1: Lasting from plating until the network displayed clear spontaneous activity, defined as a steady firing activity exhibited from most electrodes. During this period, the acquisitions consisted of spontaneous activity recordings with no stimulation delivery. Based on the results of the experiments run on dissociated rat neurons, this phase usually lasted between six to eight days in-vitro.

Phase 2: Training Phase, lasting ten days. During this phase the network underwent one hour of stimulation every two days; eight electrodes in Q1 and eight electrodes in Q3 were sequentially used to deliver the stimuli. During this phase the acquisitions were composed of:

1. a 30–minute long spontaneous activity before stimulus delivery;
2. a 1-hour long evoked activity recording during stimulus delivery;
3. a 30-minute long spontaneous activity recording;

Phase 3: Test Phase, following the training phase, lasting for ten days. The network was subjected to stimulation, but this time only the eight electrodes in Q1 were used to deliver the stimuli, in a one-hour session every two days. No stimuli were delivered to Q3.

Phase 4: The acquisition paradigm used in the Training Phase (phase 2, Q1 and Q3 stimulated) was resumed for ten more days. This phase was designed to expose differences between the two quadrants with respect to temporal changes in the stimulation delivery and to help reveal the memory (plasticity) mechanisms in the neural network.

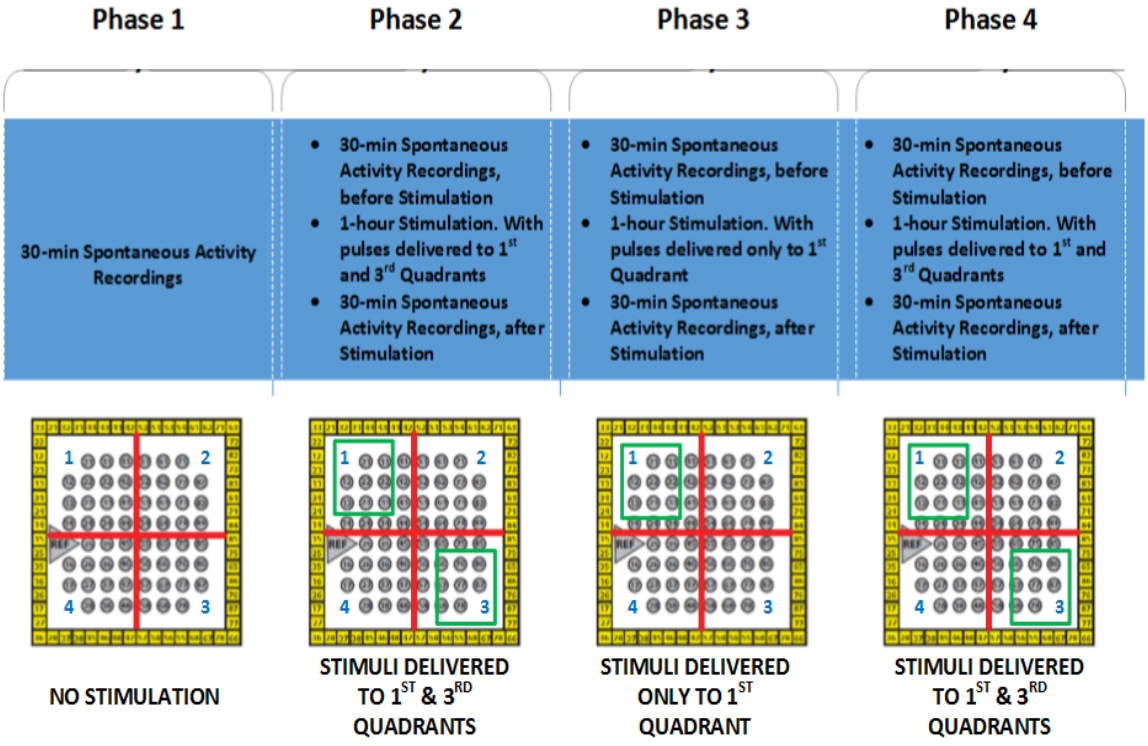

Figure 4.1: Different Experimental Phases

Dividing the experiments into four distinct phases made it possible to emphasize differences in functional responses between the test quadrant and the experimental quadrant due to stimulus delivery interruption. The temporal organization of the implemented experimental approach is shown in Figure 4.1.

4.3 Stimulation Protocol

4.3.1 Stimulation: Voltage Pulse Characteristics

The stimulation paradigm consisted of a simultaneous paired-pulse stimulus delivered to two electrodes in the two test quadrants. The paired-pulse stimulation approach was chosen to maximize the probability of excitation without over-stimulating the neuronal cultures. This is based on previous studies in which it has been shown that the use of paired pulses allows one to decrease the amount of current/voltage necessary to evoke meaningful network responses. The reason why a paired-pulse paradigm evokes higher responses with lower voltages is that the first pulse activates presynaptic voltage-gated

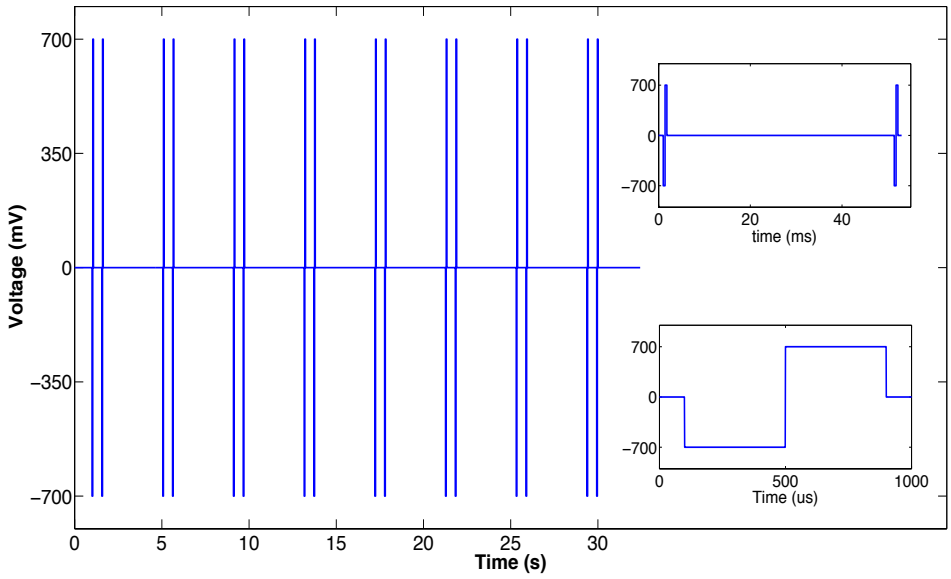

Figure 4.2: Delivered Stimulus Pulses. Left-hand side panel shows the eight paired-pulse stimulus sequence, with one pulse delivered to each of the selected stimulating electrodes every four seconds. The top inset panel on the right-hand side of the figure displays the inter-pulse time interval of 50 ms. The bottom inset panel on the right displays the characteristics of a single bipolar pulse.

calcium channels, allowing for calcium influx into the neurons. If the second pulse quickly follows the first one (by a few milliseconds) when the second pulse arrives, it raises the calcium concentration even further before the calcium released by the first pulse is reabsorbed. This generates higher neuronal responses [39]. Every stimulus pulse consisted of two bipolar voltage square waves (negative phase first) with amplitude respectively of -700 mV and 700 mV and duration 400 μs per phase. Bipolar stimulation between one electrode and a distant large ground electrode was used to minimize the effects of electrolysis. These effects can easily damage the MEA electrodes as well as the cultured neurons. It has been shown that utilizing charge-balanced stimuli, especially for long-term applications, drastically reduces the risk of generating electrolysis. Another important parameter to take into consideration is the pulse time duration. Pulse width has to be kept short, because during stimulus delivery, large artifacts hide neuronal signals, making it hard to record during stimulation [81]. To increase the probability of neuron excitation without over-stimulating the network [84], the stimulus pulses were always delivered in pairs with an inter-pulse interval of 50 ms and with an inter-pair interval of 4 s. The temporal characteristics of the implemented stimuli are shown in Figure 4.2.

4.3.2 Stimulation: Electrode Pattern

The eight electrodes in each of the test quadrants were stimulated sequentially for one hour in each experimental session. During stimulus delivery, two electrodes, one per test quadrant, were simultaneously stimulated. The stimulus pulses were always delivered between a stimulating electrode and the ground electrode. The experimental design involved the delivery of eight pulses per cycle to two quadrants in the order reported in Table 2:

Table 2: Stimulation Electrode Sequence

Pulse 1:	Ch1: 21 & Ch2: 66
Pulse 2:	Ch1: 31 & Ch2: 76
Pulse 3:	Ch1: 12 & Ch2: 86
Pulse 4:	Ch1: 22 & Ch2: 67
Pulse 5:	Ch1: 32 & Ch2: 77
Pulse 6:	Ch1: 13 & Ch2: 87
Pulse 7:	Ch1: 23 & Ch2: 68
Pulse 8:	Ch1: 33 & Ch2: 78

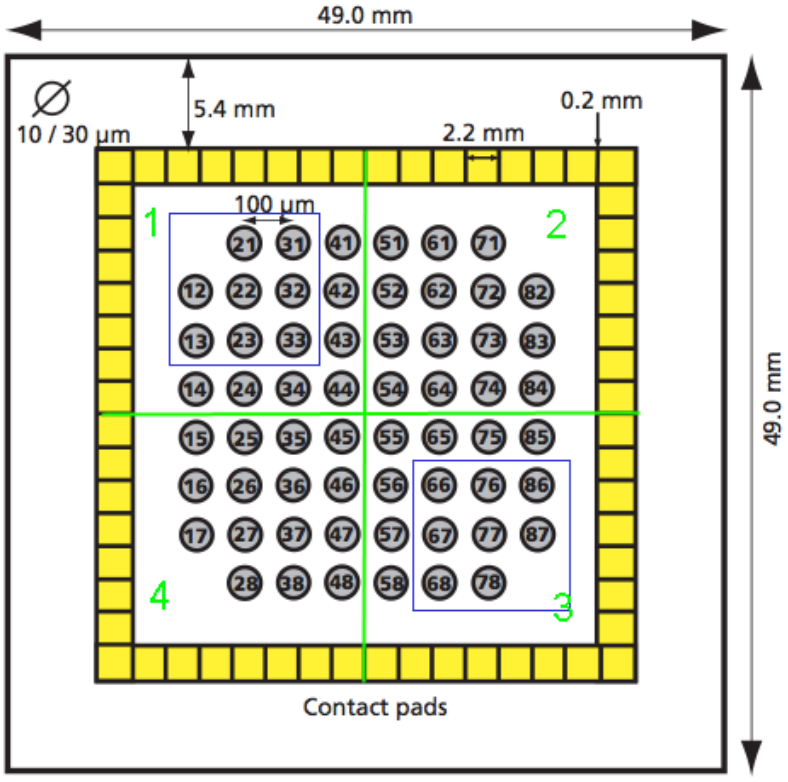

Figure 4.3: Electrode Grid, the blue rectangles show the 8 electrodes, for each quadrant, that are used to deliver stimulus pulses

The electrodes that we used to deliver the stimulus pulses, their spatial location and the quadrant division and are shown in Figure 4.3

4.4 Signal Processing

4.4.1 Spike Detection

Continuous voltage traces were band-pass filtered to enhance the spiking components of the signals. In order to implement a band-pass filter we fed our signals into the cascade of a high-pass 2nd order Butterworth filter with cutoff frequency set at 180 Hz and a low-pass 2nd order Butterworth, whose cutoff frequency was 3 kHz.

