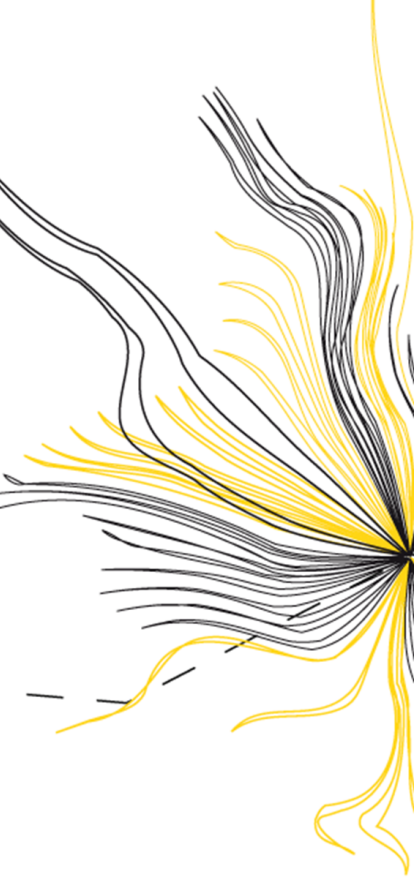

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Human neuronal *in-vitro* model of the ischemic penumbra

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Abstract

Ischemic stroke is one of the leading causes of mortality and morbidity in the world. The area that surrounds the irreversibly damaged core of a brain infarct - the penumbra - can successfully recover if the blood perfusion is restored in time. If not, massive cell death occurs. The fact that current treatments aiming at neuronal inhibition are ineffective, combined with the assumption that neuronal activity is crucial for cell survival, rises the hypothesis that neuronal activation can improve penumbral recovery. The recent advent of human induced Pluripotent Stem Cells (hiPSCs) allows studying brain dysfunctions *in vitro* and might provide more substantial results than rodent models, which cannot fully mimic the unique characteristics of human neurons. Despite the advantages of this technique, no studies on the penumbral recovery have been performed with the use of human neurons.

For this reason, the current project aims to test whether stimulation helps the recovery of human neuronal networks after an ischemic event. To do so, an *in vitro* model of the penumbra was built and the effect of different durations of hypoxia (6 to 48 hours) was assessed in networks coupled to Micro Electrode Arrays (MEAs). Three different stimulation techniques were applied - electrical, optogenetic and chemical (with the use of ghrelin) - and the responses were evaluated in terms of the culture's disperse activity and synchronicity.

Our results show that the survival of hiPSCs derived neurons decreased as a function of the hypoxia duration. Significant differences between synchronicity in the baseline and hypoxia phases were found after 24 hours of low oxygen conditions ($p < 0.05$). Furthermore, stimulation helped maintaining a higher level of activity when in comparison with untreated cultures (more than a 50% increase in the levels of synchronicity), suggesting that activation can improve recovery. Optogenetic stimulation and ghrelin presented the most significant increase after 24 hours of re-oxygenation when compared to controls ($p < 0.05$). The fact that stimulation proved to be beneficial is expected to open new perspectives on the successful treatment of patients after stroke.

Keywords ischemic stroke, penumbra, hiPSCs, electrical stimulation, optogenetic stimulation, ghrelin, neuronal networks

Contents

List of Acronyms

hiPSCs human induced Pluripotent Stem Cells

MEA Micro Electrode Array

mwMEA Multiwell Micro Electrode Array

ATP Adenosinetriphosphate

ROS Reactive Oxygen Species

ST Spike Train

MFR Mean Firing Rate

BT Burst Train

BD Burst Duration

MBR Mean Bursting Rate

NBR Network Burst Rate

NBs Network Bursts

PSTH Post Stimulus Time Histogram

SAT Stimulus Artifact Train

NGN2 Neurogenin 2

Brain Derived Neurotrophic Factor BDNF

Neurotrophin-3 NT-3

Fetal Bovine Serum FBS

SEM Standard Error of the Mean

ChR_2 Channelrhodopsin-2

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Chapter 1

Introduction

Acute ischemic stroke is the second most prevalent cause of death in the world and the principal source of disability [1]–[4]. Stroke can be defined as a sudden neurological deficiency resulting from insufficient blood supply that can be either cerebral or spinal [5], [6]. Although it is a preventable disease, its incidence is expected to rise in the upcoming years, making it one of the main health issues in the Western world [7].

Up to date, the only therapies that can improve neurological outcomes in stroke patients are intravenous thrombolysis [8] and intraarterial treatments [9]. However, the success rate of these strategies is fairly low (patients are only 30% more disposed to have no/minimal impairments), the therapeutic window is reduced (maximum of 3 to 6 hours after the onset) and the patient acceptance criteria are strict [8], [9].

The consequences of cerebral infarction, which is the result of obstructed brain perfusion, are primarily observed by the loss of neuronal functioning and viability and, within 5-10 minutes, by neuronal death [10], [11]. The area that surrounds the core of the infarct, also known as the penumbra, is electrically silent and functionally debilitated due to the lack of blood perfusion [11], [12]. However, the penumbra has the potential for full recovery if the blood perfusion is restored in time, since it initially remains structurally undamaged and viable [13]. If the levels of oxygen and nutrients are not timely restored, the areas of metabolic and functional failure will expand to its surroundings and eventually lead to massive neuronal death [10], [13]. The electrical and biochemical mechanisms behind the transition to irreversible synaptic failure are not yet completely understood.

The existing clinical methodologies to enhance the recovery of the penumbral areas are limited and resort to unspecific interventions [14]. Most of these strategies are based on the inhibition of neuronal activity (e.g. sedatives, NMDA receptor antagonists), aiming to minimize the energy needed to preserve basic cellular function. However, these treatments have not successfully shown substantially improved brain recovery in patients suffering from this condition [15], [16].

Several rodent *in vitro* models of the penumbra have been developed over the years to better understand this intriguing tissue-at-risk and to create possible treatments to improve brain recovery after stroke [14], [17]–[19]. Perpendicular to the prevailing belief that suppression of the network activity acts as a neuroprotector, certain research teams observed that it is, instead, associated with progressive neuronal damage and, eventually, cell death [20], [21]. The fact that treatments based on inhibition have failed in clinical trials and the idea that neuronal network suppression leads to severe impairments, corroborate the theory that neurons need to be active to survive and the notion that there is an ongoing adaptive brain strategy during severe hypoxia towards the increment of the global levels of activity [22], [23]. This knowledge is crucial in the process of finding new successful treatment strategies.

Human cells may respond differently to hypoxia when in comparison to animal cells and thus, their usage may provide more substantial and reliable results for the study of human brain dysfunctions. The recent advent of human induced Pluripotent Stem Cells (hiPSCs) technology and the possibility to differentiate these cells into neurons has opened the way to study human neuronal diseases *in vitro* [24]. Nonetheless, to our knowledge, no studies about hypoxia and treatment effects have been performed on human neurons derived by hiPSCs.

1.1 Research Goals and Methods

This thesis project aims to test whether stimulation helps the recovery of hiPSC derived neurons in a model of the penumbra. To accomplish the desired goal, we will couple human neurons to a 24 well Micro Electrode Array (MEA) and we will expose the networks to different durations of hypoxia (6 to 48 hours). To improve cell survival, different techniques will be applied - electrical, optogenetic and chemical stimulation (with the use of ghrelin) - and the responses of the culture will be assessed in terms of the culture's disperse activity and synchronicity.

1.2 Publications

Part of this study has been published in 'S. Pires Monteiro, J. Covelo, M. Levers, G. Hassink, J. le Feber, M. Frega, "Ischemic Stroke: Treatments to Improve Neuronal Functional Recovery in vitro", Studies in Health Technology and Informatics, pp. 313–316,2019' [25]. The introduction has been adapted from this publication.

Chapter 2

Background

2.1 Ischemic Stroke

Every year, around 15 million people suffer from a stroke and 6 million end up dying from its consequences [26]. Due to demographic changes (e.g. aging of the population, obesity, stress, lack of physical activity) these numbers are expected to increase over the following decades, mainly in the developed countries [27], [28]. Stroke represents a massive health and economic burden on a global scale, since patients suffering from this condition have high chances of developing deficiencies such as motor impairments, depression, cognitive dysfunction and dementia [5], [29], [30].

Ischemic stroke is most frequently caused by the occlusion of an arterial vessel in the brain (ultimately leading to a brain infarction), while only 15% of all stroke cases are due to vessel rupture with subsequent hemorrhage (hemorrhagic stroke) [29]. This event generates the reduction or complete loss of blood delivery (bellow 12 ml/100g per minute) in a certain area of the brain, leading to a shortage in the levels of oxygen and glucose and a scarce expulsion of the metabolic wastes. The reduced levels of adenosinetriphosphate (ATP) are incapable of maintaining ion gradients across the membrane (failure of the Na^+/K^+ pump) and eventually lead to ion influx that generates depolarization and cell swelling. Furthermore, the production of reactive oxygen species (ROS) resultant from the depolarization, causes membrane degradation, inflammation and apoptosis [14], [31]. Since cerebral tissues are extremely susceptible to changes, the abnormal functioning of the brain immediately interferes with the regular neuronal activity and, within minutes after the onset, neuronal death occurs [5].

Recovery after stroke is highly uncertain and extremely time-dependent. The therapeutic window required to avoid the transition from 'reversible ischemia' to 'permanent infarction' is limited and the concept of 'time is brain' is crucial, unveiling the susceptibility of the ischemic brain [32], [33]. For this reason, despite the numerous studies on the topic, only two therapies manage to improve the neurological outcomes of patients after stroke so far: intravenous thrombolysis [8] and intraarterial treatments [9]. However, there is a vast list of drawbacks associated with these techniques (e.g. limited therapeutic time window, low success rate) [8], [9].

The degree of brain damage (infarct size and neurological outcome) is extremely dependent on the residual perfusion. Evidence suggests that the collapse of basic cellular procedures (e.g. synaptic activity, ion pumping and neuronal metabolism) is dependent on the expansion of ancillary vessels from the neighboring areas [10]–[12]. Therefore, it is of extreme interest to study the area that envelops the core of a brain infarct were there is still some remaining perfusion - the ischemic penumbra - since it is a valuable target for therapeutic interventions.

2.2 Ischemic Penumbra

The concept of ischemic penumbra was first introduced by Astrup *et al.* [11] over thirty years ago and it was a significant paradigm shift in ischemic stroke. The penumbra can be defined as the hypoperfused viable tissue surrounding the inevitably impaired ischemic core (regional cerebral blood flow between 12 to 22 ml/100g per minute). In this tissue-at-risk, there is an impairment in the electric activity of the neurons but the residual perfusion is sufficient to preserve their structural integrity and viability [32]–[34].

The fate of the penumbral tissue is highly unpredictable due to its dynamic features. Despite this, the damage might be reversible if the blood perfusion is restored in time. Nonetheless, if oxygen and nutrients are not timely resupplied, the areas of metabolic and oxidative stress will expand and irreversible impairments will take place. The mechanisms behind the propagation of the ischemic core into the neighboring areas and the transition to irreversible damage are not yet completely understood, even though the aggravation of the clinical outcome is severely dependent on this process [10], [32], [35].

The conceptual development of the penumbra over the past 30 years was sub-dived in physiological characterization, acknowledgment of the fundamental neuronal mechanisms and clinical applications [36]. However, despite all the extensive research on this topic, there is no clear consensus on the vital processes that forecast irreversible damage. Hence, the existent strategies to promote the recovery of the penumbral areas are limited and resort to unspecific interventions [14].

Numerous neuroprotective treatments, directed at the secondary damage, have been tested. All these strategies (e.g. hypothermia [15], supportive therapy - sedation [37], magnesium [38], antioxidants [16], NMDA receptor antagonist [39]) share the same fundamental concept: diminishing activity levels to secure the remaining energy for the fundamental procedures. However, despite their success in animal testing, none of the strategies presented any benefit in clinical trials [40]. The reduced knowledge on the mechanisms that occur in this area might be the reason for the negative outcomes.

