

UNIVERSITY OF GENOA

MASTER'S PROGRAM IN BIOENGINEERING



**MEA-Induced Long-Term Depression (LTD) as
a neuroprotective strategy in ischemia models
in vitro**

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Abstract

Ischemic injury is characterized by a rapid breakdown of neuronal homeostasis, excessive network dysfunction, and activation of apoptotic cell death pathways. Because heightened neuronal activity can increase metabolic demand and exacerbate ischemic vulnerability, strategies that reduce network excitability may provide neuroprotection. In this thesis, we tested whether long-term depression induced by microelectrode array stimulation can protect human iPSC-derived neuronal cultures from oxygen-glucose deprivation (OGD). We demonstrate that patterned MEA-based electrical stimulation decreases OGD-induced apoptotic cell death and improved neuronal survival. Moreover, MEA-induced LTD mitigated the functional collapse of neuronal networks following OGD, partially preserving key features of spontaneous electrophysiological activity. These data indicate that turning down network activity after ischemic-like stress reduces neuronal susceptibility to injury. Our findings support MEA-induced LTD as a non-pharmacological preconditioning strategy and reveal activity-dependent network modulation as a promising approach to promote resilience in human neuronal models of ischemia.

Abstract in italiano

L'ischemia cerebrale è caratterizzata dal rapido collasso dell'omeostasi neuronale, ciò comporta un'attività disfunzionale della rete e l'attivazione del processo di morte apoptotica. La sovra-attivazione patologica della rete può aumentare la richiesta metabolica esasperando il danno ischemico, strategie indirizzate verso la riduzione dell'eccitabilità di rete potrebbero dunque rivelare un effetto neuroprotettivo.

In questa tesi, abbiamo valutato l'effetto protettivo della depressione sinaptica a lungo termine (LTD) indotta tramite MEAs (Microelectrode arrays) su un modello ischemico in vitro di neuroni glutamatergici, ottenuti dalla differenziazione di cellule staminali umane pluripotenti indotte.

Abbiamo dimostrato che il pattern di stimolazione elettrica riduce la morte apoptotica e sostiene la sopravvivenza neuronale. In più, la LTD indotta tramite MEAs si è rivelata efficace nel controllare il collasso funzionale della rete dopo l'insulto, parzialmente preservandone i parametri elettrofisiologici basali di rilievo. Questi dati indicano che l'inibizione dell'attività di rete, a seguito dell'insulto ischemico, riduce la sensibilità neuronale all'infortunio. Le osservazioni prodotte in questo studio indicano come la LTD indotta tramite MEA possa essere una valida strategia di pre-condizionamento non farmacologico, mostrando quanto la modulazione della rete possa essere un approccio promettente nel preservare la vitalità cellulare all'interno dei modelli umani di ischemia in vitro.

Introduction

Ischemic stroke remains one of the leading causes of mortality and long-term disability worldwide. Despite the widespread establishment of specialized hospital stroke units and the advancement of acute endovascular interventions, the global socioeconomic burden of the pathology continues to grow, particularly in ageing populations and developing countries. Current acute-phase clinical management relies almost exclusively on recanalization strategies, namely intravenous thrombolysis via recombinant tissue plasminogen activator (r-tPA), alone or combined with mechanical thrombectomy. However, these reperfusion therapies are constrained by narrow intervention windows and do not directly arrest the secondary cascade of cellular injury once metabolic failure has been triggered. At the same time, the development of neuroprotective drugs has consistently suffered from a severe translational gap between preclinical animal models and human clinical trials, highlighting the urgent need to explore non-pharmacological, bioengineering-driven therapeutic strategies.

At the cellular and network levels, an abrupt reduction in oxygen and glucose availability rapidly compromises ATP synthesis, impairing the Na^+/K^+ -ATPase pumps responsible for maintaining transmembrane ionic gradients. As neuronal homeostasis collapses, cells undergo anoxic depolarization and uncontrolled glutamate release, initiating a self-reinforcing excitotoxic loop driven by NMDA and AMPA receptor overactivation and

intracellular Ca^{2+} overload. Crucially, neuronal electrical activity is itself a major determinant of metabolic consumption: action potential propagation, postsynaptic ion fluxes, and the continuous restoration of ionic gradients absorb a substantial fraction of the cellular energy budget. During post-ischemic metabolic stress, pathological over-activation of the network discharges culminating in depolarizing block and spreading depolarization-like waves accelerate energy exhaustion, driving vulnerable neurons of the penumbra from reversible dysfunction toward apoptotic death (mediated by Caspase-3/7 activation).

Rather than relying on indiscriminate pharmacological receptor blockade, which can suppress physiological survival pathways, a promising electroceutical approach consists in selectively downscaling synaptic strength through activity-dependent plasticity. Long-Term Depression (LTD) is a physiological form of synaptic plasticity characterized by a persistent reduction in excitatory synaptic efficacy, biologically mediated by the internalization of postsynaptic AMPA receptors. Inducing an LTD-like state via low-frequency electrical stimulation during the early post-insult recovery window could dampen pathological hyper-excitability, reduce glutamate-dependent ionic loading, and lower the metabolic demand of compromised neuronal networks, thereby halting the spatial propagation of apoptotic cell death.

In this thesis, we combined human induced pluripotent stem cell (hiPSC) technology, Micro-Electrode Array (MEA) electrophysiology, and confocal fluorescence microscopy to investigate whether post-insult electrical stimulation can exert a neuroprotective effect in an in vitro human model of cerebral ischemia. Homogeneous networks of hiPSC-derived glutamatergic cortical neurons were cultured on 60-electrode MEAs and subjected to a 4-hour Oxygen–Glucose Deprivation (OGD) protocol in a custom anoxic chamber to reproduce ischemic-like metabolic stress. Following reperfusion, treated cultures received a 15-minute low-frequency (1 Hz) biphasic electrical stimulation protocol designed to induce network-wide LTD, while unstimulated OGD cultures served as controls across three independent experimental sessions. To evaluate both cellular rescue and functional preservation, we developed a multimodal quantitative pipeline integrating deep-learning nuclear segmentation (Cellpose-SAM), spatial point-pattern

analysis, and multivariate electrophysiological feature tracking in a standardized log fold-change space, evaluated through exact session-stratified permutation tests.

Confocal image analysis demonstrated that post-OGD electrical stimulation reduced the apoptotic cell fraction by approximately threefold compared with unstimulated controls (from 7.4% to 2.4%, $p=0.017$) and significantly confined the spatial spreading of residual apoptotic foci ($p=0.044$), particularly in the immediate vicinity of the stimulating microelectrodes. Concurrently, multivariate analysis of eight MEA-derived electrophysiological parameters revealed a statistically significant stabilization of the global Pre-to-Post network state in stimulated cultures compared with controls ($p=0.042$), indicating that low-frequency neuromodulation attenuates post-ischemic functional drift and preserves baseline network dynamic.

Thesis outline

This thesis is organized into six chapters. Chapter 1 reviews the state of the art on cerebral ischemia, covering its epidemiology and social burden, the cellular and network pathophysiology of ischemic injury, and the current therapeutic approaches.

Chapter 2 introduces the neuroengineering and bioengineering tools employed in this work: hiPSC-derived neuronal models and in vitro ischemia (OGD), Micro-Electrode Array electrophysiology, and fluorescence/confocal imaging with deep-learning segmentation.

Chapter 3 describes the materials and methods: the cell cultures, the experimental OGD and stimulation protocol, the imaging and image-analysis pipeline, the electrophysiological feature extraction, and the statistical framework.

Chapter 4 reports the results on cell survival, spatial distribution of apoptosis, caspase intensity, and the multivariate electrophysiological analysis.

Chapter 5 discusses the interpretation of the findings and the limitations of the study.

Chapter 6 summarizes the main conclusions and outlines future perspectives.

Chapter 1

1. An overview of cerebral ischemia

1.1. Epidemiology and extent of ischemic injury

The aim of this first chapter is to give a preliminary knowledge about ischemic stroke, from the general profile of the pathology (risks factors, pathogenesis, aetiology) to social/global burden and therapeutic strategies.

Stroke is essentially originated by cardiovascular diseases, in fact his occurrence is due to an obstruction (ischemic stroke) of the cerebral bloodstream or a brain haemorrhage (haemorrhagic stroke), this causes the loss of nutrient intake and oxygen necessary for the brain tissue. Without those important factors for the life and the metabolism of the cells, we assist to the death of the cerebral tissue and all the degenerations attributable to such an event (motor impairments, cognitive and sensory impairments, death). Three common macro categories were identified as risks factors: environmental, metabolic and behavioural [1].