Spike detection was carried out using a simple threshold mechanism. The threshold was individually set for each channel and chosen as -6 times each band-pass filtered signal's standard deviation, as measured by MC_Rack software, within a 500 ms time window. To reduce the possibility of detecting duplicated spikes, a detection refractory period of 1 ms was used, during which no multiple detection events were accepted.

4.4.2 Feature Extraction

Raw voltage signals were sampled at 20 kHz and amplified 1200x before being digitized and stored onto the acquisition computer. In order to perform off-line analysis significant parameters such as neural spikes and LFPs were extracted from the raw acquired signals, and the overall spike rate and burst parameters were also recorded.

Given their importance in characterizing the behavior of cultured neuronal networks, bursting activity patterns are characterized according to the number of bursts occurring in each recording session, the number of spikes in bursts, and the frequency of burst

occurrence. Bursts are detected using MC_Rack software, which allows investigators to select a series of parameters to perform burst detection. To detect the bursts, the software searches for instances where the interspike interval between spike trains is less than 10 ms. Events were included in a detected burst until the interspike interval became larger than 100 ms. A single bursting event included all the detected events whose interspike interval was less than 15 ms. A burst was not counted if its duration was less than 50 ms or if it contained four or fewer spikes.

4.4.3 Data Analysis

Given the high variability and randomness associated with the MEA recording features described in section 2.2, a statistical analysis procedure was implemented to investigate the network responses to the stimulation protocol described in section 4.3. This aimed to take into account the temporal spontaneous variability and the spatial spontaneous variability that MEA activity displays. This is even more crucial when considering that the presented experiments lasted 30 days, during which the temporal evolution of these neuronal cultures could play an important role in modulating their firing characteristics. In summary, short-term temporal variability within every experimental session was assessed by dividing every acquisition in 5-minute time windows and implementing statistical analysis on these time bins. The results were used to quantify the temporal variations of network activity. Spatial variability was instead measured by computing the mean firing activity and bursting parameters with respect to every quadrant in each experimental condition, pre-stimulus and post-stimulus. These values were statistically analyzed and the results used as metrics of network local activity evoked by the delivered stimulation pulses.

4.4.4 Temporal Statistical Analysis: Temporal firing stability

In order to quantify the temporal stability of the dissociated neuron networks, every recording was divided into 5-minute time windows; network spiking statistics (firing parameters) were calculated across and within time bins. Network spontaneous firing before stimulation sessions was considered as a natural characteristic of the network during a specific experimental session and it was used as a baseline to measure stimulation-induced changes. Specifically, to test whether stimulation had an effect on spontaneous activity, a series of firing parameters, such as the number of bursts and the Array-Wide Spike Detection Rate (ASDR) were measured. Such parameters were computed with respect to recordings acquired immediately before the stimulation sequence (Nbase), as well as in the recordings after stimulation (Npost). Next, the 30-min long spontaneous recording sessions were divided into six 5-minute bins. Bursts and ASDR were then measured within these time windows, for both pre and post-stimulation recording sessions. Then, we quantified stimulation-induced changes as

$$\Delta \mathrm{N_{ind}} = \frac{\mathrm{N_{post}} - \mathrm{N_{base}}}{N_{base}}$$

For the purpose of comparing results between cultures with widely varying firing parameters, it was chosen to normalize the changes using the baseline parameters Nbase, and then to evaluate the statistical significance of changes in the means of $\Delta \mathrm{N_{ind}}$ within and across experimental sessions, quadrants and experimental phases. This controlled for detected changes that were not statistically significantly larger than spontaneous changes.

4.4.5 Spatial Statistical Analysis: Stimulation evoked variability

Each MEA electrode grid was divided into four quadrants, each composed of 15 channels/electrodes, with the only exception of Q4 that has 14 electrodes plus a ground contact. The average firing parameters as measured from the electrodes within each quadrant were used as a metric of the overall neuronal activity in each dish region, also called local activity.

In addition to changes induced by the four different experimental phases, it was also investigated whether the spatial distribution of the delivered stimuli could play a role in shaping the spatial patterns of network responses. Furthermore, given the high network connectivity, another area of study was how distributed network responses related to the division into electrode quadrants as selected in the experiments.

Hence, ASDR and burst parameters were measured in the network responses in each quadrant before and after stimulation delivery; significant changes in spatial response patterns were assessed. To have an activity baseline of the network electrical behavior, the ASDR and burst parameters were also computed in each quadrant within the spontaneous recording sessions. These were taken as representative of the spontaneous spatial variations or pattern drifts in the network.

4.5 Results

It is common practice in MEA research studies to use activity-based criteria for two purposes: to select the stimulating electrodes that evoke larger network responses and to select neuron cultures whose behavior fits some predetermined conditions [38]. On the

contrary, in this work, activity-based selection criteria were not used, neither to select stimulating electrodes nor to select experimental results to be included in the analysis.

The data analysis implemented in this work focused mainly on two aspects of neuronal network activity, the first aiming to assess the spatial characteristics of firing evoked by a localized stimulation protocol and the second aiming to identify the temporal evolution of network responses to a stimulation protocol that was variable in time.

The presented experimental technique was performed on a total of eight MEA dishes plated with E-18 dissociated rat neurons. All the neuronal cultures came from the same brain tissue and the dishes were plated simultaneously. The results shown here represent the mean values of the data collected from all the MEA experiments. Averaging the results across dishes allows for discovering trends and behaviors that are common to multiple cultures, therefore yielding more general results.

The rest of this section presents the results of the analyses performed on three main features extracted from the neuronal network activity. Namely, spike count, number of bursts and mean number of spikes within bursts.

4.5.1 Spike Count Spatial Analysis

The first network activity parameter that has been analyzed was the average spike count as measured in each recording session. Figure 4.4 shows the means of network firing activity averaged across all the four experimental sessions performed within every phase with respect to each quadrant are shown.

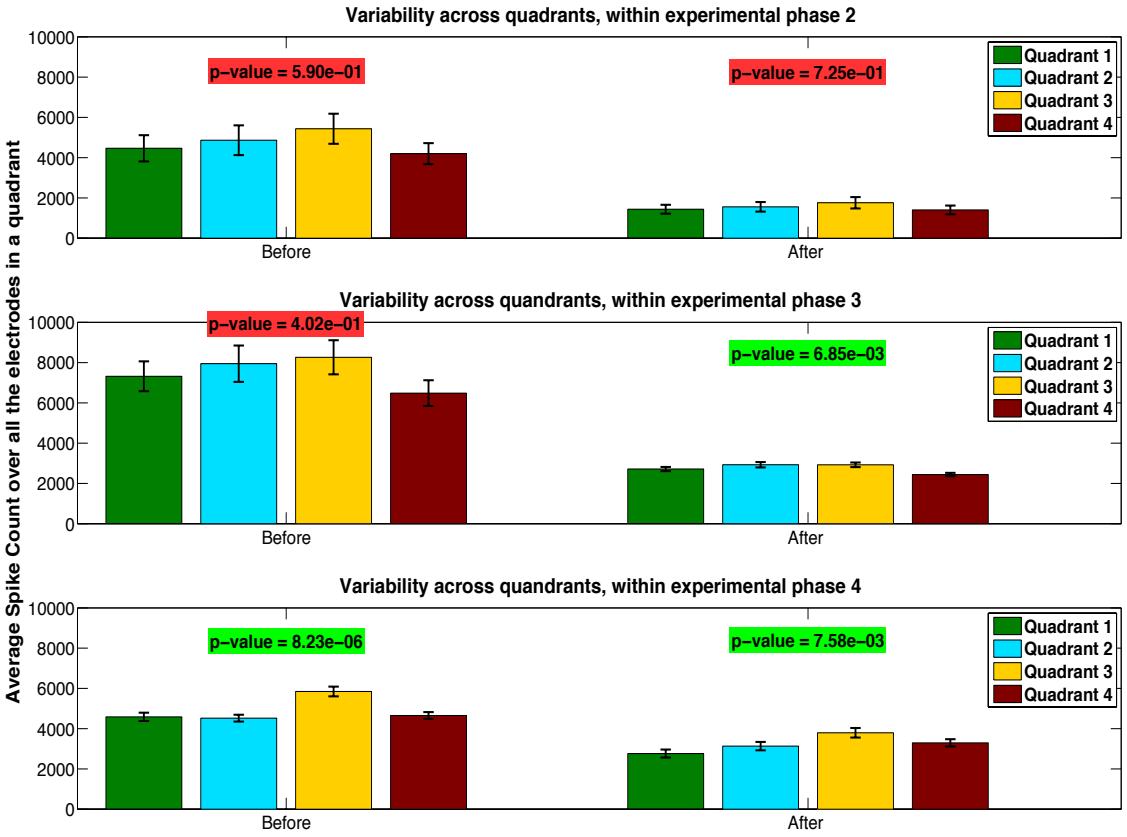

Figure 4.4: Variability across quadrants in average spike counts within different experimental phases, before and after stimulation delivery. In red are the non-significant p-values, while in green are the significant p-values (less then 5%).

To quantify statistically significant changes in quadrant activity due to the local stimulation approach used in this study, one-way ANOVA tests were carried out on spike counts averaged across all the experimental sessions within every single experimental phase. Significance threshold was set to 5%. The p-values reported in the figures measure the statistical significance of changes that occurred across quadrants within each experimental phase. Non-significant p-values are reported in red boxes, while in green are the significant ones. When comparing neuronal network activity derived from pre-stimulation recordings to post-stimulation ones, it was found that the networks responded to the electrical stimulation protocol by reducing their firing activity on average. This can

be seen in Figure 4.4 in which the activity post-stimulation was consistently lower than the corresponding pre-stimulation mean values. Furthermore, Figure 4.4 shows that firing activity was not significantly different between quadrants in phase 2 in recordings carried out both before and after stimulation. On the contrary, such changes became statistically significant in phase 3 post-stimulation sessions and in phase 4 (for both pre and post-stimulation sessions).