Studies should focus on the dynamic features of the penumbra and the factors that distinguish injury from repair, taking into account that the penumbra is not merely deteriorating with time but also vigorously repairing [36].

2.3 Stroke-on-an-array

Several *in vitro* models have been developed to better understand the complex mechanisms behind stroke. In comparison to *in vivo* strategies, these experimental *in vitro* models present various benefits such as being less time consuming and less expensive. Moreover, they provide better control on the levels of oxygen and glucose, give the ability to perform mechanistic and dose-dependent studies as well as the possibility to assess toxicity [41].

In vitro dissociated neuronal networks preserve the majority of the physiological, pharmacological and electrical characteristics of the brain and allow an easier observation and manipulation than *in vivo* measurements of the cerebrum. Therefore, it is a simpler and unequivocal way to study the effects of hypoxia and low levels of glucose on neuronal viability and to manipulate certain specificities of stroke (e.g duration and depth of hypoxia, level of glucose). Furthermore, these networks can grow on Micro Electrode Arrays (MEAs), which allows an extensive monitorization of the neuronal activity, since these devices can record electrophysiological signals up to several days in a non-invasive way.

Using the MEA technology, le Feber *et al.* (2016) [14] conducted a study on rat cortical cultures coupled to MEAs exposed to hypoxia of different durations and depths. The authors showed that, un-

der hypoxic conditions, there is an initial reversible reduction in the neuronal activity and connectivity, leading to partial recovery if normoxia is restored within 18 hours. After this period, the changes in connectivity become irreparable, but the integrity of most neurons remains unaltered. Finally, they observed that irreversible neuronal silence occurs after 30-40 hours of hypoxia, seemingly independent of the hypoxic depth. Furthermore, these experiments allowed the authors to conclude that inhibitory neurons are more susceptible to hypoxia than the excitatory ones.

More recently, the same research group suggested that the excitability of neuronal networks increases when these cells are exposed to low oxygen conditions [23]. These novel findings, combined with the previous work, stand perpendicular to the previous belief that neuronal suppression can act as a neuroprotector. Instead, they suggest there is an ongoing adaptive process during hypoxia to rise the total levels of activity. Combining this knowledge with the idea that neurons need to be active to remain alive [21], [42], suggests that hypoxia-induced neuronal inactivity can contribute to neuronal death and, consequently, gives rise to the notion that the activation of the penumbral neurons can improve cell survival.

In line with the previous studies, evidence that neuronal activation might help the recovery of the damaged brain has been evaluated by Stoyanova *et al.* (2015) [43] with the use of ghrelin. This hormone, commonly known as the 'hunger hormone', is responsible for increasing synaptic plasticity, helping in memory consolidation and promoting neurogenesis. The effects of ghrelin were addressed in an *in vitro* model of ischemic stroke, with the use of cortical cultures previously exposed to 6 hours of hypoxia. Mild excitation with ghrelin showed improved outcomes, such as increased synaptic density. Thus, this study opens the possibility of a new therapeutic procedure. Furthermore, preliminary results obtained in le Feber's group, suggest that electrical and optogenetic stimulation might help the neuronal recovery of rodent cells under hypoxic conditions.

Rodent based *in vitro* models have been essential for the understanding of certain pathological mechanisms, since they are a very stable and reliable reductionist approach [44], [45]. However, these tissues and models cannot fully retain the unique and dynamic features of human brain development. Indeed, human cells may respond differently to hypoxia (i.e. different vulnerability of specific cell types and synapses) and to drug testing (i.e. drugs tested in rodent models may fail in clinical trials). Furthermore, rodent models cannot mimic the patient genetic background and human physiology. Therefore, the use of neurons derived from human induced Pluripotent Stem Cells (hiPSCs) might provide more substantial results and ease their translation into the clinical practice [24].

Combining the concept of neurons derived from hiPSCs and MEA recordings might provide essential understanding of specific features and mechanisms of ischemic stroke. This issue will be addressed in the following sections.

2.3.1 Human induced Pluripotent Stem Cells

The advent of hiPSCs and the possibility to differentiate these cells into neurons opened the possibility to study human neuronal diseases *in vitro*. Indeed, in the last few years, this methodology has been extensively promoted as a way of generating cell lines with a specific genetic background, very promising for personalized medicine [24]. However, despite their high potential, the use of this methodology is still in its early stages since culturing hiPSCs, differentiating them into neurons and using them to model diseases is still problematic, requiring a large amount of materials, time and expertise [46].

Over the years, several authors devoted their work to the development of differentiation protocols with a high hiPSC-to-neuron conversion efficiency [47]–[50]. However, most of the studies presented severe limitations such as being time-consuming (taking up to several months), presenting a low

conversion rate and providing little control over the eventual neuronal density.

To overcome the limitations of the previously developed conversion protocols, Zhang *et al.* (2013) [51] conducted a study in which the authors obtained mature networks within 2 to 3 weeks in a highly efficient way. However, this approach still provided limited control over the final neuronal density with respect to the hiPSCs plated initially, crucial for electrophysiological studies with MEAs. Later, Frega *et al.* (2017) [52], focused on optimizing the parameters of this study, by reducing the variability concerning the number of cells existent at the beginning of the process. Thus, this new protocol is highly reproducible, stable and presents significant control over the density of the cells, providing fair consistency for neurological studies.

Neuronal networks derived from hiPSCs exhibit their electrical activity in the form of spiking and bursting. Spikes (action potentials) can already be visible in the early stages of development and are defined as changes in the electrical activity that exceed a certain threshold. This sparse activity develops into bursts, which are an extremely condensed sequence of spikes that can be globally synchronized across the culture (in the late stages of development) forming network bursts (NBs) [53], [54]. Bursting patterns can be seen as a very robust phenomenon, since they can represent the general activity of a given culture. These patterns are deeply shaped by several external factors such as the age of the cells and the presence of electrical/chemical stimulation [55]. Network bursts play a relevant role in synaptic plasticity and may give a clearer insight into the activity of the network, rather than single spikes alone [56].

To analyze the particular mechanisms of *in vitro* neuronal networks, the bursting and spiking patterns need to be examined at multiple locations within the same culture [57]. Human neuronal cultures coupled to MEAs can be seen as a straightforward approach to monitor and record network activity, thereby tackling the dynamic functioning of the brain [58].

2.3.2 Micro Electrode Arrays

Electrophysiology activity measurements are able to capture a broad spectrum of neuronal phenomena [59], [60]. MEAs offer the opportunity to investigate the response patterns of the neuronal cultures in a non-invasive and simple way, by performing real-time multi-site electrophysiology recordings (considered to be a suitable reductionist model) [44], [45]. These extra-cellular recordings do not interfere with the integrity of the cells and, therefore, allow evaluating neuronal networks for a long period, providing more information than other traditional single electrode electrophysiology practices, such as patch-clamp [61], [62]. MEAs are generally seen as a trustworthy method to perform extensive electrophysiology analysis due to their high signal quality and spatio-temporal resolution [63]. Therefore, this technology is widely used for different practices including pharmacological tests [62], [64], [65], neuronal network development [66], [67] and disease modeling [68], [69].

Recently, there was the need to push MEA standard technology (single MEAs with 60 electrodes) into multiwell MEAs (mwMEAs) suited for high-throughput experiments [70]. This approach is crucial for hiPSCs disease modeling, since a substantial amount of recordings is needed to diminish the conversion variability and to generate strong and trustful results. This new system provides the possibility to stimulate and record neuronal networks, at the same time, in 24, 72 or 96 different wells (with 12, 4 and 3 electrodes each, respectively), allowing the user to investigate different treatment types and cell lines significantly faster.

The features of the mwMEA make it possible to monitor the same neuronal network for a long period of time (up to weeks), to see the effects of the different treatments and to assess the effect of low oxygen conditions in different parallel experiments. Thus, this system, in combination with human derived neurons, provides the necessary support to build a consistent reductionist model of stroke.

2.4 Open issues

Ischemic stroke is a very complex and heterogeneous disease and so is the area that surrounds the core of the event - the penumbra. Notwithstanding, despite the extensive effort taken over the years on its understanding, the successful treatment of an injured penumbra after stroke is still far from optimal. Neuronal activation might be the key aspect in the recovery of this tissue-at-risk and different stimulation techniques (e.g. electrical, chemical and optogenetic) might be useful in the process of obtaining the desired activation.

The possibility to differentiate hiPSCs into neurons and to assess their electrophysiological activity on MEAs gives the possibility to study this disease *in vitro* and might provide an easier extrapolation of *in vitro* findings to the clinic, when in comparison to rodent models. However, the comportment of these cells is still not fully understood and thus, their reaction to hypoxia is uncertain. We hypothesize that the combination of different stimulation protocols with these promising cell lines might provide substantial new information on the topic. Therefore, in the current project, we aim to create an *in vitro* model of the penumbra and to:

- determine the effect of different durations of hypoxia (6-48h) on the human neuronal cultures;
- evaluate the maximum duration of hypoxia that is still associated with a (partial) recovery of the network's electrophysiological activity;
- test whether different types of stimulation (electrical, optogenetic and chemical) help the recovery of hiPSC derived neurons.

The following chapter will address the methodology required to achieve all the previously mentioned goals.

Chapter 3

Materials and Methods

3.1 Cell Cultures

3.1.1 hiPSC generation

hiPSCs required for this thesis project had already been generated from fibroblasts obtained from healthy subjects, through induced reprogramming by retroviral vectors expressing the transcription factors Oct4, Sox2, Klf4 and cMyc as previously explained [52]. Lentiviral vectors (Ngn2 and ASCL1) were required to include the transgenes into the genome of the stem cells, which was necessary for the formation of two hiPSCs populations: Ngn2 and ASCL1 positive hiPSCs. hiPSCs cells were supplemented with E8 medium and selection factors (G418 and with puromycin), to select the ones that were successfully transduced with the vectors. The two stable hiPSCs lines (Ngn2 and ASCL1 positive hiPSCs) were the starting point for the neuronal differentiation.

3.1.2 Neuronal differentiation

The study was performed on networks composed by only excitatory (glutamatergic) neurons and networks combining both excitatory and inhibitory (GABAergic) neurons.

Excitatory neuronal networks

Approximately 20.000 Ngn2-positive hiPSCs were plated as single cells into a sterile 24-well MEA with an area of 32 mm^2, precoated with polyornithine and laminin. This resulted in a final cell density of around 2000 cell/mm^2. Doxycycline was added to medium to force neuronal differentiation in the Ngn2-positive hiPSCs, as performed by Frega *et al.* (2017) [52]. The day after plating, cells were supplemented with DMEM/F12 with 1% (v/V) N-2 supplement, 1% (v/v) non-essential amino acids and two human factors: human recombinant neurotrophin-3 (NT-3) and human recombinant brain-derived neurotrophic factor (BDNF) to help neuronal maturation.

Two days after plating, embryonic (E18) rat cortical astrocytes were added to the hiPSCs culture in 1:1 ratio, to support neuronal maturation. These cultures were obtained by sacrificing a pregnant rat, harvesting the embryos from its uterus and isolating the brains. The procedure involving rats was conducted in agreement to Dutch and European laws and the guidelines of the Dutch Animal Use Committee.

The day after the addition of astrocytes, the neurobasal medium was supplemented with cytosine β-D-arabinofuranoside to obstruct the proliferation of astrocytes and to kill the existent non-

differentiated hiPSCs. From this day onwards, each well was filled with $\sim$ 500 μl of R12H (neurobasal) medium as previously defined for rodent cultures, including 1.24 g/L of D-Glucose (6.9 mM) [71], plus four other components specific for iPSCs: BDNF, NT-3, fetal bovine serum (FBS), doxycycline. The medium was changed every 2-3 days - in each well 200 μl were removed and 250 μl were added to compensate evaporation loss. Cells were kept in an incubator with a controlled atmosphere (temperature of 37°C, 100% humidity and 5% of CO_2), until mature networks exhibiting spiking and bursting activity were established.