Factors like air and soil pollution, excess of body weight, smoking habits, hypertension, physical inactivity and wrong diets (processed meat, industrial foods, diets poor of fruits and vegetable, excess of sodium) were studied as well as high fasting plasma glucose and high outdoor temperatures. All those factors together are responsible of more than the 80% of stroke cases showing that stroke can be highly predictable, so enhancing actions of prevention and sensibilization could be decisive, anyway, this represents a public health challenge [2].

1.1.1. The impact of cerebral ischemia worldwide

Nowadays stroke is still one of the most devastating of all neurological disease. According with the Global Burden of Diseases studies, stroke is the second cause of death worldwide by producing million deaths every year (~7 million deaths in 2025) [2], being a disease of aging, with the growth of the number of people older than 65 years also the statistics related to stroke are expected to increase exponentially. Ischemic stroke it's not always fatal, it's possible to survive with different degrees of disability [3].

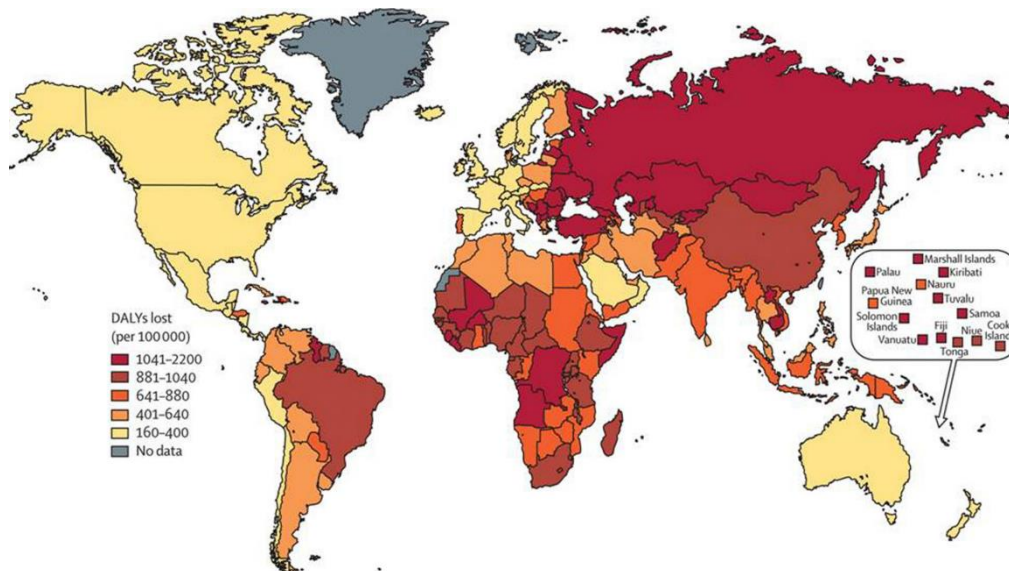


Figure 1. Rates of age-adjusted and sex-adjusted DALY loss as the result of stroke. Rates are greatest in Eastern Europe, North Asia, Central Africa, and the South Pacific. From: [2].

The debilitating side, plus the increasing deaths for year related to this pathology, gives rise to social and economic costs that brings to the necessity of appropriate measures to be taken, from prevention to rehabilitation and health care.

Pathologies as ictus are characterized by leading mortality and disability combined, a parameter named DALY (Disability-Adjusted Life Years) it's used to measure global burden of disease, evaluating the years lost due to premature death plus years spent with a disability. The first factor of this parameter, that is death related, thanks to new technologies is quite sustainable in developed countries, while the disability is still an issue in terms of

efficiency of the treatments and healthcare costs, considering that stroke survivals are going to increase in the next years. The estimated global costs for stroke are about 890 billion of US dollars per year, with a tax of growth of the 50 % by the 2050 [2].

The biggest part of the global DALYs related to stroke are produced by low-income and low-middle income countries, the stroke burden varies a lot by one country to another showing great disparities. The 89% of the global DALYs is in fact produced by low-to middle income countries with a special emphasis on Asiatic, North-east Europe and central African countries [2].

In conclusion, without some planned and geared policies the burden of stroke will continues to grow, thus threatening the sustainability of the health system across the globe.

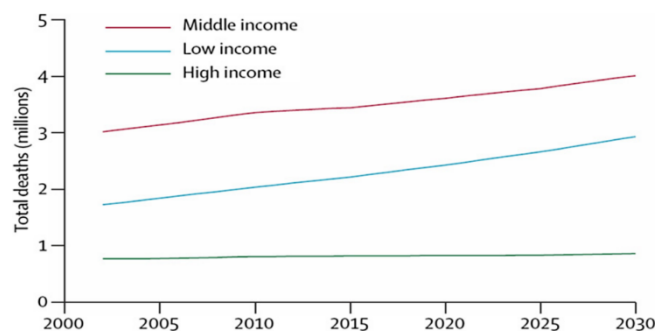


Figure 2. Projected trends for stroke death by World Bank income group 2002-2030. From [1]

1.1.2. Extent of ischemic injury in patient everyday life and social cost

Beside the direct costs of the pathology (hospitalization, rehabilitation, medication) there are also indirect costs that involves the productivity and the social fulfilment of the patient and his family, or caregivers.

Stroke is the third cause of disability in the world [3], for the people who survive is hard to come back to a full and satisfying life. The stroke in certain brain areas can produce severe motor disfunction as well as cognitive disfunction, obviously this would affect the patient everyday life. There is a certain margin to recover the altered functions but is a difficult way, for different reasons: costs and times of rehabilitation (since a precise and monitored rehabilitation requires lot of attention for a single individual time it's rationed), emotional setting (efficacy of the rehabilitation depends also on the psychological disposition and on

the point of view of the patient), cooperation is required (the patient is not alone, also the quality of life of the caregivers and their approach to the assistance is an element of the healing) [4].

As we said psychological impact of stroke is an important factor, and a correct rehabilitation program must consider all those complications, but this not always happen. What is hard to handle for those patients is the loss or the changes of their social/familiar role and the discontinuity of their self. In some recent studies post-stroke patients reports to feel disconnection inside their identity being unable to understand and control their body, furthermore they report struggling on their role switching and some agreements about uncertainty and ambiguity after discharging from rehabilitation [5] in specialized structures or hospitals.

The necessity to manage all those aspects is also required to be recognized by health care interventions that must acquire more psychological sensibility to approach complex socio/individuals needs of the patient to really adopt an efficient rehabilitation program.

Another impairing effect typical of this disease is the after-stroke depression. To deal with this condition self-management interventions are indispensable as integrating part of rehabilitation to truly increase the quality of life after stroke [6].

Known the psychophysical profile of the patient who survived to the stroke we can address some thought about the importance of rehabilitation, especially in a world in which the impaired people will be more, looking at the decreases of the death rate. Programs for home-based rehabilitation and early therapeutics approaches will be crucial in order to contain costs and improve health care efficiency.

1.2. Pathogenesis

In this section we will look at the neurons, in particular to their generic functional and biological characteristics and how they are impaired by the ischemic event.

1.2.1. Basics on electrophysiology of neurons

If we can read, move in the environment, make experiences and have a coherent perception of ourselves is because we have an advanced nervous system that is composed by billions of neurons.

Neurons are electrically excitable cells that act as biological transistors by elaborating and transmitting flux of information through electro-chemical signalling cascades. They can receive input signals from the external world or other neurons, and they can produce an output sequence to neurons or actuators (like muscles or glands). Output sequence for a neuron means an action potential, this response is produced when a neuron takes a certain input amount from other units and so it triggers an action potential [7] that can activate or inhibit another neuron depending on their functional role.

Before taking a look on the mechanism behind these neuronal responses, is necessary to understand the morphological and functional elements of a neuron.