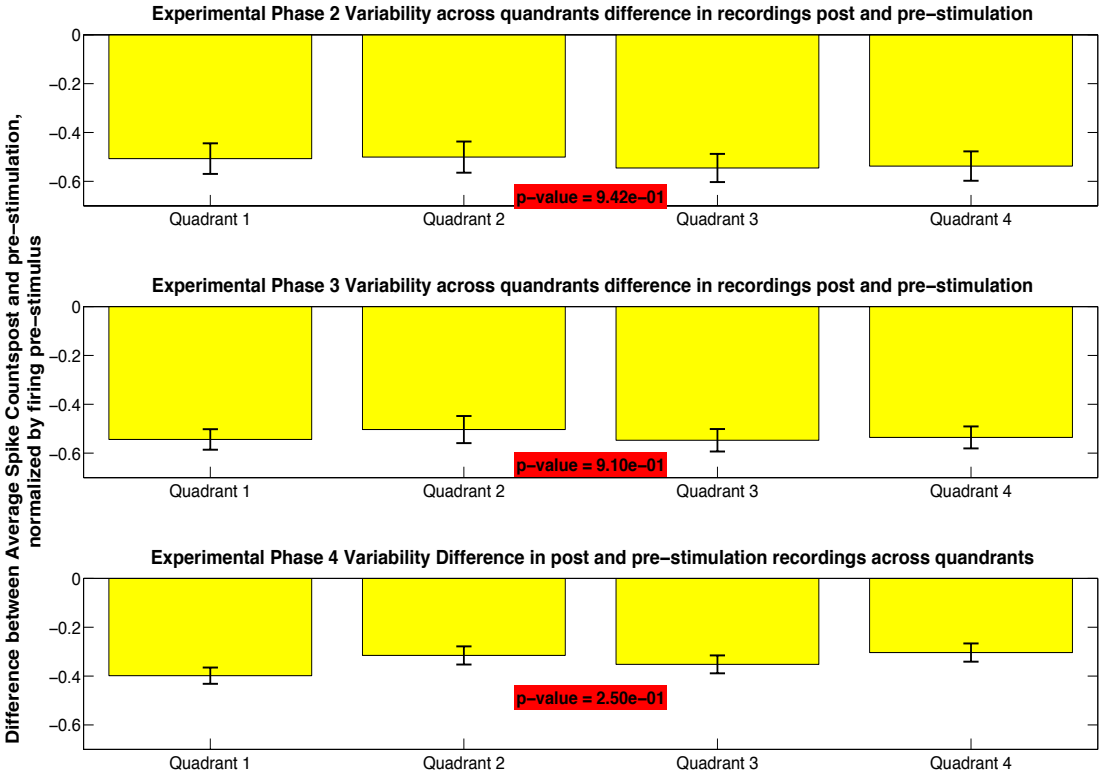

Figure 4.5: Variability across quadrants in average spike counts within different experimental phases, measured as normalized differences between post and pre-stimulation firing activity. Non-significant p-values are reported in red.

Figure 4.5 shows the differences between the average spike counts post and pre-stimulation (normalized by the pre-stimulation average spike count) and grouped by quadrant. The spike count differences between quadrants were not significantly different,

as proven by a one-way ANOVA test results performed on these parameters (p-values were all above the 5% significance level). The corresponding p-values are displayed in Figure 4.5.

The first significant result of this Aim is that the local stimulation protocol implemented in this work was not able to evoke any localized changes in network activity. This is proven by the fact that, when comparing activity pre and post-stimulus (see Figure 4.5) all the quadrants display similar trends in firing activity independently of the delivered stimulus location (position). Furthermore, the suspension of stimulation in Q3 during experimental phase 3, and then its resumption in phase 4, did not induce any detectable activity changes, neither in that specific quadrant nor in the others.

Another approach that was used as a measure of spatial changes in network activity evoked by local stimulation is to measure the changes that occur between experimental phases, within each quadrant.

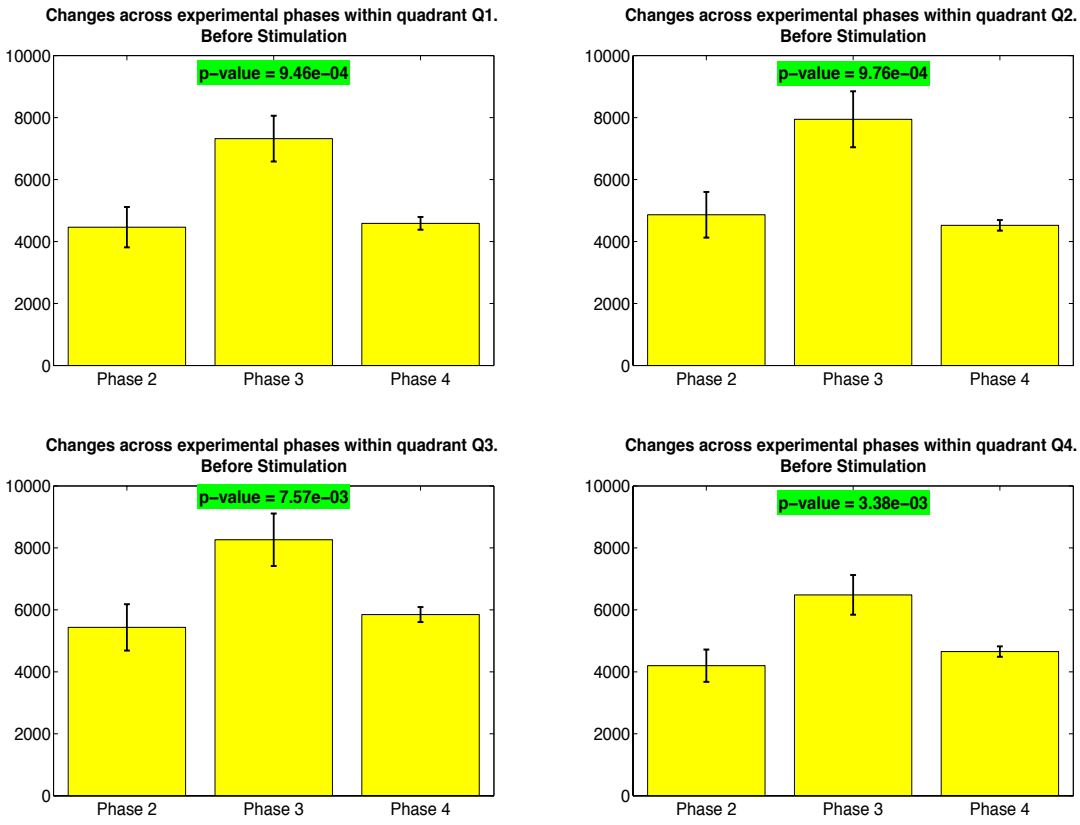

Figure 4.6: Variability across the three experimental phases with average activity grouped by quadrant. Activity from pre-stimulation recordings. Significant p-values are reported in green.

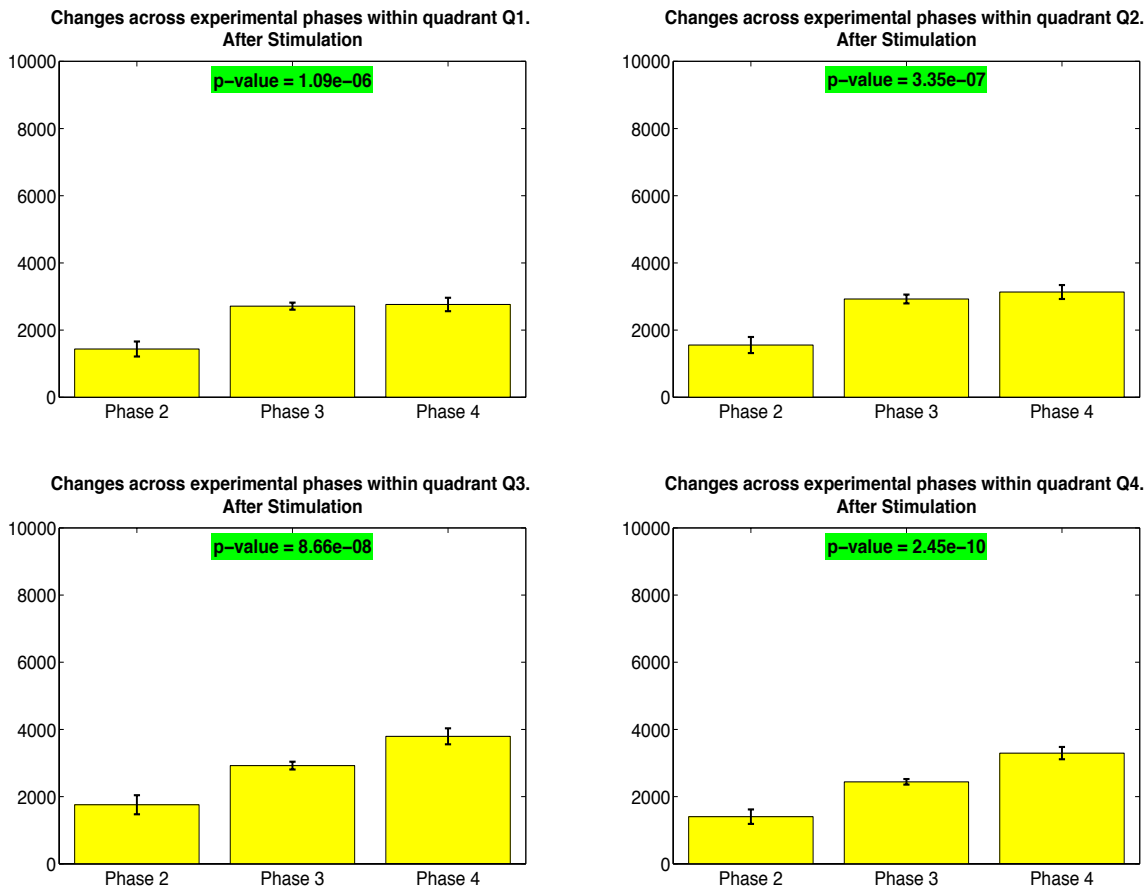

Figure 4.7: Variability across the three experimental phases with average activity grouped by quadrant. Activity from post-stimulation recordings. Significant p-values are reported in green.