Excitatory and Inhibitory neuronal networks

In the late stages of the research carried out in this work, the differentiation protocol was improved with the inclusion of GABAergic neurons in the excitatory neuronal network. These cultures were obtained by plating together ASCL1 and Ngn2 positive hiPSCs at a 40/60 ratio. Doxycycline was used to force expression of ASCL1 in combination with forskolin (to increase efficiency) as described in a previously developed protocol [72] and Ngn2 as described in the previous section. This way, two populations of neurons could grow independently while plated in the same well. The final inhibitory/excitatory ratio was $\sim$ 20:80 (as reported in literature [73], [74]). A schematic overview of the process is presented in Figure 3.1.

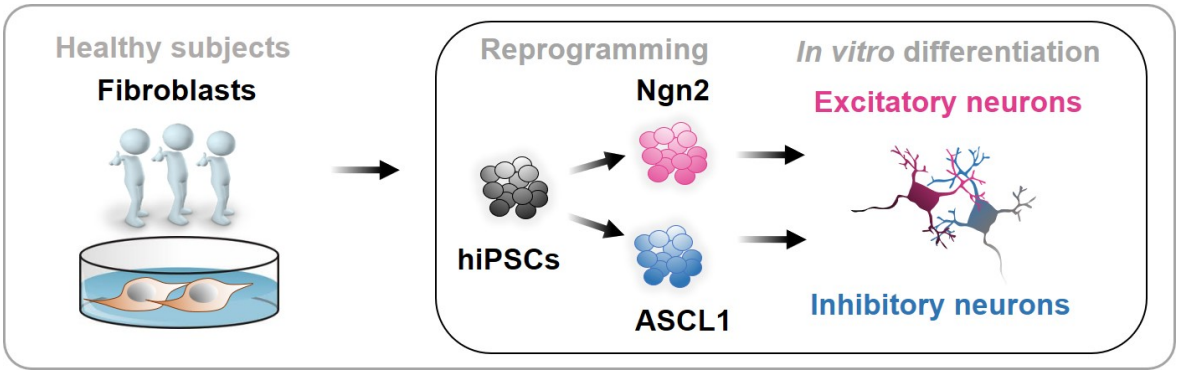

Figure 3.1: hiPSCs generated from skin cells (fibroblasts) differentiated into excitatory and inhibitory neurons through the overexpression of NGN2 or ASCL1 with doxycycline treatment.

3.2 Immunocytochemistry

Human neurons were fixed at 21 DIV with 4% paraformaldhyde and incubated for 15 minutes at room temperature. Cells were washed with PBS (5% normal goat serum, 1% bovine serum, 0.2% triton) to block non-specific binding sites and stored in PBS overnight at 4°C. The cultures were stained for rabbit anti-MAP2 (1:1000; Sigma M3696), rabbit anti-GFAP(1:500, Abcam ab7260) and DAPI (1:1000). The secondary antibodies used were goat anti-mouse Alexa Fluor 488 (1:2000, Invitrogen A-11029) and goat anti-rabbit Alexa Fluor 568 (1:2000, Invitrogen A-11036). Epifluorescent images were taken at a 40x magnification with the use of a Nikon Eclipse 50i Epi-Fluorescence microscope (Nikon, Japan).

3.3 Induction of hypoxia

To model the penumbra *in vitro*, neuronal networks derived from human iPSCs were exposed to low oxygen conditions. Two mass flow controllers were used to establish different mixtures of air and N_2, which were conveyed to a plexiglass cover containing the mwMEA at a flow rate of 0.2 L/min. During normoxia, the values were set to 100% air / 0% N_2 and 10% air / 90% N_2 during hypoxia. CO_2 (5%) was added constantly to the gas mixture.

To prove that neurons were being exposed to hypoxia, the level of oxygen was measured in the medium using a Neo-Fox-GT optical oxygen sensor (Ocean Optics, Largo, FL, USA). Prior to the measurements, the sensor was calibrated in air (pO_2 = 21.0 x atmospheric pressure) and N_2 (pO_2=0). As seen in Figure 3.2, the oxygen level dropped from normoxia (21%) to the 2% defined in the onset of hypoxia in about one hour and took around the same time to go back to the atmospheric values, when normoxia was restored.

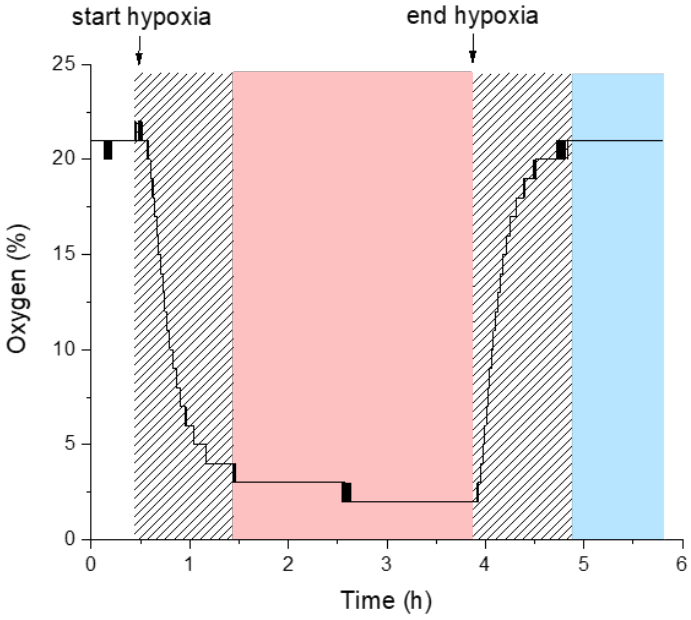

Figure 3.2: pO_2 measured in a mwMEA with neurobasal medium. The background in pink represents the hypoxic period while the blue shows the normoxia. The black strips outline the transition period. Oxygen took 1 hour to reach the value set. Hypoxia: 10% air/90% N_2; Normoxia: 100% air. Constant flow of CO_2: 5%.

3.4 BDNF immunoassay

To measure the release of BDNF from hiPSCs derived neuronal cultures, six GABAergic and glutamatergic cultures plated on coverslips were exposed to 48 hours of hypoxia, followed by 24 hours of normoxia. A part of the supernatant medium (500 μl) was retrieved before the start of hypoxia and 6, 12, 24, 48 hours after hypoxia, as well as 24 hours after re-oxygenation, in each one of the 6 coverslips. Differences in osmolarity were accounted with the use of a K-7400S Semi-Micro Osmometer (Krauer, Berlin, Germany). To measure the quantity of BDNF in the medium, a BDNF ELISA Kit (Chemicon International, Temecula, CA, USA) was used.

3.5 Experimental Protocol

The electrophysiological recordings of the neuronal networks were performed when the excitatory cultures were 5 to 6 weeks *in vitro* while the inhibitory and excitatory cultures were 7 to 9 weeks *in vitro*. This choice was made based on the different developmental times of the cultures. Before the start of each experiment, the 24-well mwMEA was covered with a paper lid to reduce evaporation during the long lasting recordings, while allowing gases to flow through. The experiment was only conducted on wells presenting a minimum of 4 synchronous electrodes, representative of a healthy network. The recording was started after a period of accommodation of 20-30 minutes after moving the cultures into the recording setup, to allow the stabilization of the neuronal networks before the beginning of the experiment. Furthermore, the initial recording of hypoxia was performed only when the oxygen reached 2% (1 hour after). The same time scale applied for the hypoxia-normoxia transition.

In each experiment, hiPSC derived neurons were continuously exposed to normoxia, and 10 minutes of activity were recorded. This step was followed by exposing the cultures to different durations of hypoxia (6, 12, 24, 30 and 48 hours) and, finally, by 6 hours of normoxia. The cultures remained in the recording setup throughout the entire experiment but the activity was only recorded for 10 minutes, every two hours, due to technical limitations (e.g. heavy data and software overload). Afterwards, the cultures had their medium changed and were stored in the incubator for 24 hours. Then, after this period, the activity was recorded for another 10 minutes to further assess their recovery after being exposed to long periods of hypoxia.

In the end of each experiment (after 24 hours of re-oxygenation), if the cultures were still alive and presented synchronous events, networks were then exposed to a second period of 24 or 48 hours of hypoxia, repeating the experimental protocol explained above.

To promote neuronal recovery, different stimulation techniques were applied to the cultures.

3.5.1 Electrical Stimulation

Before the start of the experiment, different electrodes were tested, in a random order, to select one that induced clear stimulus responses. Afterwards, electrical stimulation was delivered to the chosen electrode (biphasic voltage pulses starting with the negative part, 250 μs for phase, 750 mV), either during the entire period of the experiment (*elec stim all*) or only after normoxia was restored (*elec stim after*). The inter pulse interval was set at 5 seconds so each stimulus would not influence the subsequent one. Stimulation was applied for 3 minutes every 2 hours, since it could only be delivered during recordings.

3.5.2 Optogenetic Stimulation

On the seeding day, inhibitory and excitatory neurons were infected with Channelrhodopsin-2 (ChR_2) by the addition of 0.75 μl of virus per well. Therefore, neurons acquired membrane proteins (opsins) sensitive to the blue light (λ = 470 nm), becoming excitable if the cells were exposed to light of this wavelength.

Blue light stimulation was delivered to the cells, during the entire experimental period (*opto stim all*), with the use of a Multiwell-Optogenetic prototype (Multi Channel Systems, Reutlingen, Germany). Light pulses were applied for 3 minutes every 2 hours (200 ms, 10 mA) by putting the device on top of the plexiglass cover. The inter pulse interval was set at 5 seconds so each stimulus would not influence the subsequent one. The experiment was conducted in the dark to avoid light interference.

3.5.3 Chemical Stimulation

Cultures underwent pharmacological manipulation, with the use of ghrelin, either before the start of hypoxia (*ghrelin all*) or immediately after normoxia was restored (*ghrelin after*). Ghrelin in R12H was added to the desired wells (4 μl) at a final concentration of 2 μM as previously described [75]. Every time the medium was changed, 2 μl of ghrelin were added extra, so that the final concentration would remain 2 μM.

All the treatments strategies applied are schematically represented in Figure 3.3.

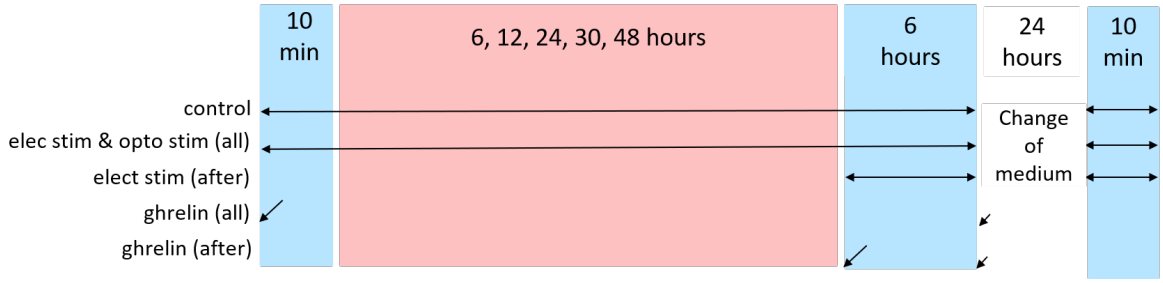

Figure 3.3: Schematic representation of the experimental protocol. The different arrows represent the time at which the specific condition was applied, either regular hypoxia (control), optogenetic stimulation (opto stim), electrical stimulation (elec stim) or ghrelin. The big arrow on the ghrelin treatment represents the introduction of 4 μl in the medium while the small arrow stands for the 2 μl added extra during the change of medium.