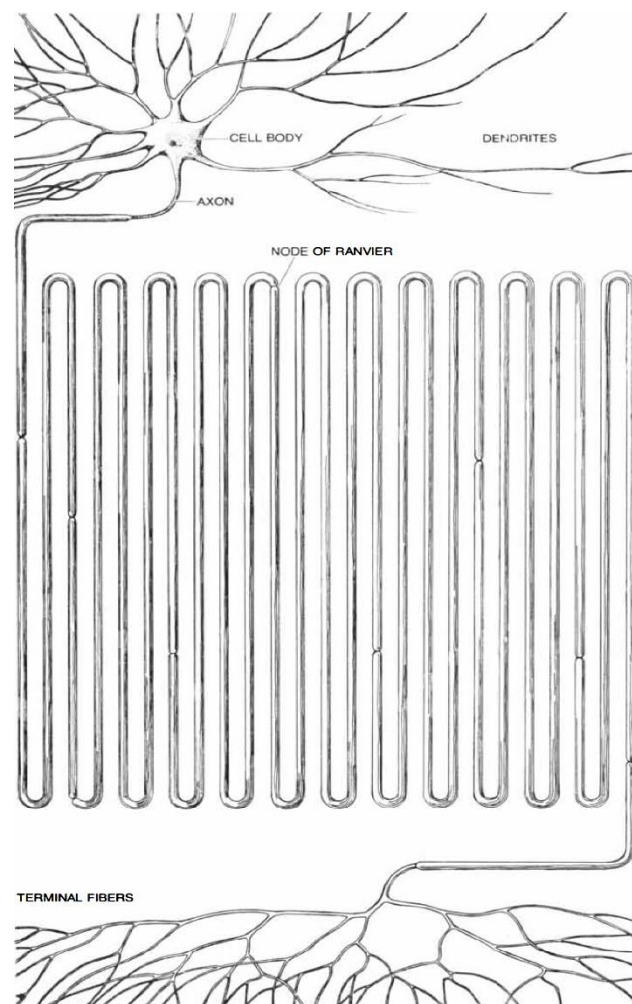


Figure 3. Illustration of structural components of the neuron. From [7]

There is a wide range of phenotypic expression also in nerve cells, so their morphologies can vary between different shapes based on their function and anatomical position, but we can distinguish three main structural components: soma, dendrites and axon.

Soma is the cell body, the main part of the neuron in which we have the nucleus and other various cell organelles; it contains the molecular machines for protein synthesis and all the functions to preserve cell viability.

Dendrites are branched cellular extension that are located near the soma, their complex is known as dendritic tree. Those filaments are in general the main input source for the neuron, in fact along dendrites we have junctional complexes named dendritic spines.

From the soma we have a point, the axon hillock, from which the axon is extended. Axon is a finer, cable-like structure that can extend tens, hundreds, or even tens of thousands of times the diameter of the soma. The axon is the output of the neuron and carries away electrical signal all along its length by propagation, it can also give some sort of informative feedback to the neuron, they cover a great part of the nervous tissue linking neurons also very far one from each other. At the final section of the axon, in which emitting neuron contact other receiving neurons, we find the synaptic cleft (in case of chemical synapses) or gap junction (in case of electrical synapses). Axons at their distal segment present branched structures with a swelled terminal that are called synaptic buttons, in this place the electrical signals are converted into the release of chemical molecules that can excite or inhibit the receiving neuron [8].

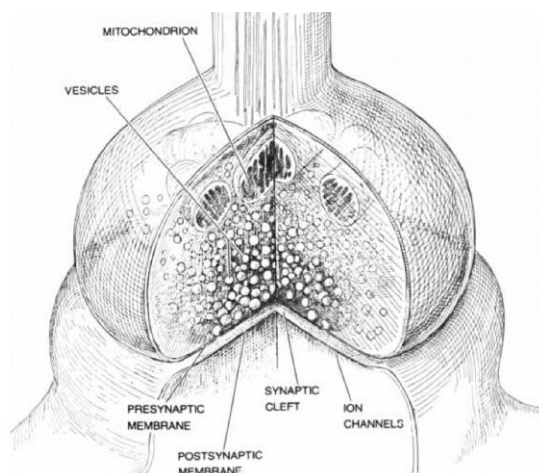


Figure 4. Illustration of the synaptic terminal. From [7]

To understand how neurons can be excited and how they can propagate electrical signals we have to take under examination their cellular membrane.

Every cell has a membrane that isolate the inner space of the cell, the cytoplasm and nucleus, from the outer space, the extracellular liquid.

In all cells the membrane is composed by phospholipids, that are amphiphilic molecules capable to separate two aqueous solutions, thanks to their spatial organization, they act like semi-permeable barriers.

Between the two solutions, so from the inner and outer cellular spaces, there is a dynamical equilibrium that is maintained constant to keep the cell alive. This because the cell needs to keep an osmotic equilibrium of the ionic species that are present in extracellular and intracellular fluids. This mechanism has two important consequences, the first is that this equilibrium needs energy (ATP) to be maintained, because often the molecular structures designed for keeping this equilibrium act also in an active way, by moving ions in non-preferential direction (for example positive ions travels to a negative potential), the second is that it establishes an electrical potential across the membrane due to the different concentration of ionic species inside the inner and outer liquid. Said so, every cell maintains a certain membrane potential referred to as resting potential (-70 mV for neurons), that is in general more negative with respect to the external potential. Neurons are specialized cells that express on their membrane specific molecular machines able to generate variations of their membrane potential to transmit and extract a coherent informative representation, which is originated from external inputs (light, soundwaves, tactile) or internal processes (like memory, motor commands, emotions).

The molecular machines cited before are known as ion channels, ion pumps and membrane receptors. These components are in general constituted by active complexes of proteins embedded in the membrane and they could be free to move inside and continuously replaced, added or removed by the cell.

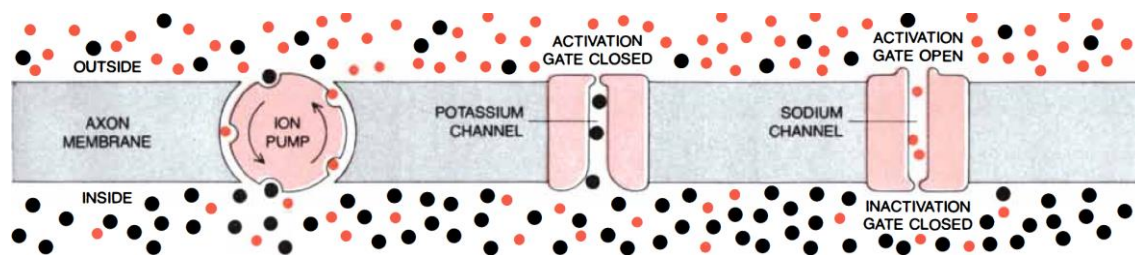


Figure 5. Schematic representations of the neuronal membrane (K^+ ions in black, more abundant in cytoplasmic side, Na^+ in red). Adapted from [7].

These molecules can be activated by a change of the membrane potential to a specific threshold value (voltage-gated) or through the bind of a chemical transmitter on a distinct binding site (chemically gated), their activation determines an ion flux that could be inward or outward, pushing the membrane potential to higher values (depolarization) or lower values (hyperpolarization), these voltage variations on neurons corresponds respectively to excitation and inhibition.

The first studies that elucidated those mechanisms has been conducted by A.L. Hodgkin, A.F. Huxley and B. Katz. Studying nerve-impulse transmission on axons from giant squid they discovered and demonstrated that the propagation of a nerve impulse coincided with sudden changes in the permeability of the membrane to sodium and potassium ions. This is due to the presence of two important channels one relative to sodium, one to potassium, their interplay causes the classical phenomena of the action potential, which is a transient pulse due to an increase of the membrane potential that is propagated through the axon. This phenomena starts with the activation of sodium channels through a supra-threshold stimulation, when the membrane potentials reaches values about $-55mV$ those channels assumes a morphological configuration that allows sodium ions to access the cell thanks to the fact that extracellular concentration of sodium is about tenfold higher than the cytosolic one (electrochemical gradient it's the driving force). The effect produced by this influx is an amplification of the phenomena (more and more sodium channels are recruited) and the depolarization of the cell, this brings the membrane potential to positive values near to sodium equilibrium potential. Once that sodium flowed into the cell the channels switch to their deactivated shape, reducing the sodium permeability, the excess of sodium ions that penetrate the cell is then actively transported back out of the plasma membrane. The same raise on voltage that activates sodium channels, with a certain delay opens the potassium channels. In this case potassium concentration is higher in cytosol more than in the external

solution, so we assist to an outward potassium current that rapidly takes back the membrane potential to the resting value [9]. After that there is often an ulterior hyperpolarization that takes the potential below the resting potential of the cell, due to the overactivation of potassium channels. When potassium permeability returns to its usual value the action potential ends and the sodium channels undergoes a refractory period that leaves the channel in their deactivated state. The time interval during which no new action potential can be fired by the cell is called the absolute refractory period (1-2 ms) [8]. At longer times, after some but not all of the ion channels have recovered, the axon can be stimulated once again but it requests a higher voltage threshold value for the generation of an action potential. This is known as the relative refractory period. The absolute refractory period is necessary to ensure the unidirectionality of the signals which travels along the axon, in fact a new action potential can be initiated by the subsequent portion of the axon while the previous is in his refractory period.