In Figure 4.6 and Figure 4.7, changes in activity, before and after stimulation delivery, respectively, across experimental phases and within quadrants are shown. The four panels represent the spike counts for the three experimental phases in every quadrant averaged across the four experimental sessions that took place during each phase. These figures confirm the previously mentioned finding, that is the implemented stimulation protocol did not generate spatial differences in quadrant activity. It is worth noting that during the different experimental phases the neurons in the different quadrants responded to stimulation with similar trends. This is shown in results derived from recordings carried out both before and after stimulation delivery. Figure 4.6 shows that in all four

quadrants, average spontaneous network activity in phase 3, recorded in pre-stimulation sessions, was significantly larger than those in other phases. Furthermore, it can be seen how similar the results concerning the different quadrants are, both in terms of standard errors and spike count means.

Figure 4.7 displays average network activity recorded in post-stimulation sessions. These results demonstrate that the networks on average responded to stimulation (data recorded after stimulation delivery) on average with two distinct trends in two areas of the network. These two regions had similar post-stimulus responses: namely, the top area of the electrode grid, composed of Quadrant 1 and Quadrant 2 and the lower area, composed of Quadrant 3 and Quadrant 4. This could indicate that the neurons had a functional evolution that was symmetric with respect to the ground electrode position and thus the stimulation current pathway. The large reference electrode lies right in the center of MEA electrode grid and the effects seen in Figure 4.7 highlight such a top/bottom difference in neuronal activity.

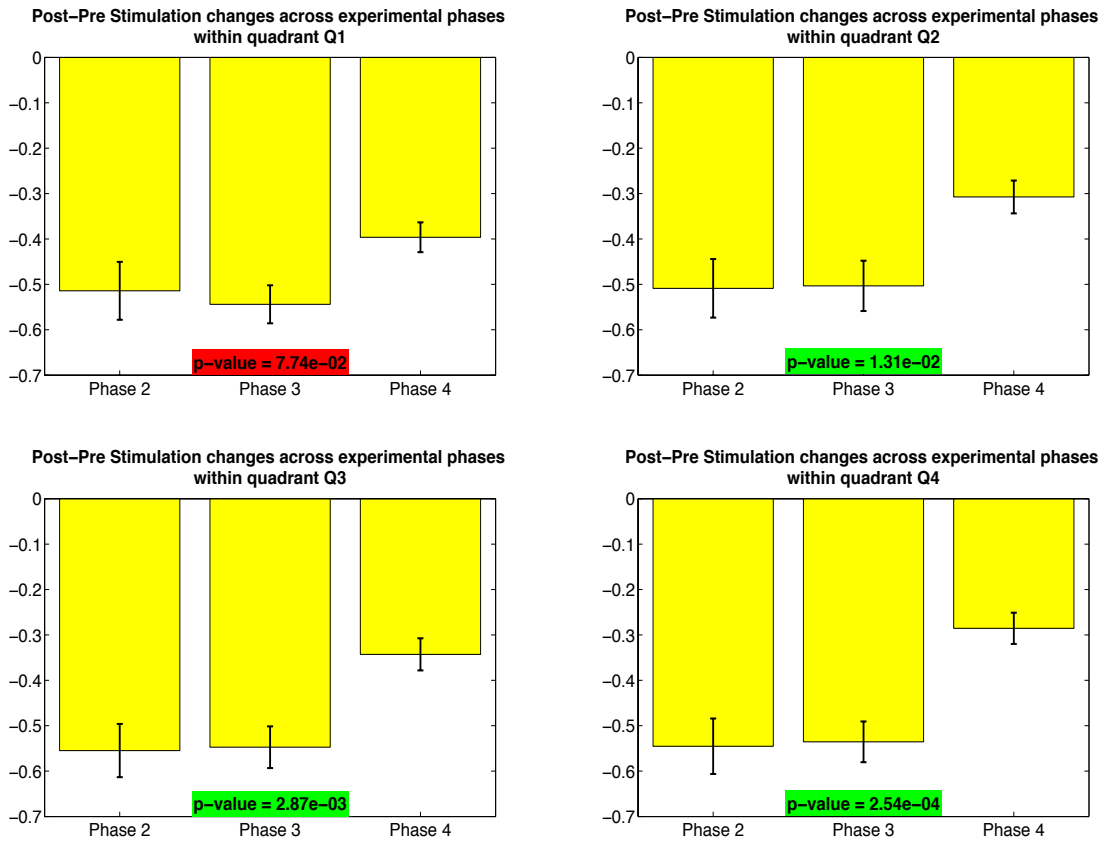

Figure 4.8: Normalized post-pre stimulation difference variability across the three experimental phases with average activity grouped by quadrant.

Figure 4.8 shows normalized post-pre stimulation difference variability across the three experimental phases with average spike counts grouped by quadrant. Differences observed between the three experimental phases are not statistically significant for Quadrant 1. On the contrary, Quadrants 2-4 display significant variations during phase 4 compared to phases 2 and 3. The differences between the groups were assessed using p-values computed using a one-way ANOVA test followed by a multi comparison test based on the Tukey-Kramer difference criterion, also known as Tukey's Honest Significance Difference Test (HSD test).

Interestingly, in Figure 4.8 it can be seen that average network responses were significantly different during different experimental phases, as demonstrated by the reported ANOVA p-values.

Moreover, looking at the corresponding p-values, it can be seen that Q1 did not display significant differences, while Q2, Q3 and Q4 display significant differences. More specifically, in both quadrants phase 4 variations are the significant ones compared to other phases.

4.5.2 Spike Count Temporal Analysis

Another important method used to investigate the effects of the electrical stimuli on dissociated neuronal cultures is to quantify the mean overall firing activity, that is spike count over all the electrodes or Array-Wide Detection Rate (ASDR). This metric has been extensively used in the past to evaluate the network total activity [14], [85]. In this work the ASDR has been combined with statistical analysis to capture changes in activity due to stimulation throughout the different experimental phases that the neuronal networks have undergone. To investigate short-term and long-term temporal synaptic plasticity, two methods have been utilized. The first is statistical assessment of changes that occurred over different experimental days during a single experimental phase. This quantifies how the networks evolved over time when presented with a constant stimulation protocol (short-term plasticity). The second is statistical assessment of changes that occurred across the three different experimental phases. This allows for studying the network reaction to a stimulation protocol that varied in time and that was designed to induce memory effects in neuron responses (long-term plasticity).

Figure 4.9 and Figure 4.10 show the overall ASDR, averaged across 5-min time bins within experimental sessions. Every panel shows a different experimental phase. The data displayed in the figures were recorded respectively in pre-stimulation and post-stimulation sessions.

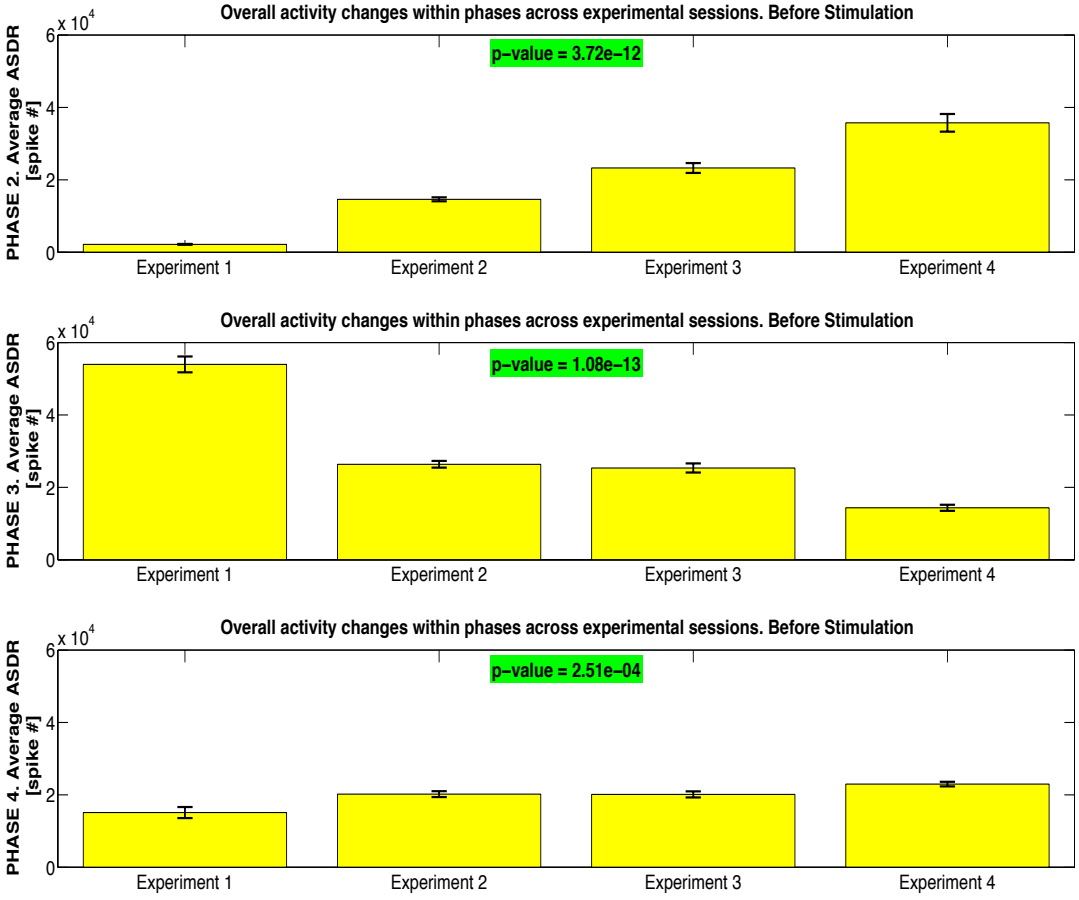

Figure 4.9: Overall ASDR, averaged across 5-min time bins within experimental sessions. Every panel shows a different experimental phase. Data recorded from pre-stimulation sessions.