3.6 Micro Electrode Array recordings and data analysis

Electrophysiological activity was measured by the Multiwell-MEA system using the Multiwell-Screen software (Multi Channel Systems, Reutlingen, Germany). All the recordings were conducted using the 24 MEA well-plate device with 24 independent wells presenting 12 embedded electrodes each (electrode diameter = 30 μm and distance between electrodes = 200 μm) in a 4x4 disposition excluding corners. The temperature of the mwMEA headstage was set at 37°C during recordings, granting a stable environment for the experiments. An overview of the experimental setup is presented in Figure 3.4.

Data were acquired at a frequency of 10 kHz and the signal was primarily filtered throughout the recording with a high-pass filter (2nd order Butterworth filter, 100 Hz) and a low-pass filter (4th order Butterworth filter, 3.5 kHz). An electrode was considered active if exhibiting at least 0.1 spikes/second. Spikes were considered if exceeding 4.5 times the standard deviation noise threshold (based on the activity of hiPSCs), while bursts were detected if lasting more than 50 ms, containing a minimum of 4 spikes. The minimum interval between bursts was set at 100 ms. Network bursts were considered if at least 50% of active the channels were participating on the burst and if a minimum of 3 were simultaneous.

Data analysis was performed with the use of the Multiwell Analyzer software, which allowed the extraction of the spike and burst trains from the mwMEA system, in combination with different MATLAB scripts (The Mathworks, Natick, MA, USA). Additional parameters were obtained through the adaptation of a custom software package named SPYCODE developed in MATLAB [76].

Both qualitative and quantitative analysis were performed on the activity of the neuronal cultures. Raster plots were used to assess the activity patterns in a visual way.

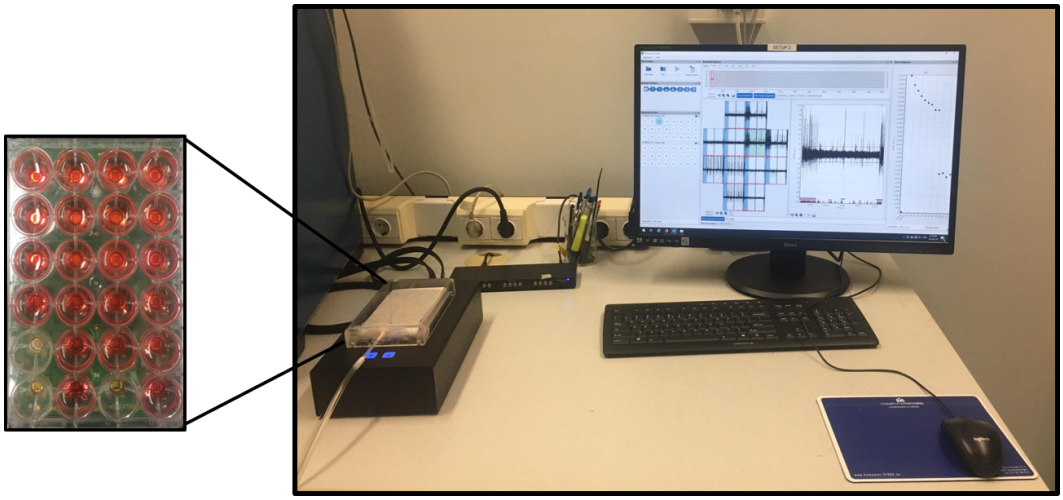

Figure 3.4: Experimental Setup. The headstage contains a 24-well plate MEA (zoomed in) covered by a paper lid. The gases (O_2, N_2 and CO_2) are conveyed to a plexiglass cover containing the MEA. The interface board makes the connection between the MEA and the computer. The Multiwell-Screen software is displayed on the computer's screen.

Raster Plot. A raster plot presents the activity of a group of neurons recorded by each channel (y-axis) over time (x-axis). Therefore, each dot implies that the electrode corresponding to that row registered a spike, at the time stamp seen in the time axis. Furthermore, the picture as a whole gives a clear insight on the entire culture's activity during the time slot designated by each phase of the experiment.

The spontaneous activity of the neuronal network was computed and analysed quantitatively with the use of two different descriptors: mean firing rate (MFR) and network burst rate (NBR). The evoked activity was evaluated with the use of the post stimulus time histogram (PSTH). All the values of the descriptors obtained throughout the different phases of the experiment were normalized with respect to their baseline values to allow comparisons.

Mean Firing Rate. The Firing Rate (FR) is defined as the number of spikes detected through a specific electrode, during the time window of the experiment. The final result of the spike detection is a spike train (ST), a structure containing all the time stamps related to each spike detected, according to

$$ST(t) = \sum_{s=1}^{N} \delta(t - t_s), \tag{3.1}$$

where t_s is the time at which the spike occurs, N is the number of acknowledged spikes and $\delta(t)$ is the Kronecker delta function [76].

FR is defined as

$$FR = \frac{\int_0^T ST dt}{T}, \tag{3.2}$$

where ST is the spike train obtained by Equation 3.1 and T is the time window of the experiment. The MFR is calculated by averaging the FR of every electrode among all the electrodes.

Network Burst Rate. The NBR was calculated by dividing the number of network bursts detected on a well for a defined time (10 minutes of recording).

Post Stimulus Time Histogram. The PSTH presents the probability of a spike to occur (as a function of time) after a certain stimulus is applied [76]. PSTH is defined as

$$PSTH(t) = \frac{1}{N}\sum_{i=1}^{N} ST(t - t_{stim_i}), \tag{3.3}$$

where *N* is the total number of stimulus provided to the culture, t_{stim_i} is the time stamp of each stimulus *i* and *ST* is the spike train as previously defined.

It is also of great importance to store the artifacts that result of the electrical stimulation in order to coordinate recording with stimulation. The Stimulus Artifact Train (SAT) saves the stimulus artifacts, as it is conveyed bellow:

$$SAT(t) = \frac{1}{N}\sum_{i=1}^{N} A_{ST_i}\delta(t - t_{stim_i}), \tag{3.4}$$

where AST_i and t_{stim_i} are the amplitude and time stamp of artifact *i*, respectively.

Stimulus responses were quantified in each experimental phase by the area under the curve of the PSTH of each electrode. A time window of 300 ms was chosen, since this interval was defined as the time in which evoked activity occurred (5 ms bins). The area under the PSTH curve of each electrode was then averaged among all active electrodes - PSTH mean area. A blanking period of 4 ms was considered in all the experiments to avoid mistaking the stimulation artifact with real electrophysiological activity.

3.7 Statistical Tools

In order to study the relevance of the results obtained with this research project, different statistical tests were applied. All the data used for the statistical tests were checked for normality with the use of a Shapiro-Wilk test. In case the data were normally distributed, a two-tailed Welch's t-test was applied. A two-tailed Mann Whitney test was applied in case the data were not normally distributed. The parameters chosen for the statistical analysis were the MFR and the NBR.

These tests were used to perform the following comparisons: baseline and the activity immediately after different periods of hypoxia (6h, 12h, 24h, 30h and 48h); baseline and the results after 24h of re-oxygenation; the values after 24 hours of re-oxygenation in the first and the second exposures to hypoxia; the values after 24 hours of re-oxygenation in excitatory cultures and both inhibitory and excitatory cultures undergoing 24 hours of hypoxia.

Differences between the effects of the treatment strategies in comparison to controls, at 6 and 24 hours post-hypoxia, were also assessed.

In all graphs, the results presented show the mean and respective standard error of the mean (SEM). Statistical tests were performed with the Graph Pad Prism 8 software (San Diego, CA, USA) using a level of significance of 5%.

Chapter 4

Results

4.1 Excitatory Neuronal Networks

The effects of hypoxia were assessed in 78 glutamatergic neuronal cultures. hiPSC MAP2-positive neurons were differentiated, in a regulated way, from control lines (Figure 4.1).

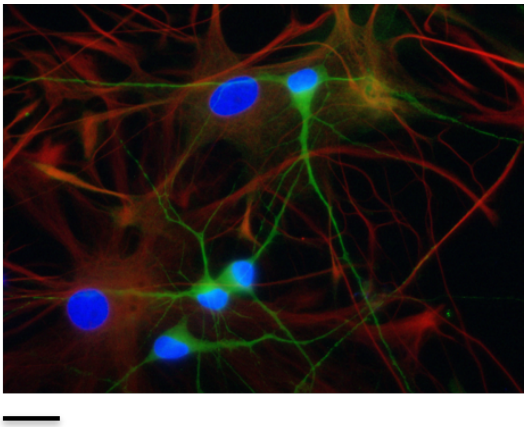

Figure 4.1: Staining of neurons (MAP2, green), astrocytes (GFAP, red) and nuclei (DAPI, blue) at DIV 21. Scale bar: 10 μm.

Furthermore, during the 5-6 weeks *in vitro*, neurons formed a mature neuronal network exhibiting spiking and bursting activity. Network bursts were also detected, indicating that neurons were organized in a functionally connected network (see outlined boxes in the Baseline raster plot of Figure 4.2).

4.1.1 Spontaneous activity

The electrophysiological activity of the cultures was measured as they were exposed to 6 (N=4), 12 (N=6), 24 (N=28), 30 (N=20) and 48 (N=20) hours of severe hypoxia (10% air, 90% N_2, as previously defined). Three representative raster plots of the 10-minute recordings at different time-points of the same experiment are presented in Figure 4.2. It can be seen that the activity of the culture decreases as a function of hypoxia duration. The synchronicity is lost within the first 24 hours since the network bursting activity disappears, while after 48h the network is almost silent. These qualitative results are solidified by the analysis of the two quantitative parameters (MFR and NBR), present in Figure 4.3.

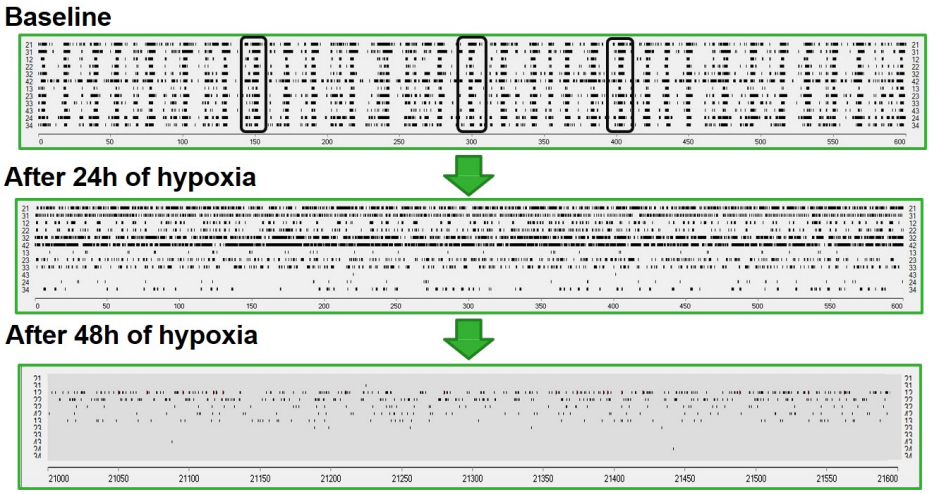

Figure 4.2: Raster plots showing 10 minutes of activity at baseline, 24 hours of hypoxia and 48h of hypoxia. Three representative NBs are identified in the top plot. The activity and synchronicity of the culture are lost as a function of hypoxia duration.