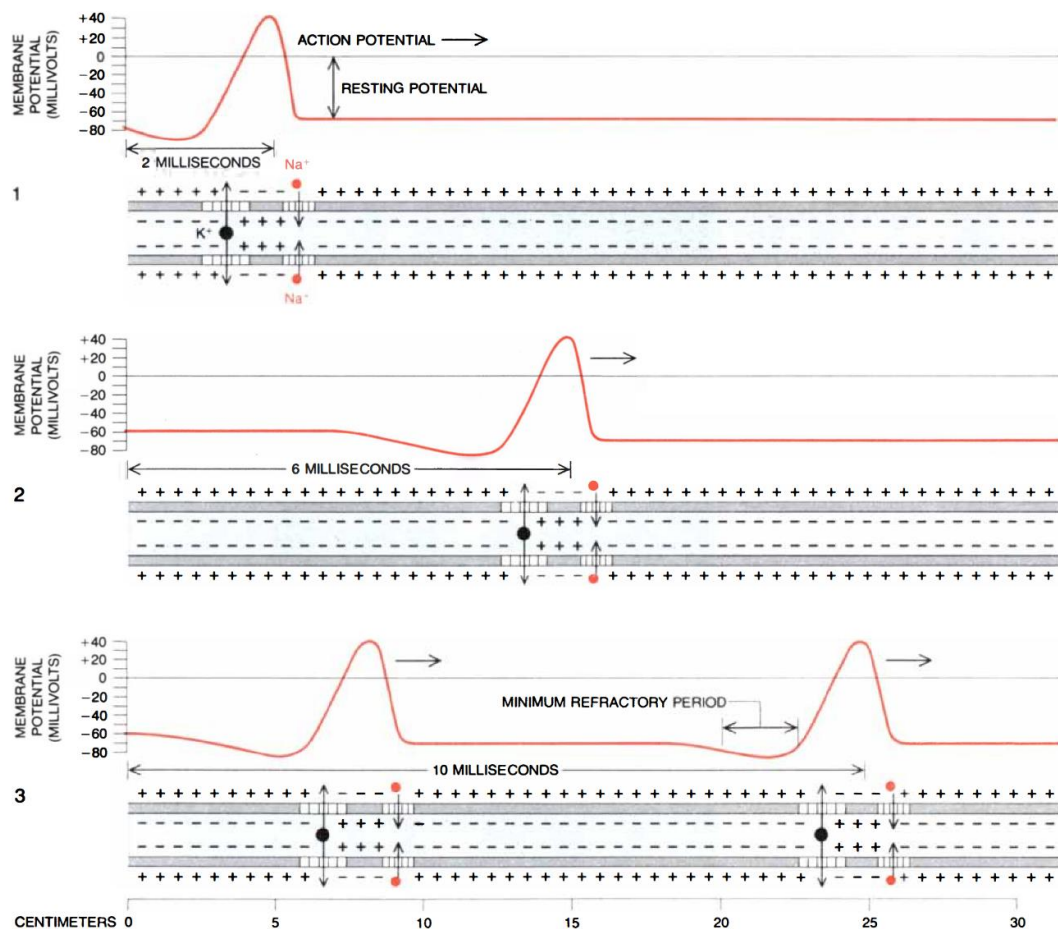


Figure 6. Example of a travelling action potential propagated through the axon. From [7].

Furthermore, there are other types of action potentials related to calcium ions rather than sodium. Calcium action potentials have longer dynamics (10-100 ms) and different activation threshold, this phenomenon is often observed in dendrites, in fact a depolarization near the axon hillock can be back-propagated from the soma to dendrites thanks to calcium voltage-dependent channels, this gives rise to a complex chemical cascade in the neuron that is one of the mechanisms that allows memory and learning by regulating neural plasticity; back-propagated action potentials could be seen as a feedback signal that can modify dendritic spines permeability, increasing or decreasing their contribution to generate an action potential. Other types of action potentials are given by the mixed contribution of calcium and sodium channels, in which calcium act as a driving force creating a persistent depolarization (plateau) on which sodium action potentials are rapidly generated, this is in general the representation of a bursting regime [10]. Bursts are rapid sequences of action potential (or spikes) fired by a neuron.

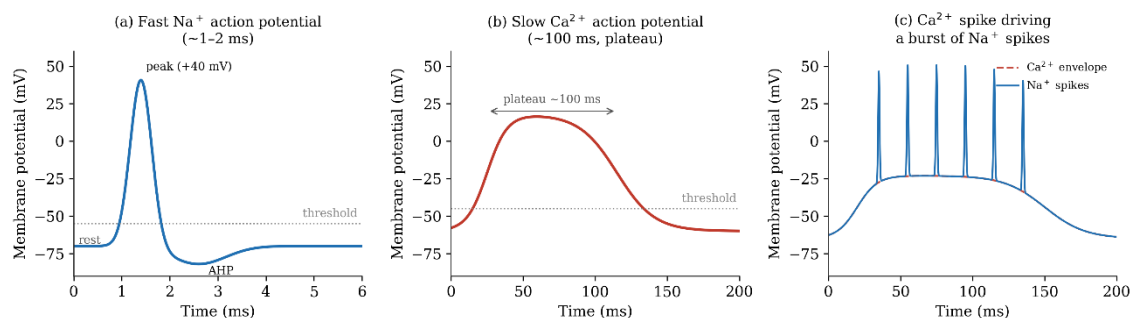


Figure 7. Main types of neuronal action potential. (a) Fast Na^+ -based action potential (~1–2 ms), with threshold, overshoot and afterhyperpolarization. (b) Slow Ca^{2+} -based action potential, lasting up to ~100 ms. (c) A slow Ca^{2+} spike can drive a burst of fast Na^+ spikes.

1.2.2. Chemical and electrical synapses, glutamatergic synapses and plasticity

After that a neuron is triggered to produce an action potential, this signal needs to be propagated and then transmitted to other neurons, this happens thanks to synapses. Synapses as we said, are specialized cellular structures that put in communication two neurons, there are two fundamental types of synapse, chemical and electrical (some synapse also have a mixed contribute of the two).

In chemical synapses, the action potential of the pre-synaptic neuron is converted (via the activation of voltage-gated calcium channels) into the release of a chemical called neurotransmitter that binds to receptors on the membrane of the post-synaptic neuron. The neurotransmitter may initiate an electrical response or a secondary messenger pathway that may either excite or inhibit the postsynaptic neuron. Chemical synapses can be classified starting from the type of neurotransmitter involved in the process, glutamatergic synapses use glutamate (often excitatory), GABAergic (often inhibitory), cholinergic use acetylcholine and adrenergic (releasing norepinephrine). The wide diversity of neurotransmitters and receptors and their capability to generate different characteristics of responses by the neuron, is indicative of how complex could be to understand and clarify neural processes, chemical synapses are a powerful tool to modulate neural activity [11].

In electrical synapses instead the cytosol of the post-synaptic cell and pre-synaptic cell are directly connected through some proteic channels called gap junctions that are trans-cellular channels contained in the membrane of the cells. In this case the channels allow the passage of electrical currents, causing a direct depolarization on the post-synaptic neuron, this is translated in a rapid communicative mechanism [12].

These two synaptic modalities are not independent, they have not a dichotomic relation, but they are both expressed (with some prevalence for one or the other modality for some anatomical structures) and related to determine neural activity.

Chemical synapse can have ionotropic (the neurotransmitter bound to the ionotropic receptor opens a specific ionic channel causing variations of the membrane potential) or metabotropic effects (neurotransmitter binds a metabotropic receptor that activates a signalling cascade by second messengers, their activation is translated into complex phenomena like changes in the membrane conductance and so composition). These effects have different time dynamics and can affect gene expression (long time) as well as variations in membrane potential (short time), increasing complexity in neural communication and modulation. Differently from chemical synapses, electrical synapses are very fast and bidirectional, allowing synchronous firing and furthermore they present a different type of synaptic regulation; putting the two cells in communication, these type of synapse offers also the possibility to exchange some small metabolites [12].