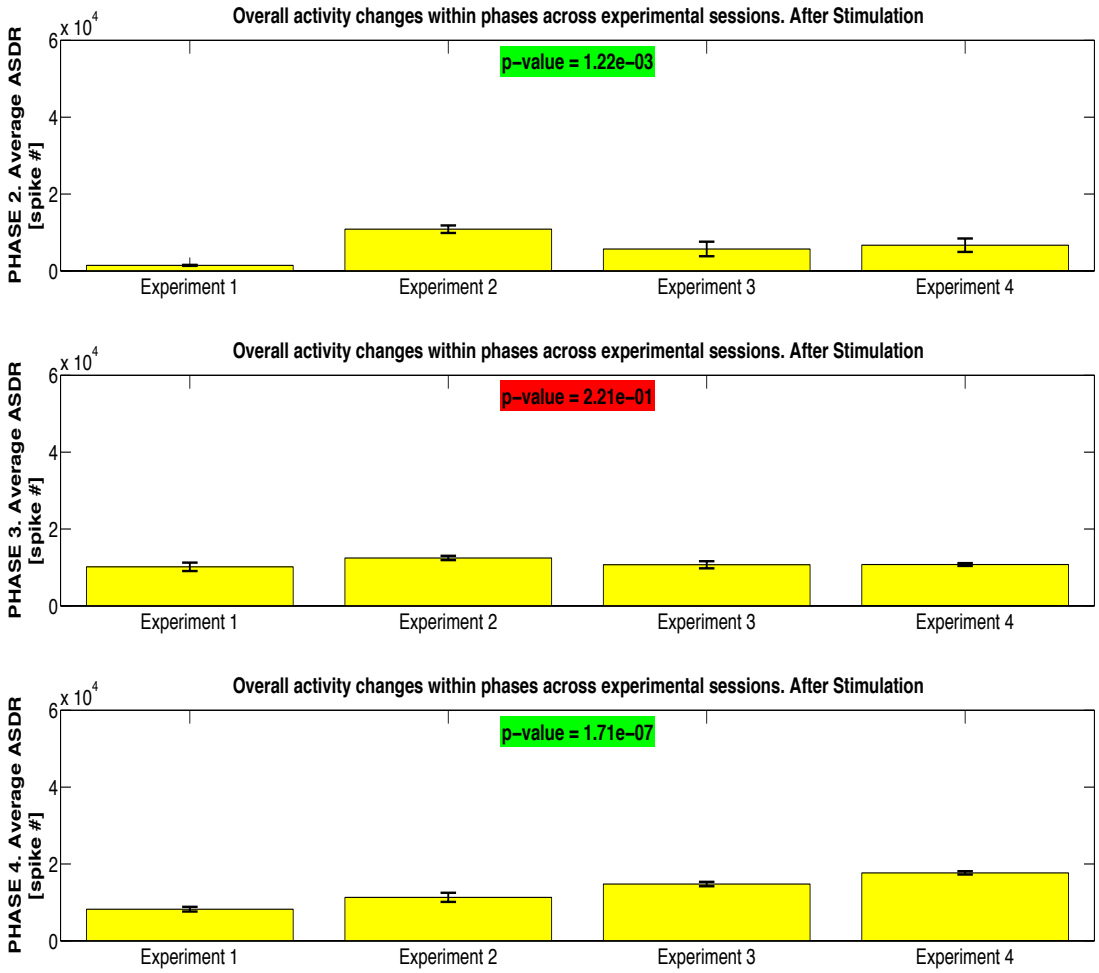

Figure 4.10: Overall ASDR, averaged across 5-min time bins within experimental sessions. Every panel shows a different experimental phase. Data recorded from post-stimulation sessions. Significant p-values reported in green, non-significant p-values are in red.

It is important to emphasize that variability across 5-min time windows is small meaning that within an experimental session the changes due to spontaneous fluctuations of the neuronal activity are not significant. This is especially apparent they are compared to the magnitude of the changes that occurred between pre and post-stimulation recordings. This demonstrates that the effects of the external stimulation are much larger than natural variability in the neuronal network activity.

Moreover, all the observed differences are statistically significant and a clear trend appears: throughout the different pre-stimulation experimental sessions in phase 2, the

network's overall activity increases, then starts to decrease in phase 3 and finally becomes stable in phase 4, (see Figure 4.9).

It is interesting to notice how different the post-stimulation and pre-stimulation recordings are. As already shown in the previous section (4.5.1 Spike Count Spatial Analysis), chronic stimulation inhibited activity, as evidenced by post-stimulation ASDR levels being consistently lower than in pre-stimulation acquisitions (see Figure 4.10). More importantly, the trend that can be seen in pre-stimulation activity was not present in post-stimulation recordings. Figure 4.10 results show that network activity after stimulation delivery was much more stable over experiments and across phases, with the mean values across experiments that stayed more stable than corresponding mean values derived from pre-stimulation recordings.

In Figure 4.11, "post-minus-pre" average overall ASDR differences are shown. Remarkably, the evolution of the stimulation effect can be evaluated using the changes in the means of the post-pre activity differences. During phase 2 the changes became more negative over time, until they began to become less negative in phase 3 and were more consistently low during phase 4. All the observed variations are statistically significant based on a one-way ANOVA hypotheses test. In Figure 4.11, it can be seen that differences between average post and pre-stimulation spike counts had a decreasing trend in phase 2, reached a negative peak on the first experimental day of phase 3, after which activity differences started to become less negative throughout phases 3 and 4. During the last two experimental days of phase 4, the differences between post and pre-stimulation spike counts steadily reached their closest values to zero (highest values, that is smallest changes). This suggests that over repeated experimental sessions neurons became less

sensitive to the stimulation delivery and that the long-term depression effects linked to electrical stimulation faded. This finding confirms what previously found in [21], [86].

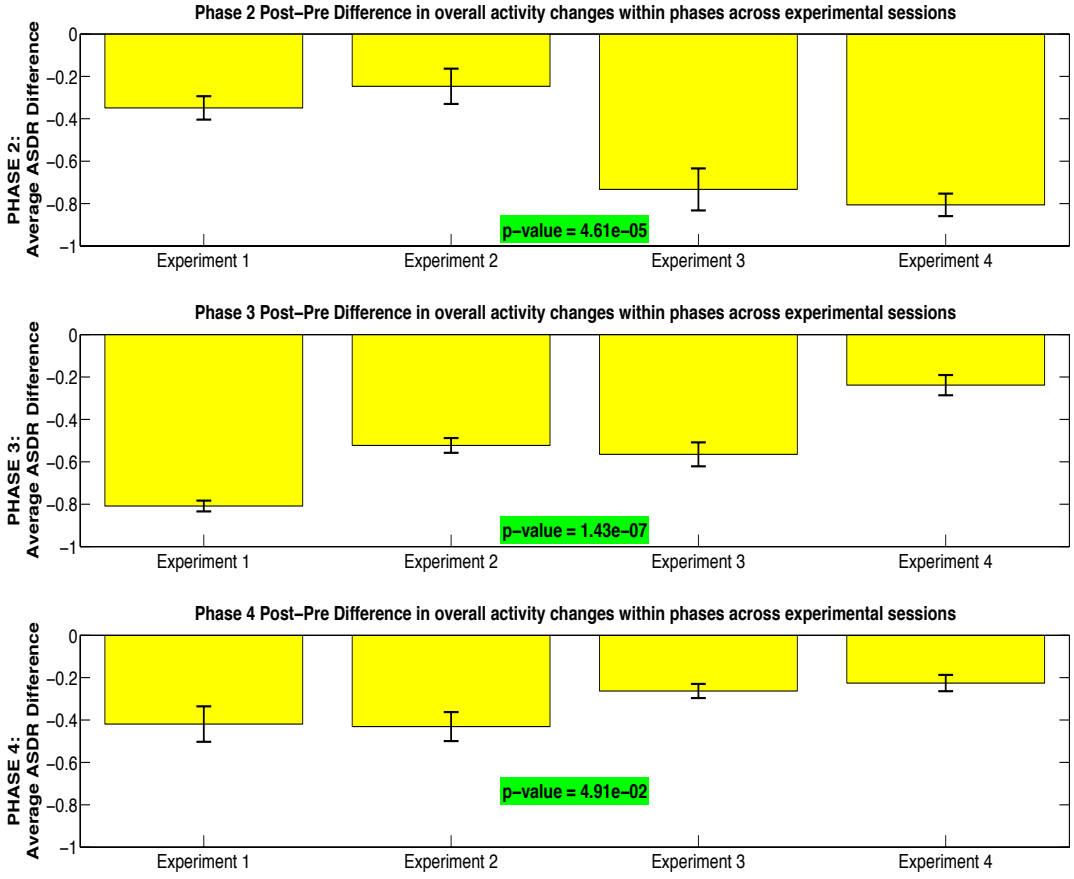

Figure 4.11: Overall normalized mean ASDR, averaged across 5-min time bins within experimental sessions. Every panel shows a different experimental phase.

The final network evolution characteristic measured in this work is how the average overall ASDR evolve across different experimental phases (long-term induced plasticity). Figure 4.12 shows the overall ASDR mean changes across experimental phases. The first two panels from the top display results respectively for pre-stimulation, post-stimulation recordings. The bottom panel displays post-pre normalized differences. All differences

between phases scored significantly different (one-way ANOVA test) and the decreased effects of the applied stimulation due to the fact that neuronal networks are habituating to the presented stimulation can be seen.

