EXECUTIVE SUMMARY OF THE THESIS

HiPSC analysis with Multi-Electrode Array technology: setup and methods for a case-control study

LAUREA MAGISTRALE IN BIOMEDICA ENGINEERING - INGEGNERIA BIOMEDICA

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Academic year: 2022-2023

1. Introduction

The present thesis has been carried out within the context of a collaboration between the Neuroengineering and Medical Robotics Laboratory in Politecnico di Milano and the Experimental Imaging Centre in IRCCS San Raffaele Hospital. The collaboration aims at adopting some among the most advanced techniques in the field of electrophysiology to study the brain. In fact, in recent years, brain research has experienced significant progress with the emergence of new discoveries, methodologies, and available resources. Understanding the normal functioning of the brain and unravelling the mechanisms behind brain diseases are major goals. To achieve this, it is crucial to develop accurate functional models of the human brain's pathological aspects. While animal cells have been used historically, they often fall short in representing the complexities of human physiology. Therefore, there is a need for more relevant models to study brain diseases effectively [5].

Induced pluripotent stem cells (iPSCs) can be derived from somatic cells and possess the important ability to differentiate into various cell types, including neurons. As known, iPSCs retain the identical DNA of the original individual, making them particularly valuable in studying pathological conditions [5]. In this way, iPSC-derived neurons express an electrical activity that can be hypothesized as similar to the pathological case and can be studied both at the single cell level and at a network level.

When it comes to studying the behaviour of individual cells, one of the key measurements used is the spiking activity. By observing and analyzing the patterns of electrical impulses, or spikes, generated by a single cell, we can gain insights into its functional characteristics and responses. However, understanding the broader dynamics of neuronal networks requires a different approach. In order to examine the interconnections and maturation of these networks, researchers often recur to a measurement known as bursts. Bursts refer to clusters of spikes that occur in rapid succession, representing coordinated activity within a network of interconnected cells. [7] This synchronized activity is fundamental to various aspects of neural processing, including learning and synaptic plasticity.

Learning, the process by which neural circuits adapt and acquire new information, relies on the ability of individual neurons and networks to undergo changes in their synaptic connections.

Synaptic plasticity, the strengthening or weakening of synaptic connections, is a key mechanism underlying learning and memory formation. [2]. If present in iPSC-derived neurons is interpreted as a sign of functional circuit formation between cells [2].

A valuable approach to studying the behaviour of neuronal networks, including those derived from induced iPSCs, is through the utilization of multi-electrode arrays (MEAs). MEAs consist of electrodes that record extracellular voltage, which correlates with neuronal action potentials. The advantages of MEAs include their non-invasive nature, allowing long-term measurements without disrupting the cellular environment, the ability to culture cells on them, enabling observation of cellular changes over time, after ensuring the proper environmental conditions for the cells' survival. [1]

1.1.   Aim of work

In the present thesis, we present the setup and analysis protocol for a comparative study of human iPSC (hiPSC) from one control and one pathological subject affected by a genetic disorder, namely Prader-Willi syndrome (PWS). PWS is a multisystemic complex disorder caused by a lack of expression of genes on the paternally inherited chromosome 15q11.2-q13 region, a region that comprehends genes expressing, among the many, $GABA_A$ receptor subunits, present in neurons. In particular, the work was focused on assessing differences between the control and pathological subject in terms of

1. maturation of the culture, analysed through the basal activity of the sample;
2. response to high frequency stimulation;
3. response to glutamate stimulation.

2.   Materials and methods

The biological material used in the experiments was obtained from the Neurogenesis and Stem Cell Unit Laboratory of IRCCS San Raffaele Hospital. The procedure to obtain the hiPSCs used in this study is a reference method [3] with a slight modification: human peripheral blood mononuclear cells (PBMCs) were used, instead of human skin fibroblasts.

Once differentiated, the 250,000 iPSC-derived neurons were seeded onto a coated MEA chip details of the samples can be found in Table 1

and their activity was recorded.

2.1.   Instrumentation setup

The MEA system employed in the study consisted of a MEA1060-inv-bc mounting chip with 59 recording electrodes and one grounding electrode, a 200/30iR-Ti-gr MEA chip. It also included a pre-amplifier, power generator, temperature-controlled plate, and a stimulus generator. This comprehensive setup enabled the accurate recording of neuronal activity, controlled stimulation, and maintenance of optimal environmental conditions for the cells under investigation.

2.2.   Basal activity in weeks

An analysis of the maturation of the cultures in terms of activity of both control and pathological subject was performed. The objective is to access when, in terms of weeks from seeding, the activity is higher and to highlight possible differences in the maturation of the cells in the two types of samples. The recording used was the first basal of the day for each chip, which lasts 5 minutes.