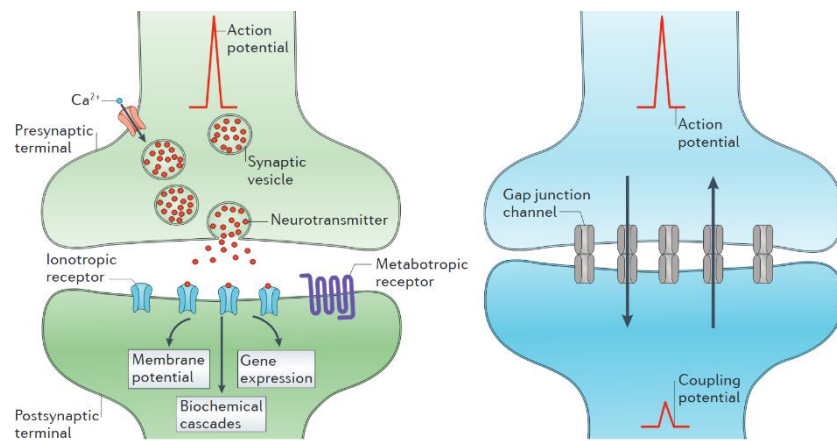


Figure 8. Schematic representation of the main functional elements in chemical (left) and electrical (right) synapses. From [12].

Chemical and electrical synapses are both impaired by the ischemic insult, being chemical energy (ATP) indispensable for every type of neuron and cell more in general. But to understand how to operate in therapeutic or rehabilitative contests we must develop a clear vision of how the synaptic transmission works and how it could be impaired. This bonds with different concepts as neuronal calcium signalling, glutamate effects on neurons and neural plasticity.

Chemical synapses, in particular, glutamatergic synapses are the most abundant inside an adult mammal brain. These synapses use a complex electro-chemical cascade to transmit signals, we can distinguish two main phases for this process, the first is the pre-synaptic phase. When an action potential reaches the pre-synaptic axon terminal, the membrane depolarization causes the activation of voltage-dependent calcium channels generating a calcium influx that increases inner calcium concentrations near the pre-synaptic membrane. Here there are lot of cellular vesicles in which neurotransmitters (in this case glutamate) are contained, calcium ions interact with the endoplasmic reticulum (an important cellular component that act as a calcium source and buffer) starting to release neurotransmitters on the synaptic cleft by exocytosis, so vesicles are completely fused onto the membrane releasing all the neurotransmitter in the cleft, another mechanism of release is the “kiss and run” where the vesicles are in contact with the membrane just in a point, generating a small pore from which a fraction of neurotransmitter is released. Vesicles can then be recycled and the excess of neurotransmitters in the synaptic cleft is actively reuptake by the

Excitatory Amino-Acid Transporters (EAATs) or by glial cells (astrocytes), glutamatergic synapse are therefore said tripartite synapses [13]. Once the neurotransmitters are released, they binds some specific receptors on the post-synaptic membrane, depending on their effects on the post-synaptic neuron that have been classified as ionotropic or metabotropic, although there is a wide range of receptors families and they can have different role also by changing their localization on the cell. In the case of glutamatergic synapses those receptors are NMDA, AMPA (ionotropic) and mGluR (metabotropic) [14].

NMDA and AMPA are two complementary ionic channels and their activation causes an influx of calcium (NMDA) and sodium (AMPA) ions that are in general responsible of the generation of an excitatory post-synaptic response (EPSP). NMDA are double gated channels, their activation in addition of the presence of glutamate (and glycine) needs an already depolarized membrane potential (could be initiated by AMPA channels) to remove a magnesium ion that act as gating particle. This is because the influx of calcium generated by NMDA receptors is a second messenger that inside the cell can promote the synthesis of new NMDA receptors on the membrane, enhancing the strength of the synapse, based on the principle that activity of NMDA is indicative of some synchronous and causal activity between pre-synaptic and post-synaptic neurons. This opens the field to the concept of neural plasticity, that is the capability of our neurons to modulate the strength (both increasing or reducing) and the number of connections, relying on informative contents like spike timing (Spike Timing Dependent Plasticity) and frequency of firing of the pre-synaptic neuron. Those changes in the strength of the connections can be momentaneous (Short term potentiation/depression) or long standing (Long term potentiation/depression). These principles are strongly related to intra-cellular calcium signalling dynamics and are at the basis of the development for biological memory and learning [13], furthermore the possibility to better understand and control plasticity in the human brain opens new perspective in the field of rehabilitation and therapy for different neuropathologies.

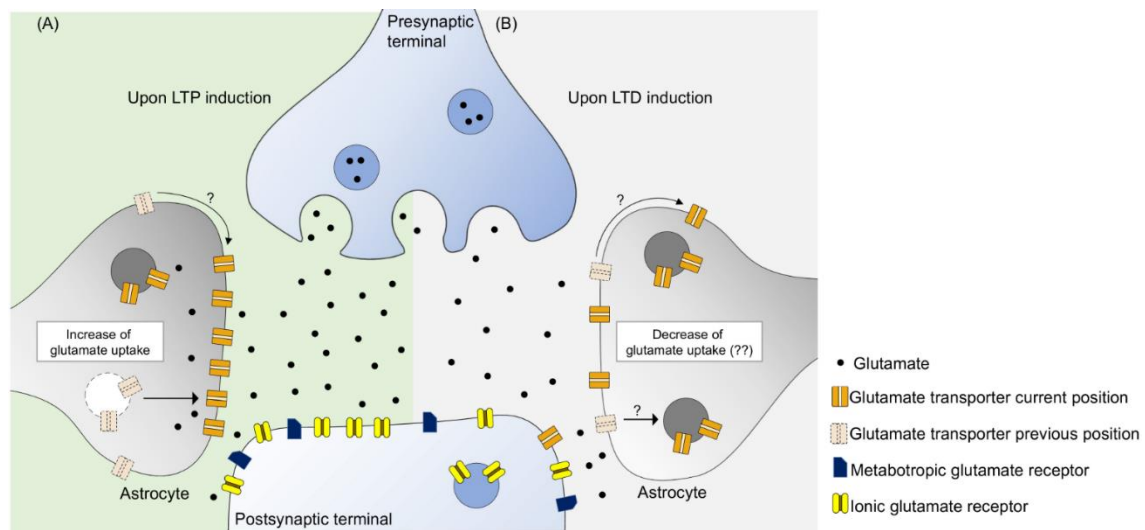


Figure 9. Schematic representation of the tripartite glutamatergic synapse, showing the activity-dependent relocation of astrocytic glutamate transporters (EAATs) and of postsynaptic receptors upon LTP (A) and LTD (B) induction. From [13].

1.2.3. Ischemic damage: ATP depletion, excitotoxicity and cell death

Fine regulation of the cellular machinery depicted in the previous sections, requires intrinsically a lot of energy, chemical energy in the brain is produced starting from the oxygen and glucose supply that is provided by the cerebral bloodstream.

To understand the pathophysiological profile of cerebral ischemia we have to look to all the alterations caused by this condition on the neurons (microscopic alteration) and on the functional connections that are present between different brain areas (macroscopic alterations).

Once the cerebral bloodstream is interrupted by an ischemic insult, neurons are rapidly deprived of oxygen and macronutrients. This event results in severe metabolic stress and in a deep impairment of the electrochemical balance of the cell. The ischemic damage is in general difficult to evaluate in timing and extension but from other studies it was demonstrated that the cerebral concentrations of glucose and ATP (adenosine triphosphate) undergoes to a rapid depletion, being almost completely exhausted in 10-12 minutes.

Without ATP the cell membrane can't provide chemical energy to all the machinery necessary to maintain the electrochemical balance of the ionic species, ion pumps and ion channels can't be activated, and the membrane becomes a passive component in which ions

can penetrate following their electrochemical gradient. This is the earlier phase (60-180 sec after the insult) and it's known as anoxic depolarization, during this period sodium, calcium and chloride ions (that are abundant on extracellular space) get inside the cell through the membrane while potassium is released, generating a huge and disordered amount of charge movement. As a result of the influx of ions also water is recruited inside the cell to balance osmotic equilibrium generating cellular swelling (cytotoxic oedema) [15].

This strong and continuous depolarizing current causes an uncontrolled neurotransmitters release in the synaptic cleft. Glutamate is so released in a massive dose triggering NMDA and AMPA receptors in an anomalous way generating excitotoxicity phenomena.

NMDA receptors in particular can promote different cellular signalling cascades and pathways in function of their composition (different protein subunits form the receptor and some of them can promote cell death or survival) but also in function of their spatial location (synaptic or extra-synaptic).