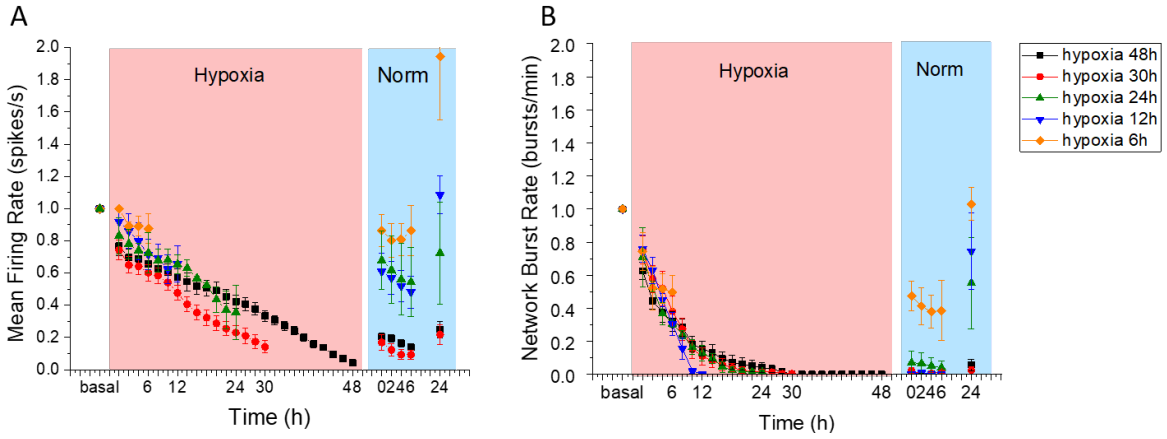

Figure 4.3: Temporal evolution of the MFR and NBR in glutamatergic neuronal cultures exposed to different durations of hypoxia: 6h (orange, N=4), 12h (blue, N=6), 24h (green, N=28), 30h (red, N=20) and 48h (black, N=20); N_{total}=78. There is a general decrease in the spontaneous activity of the neuronal cultures as a function of time and exposure to low oxygen. The synchronicity is the parameter that is mostly impaired after long exposures to hypoxia. Error bars indicate SEM.

From 6 to 12 hours of hypoxia, there is a non-significant decrease in the spontaneous disperse activity of the cultures when compared to baseline (Welch's t-test; $p > 0.05$ for the MFR values of the last recording of hypoxia), while the synchronicity becomes significantly impaired as soon as 6 hours after the onset of hypoxia when compared to the initial values (Welch's t-test; $p < 0.05$). All the cultures lose their synchronicity within 24 hours of low oxygen exposure (Mann-Whitney test; $p < 0.0001$).

After the different periods of hypoxia, the first 6 hours of normoxia are not enough to observe neuronal recovery, since significant improvements can only be seen after 24 hours of re-oxygenation. After this period, networks that underwent 6-12 hours of hypoxia remain active - even hyperactive in the 6-hour scale when compared to baseline (Welch's t-test; $p < 0.05$ for the MFR), while the ones that were exposed to 24 hours of low oxygen conditions start to become impaired (50% decrease in

the levels of synchronicity). The results of longer periods of hypoxia (30 and 48 hours) are clearly visualized by the NBR (Fig 4.3 C) that drops to zero and remains at this level (Mann-Whitney test; $p < 0.0001$ when compared to baseline, for the two time scales).

The effects of electrical stimulation were assessed in these neuronal cultures on the 12 and 24-hour hypoxia durations. However, since our model lacked inhibitory neurons, networks ended up being over-excited and no positive effects on the recovery were seen. These results are presented in appendix A.

4.1.2 Second exposure to hypoxia

In order to further assess the vulnerability of the cultures to low oxygen conditions, we submitted the neuronal networks to a second encounter with hypoxia. Cultures that underwent 6 (N=11) and 12 hours (N=6) of hypoxia were subjected to a second exposure of 24 hours (Figure 4.4), while 22 cultures that were exposed to 24 hours of hypoxia underwent an extra 48 hours (Figure 4.5).

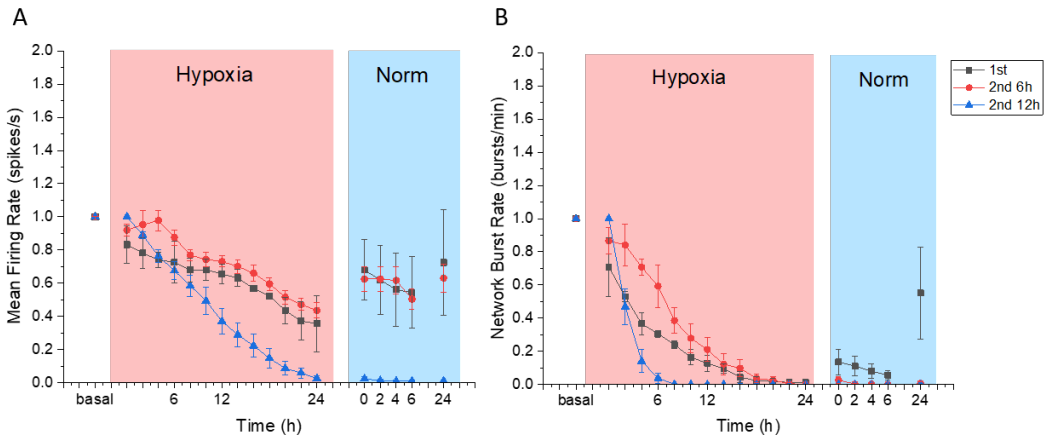

Figure 4.4: Temporal evolution of the MFR and NBR in excitatory neuronal cultures (1^{st} exposure to 24 hours of hypoxia: N= 28; after 6 hours: N=11; after 12 hours: N=6. A second exposure to hypoxia appears to increase the vulnerability of the cells to low oxygen conditions, since the synchronicity is lost, in both cases, after 24 hours of re-oxygenation. Error bars indicate SEM.

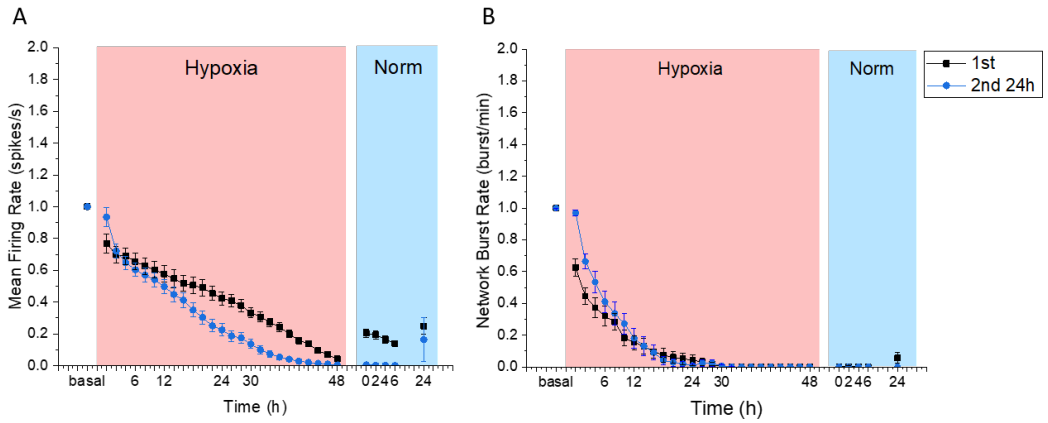

Figure 4.5: Temporal evolution of the MFR and NBR in excitatory neuronal cultures (1^{st} exposure to 48 hours of hypoxia: N=20; after 24 hours: N=22). In both cases, after 24 hours of re-oxygenation, the network's synchronicity is completely lost. Error bars indicate SEM.

Cultures that underwent a first exposure of 6 and 12 hours of low oxygen, when submitted to another 24 hours of hypoxia, are not capable to restore the levels of synchronicity after 24 hours of re-oxygenation, dropping to zero (Fig 4.4 B). These activity values significantly differ from the cultures that were exposed to hypoxia just once, where synchronicity rises up to $\sim$ 50 % baseline 24 hours after restoring the normal oxygen levels (Mann-Whitney test; $p < 0.05$).

Regarding the hiPSCs derived neuronal networks that were submitted to 48 hours of hypoxia (after a first exposure of 24 hours), there is no statistically significant difference after 24 hours of reoxygenation when in comparison to cultures that suffered their first 48-hour encounter with hypoxia, since the synchronicity is completely lost in both cases.

Overall, the cultures appear to be more fragile to the second encounter with hypoxia.

4.2 Excitatory and Inhibitory Neuronal Networks

To increase the reliability of the model, inhibitory neurons were included in the cultures, creating an environment more similar to the human brain. Then, hypoxia was induced in fifteen neuronal cultures (Figure 4.6).

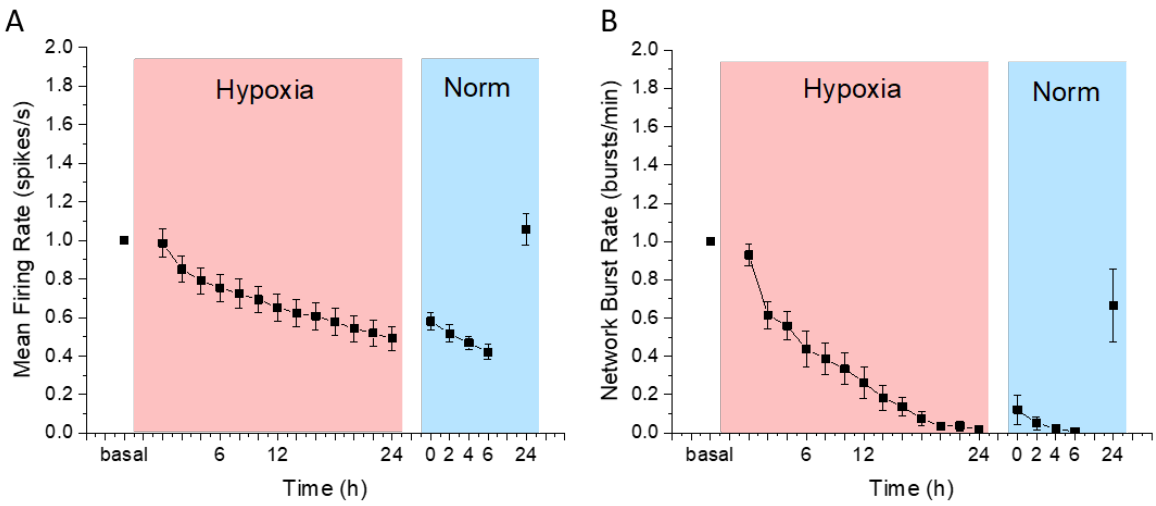

Figure 4.6: Temporal evolution of the MFR and NBR in GABAergic and glutamatergic neuronal cultures (until the end of hypoxia: N= 15; after hypoxia: N=8). Similarly to what was observed in the excitatory neuronal cultures, these networks respond to hypoxia with a decrese in the levels of activity as a function of time. Error bars indicate SEM.

In comparison to the excitatory neuronal networks, these cultures appear to have a similar response to low oxygen conditions (Mann-Whitney test; $p > 0.05$ for the two parameters after 24 hours of re-oxygenation). Since the synchronicity of the cultures is not completely restored after normoxia ($\sim$ 35% decrease), different strategies aiming at neuronal recovery were tested at this time scale of hypoxia.

4.2.1 Second exposure to hypoxia

We assessed the result of a second exposure to hypoxia in the GABAergic and glutamatergic neuronal cultures to further understand their vulnerability to hypoxia (Figure 4.7).

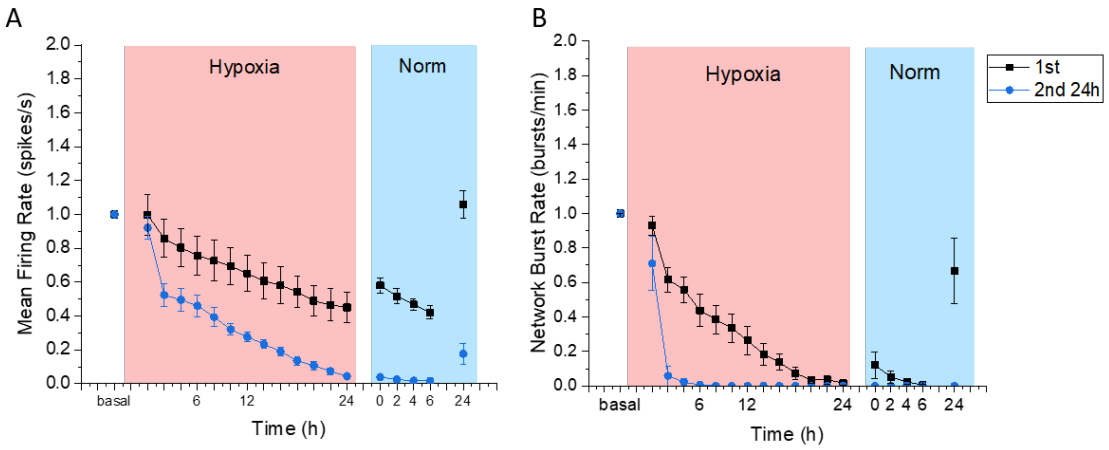

Figure 4.7: Temporal evolution of the MFR and NBR excitatory and inhibitory neuronal cultures (1^{st} exposure to hypoxia: N= 8; after 24 hours: N=6). A second exposure to hypoxia appears to increase the vulnerability of the cells to low oxygen conditions, since the synchronicity is lost after 24 hours of re-oxygenation. Error bars indicate SEM.