2.3.   High frequency stimulation (HFS) protocol

Besides the maturation of the cultures, we wondered if there was a difference in the network organization of the network and response to external stimulation. To do so, a high frequency stimulation protocol was used to see if the cells showed long-term potentiation. The HFS analysis was performed by comparing the first 5 minutes of basal activity and the activity in the 5 post stimulation. In between HFS was provided for 5 minutes. A biphasic 500 mV pulse was used, each phase lasts 300 μs, the negative phase before the positive one. This stimulation unit was repeated four times every 9400 μs, so to result in 100Hz. This 100Hz stimulation was repeated every 200 ms ten times. This block was repeated ten times every 30 seconds. This pattern was inspired by previous works [2, 4].

2.4.   Glutamate

Following the HFS protocol, we aimed to test another type of stimulation, namely chemical stimulation with glutamate. Glutamate is an excitatory neurotransmitter, its effect on neurons is

	1 week	2 weeks	2 weeks	4 weeks	5 weeks	6 weeks
Control	3	5	5	7	7	2
Pathological	3	5	5	8	8	3

Table 1: Numerosity of the group used for the time course analysis for the control and the pathological.

to make the generation of action potential possible and easier. For the glutamate stimulation protocol one single recording was performed: 5 minutes of basal recording, glutamate addition and lastly another 10 minutes were recorded. To evaluate the effects of this stimulation the basal activity was compared to the one of the first 3 minutes post-stimulation.

2.5. Software comparison

For data analysis a custom SW was developed, it performs event detection based on thresholding methods and extracts the most relevant features related to the culture activity. It has been coded to obtain a fully open source SW, with open access to the parameters used in the analysis and can be changed easily to the operator's needs and to new algorithms. The developed SW was compared to MC Rack, commercial software commonly used with the same instrumentation. The decision to compare the developed software to it was motivated by the intention to establish a comparative benchmark within the existing literature. Two cases are considered, the first signal has clear and well visible spikes, and the second present some background noise during spiking activity. The standard deviation (STD) of the entire signal was considered to somehow characterize numerically this difference. The STD of the signal in case one is 3.06 μV and for case two is 3.94 μV.

2.6. Feature extracted

Events detection was done using a thresholding method [6] using the developed SW. The selected features extracted from the signals and used for the analysis [5] are:
- total events per minute
- bursts per minute
- number of active channels
- mean amplitude of events
- number of channels with bursts
- mean length of bursts

In particular, the features related to the single events describe the general activity of the sample while the ones related to the burst provide an indication of the grade of connection and interaction within the network.

3.   Results

3.1.   Software comparison

Two channels were taken into consideration and present two different conditions: the first one has visually distinguishable spikes, and the second one presents significant background noise along with the firing activity. The result of the threshold used for spike identification, number of events detected, mean ISI and number of bursts are reported in Table 2.

3.2.   Basal activity in weeks

The first comparison was related to the maturation of the cell cultures. In figure 1a the data of the total spiking activity is reported: the pathological subject expresses a higher activity w.r.t. the control one from the 3rd week with a further evident increase at the 4th and 5th weeks. The same pattern can be seen in figure 1b which reports the number of bursts per minute. Although these differences seem promising, the Mann–Whitney U test tells us that there is no significant difference between the control and the pathological groups in both the features reported.

3.3.   High frequency protocol

From the plots and the statistical analysis, no relevant difference between the pre and post stimulation emerge in all the features considered in the HFS protocol.

3.4.   Glutamate

Given that the post stimulus response in the case of chemical stimulation was faster, we opted for a different analysis of such a response. In par-
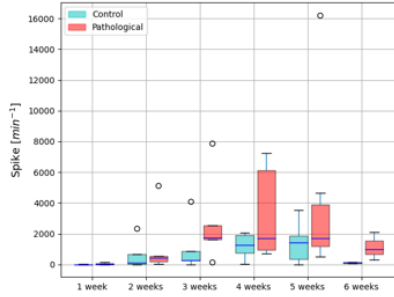
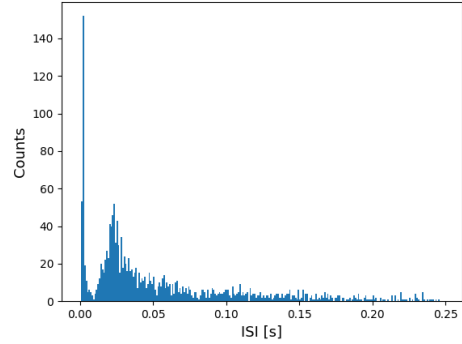(a) Total spike per minute in pathological and control subjects.
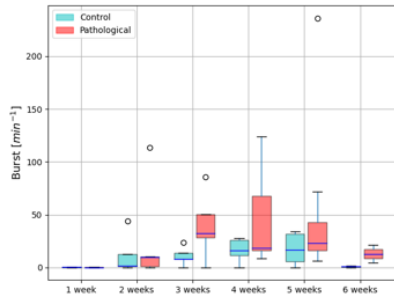
(b) Total burst per minute in pathological and control subjects.