The overactivation of extra-synaptic NMDA receptors causes a big influx of Ca^{2+} ions that is also amplified by the release of the Ca^{2+} that is stored in the endoplasmic reticulum of the cell. Endoplasmic reticulum in physiological condition acts as a calcium buffer but in the ischemic condition it participates to a positive feedback of calcium release triggered by the calcium itself. Calcium influx and glutamate excitotoxicity are correlated and are a crucial component of the event cascade that leads to cell death. In the condition of calcium overload there is also an anomalous activation of calpain (calcium dependent protease) that is translated into degradation of membrane components (NMDA receptors) and of the cytoskeleton. Furthermore, protein synthesis and mitochondrial metabolism are compromised, in particular, mitochondrial disfunction is responsible of the production of superoxides like peroxynitrite, an extremely toxic molecule [16].

This chain of events is accompanied by an overall metabolic stress due to the state of hypoxia. Without oxygen the cell it's forced to produce energy through anaerobic glycolysis producing huge quantities of lactic acid generating a general condition of acidosis, that if prolonged in time, contributes to compromise cell viability.

After the ischemic event it's possible to observe two typologies of cellular death, if we have a critical impairment of the energetic metabolism and a huge overload of calcium (typically it happens on the ischemic core) the cells in the tissues undergoes to necrosis,

this is a traumatic condition in which cells undergoes swelling and are degraded in the environment. In the second case where the ischemic damage is less severe (ischemic penumbra) or when we are in a tardive phase of the recovery, other cellular pathways are activated promoting apoptosis, a natural mechanism of cellular death in which cells are slowly degraded by capsases activation (enzymes that can digest the cell protein, organelles and genetic material) and then are eliminated through the intervention of macrophages [16], [17].

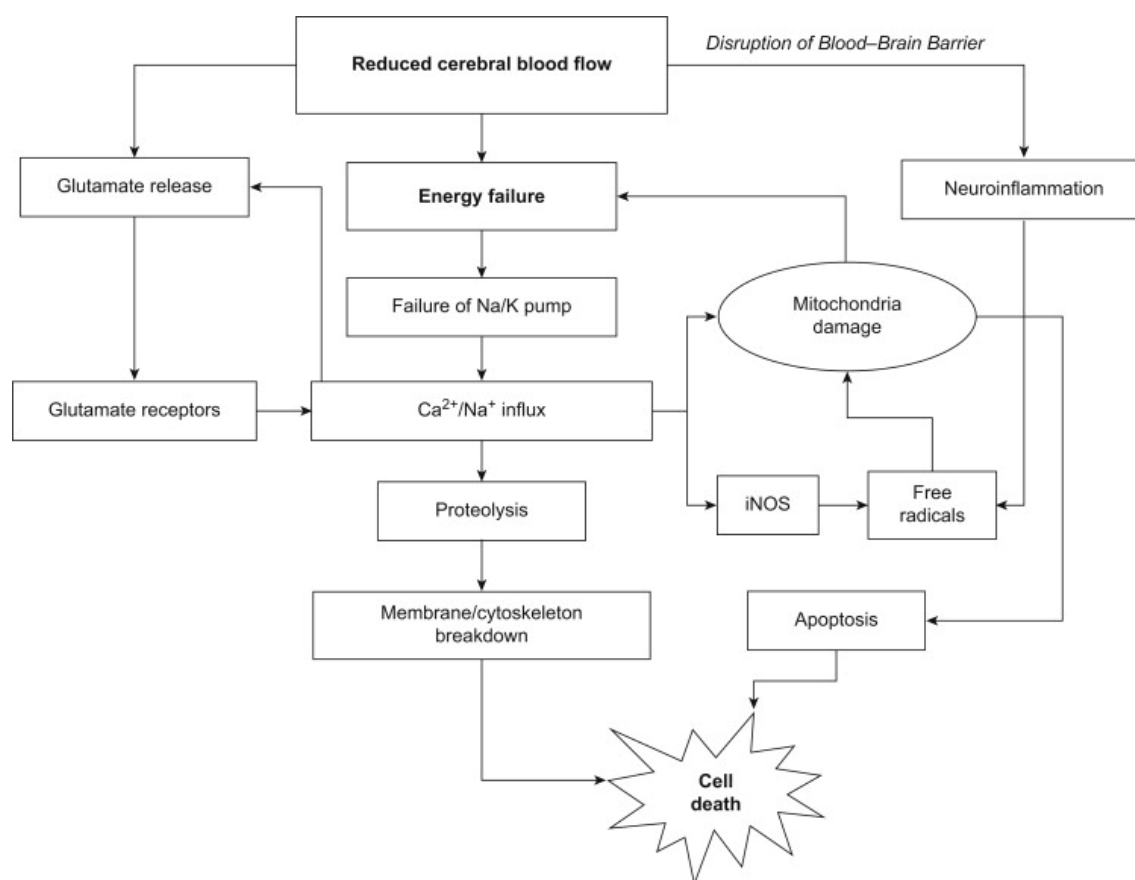


Figure 10. The ischemic cascade; a simplified illustration of the major steps in the ischemic cell-death pathway. From [17].

As discussed in the previous sections, at the microscopic level, the lack of macronutrients and the ensuing metabolic stress cause severe damage to the cellular machinery. This damage is further worsened by a positive feedback loop driven by calcium and glutamate overload, which spreads excitotoxic stress to surrounding neurons through peri-infarct depolarizations. But what happens at the macroscopic scale of the Central Nervous System (CNS)?

At the macroscopic level, the affected tissue can be divided into two main regions. The ischemic core undergoes rapid necrosis and is essentially irreversibly lost. Conversely, the surrounding ischemic penumbra, while preserving its cellular viability, exists in a highly vulnerable state. Neurons within the penumbra remain synaptically connected not only to the depolarizing dying cells but also to distant, structurally intact regions of the CNS, including contralateral areas via the corpus callosum.

Consequently, a focal stroke does not merely cause localized damage; it compromises the excitation/inhibition (E/I) balance of the entire brain network involved. This systemic disruption is best described by the concept of diaschisis. Originally proposed by von Monakow in 1914, diaschisis refers to the neurophysiological changes and loss of function that occur in structurally intact brain areas distant from the focal lesion. Modern neuroimaging has expanded this into the concept of connectomal diaschisis, highlighting how a localized infarct alters the structural and functional connectivity between remote brain networks. A clinically significant manifestation of this network imbalance is the abnormal interhemispheric inhibition. Following a unilateral stroke, the infarcted hemisphere loses its ability to balance the contralateral healthy hemisphere. As a result, the intact hemisphere generates a strongly inhibitory reaction against the lesioned side via transcallosal connections [18], [19].

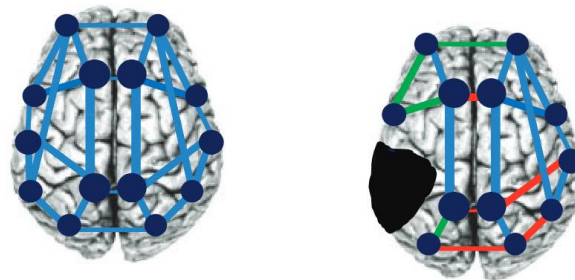


Figure 11. Example of connectomal diaschisis: a lesion of the connectome induces widespread changes in brain network organization including decrease (red) or increase (green) in connectivity. From [18]

While some of these network disruptions may spontaneously subside over time, they often persist, severely hindering behavioural recovery and limiting the patient's ability to perform specific tasks depending on the affected areas. Therefore, understanding and therapeutically intervening in the network dynamics triggered during the post-lesion

period is of great importance. Interventions that aim to reverse these maladaptive processes can significantly enhance rehabilitation outcomes by promoting cortical rewiring facilitating the creation of new functional networks, the restoration or the preservation of pre-existing ones.

Besides those wider observation, in this thesis project the focus for the therapeutic treatment is on the acute phase of the ischaemic insult, so the approach is concentrated on the depolarizing blockade produced by excitotoxic effect of glutamate and its self-reinforcing loop with calcium dysregulation in glutamatergic synapses. The excitotoxic effect produced by glutamate pushes physiological plasticity mechanisms of our brain to abnormal and acausal reinforcement (LTP); the idea it's to look at long-term depression mechanisms as a tool to reduce excitotoxic damages.

1.3. Therapeutic approaches

Currently there are few therapeutic approaches of which the majority are still precluded to non-clinical usage. The canonical treatment for acute ischemic stroke is focused primarily on blood flow recovery via intravenous thrombolysis, this happens with drugs like recombinant tissue plasminogen activator (rtPA), that is in general administered alone or in combination with endovascular treatments with mechanical thrombectomy (in case of large vessel occlusion). Reperfusion interventions are just the primaries and indispensables procedures to save the life of the patient but are strictly limited on their efficacy [20]. The first limiting factor is the time window for intervention, if the reperfusion is not performed on the first 4.5-6 hours the restoration of blood flow produce a strong inflammatory response on the brain due to the switch to hypoxic metabolism from the infarcted cells; this is translated in less effective rehabilitation and recovery and it's a critical condition for the health care system for a lot of countries worldwide in which specialized structures are not available. Other factors are instead related to the residual damage of the cerebral tissue injured by the stroke.