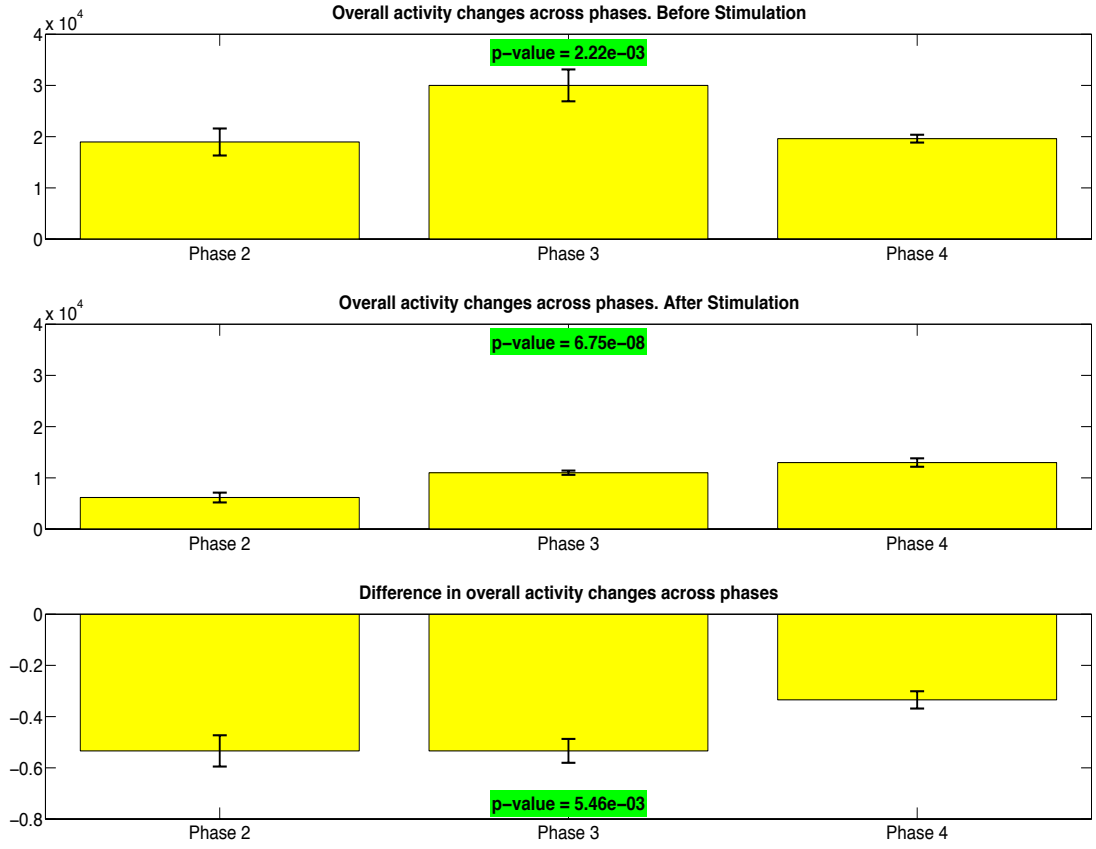

Figure 4.12: Overall ASDR mean changes across experimental phases. The first two panel from the top display results respectively for pre-stimulation and post-stimulation recordings. The bottom panel displays post-pre normalized average differences.

4.5.3 Burst Parameter Spatial Analysis

Burst parameter analysis is a well-known technique to investigate the activity characteristics of neuronal networks [86]. In this section the average (across eight neuronal cultures) burst parameter spatial evolution is presented. Considering that the

experimental sessions for different cultures did not happen on the same day, a moving average boxcar filter with a 3-day sliding window has been used to average the results derived from different neuronal networks. This filtering approach smoothed some of the activity events, on the other hand it allowed for averaging data points that would not line up otherwise.

Figure 4.13 shows the average number of bursts during pre and post-stimulation delivery sessions. Burst detection was performed utilizing the algorithm introduced in section 2.5.3. Blue curves and red curves represent spontaneous bursting parameters derived respectively before and after the delivery of the one-hour stimulation session. Every panel displays bursting activity within each of the four quadrants. This is the same quadrant breakdown used to analyze network activity spatial distribution in section 4.5.1. Interestingly, the burst analysis results confirm the finding obtained from analyzing the mean spike counts, in which it was highlighted that the utilized stimulation protocol did not generate any kind of spatial effects in the network responses. This is demonstrated by the fact that even the mean number of bursts across the four experimental phases did not display meaningful variations between quadrants.

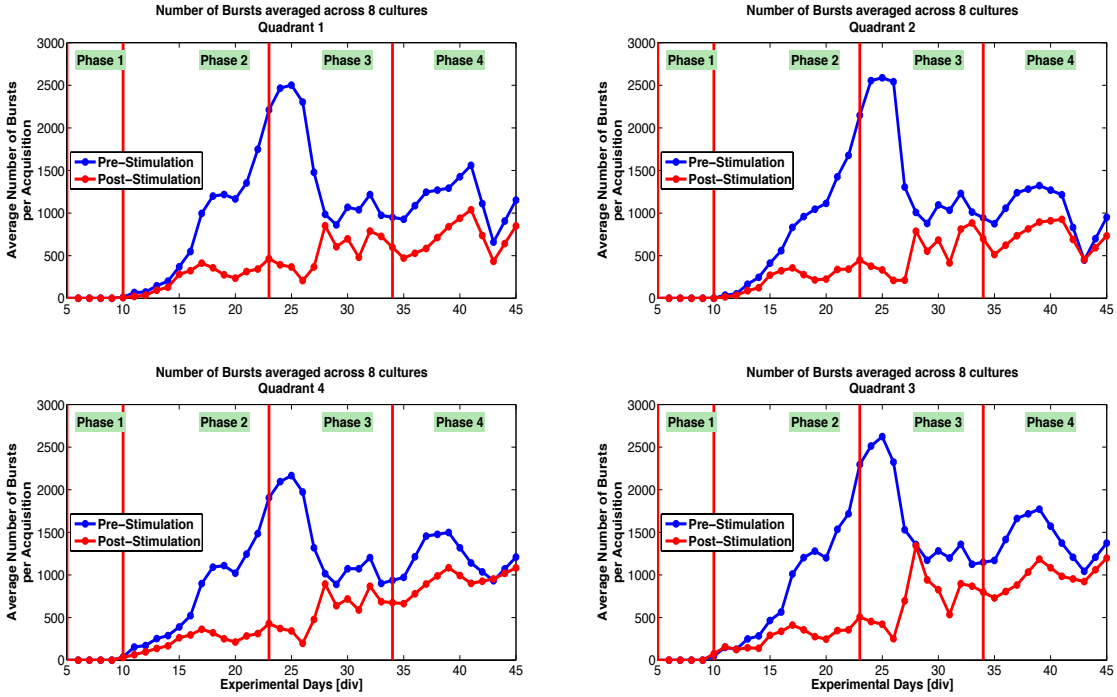

Figure 4.13: Average Number of Bursts grouped by quadrant. Blue and red lines represent respectively pre-stimulation and post-stimulation curves. Four experimental phases are shown.

In Figure 4.13, it can be seen that both pre and post-stimulation number of bursts display a similar behavior across quadrants. To better assess the changes due to the applied stimulation, the normalized differences of the mean number of bursts have been calculated and displayed in Figure 4.14, that shows how the normalized differences in average burst number across the four experimental phases. Such results demonstrate that across the four quadrants independently of the stimulation phase, the dish responded to electrical stimulation as a single neuronal ensemble. This again confirms what found when analyzing spike count results.

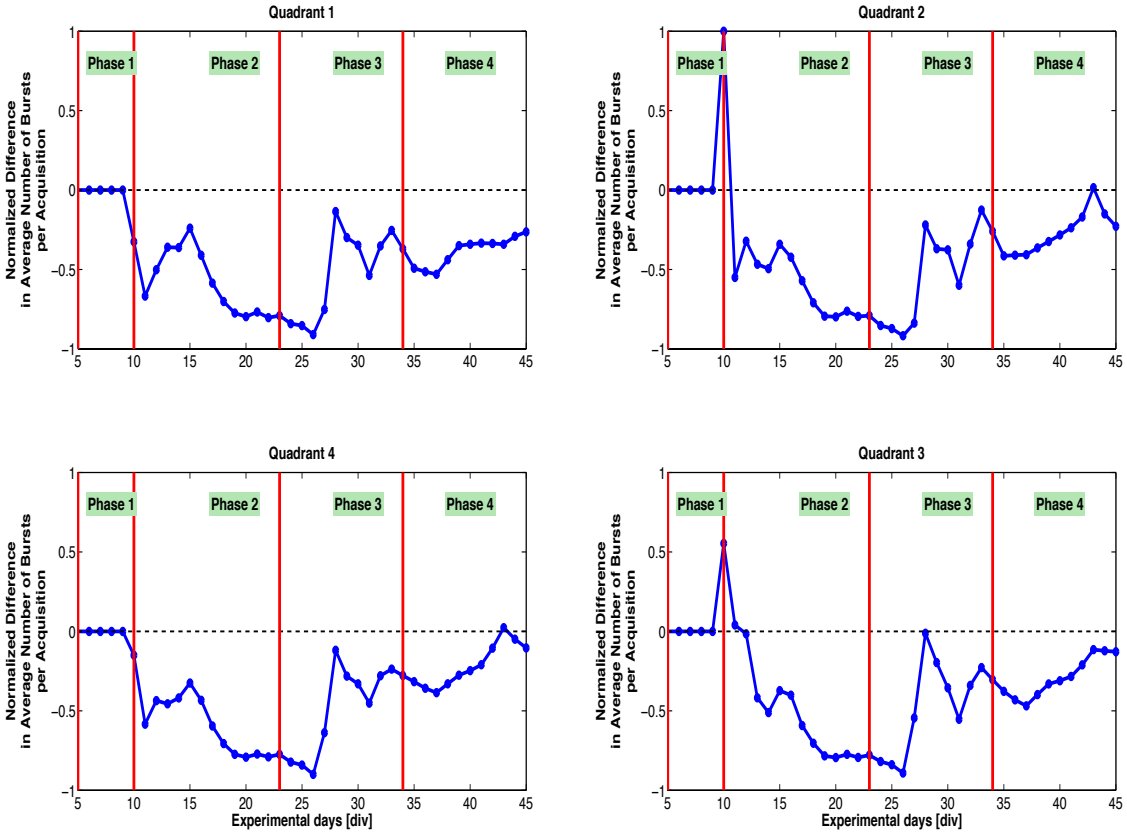

Figure 4.14: Normalized Difference of the average burst numbers, across experimental phases and divided by quadrant.

Another burst parameter that is usually associated with changes in network activity levels is the average number of spikes detected during bursting events. This differs from the ASDR or overall spike counts, because the mean number of spikes in a burst does not include spikes that occurred outside bursting activity events. Results with respect to pre and post-stimulation session recordings are shown in Figure 4.15.

To quantify changes that occurred because of the applied stimulation, in Figure 4.16 the normalized differences between "post-minus-pre" parameters are shown. It can be seen that the differences of the means of spikes in bursts, after reaching their negative peak in the beginning of phase 3, they became less negative and approached less negative values (meaning smaller changes between pre and post) towards the end of phase 4.