Similarly to what was observed in the excitatory neurons (Section 4.1.2), the GABAergic and glutamatergic were less resistant to a repeated hypoxia episode. This tendency is pictured by the complete loss of synchronous events after 24 hours of re-oxygenation when compared to cultures that were only exposed to hypoxia once (Fig 4.7 B), which remained at $\sim$ 65% baseline synchronicity.

4.2.2 BDNF immunoessay

To understand whether there were any fluctuations in the BDNF levels during the experiments, we determined the changes in the amount of BDNF at the different time-scales of hypoxia evaluated (Figure 4.8). We performed the assay twice, using the same 6 cultures.

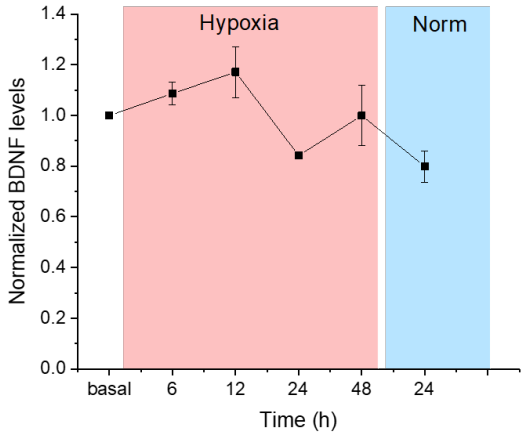

Figure 4.8: BDNF levels normalized with respect to baseline, in six different cultures (one for each time scale). Two different analysis were performed for the same culture at each time-scale. Error bars indicate SEM.

By the analysis of Figure 4.8, it can be seen that the levels of BDNF tend to increase until 12 hours of hypoxia. From 12 to 24 hours, there is a $\sim$ 30% decrease, followed by an increase to baseline

values 48 hours from the beginning of hypoxia. The last value of BDNF measured, 24 hours after re-oxygenation, is 20% lower than the initial value measured.

4.2.3 Treatment Strategies

We delivered pulses of electrical and optogenetic stimulation throughout the entire experimental period to four and three neuronal cultures, respectively, and we added ghrelin to another three networks before the beginning of hypoxia. In addition to these three stimulation paradigms, we also applied electrical stimulation (N=4) and added ghrelin (N=3) to neuronal cultures only after the end of hypoxia. The responses of the cultures to the treatments that we started before hypoxia are presented next (Figure 4.9).

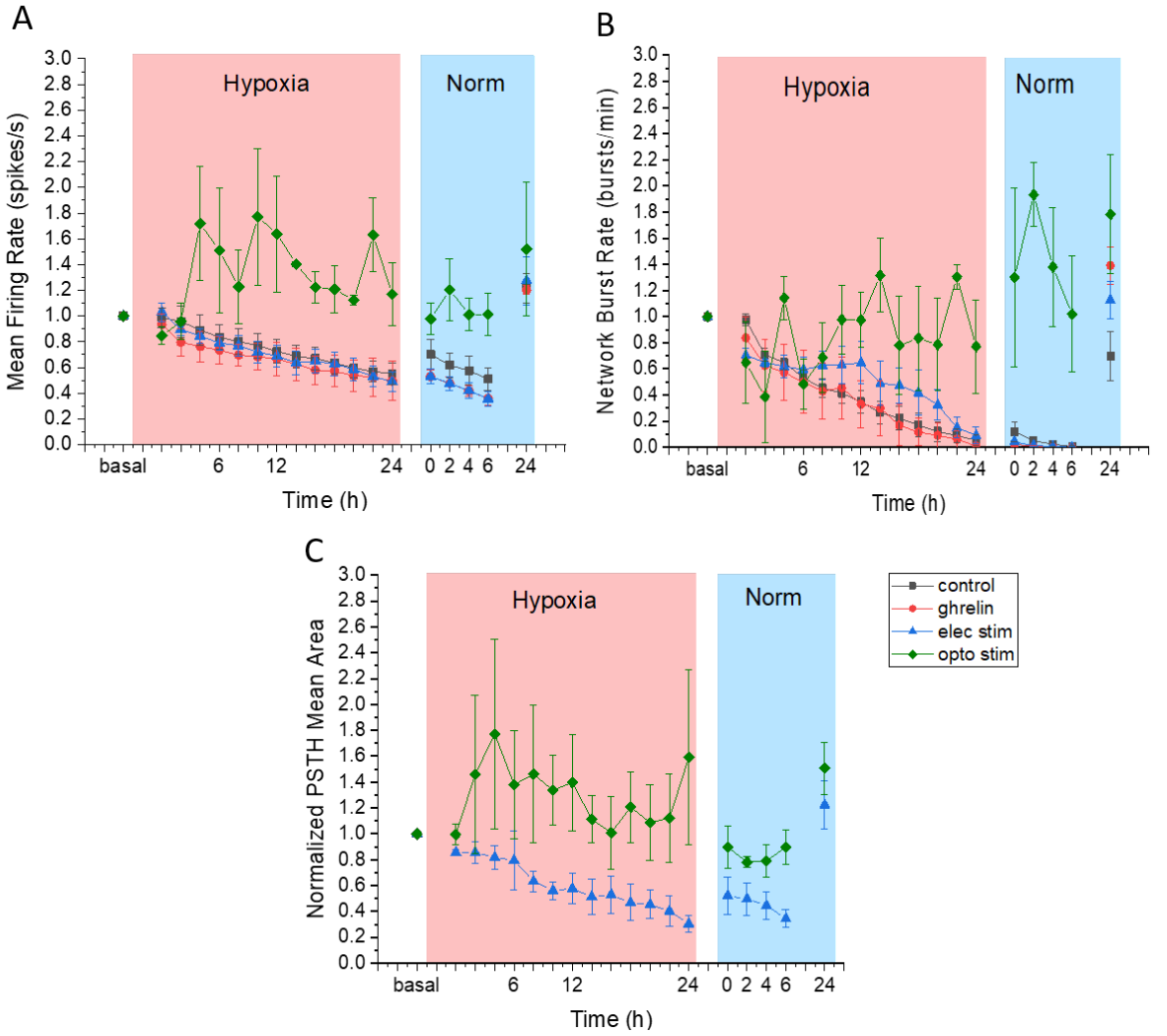

Figure 4.9: Temporal evolution of the MFR, NBR and PSTH in GABAergic and glutamatergic neuronal cultures exposed to three different treatment strategies: electrical stimulation (blue; N=4), ghrelin (red; N=3) and optogenetic stimulation (green; N=3). Controls (black) until the end of hypoxia: N= 14; after hypoxia: N=8. Error bars indicate SEM.

Overall, the temporal evolution of the cultures' activity and synchronicity (Fig 4.9 A and B, respectively), when treated with ghrelin, remains very similar to controls (Welch's t-test; $p > 0.05$ after 24 hours of hypoxia).

Regarding the responses to electrical stimulation, this strategy shows a positive effect on the neuronal networks, keeping synchronous activity at higher levels until around 20 hours of hypoxia (Fig 4.9 B). The responses to electrical stimulation show a tendency to decrease with time as seen by the PSTH (Fig 4.9 C).

The optogenetic stimulation was the only paradigm that allowed networks to remain synchronous throughout the entire period of the experiment (Fig 4.9 B), despite the high standard error.

Post-hypoxic recovery

The responses of the cultures, 6 and 24 hours after normoxia is restored, to all the treatments strategies applied, are presented in Figure 4.10.

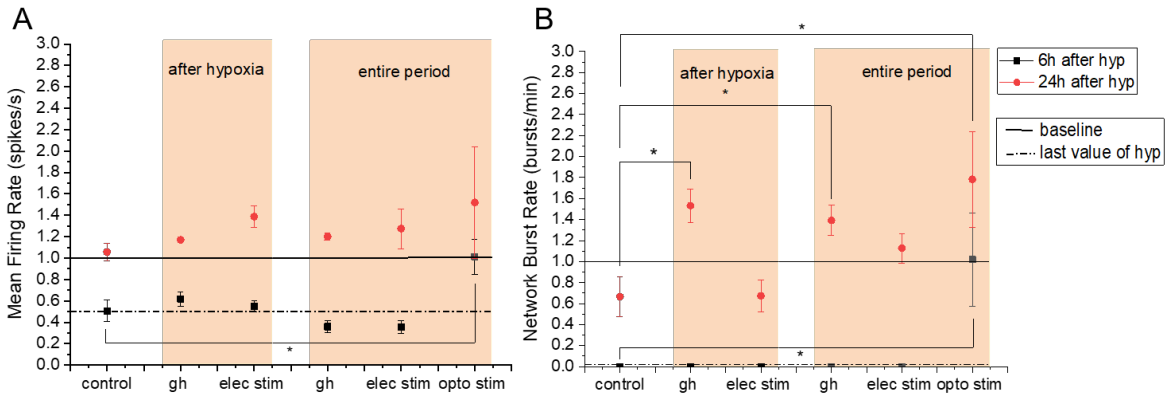

Figure 4.10: Values of MFR and NBR for the five different treatment paradigms applied in comparison to the controls (gh = ghrelin; elec stim = electrical stimulation; opto stim = optogenetic stimulation), 6 and 24 hours after the end of hypoxia (24 hours of exposure). The first shaded area comprises the treatments that were applied after hypoxia while the second one presents the strategies that were delivered as soon as the experiment started. The black line represents the initial baseline value. The doted line stands for the last value of each parameter in the hypoxia phase of the control. Error bars indicate SEM.

Overall, most of the stimulation techniques did not show any significant effects on the disperse spiking activity of the neuronal cultures, neither 6 hours nor 24 hours after the end of hypoxia (except optogenetic stimulation after 6 hours), since these patterns are the simpler forms of activity and, thus, more easily reestablished (Fig 4.10 A).

When looking at the most complex patterns of neuronal activity (Fig 4.10 B), it can be seen that only the cultures that were optogenetically stimulated present improved synchronicity after 6 hours of re-oxygenation, when compared to controls at the same time-scale (Mann-Whitney test; $p<0.05$). Significant differences were also found between the control and the optogenetic stimulation groups after 24 hours or re-oxygenation (Mann-Whitney test; $p<0.05$). At this time scale, the NBR was also significantly higher in the two ghrelin groups when compared to controls (Welch's t-test; $p<0.05$). The baseline synchronicity was restored when electrical stimulation was delivered trough the entire experimental period, reflecting the culture's tendency to increase the synchronous activity.

Overall, by the analysis of these two descriptors, it can be seen that the NBR is the most sensitive parameter to hypoxia, since the synchronicity after 6 hours of post-hypoxia is completely lost in controls.

Chapter 5

Discussion

In this thesis project, we aimed to test whether stimulation helps the recovery of hiPSC derived neurons after an ischemic event. To accomplish the desired goal, a human model of the penumbra was built and the effects of low oxygen were assessed in the human neuronal networks.