Tissutal damage associated to the lesion can be expressed in a wide range of symptoms and dynamics that are different from case to case, but in general, these additional damages are the consequences of the inflammatory response on the post reperfusion period and are associated with the cerebral oedema.

Treatment of the cerebral lesion implies the necessity to understand in deep the pathophysiological profile of the lesion, this is still a big scientific question that involves the study of cerebral plasticity, neuronal death, pathways and neuroprotective or neuro-regenerative drugs and therapies [21].

1.3.1. Drugs used for reperfusion (r-tPA)

First treatments of stroke are aimed to remove the clot with chemical or mechanic agents to clear the endoluminal space allowing blood to reach the tissue.

As it has been exposed before, the “gold standard” for acute ischemic stroke treatment is the intravenous thrombolysis with alteplase (rtPA). From the clinical protocol this therapy is time constricted, the drug must be administered in a time window of maximum 4.5 hours after the ischemic insult has started. Furthermore, the efficacy of the treatment is strongly dependent about the clot location, age of the clot and time of hospitalization. Large vessel occlusion for example is a critical condition in which rtPA alone is insufficient, in these cases mechanical disruption (mechanical thrombectomy) of the clot is accompanied to the chemical degradation [20].

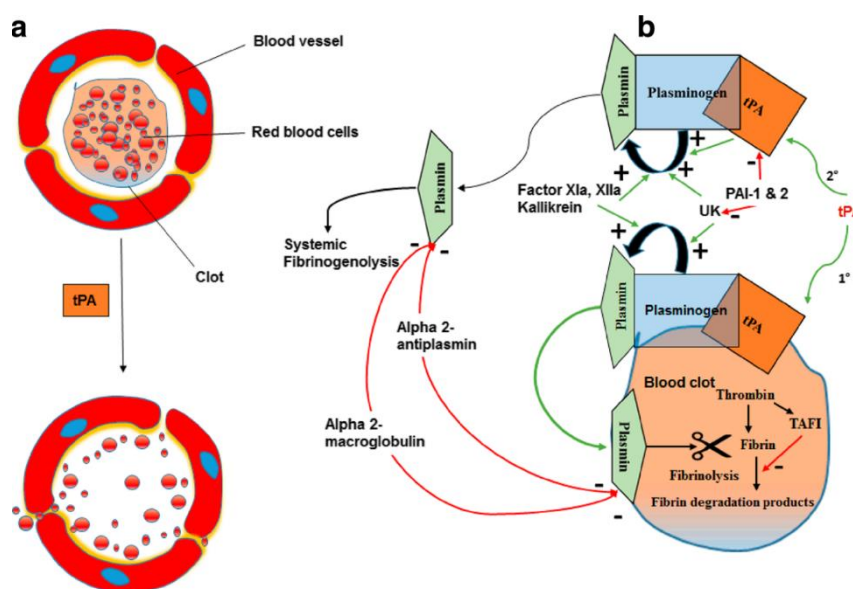


Figure 12. The illustration of the fibrinolytic mechanisms: (a) tissue plasminogen activator (tPA) causes breakdown of the clot, and (b) detailed mechanism of fibrinolysis. Green arrow denotes activation/stimulation, and the red arrow indicates inhibition. tPA = tissue plasminogen activator; UK = Urokinase; PAI = plasminogen activator inhibitor. From [20].

Regarding the time window of intervention, big changes have been introduced thanks to modern imaging techniques (as CTP and DWI-FLAIR), allowing the possibility to look in details the pathological damage and his temporal evolution directly on the tissue the time window for the intervention has been extended up to 9 hours.

Thus it is now possible to change the window of intervention from a time based paradigm to a tissue state paradigm by looking and distinguishing ischemic core and penumbra letting the treatment for “wake-up strokes” [22], the condition in which patients notices the stroke upon awakening, so with uncertainty on the time of occurrence.

New advancement on the reperfusion therapies is mainly on more effective and specific meds to dissolve the clot avoiding rtPA post reperfusion problematics as haemorrhagic transformation and half-life of the drug. A new proposal is Tenecteplase, a genetically modified version of rtPA that has higher safety and better affinity for fibrine (plasminogen activators starts an enzymatic reaction that is aimed on breakdown of the scaffold crosslinked structure of the clot composed by fibrine), but it’s still not officially approved [21].

1.3.2. Drugs for neuroprotection

A significant advancement in stroke clinical treatment could be achieved by supporting the standard reperfusion protocols with neuroprotective drugs (rtPA helpers) that can act on the excitotoxic mechanisms and on pro-death signalling cascade, targeting the preservation and restoration of ischemic penumbra [23].

In the recent years several studies have been conducted around this topic, contributing to extend our knowledge about the complex pathophysiological profile of stroke and proposing a variety of molecules or agents that can have neuroprotective properties.

The drugs and agents proposed by the research are specific elements that targets the physiological alterations underlying the ischemic brain injury, intervening by blocking or mitigating the effects of the events chain that leads to cell death.

Excitotoxicity pathways are one of these targets, mainly involving glutamate-dependent channels (Ca²⁺ channels, NMDA receptors, PTEN receptors) and glutamate-independent Ca²⁺ channels (ASICs, TRP and Na/Ca exchangers). Excitotoxicity focused treatments

acts in general by regulating channels through agonist or antagonist (activating or inhibiting molecules), they require high specificity, to avoid for example that a drug totally blocks NMDA receptors producing highly non-physiological inhibition and unacceptable consequences. One such molecule is the peptide Nerinetide (NA-1), this is one of the more promising experimental drugs (phase III trial), it's an interfering peptide that has great affinity for the protein PSD-95, by binding this protein it protects from the toxic production of nitric oxide that in the ischemic lesion happens during the interaction between an NMDAr subunit (GluN2B) and PSD-95, furthermore it doesn't affect the normal synaptic function of the receptor [24].

Another process in which it's possible to intervene it's the state of oxidative stress and subsequent reperfusion damage by regulation of Hypoxia-inducible factor-1 α (HIF-1 α) or through free radical scavengers. Two powerful drugs proposed as free radical scavengers are the Edaravone (phase III trial), its recent evolution Edaravone dexborneol and uric acid (phase III trial) that is an endogenous antioxidant and a scavenger for peroxynitrite (ONOO⁻). Other treatments are instead focused on attenuating the neuroinflammatory response, to prevent haemorrhagic transformation after thrombolytic therapy and to preserve integrity of the blood-brain barrier. Otaplimastat (SP-8203) is a proposed drug that act on collateral effects of rtPA on the cellular matrix, another one is instead Fingolimod, already approved by FDA in 2010 [24] for multiple sclerosis.

More recently some studies on neuro-regeneration and systemic modulation have led scientists to explore alternative solutions like mesenchymal and neural stem cell therapies. These therapies are more concentrated on the paracrine effects like neurotrophic factors (BNDF) production, rather than in neurons replacement, thereby enhancing synaptic plasticity, neurogenesis and angiogenesis [23].

On the other hand, recent evidence has highlighted the crucial importance of the gut-brain axis: stroke induces rapid intestinal dysbiosis that exacerbates cerebral infarction by modulating peripheral immune responses (e.g., via gamma\delta T cells) and neuroinflammation. The correction of this dysbiosis and the use of microbiome-based immunomodulatory approaches represent a translational therapeutic target [25] to improve prognosis and post-lesion recovery.

Anyway, all these future drugs and therapies are meeting some difficulties in translating results deriving from trials performed on animal models to human clinical trials. This “translational-gap” is attributable to various methodological and biological causes, including the significant discrepancy between animal models (typically young and lacking risk factors) and the clinical population (generally elderly and affected by vascular pathologies) [23].

1.3.3. Rehabilitation

Despite progress in the acute treatment of stroke through thrombolysis, alteplase and the concept of specialized stroke units, the neural damage is still a critical issue and the recovery of damaged areas is almost totally dependent on spontaneous biological recovery mechanisms. Recently, new knowledge about those principles of spontaneous neural recovery has set the basis for an effective neurorehabilitation process. After the acute treatments, stroke patients are impaired by severe neurological deficits and in general there is the need for an early initiation of neurorehabilitation (rehabilitation efficacy decreases with time after the lesion) , the rehabilitation requires a specialized and multidisciplinary team [26], [27] in order to cover all the facets of what a rehabilitation process implies, from the programs and the efficacy of exercises to the psychological approach to the pathology and the therapy.