Figure 1: Boxplots of the total spike per minute and total burst per minute, considered for the general activity level of the cells and for the interconnection respectively.
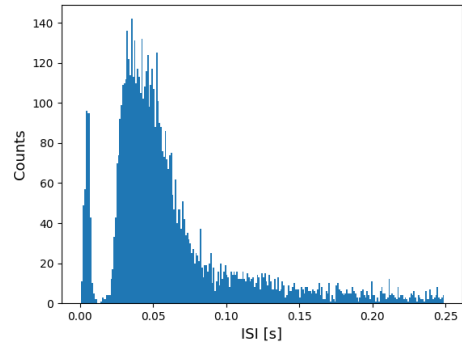

(a) ISI histogram before adding glutamate to the control subject.
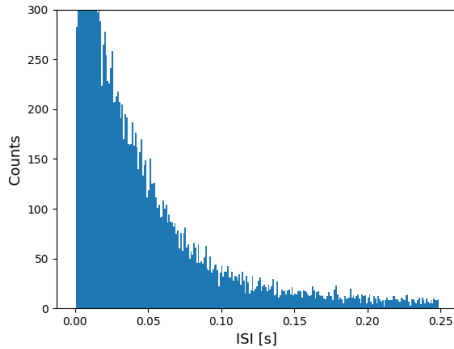

(b) ISI histogram after adding glutamate to the control subject.

Figure 2: Control ISI histogram, on top before the addition of glutamate, bottom right after.

ticular, histograms of Inter-Spike Intervals were chosen as a suitable representation. Pre and post stimulation results are reported in 2, in the basal histogram a low activity is shown, adding the glutamate the distribution of ISI doesn't seem to change but the number increased. A different result is found in the pathological case, the activity decreased after the injection of glutamate and the shape of the ISI histogram changed, as seen in 3.

4.   Conclusions

In this work, we defined and implement a pipeline for the recording and analysis of iPSC cultures. We tested out the pipeline in the context of a comparative study aiming at accesses differences between a pathological, affected by the PWS, and a control subject. The cellular preparation yielded positive results, as it led to a favourable maturation process. On the other hand, the electrical stimulation protocol provided unclear results and suggest further investigation. This is different for the chemical stimulation, in fact from the results (Figure 2)

the iPSC-derived neurons respond promptly to glutamate where the expected response of an increase in the activity is found. In general, the processing pipeline was deemed satisfactory; additionally, through a preliminary comparison, the event detection resulted comparable to MC Rack. From the testing side, we found that the pathological subject showed overall higher activity than the control one. In particular, it has shown higher spiking activity on the 3rd, 4th and 5th weeks, with a particular predominance in these last two. This conclusion tends to align with the fact that the PWS comports less expression of genes that encode for the $(GABA_A)$ β_3 sub-unit. This is also reflected in features regarding the burst, both of these elements suggest that the pathological subject has a major grade of interconnection between hiPSC-derived neurons.

	Threshold value [μV]	Events detected	Mean ISI [s]	Burst detected
MC Rack 1	13.9	400	0.750	14
SW 1	12.53	418	0.718	11
MC Rack 2	17.5	4143	0.072	20
SW 2	15.11	5982	0.050	24

Table 2: Comparison of the threshold value used for spike detection, values of features extracted from MC Rack and the developed SW. In the first case (1), the signal is clean (the STD of the signal is 3.06 μV) and spikes are easily detectable by eye. In the second case (2), the channel is noisier, in particular, while there is a spiking activity the noise slightly increases (STD is 3.94 μV).

(a) ISI histogram before adding glutamate to the pathological subject.
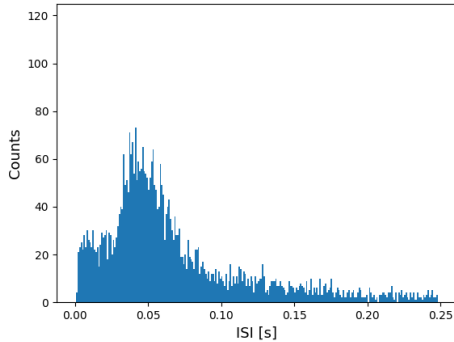
(b) ISI histogram after adding glutamate to the pathological subject.

Figure 3: Pathological ISI histogram, on top before the addition of glutamate, bottom right after. Pay attention to the different scales of the histograms.

4.1. Limitations

The first and important thing to notice is linked to the sample size, the dimension of the two groups suggests that to reach a more reliable result more experiments must be done. Also,

deeper testing of different stimulation protocols should be performed to access the efficacy of the intervention.

5. Acknowledgements

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