In line with some authors [14], [75], we hypothesize that neuronal activation is crucial for cell survival under low oxygen conditions and that the proper activation should be achieved for a better outcome. Our results showed, for the first time, that severe hypoxia leads to a substantial decrease in the activity of the hiPSCs derived neuronal cultures and that different types of stimulation increase the recovery of these cells after a period of hypoxia. These discoveries go against the common assumption that the inhibition of neuronal activity can preserve the energy required for basic cellular procedures and, thus, act as a neuroprotective agent.

5.1 Effects of hypoxia in Excitatory Neuronal Cultures

The possibility to differentiate hiPSCs into neurons (in a controlled way and in a short period) and to measure their neuronal activity with the use of the MEA technology provided a solid ground for this research. hiPSCs derived neuronal cultures showed electrophysiological activity and synchronous patterns in agreement with the ones measured in rodent cultures [14].

The analysis conducted on the hiPSCs derived excitatory neuronal cultures revealed that these cultures are sensitive to hypoxia, since their level of activity decreased along with the synchronicity under lower oxygen conditions (Figure 4.3). The results of the exposure to different durations of hypoxia will be addressed in the following paragraphs.

In the 6 and 12-hour hypoxia groups, neurons remained active and only the synchronicity was reversibly impaired during the hypoxic period (Welch's t-test; $p < 0.05$), showing that neuronal integrity remained undamaged. After 24h of normoxia, the networks that were kept 6 hours under hypoxia showed increased activity, as indicated by MFR (Fig 4.3 A), revealing the probable presence of homeostatic activity regulation by synaptic scaling (up-regulation of glutamatergic neuronal networks), making the culture more excitable with less input. This increased excitability had already been observed in rat models of ischemic stroke presenting neuronal impairments [77]. Moreover, this change was also addressed in a recent study, where the authors showed that the synaptic density of the inhibitory neurons decreased within the first 12 hours of hypoxia, increasing the excitatory/inhibitory ratio (which might also be related to impairments in the inhibitory synaptic transmission) [23]. Furthermore, preliminary results obtained in the CNPH laboratory on hiPSCs derived neurons, with the use of immunocytochemical stainings, showed that the excitatory puncta are increasing during expo-

sure to low oxygen conditions. All this information combined suggests that the results obtained with hiPSCs sustain all the previous observations on rodent cells.

If the low oxygen persisted for 24 hours, the electrophysiological activity of the cultures significantly changed in the last period of hypoxia, when compared to baseline (Mann-Whitney test; $p < 0.0003$ for the two descriptors). Twenty four hours upon the restoration of normoxia, all parameters presented high variability, picturing that only some cultures partially recovered. For this reason, we can consider the 24-hour time scale a critical period of hypoxia. The fact that the firing rate was not significantly altered in the post hypoxic period comparing to baseline (Mann-Whitney test; $p > 0.05$) most likely represents networks that manage to keep their neuronal integrity. However, since there is a 50% decrease in the levels of synchronicity, cultures probably experienced a weakening of their internal connections due to synaptic failure caused by the low energy levels.

Beyond 24 hours of hypoxia, the NBR dropped to zero within the hypoxic period (Mann-Whitney test; $p < 0.0001$) and remained at this level after 24 hours of normoxia (Mann-Whitney test; $p < 0.0001$ compared to baseline). Furthermore, the disperse activity was significantly impaired, as translated by the drop in MFR in comparison to baseline (Mann-Whitney test; $p < 0.05$), suggesting permanent alterations (e.g. membrane depolarization and reduced excitatory input) that lead to cell death. The human neuronal networks most likely faced severe alterations in their internal connections that were not entirely reversible, even though certain neurons remained alive. The long exposure to low activity levels might be in the basis of these behavioral changes rather than the prolonged exposure to low O_2. These results are sustained by a previous study, where increased apoptotic levels were found in cells silenced with tetrodotoxin (ttx), suggesting that apoptosis is happening not only when cells are exposed to prolonged hypoxia but also when they are silenced for the same period of time [78].

To further assess the responses of hiPSCs derived neurons to hypoxia, the effects of a second exposure to low oxygen conditions were assessed. Overall, regardless of the duration of the first exposure to hypoxia, neuronal networks were less resistant to a second period of low oxygen conditions (synchronicity was completely lost after 24 hours of re-oxygenation in all cases). Furthermore, results showed that the susceptibility tended to increase along with the duration of exposure. However, the majority of the *in vitro* studies on rat brains claim that a prior exposure to hypoxia reduces the vulnerability of the brain, suggesting that there is a decrease in the distance between capillaries as a consequence of angiogenesis [79]–[81]. In our model of the penumbra, we only included neuronal populations, so this process is not taking place. Moreover, the authors state that the efficiency of these mechanisms deteriorates with age [81]. The cultures used for the second exposure were normally older than the ones undergoing the first exposure, so the cells might be less resistant than younger ones, due to the loss of some of their properties. These two hypotheses combined sustain the perpendicular observations obtained with our study.

All the previous findings combined suggest that hiPSCs derived neurons behave similarly to the rodent ones. However, the human cells appear to be more resistant to low oxygen conditions, since irreversible neuronal silence was never achieved at the time-scales of hypoxia tested, while it was observed after 30 to 40 hours in rat cells [14]. Since neuronal silence is probably caused by the low activity levels (that promote the silencing of synapses and, eventually, their elimination) dependent on the calcium intake, it is likely that Brain Derived Neurotrophic Factor (BDNF) might influence this process [21]. However, in cultures exposed to 24 hours of hypoxia, we only saw a 20% decrease in the levels of BDNF from baseline to 24 hours after re-oxygenation (Figure 4.8). The reduced number of neurons in our model might be insufficient to notice greater changes and may hamper further conclusions. Furthermore, other mechanisms might be involved in the process such as the glucose levels. The fact that we could only see significant improvements in the neuronal networks

after changing the medium sustains this hypothesis.

To test the hypothesis that neuronal activation improves recovery after stroke, the results of electrical stimulation were assessed in excitatory cultures at the 12 and 24-hour exposures to hypoxia, as presented in appendix A. Treatment with electrical stimulation did not show any benefits in the recovery of the cells. A possible explanation for this behavior is the fact that the cultures did not have any inhibitory neurons and, thus, the balance was compromised, which probably lead to the over-excitation of the cultures. Therefore, GABAergic neurons were included in our model of stroke to create a more complex system resembling the cell composition of the brain and to effectively test different treatment strategies.

5.2 Effects of Hypoxia in Excitatory & Inhibitory Neuronal Cultures and Treatment Strategies

The GABAergic neurons were successfully included in the networks, representing around 20% of the total neuronal population. The effects of 24 hours of hypoxia were assessed in these cultures. In comparison to the glutamatergic neuronal networks, there was no statistically significant change in any of the descriptors neither at the last moment of hypoxia, nor 24 hours after pO_2 was set back to normal values (Mann-Whitney test; $p > 0.05$). This clarifies that the cultures behaved similar in response to an ischemic event. These neuronal networks were also exposed to a second period of low oxygen conditions and, again similarly to the excitatory neurons, they showed higher vulnerability to hypoxia.

Since the synchronicity of the cultures did not go back to baseline values after 24 hours of hypoxia, different strategies aiming at neuronal recovery were tested for this time-scale, to assess the hypothesis that neuronal activation leads to improved neuronal recovery.

Considering the chemical stimulation, ghrelin generated a better recovery in the post-hypoxic period, both when added before and after hypoxia (Figure 4.10), as seen by the significantly improved synchronicity compared to controls (Welch's t-test; $p < 0.05$). These findings are in line with previous studies suggesting that ghrelin might be a useful therapeutic procedure to treat stroke patients for its positive effects on synaptic impairment, triggering the formation and agglomeration of neurotransmitter receptors, which has proven to be very beneficial for the recovery after hypoxia [43], [75].

Regarding the effects of optogenetic stimulation, it can be seen that cultures kept responding to the activation and that activity levels remained higher throughout the entire period of the experiment (Figure 4.9). Great differences were found between experiments (reflected by the high standard error of the parameters), suggesting possible differences in the efficacy of the transfection, proportion of excitatory and inhibitory neurons or simply the instability of the cultures. The low sample size might also be responsible for the high standard error. This therapy was the only one allowing statistically significant improvements within the first 6 hours of re-oxygenation (Mann-Whitney test; $p < 0.05$ when compared to controls) reflecting that stimulation can be beneficial to neuronal networks after ischemia. In a recent unpublished work on a closed loop of optogenetic stimulation conducted by our research group [82], [83], the authors had already visualized the beneficial effects of light stimulation. Optogenetic stimulation managed to keep rodent networks at higher levels of activity, thus, suggesting once more that avoiding neuronal inactivity is crucial for survival.

Cultures that were electrically stimulated did not present any statistically significant effects in improving neuronal recovery, despite showing higher levels of synchronicity when stimulation was delivered to the entire period of hypoxia (Figure 4.10). The difference in response behaviors between

the two different types of stimulation performed (optogenetic and electrical) might be related to the difference in the way cells are activated. While in electrical stimulation only one electrode is stimulated and thus, the activation is only delivered to the cells in its surroundings, with light stimulation, all the network is simultaneously activated. Since the recordings were only performed in mwMEA containing 12 electrodes, it is possible that neurons did not stand exactly on top of the stimulated electrode, generating fewer responses. Furthermore, the fact that neurons are being exposed to hypoxia may hamper the propagation of the response to stimulation, due to synaptic failure, as observed in previous studies [14], [22], [84]. All these factors combined sustain the positive effects of optogenetic when in comparison to electrical stimulation.

Regarding the same treatments when started before or after hypoxia, it can be seen that the response to ghrelin after 6 and 24 hours of re-oxygenation does not change based on the moment of delivery. However, the responses to the two different paradigms of electrical stimulation differ: electrical stimulation after hypoxia appears to have minimal effects when in comparison to controls (Welch's t-test; $p > 0.05$), while the cultures that were stimulated throughout the entire experiment show a tendency to increase their levels of synchronicity surpassing the initial baseline values. The fact that ghrelin added before and after hypoxia showed similar effects on the cultures suggests that stimulation can be helpful at all time-scales. However, the difference between the two electrical stimulation methods is in agreement with the concept 'time is brain' and suggests that neuronal activation should be promoted as soon as possible after an ischemic event [32], [33].

The majority of the treatments applied, after 24 hours of normoxia, lead to increased levels of activity when compared to the baseline value. The fact that the cells were over-excited might not be beneficial for survival. Keeping cells at higher levels of activity is not healthy since it generates the consumption of ATP, which is scarce in an ischemic event. However, this does not invalidate that stimulation is beneficial. Instead, these results suggest that the stimulation paradigm should be adjusted.

Overall, neuronal activation could be achieved with the use of the different stimulation strategies, supporting the theory that activation is essential to reach valuable effects and to maintain a higher level of activity [14], [42], [75]. However, a set of limitations to this study should be discussed.

5.3 *In vitro* model of the penumbra: strengths and shortcomings

Modeling the ischemic penumbra *in vitro* is a challenge since great effort is required to preserve the most significant characteristics of this dynamic tissue-at-risk, necessary to sustain the desired conclusions.

The use of hiPSCs derived neurons is a great advantage of this model since human cells provide an easier translation into clinical practice than animal cells. The use of MEAs enables monitoring of neuronal activity to occur over time in a non-invasive way and thus, provides solid ground to investigate the unique dynamics of these neuronal networks.

Despite promising, there are some shortcomings associated with this model. For instance, the fact that only the oxygen levels were restricted and not the glucose ones. However, during the long periods of hypoxia performed in this research project, cells probably consumed the glucose present in the medium and, consequently, the levels became naturally restricted. This hypothesis is sustained by the fact that cells did not recover within the first 6 hours after oxygen was restored, and only after the medium was changed they showed signs of improved recovery.

Another possible limitation of this model is the lack of human brain architecture and the limited

number of neurons. Nevertheless, since this study aims to give a general insight into the mechanisms that take place in the penumbra of an ischemic stroke, a 2D network of human cells might provide enough information to make solid conclusions and to extrapolate results. Furthermore, in comparison to brain slices, these neurons can survive for longer periods, allowing reliable long-lasting recordings to occur.