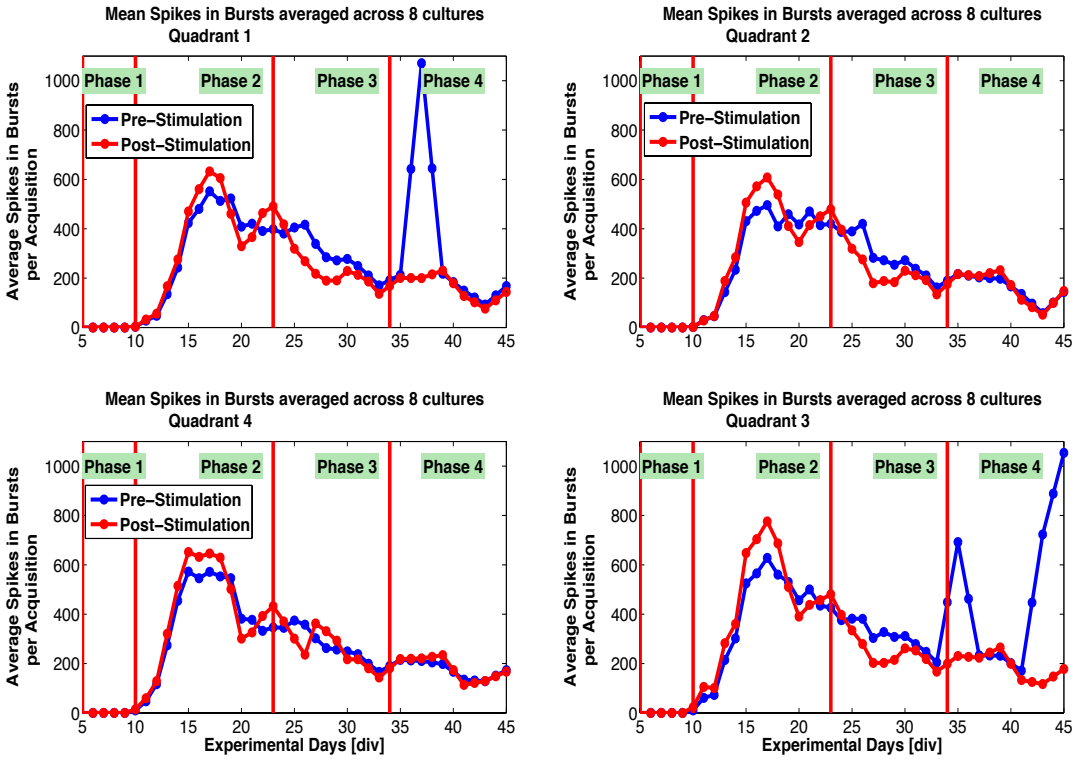

Figure 4.15: Average Number of Spikes in Bursts grouped by quadrant

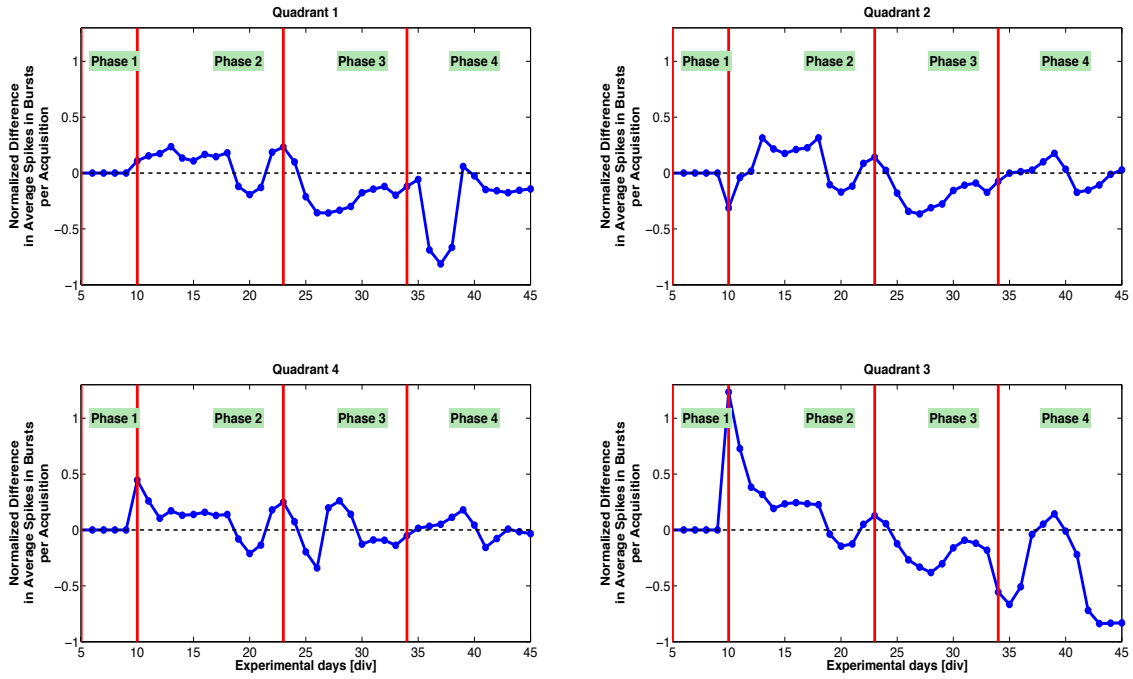

Figure 4.16: Normalized Post-Pre Differences of Mean Spikes in Bursts in each MEA quadrant.

Remarkably, Figure 4.14 and Figure 4.16 suggest that quadrants on the left-hand side of the MEA electrode grid (Q1 and Q4) display similar temporal trends. A similar effect can be seen in quadrants on the right-hand side of the MEA dish (Q2 and Q3).

4.5.4 Burst Parameter Temporal Analysis

To measure the stimulation effects on the temporal network activity evolution the number of bursts and the mean number of spikes in bursts have been measured over all the MEA electrodes and then averaged across neuronal cultures. As described in the above section, even for the temporal analysis a moving average boxcar filter with a 3-day sliding window has been used to average the results derived from different neuronal networks.

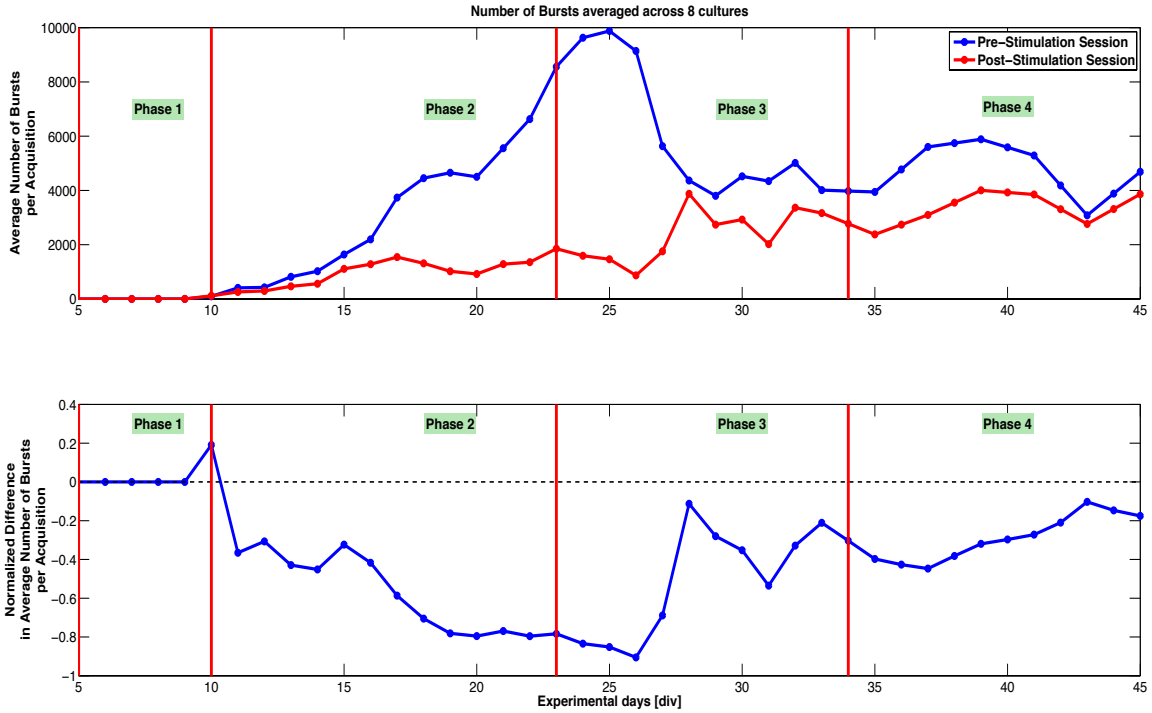

Figure 4.17: Overall Average Number Bursts in acquisition. Top panel: blue curve represents pre-stimulation results. Red curve represents post-stimulation results. Bottom panel shows the Normalized Post-Pre difference between the average number of bursts.

Figure 4.17 shows the temporal analysis of the average number of detected bursts. Once again, these results confirm that the stimulation is inhibiting the network activity, as demonstrated by the negative changes in number of bursts. Moreover, the inhibitory effects reached their negative peak at the beginning of phase 3 before starting to become less negative towards the end of the experiments in phase 4.

Figure 4.18 shows the temporal analysis for the mean number of spikes detected within bursting events. The top panel shows pre and post recording results, while the bottom panel shows the normalized differences between Post and Pre. These results did not indicate the same inhibitory effects that were revealed by the analyses performed on spike count and number of bursts. Moreover, the effects of the stimulation did not decrease towards the end of the experiments in phase 4 during.

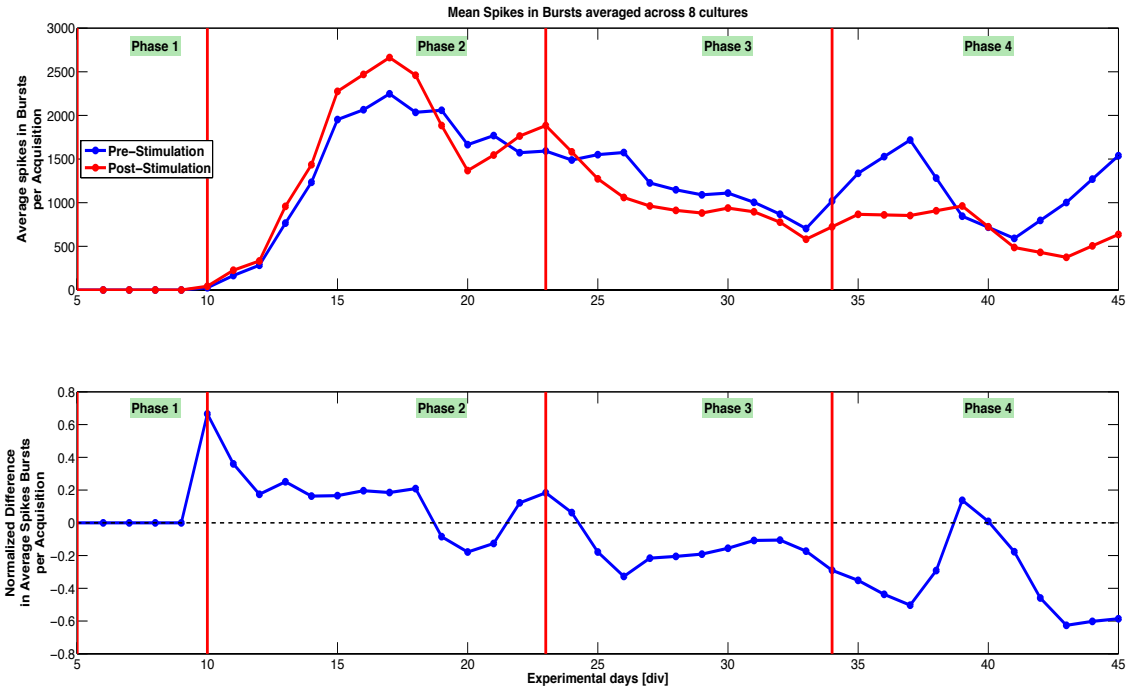

Figure 4.18: Overall Average number of Spikes in bursts. Top panel: Blue curve represents Pre-stimulation recordings, red curve Post-stimulation recordings. Bottom panel: Normalized Post-Pre difference in average number of spikes in bursts.