There are several other components and different mechanisms taking place in the penumbra of a brain infarct that might be distinct from the ones occurring in this model. Additionally, the single layer of hiPSCs derived neurons may provide distinct time scales of neuronal impairments when in comparison to real measurements. However, these differences might not affect the general observations obtained with this study.

The present *in vitro* model consisted of the penumbral areas alone. However, the core of the brain infarct may promote different mechanisms and release substances that are transferred into the penumbra (e.g. release of glutamate and cytokines), altering the response of these areas to low oxygen conditions [32].

Regarding the different treatment strategies tested, it might not be trivial to implement the stimulation processes throughout the entire period of hypoxia in the clinical practice, since stroke patients usually take a few hours since the onset of the event until they are treated in the ICU. Furthermore, the translation of the optogenetic stimulation technique to the clinical practice might not be easy, since cells need to be genetically manipulated to respond to light.

5.4 Conclusion

In hiPSCs derived neuronal cultures, within the first 12 hours of hypoxia, the cultures try to compensate for the lack of input by becoming over-excitable to go against the tendency for low activity. From 12 to 24 hours of PO_2 = 20 mmHg, the networks completely lose their synchronicity, which can be partially recovered when the oxygen is restored, picturing undamaged neuronal integrity. After 24 hours of low oxygen, the damage becomes irreversible (absent synchronicity) suggesting that connections between neurons are permanently lost.

Cultures undergoing 24 hours of hypoxia and exposed to different treatment strategies aiming at neuronal activation successfully manage to establish higher levels of activity after re-oxygenation, especially with the use of ghrelin and optogenetic stimulation ($p<0.05$ when in comparison with controls). Therefore, our study represents the first step towards the acceptance of neuronal activation as a feasible way to improve recovery after stroke.

5.5 Future Recommendations

This study further elucidates the concept that energy levels are a determinant factor for cell survival and that neuronal activation can improve neuronal survival after stroke. However, certain steps should be taken before the acceptance of this concept and implementation into the clinic.

Firstly, since hiPSCs are known for their high variability, further experiments should be conducted with neurons derived from different donors. Furthermore, the number of cultures being exposed to the different treatment strategies should be increased to further validate the results. Moreover, a control should be performed to assess the basal variations of the cells when exposed to all the external experimental conditions, except the low oxygen. This will guarantee that all the activity fluctuations observed are indeed caused by the low oxygen conditions.

In all the experiments, the medium of the cells was changed after 6 hours of normoxia. Future studies should investigate the recovery of the neurons after 24 hours of re-oxygenation, in case no glucose is added to the cells. Moreover, cultures could be recorded after longer periods (e.g. a week from the start of the experiment) to assess any behavioral changes after this period.

The possibility to differentiate hiPSCs from brain infarct patients' opens the way to create personalized neuroprotective treatments to promote recovery after stroke. The use of this technique will allow treatments to be custom-made to each patient, thus providing higher chances of success. In addition to this, to further solidify our conclusions, the responses to ischemia should be studied in terms of synaptic density, cell swelling and apoptosis, with the use of immunocytochemical staining.

Future studies should focus on the effects of different stimulation patterns, diverse hypoxia durations and in-depth research with the use of high-density MEAs to obtain higher resolution. Both the optogenetic and the electrical stimulation protocols should be optimized with the creation of a closed loop, in order to stimulate the cultures only when the activity drops a certain threshold (as performed by previous researchers in our group with rodent cells). Regarding the electrical stimulation, instead of stimulating one electrode alone, different combinations should be assessed in order to obtain the desired global neuronal activation. The use of hiPSCs and the creation of specific stimulation patterns will ease the translation of these results into the clinic, for example by adapting the previously defined patterns to the Transcranial Magnetic Stimulation (TMS) technique. However, this translation will not be straightforward since it is necessary to understand how the time scales change from our model into humans and which stimulation specificities should be used (e.g. the starting point, location and frequency of the stimulus).

In conclusion, several steps should be taken before the endorsement of this procedure in the clinic. However, our study provides solid evidence that neuronal activation can improve neuronal recovery.

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Appendix A

Effects of Electrical Stimulation in Excitatory Neuronal Cultures

A.1 Hypoxia 12 hours

The effects of electrical stimulation were evaluated in six excitatory neuronal cultures undergoing 12 hours of hypoxia. To clarify the general response of the culture to electrical stimulation, the PSTH relative to one representative well (A1) in the first phase of the experiment is presented in Figure A.1.

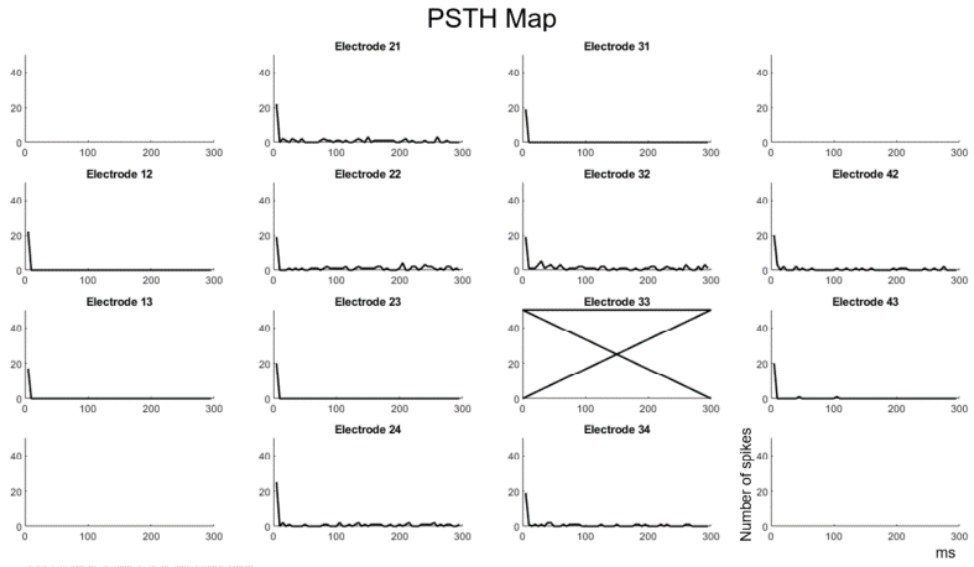

Figure A.1: PSTH 4x4 map for well A1 in the basal phase of the recordings. Each rectangle represents an electrode and the one marked with an 'X' represents the electrode being stimulated (electrode #33 in this particular case).

There is no clear response to electrical stimulation in any of the 12 electrodes. To gain further insight, the culture's disperse and synchronous activity is presented in Figure A.2.

Cultures that underwent electrical stimulation appear to be more vulnerable to hypoxia, presenting lower values of MFR and NBR throughout the entire hypoxic period. After re-oxygenation, the pattern remains the same.

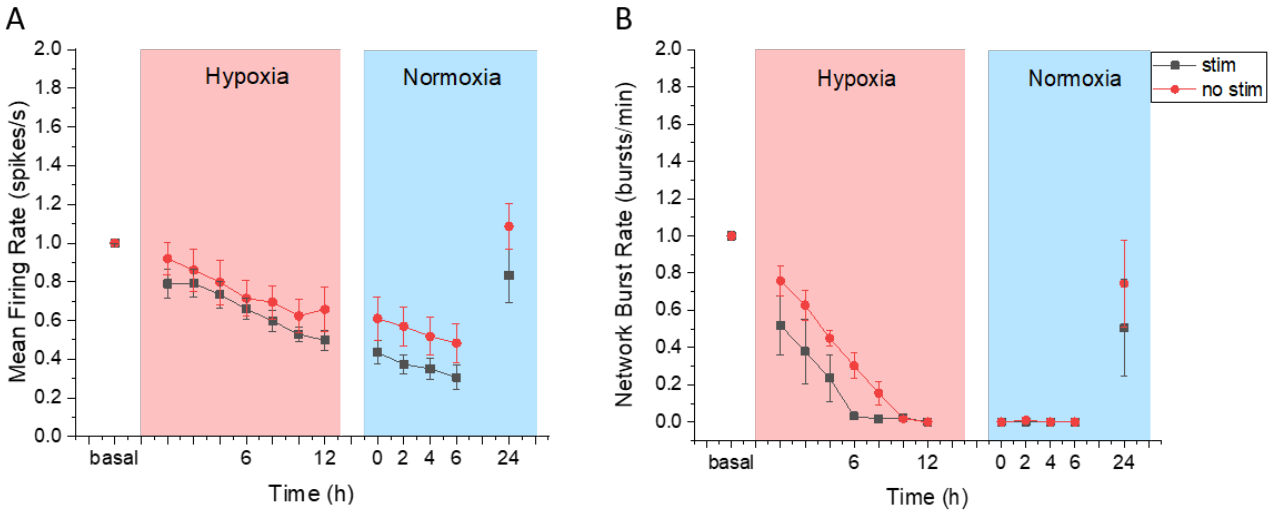

Figure A.2: Temporal evolution of the MFR and NBR excitatory neuronal cultures (electrical stimulation: N= 6; control: N=6).

A.2 Hypoxia 24 hours

The effects of electrical stimulation were evaluated in seven excitatory neuronal cultures undergoing 24 hours of hypoxia (after being previously exposed to 6 hours of hypoxia). The results of these experiments are presented next (Figures A.3).

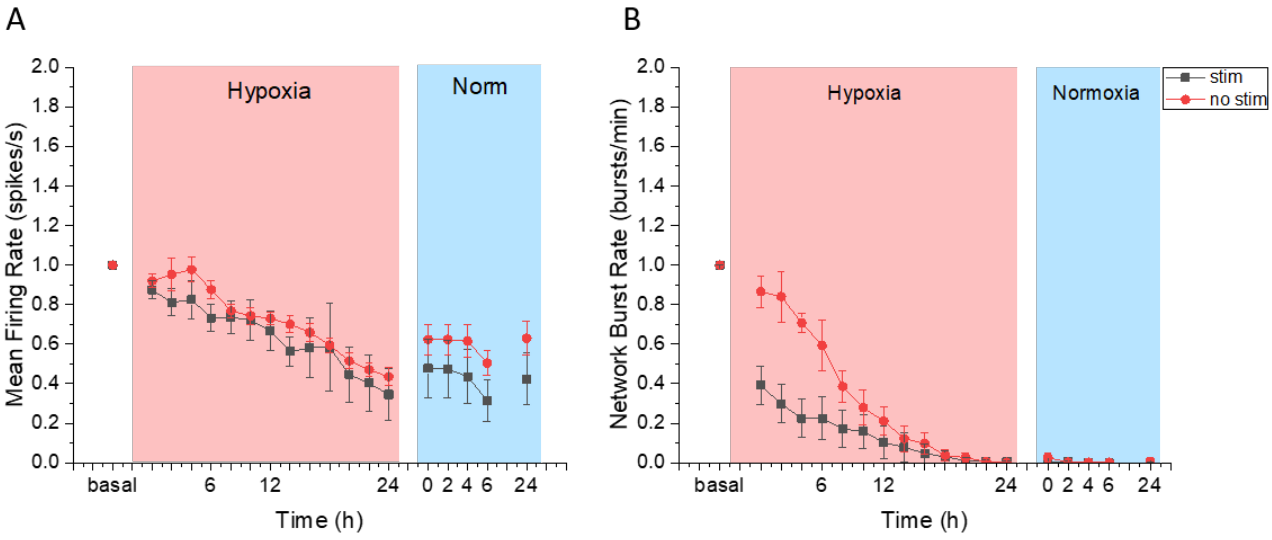

Figure A.3: Temporal evolution of the MFR and NBR excitatory neuronal cultures (electrical stimulation: N= 7; control: N=11).

Once more, neuronal excitation appears to be harmful for the excitatory neuronal cultures decreasing their activity and impairing their recovery in comparison to the non-stimulated cultures.