4.6 Discussion

The spatial analysis of network activity shows that the local stimulation protocol implemented in this work was not able to evoke any localized changes in network activity. This is proven by the fact that when comparing activity pre and post-stimulus, all the quadrants display similar trends in firing activity independently of the delivered stimulus location (position), as seen in Figure 4.8. This rejects the hypothesis that neurons that are closer to the stimulating electrodes respond with stronger activity. This also means that network connectivity is stronger than expected, that the neurons are so well-connected that even the application of localized stimulus pulses made the networks respond as a whole with activity that was widespread over all the electrodes. Another explanation could be that using the larger reference electrode as the negative one to deliver the stimulus voltage pulses might induce currents in the MEA dishes that are not localized as previously thought.

Despite the fact that the stimulation parameters we chose in this work were either adapted from previous research studies [81] or justified by physiological reasons (see paragraph 4.3), they do not appear to be optimal for driving spatial changes in network activity patterns. Before concluding that local effects cannot be generated further testing is required using different voltage pulses and changing the stimulation parameters.

Furthermore, the suspension of stimulation in Q3 during experimental phase 3, and then its resumption in phase 4, did not induce any detectable activity changes, neither in this specific quadrant nor in the others. Results can bee seen in Figure 4.5 shows the average differences in firing activity before and after stimulus delivery measured in the four quadrants. No evident changes can be detected among network responses across

different experimental phases, as demonstrated by the non-significant ANOVA test p-values for all of the three phases.

Figure 4.8 results again show two important findings, 1) it is not possible to identify any stimulus-evoked spatial changes in the network; 2) over time, the neuronal networks tend to respond with smaller variations to the presented chronic external stimulation, as demonstrated by the smaller differences in average activity recorded in phase 4. In other words, stimulation produces smaller inhibitory effects on the network behavior over time, meaning that the neurons are habituating to the presence of the electrical stimuli. Inhibitory effects due to the presence of external stimulation in these neuronal preparations make sense. Most components of the nervous system modulate activity and propagate information by means of inhibition and the absence of inhibition is what causes the system to become more active [83], [86]. In this view, it is to be expected that the presence of external electrical input signals induced the network to be less active compared to the absence of stimulation. Furthermore, results derived from the analysis of network bursting activity patterns did confirm what observed in spike count analysis (see Figure 4.14 and Figure 4.16). Suggesting that these dissociated neuronal network features are more interrelated than previously thought and that their combined use could lead to a better understanding of neuronal network connectivity evolution.

An unstimulated control for these experiments could have been useful to prove the different activity patterns generated by the input presence. Although, in MEA studies there are so many variables that take place while running the experiments, that it is arduous to have a good experimental control. In [14] the authors demonstrated that neuronal network temporal spontaneous activity evolves changing its activity level.

Precisely, neuronal networks exhibited a steady ASDR increase during the first three weeks in vitro, then their activity leveled off, while the degree to which culture-wide bursts dominated the activity kept increasing.

The temporal analysis results presented in this dissertation show that daily network activity varied in a statistically significant manner (see Figure 4.11), suggesting that short-term plasticity phenomena took place. In addition, the meaningful differences in activity between pre and post-stimulation sessions compared to changes within single sessions demonstrate that our stimulation protocol was successful in generating stimulus-evoked responses. Moreover, a stimulation habituation effect was highlighted in the neuronal networks over the course of 30 days, with network stimulus-evoked responses growing in phase 2, reaching their maximum in phase 3 before decreasing in phase 4, when the network had become desensitized to external voltage pulses. Long-term plasticity phenomena are exposed in Figure 4.11 and Figure 4.12 that display how differently the neuronal networks reacted to the same stimulation protocol that was delivered in phases 2 and 4. One last consideration regards differences across experimental phases. They were significant, but they did not reflect the amount of delivered stimulation within each phase. For instance, in Figure 4.12 differences in average activity are not higher in phases 2 and 3 during which the largest amount of electrical stimulation was delivered.

In the presented experiments, chronic stimulation was applied for 30 days and although this represents a considerable period of time in comparison with the lifespan of MEA neuronal preparations (up to 3 months), the time range utilized in the presented experiments could not have been optimal to elicit temporal changes in neuronal activity.

In future work, other time scales should be tested to investigate if further network dynamics can be identified.

CHAPTER 5

CONCLUSION

This work has studied how dissociated cortical neurons respond to chronic electrical stimulation. In particular the temporal evolution of neuronal activity has been investigated in response to a constant electrical stimulation protocol over the first five weeks of neuronal development. The goal was to quantify changes in neuronal network functional connectivity, in dissociated cortical neurons. This work hypothesized that both external stimuli and network functional evolution were fundamental in neuronal development as shown in [1]. In fact, the results show an evolution in network activity in two ways. Neuronal connectivity tends to evolve over time, with changes in both the number of statistically significant stimulus/recording pairs as well as the average length of connections and the number of connections per active node. It was therefore proposed that the FDR analysis combined with two metrics, the average connection length and the number of highly connected "supernodes" is a valuable technique for describing neuronal connectivity in MEA dishes.

Furthermore, the results indicate that cultures dissociated from the same brain tissue display trends in their temporal evolution that are more similar than those obtained with respect to different batches, as quantified by the one-way ANOVA tests run within and across batches. This suggests two hypotheses that could help explain the observed

phenomena: 1) Cultures derived from the same brain tissues were cultured and exposed to experiments in the same time periods and under very similar experimental conditions, this could have induced the similarities in the observed results. 2) Even after dissociation, the neurons preserved some of the properties and characteristics of the original brain tissue they were harvested from. This would indicate that genetic information and genetic programs control neural development and neural firing more than previously hypothesized. [19]

The second aim of this dissertation was to investigate long and short-term plasticity responsible for memory formation in dissociated neuronal networks. In order to address this issue, a set of experiments was designed and implemented in which the MEA electrode grid was divided into four quadrants, two of which were chronically stimulated, every two days for one hour. This stimulation paradigm was applied for ten days, while the remaining two quadrants (Q2 and Q4) were never stimulated and could then be used as an on-dish control. After this period of chronic stimulation, the stimulus delivery was suspended in Q3 for ten days; Q3 is therefore the "experimental" quadrant. During this period only Q1, defined as the "test" quadrant, was stimulated. Ten days later, stimulation delivery was resumed into both quadrants (Q1 and Q3). Overall network and quadrant responses were then analyzed to quantify what level of plasticity took place in the network and how this was due to the stimulation interruption. The experimental paradigm was chosen to study differences in the test quadrant's stimulus-evoked activity, once the chronic stimulation was resumed, and to determine if the activity in the experimental and test quadrants eventually went back to the activation levels they displayed before suspending the stimulation. Even though the electrode grid was divided

into different areas, such a division does not apply to the underlying neuronal networks. In fact, the cultured neurons are capable of generating connections that spread out across any area of the MEA dish. This implies that stimulation, even if localized to a specific site, could evoke responses and modifications in different locations of the dish. As a result, the observed spatial changes in the neuron activity were not exclusively contained in a quadrant.

One of three possible outcomes was expected. The experimental quadrant responds to the resumed stimulation with a lower activity compared to the test quadrant to which chronic stimulation was regularly applied. The experimental quadrant responds with higher activity compared to the test quadrant. The experimental quadrant shows no significant difference from the always-stimulated quadrant.

Our results demonstrate three main findings:

1. There were no spatial differences in the stimulus-evoked activity within quadrants and that localized stimulation evoked responses whose effects extended well beyond the quadrants in which they were delivered. Data shown in Figure 4.8 emphasize how similar normalized average differences between post and pre-stimulation recordings are for the four quadrants. This demonstrates that there were no spatial effects due to the localized stimulus delivery.
2. The chosen stimulation protocol induced depression effects in the neuronal networks as demonstrated by the consistently lower network activity following stimulation sessions. It is interesting to notice how the post-stimulation and pre-stimulation recordings followed different behavioral

trends. ASDR post-stimulation was consistently lower than in pre-stimulation acquisitions (see Figure 4.10). More importantly, the trend that can be seen in pre-stimulation activity was not present in post-stimulation recordings. Figure 4.10 results show that network activity after stimulation delivery was much more stable over experiments and across phases, with the mean values across experiments that stayed more stable than corresponding mean values derived from pre-stimulation recordings.

3. The inhibitory effects of the stimulation decreased over time, thus suggesting a habituation phenomenon (see Figure 4.11 in which the temporal evolution of the inhibitory effects due to stimulation is presented).

The results presented in this work demonstrate that external stimulation has a fundamental role in modulating the electrical activity of dissociated neuronal cultures. Stimulation was able to consistently evoke stimulus-induced responses in these neuronal preparations that were significantly different than spontaneous variations within single acquisition sessions. Moreover, long-term plasticity effects have been revealed by the fact that when exposed to the same stimulation protocol twice at different times, the networks on average responded with activity patterns that were statistically different, proving that the applied stimulation approach had induced long-term synaptic plasticity. Another interesting finding is that the implemented stimulation induced inhibitory effects on network firing, emphasized by the fact that post-stimulation activity was steadily lower than pre-stimulation. Moreover, this inhibitory effect proved to become less evident over time, meaning that networks tended to habituate to chronic stimulus delivery. Furthermore, firing activity was

more stable in recordings carried out after stimulation delivery sessions compared to pre-stimulus recordings.

Such findings are enough to conclude that electrical stimulation is an important tool to interact with dissociated neuronal cultures, but localized stimuli are not enough to drive spatial synaptic potentiation or depression. On the contrary, the ability to modulate synaptic temporal plasticity was a feasible task to achieve by chronic network stimulation.

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