As stated above neurorehabilitation is built around the neuroscientific findings behind brain recovery and organization, in the last years many studies have been conducted on these topics confirming that the adult human brain also has potential for a selective promotion of neurogenesis and adaptable re-organization after a lesion [28].

Those properties in stroke are limited or influenced by many factors, like the initial size of the lesion or the location as well as the first response after the reperfusion. The biological mechanisms behind neural recovery are described by synaptic plasticity, which refers to the capability of neurons to create functional connections and to modulate their strength and function in a continuous experience-driven process. As a consequence of neural plasticity, the brain is constantly re-organized by the creation of new anatomical connections (synapses) or the potentiation/suppression of the pre-existing ones by gene regulation of protein synthesis (and other functions) that is responsible for membrane channels production (such as NMDA channels). These wirings are indicative of recurrent

neural pathways that could be representative for acquired experiences such as memories, behavioural schemes or specific motor commands, providing a neuro-rehabilitative target. Neural recovery after stroke is a broad concept and is described by multiple overlapping concepts as plasticity, vicariation, sprouting of axons and dendrites and diaschisis resolution [18], [29].

The aim of neurorehabilitation is to induce and promote neural plasticity through physical, or also mental/psychological, activities specifically designed to reach a personal objective for patient needs or deficits, ensuring individual independence and improving quality of life.

Given that we learn on the basis of repetition, integration of informative feedback signals and interaction with the environment, rehabilitation is based on different procedural paradigms that states its efficacy, aspects that are determinant in that are the massed practice (how many repetitions), spaced practice (what are the intervals of rest), increasing difficulty (gradual approach to the desired goals), task specific practice, external/internal feedbacks, mirror therapy and social interaction [30]. These principles cannot be applied in a unique standardized way but are rather dependent on the clinical and psychological state of the patient, furthermore, some of them are associated with changes in a specific brain area while others seem to be addressed to network changes, thus an adequate rehabilitation should take into consideration both aspects.

Limits of rehabilitation may arise when the patient is in a critical condition like paresis or plegia so when the patient has no residual motor capability to undertake the rehabilitation process to enhance plasticity. Other issues are related to maladaptive plasticity expressed through the theories of interhemispheric inhibition and diaschisis [19], [31], in fact very often the cortical activity of stroke patients seems to be highly abnormal and non-functional, hindering the recovery of the patient. Driven by the need to normalize pathological cortical activity and create a favourable microenvironment for plasticity, the concept of neuroaugmentation was developed in recent years. Therefore, research for neurorehabilitation is now working on the possibility to facilitate plasticity through increasingly multi-modal approaches. One discussed approach is the combination of the motor tasks with electrical stimulation of the tissues (in general transcranial magnetic or direct current stimulation) or with the integration of pharmacological interventions [32], [33]. Other future improvements requested by rehabilitation field regards the needs of

standardized measures for the various paradigms (or “dosages”) described above and the necessity to collect more heterogeneous studies.

Chapter 2

2. Neuroengineering and bioengineering as experimental tools

Besides the pharmacological approaches the new type of technologies introduced in the past few years has extended our therapeutic strategies. Electroceutical approaches are a novel research field around which there is a growing interest thanks to their promising results in clinical trials and to the possibility to intervene in the pathology by enhancing in a controlled way the physiological mechanism that guides brain growth and organization. This is still a complex and multidisciplinary experimental practice that could lead to new therapeutic approaches and also to a deeper understanding of concepts like plasticity and neural organization.

2.1. Ischaemic stroke models

Nowadays cell cultures are a valid and relatively accessible tool to develop pathological models in which we can measure the damage generated by the pathology. It's possible to replicate a specific tissue and to assess the goodness of a therapeutic treatment in controlled conditions reducing the necessity of in vivo tests. Thus, cellular models offer the possibility to measure and observe a pathological tissue under different conditions, allowing to investigate in depth the pathophysiological mechanisms associated to the pathology [34]. Cellular stroke models are the approach utilized in this thesis to evaluate the ischaemic insult and the effects of the electrical stimulation.

2.1.1. iPSC-derived neurons

Human induced pluripotent stem cells (hiPSCs) are a powerful tool that enables the replication in healthy or pathological tissue in vitro, bringing the concept of “disease on a dish” avoiding (partially) the issues related to the translational gap showed by animal models and overcoming ethical limits imposed by embryonic stem cells and animal trials [35].

Stem cells are undifferentiated cells, so they have not a specific function other than the development and the regeneration of the tissues of an organism. Their role is fundamental

in the first phases of formation of the organism, in fact stem cells have the capability (different in the various step of development) to differentiate in many cellular phenotypes (neurons, glial cells, cardiac cells, somatic cells etc.). In general, their presence in adult organisms is limited to tissutal areas (stem cell niches, bone marrow) and their power of differentiation is limited.

However, S. Yamanaka's recent discovery has revolutionised the field of stem cells introducing the possibility to obtain pluripotent stem cells by "reprogramming", through the transfection of four transcriptional factors (retroviruses containing human Oct3/4, Sox2, Klf4, and c-Myc) in simple and accessible somatic cells (fibroblasts) from dermis of adults subjects [36]. Once obtained, hiPSCs can be cultured in vitro with dishes superficially treated with adhesion proteins mimicking the extracellular matrix, in this way the cells are expanded while maintained in the undifferentiated form preserving their pluripotent state. Once that the hiPSCs culture is stabilized, the cells are driven to a controlled differentiation in order to obtain a desired phenotype by providing an appropriate culture medium (e.g., DMEM/F12 supplemented with specific growth factors and support molecules).

To model the ischaemic cascade in vitro it's necessary to obtain glutamatergic neurons from hiPSCs, to do this there are different protocols. The canonical way to do it is to firstly differentiate the hiPSCs into neural progenitors and then into the final phenotype, this requires a broad time window (2-3 months) [37] to develop a mature network in vitro with a well-defined functional activity. To overcome these limitations, recent advanced protocols (such as the one utilized in this study) rely on transcription factor-mediated forward programming. As showed by Frega et al. (2017), a rapid and highly efficient neuronal differentiation can be achieved by forcing the expression of the neuronal determinant Neurogenin-2 (Ngn2) in hiPSCs [38].

Through lentiviral transduction of the Ngn2 (neurogenine-2) and rtTA genes with the subsequent addition of doxycycline to the culture medium to activate its Ngn2 transcription, the stem cells rapidly exit the pluripotent state and are directly driven toward a mature cortical neuronal fate. This protocol yields a highly homogeneous population of excitatory (glutamatergic) upper-layer cortical neurons within a timeframe of 3 to 4 weeks.

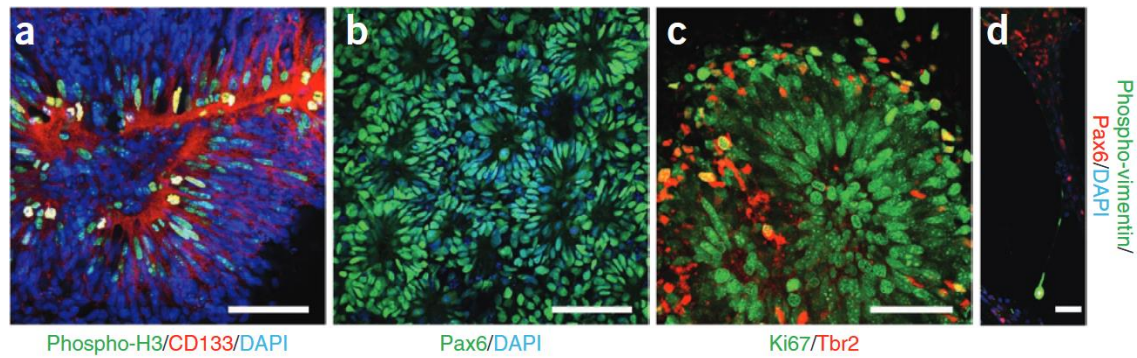


Figure 14. Confocal microscopy images of human iPSC-derived neocortical cortical stem/progenitor cells. From [37].

2.1.2. Ischaemic models (OGD) and NMDA treatment

The cell culture of hiPSC derived neurons is the starting point to successfully model an ischaemic insult in vitro. Once established a culture with fully human genetic background and a coherent electrophysiological activity, the next step is to replicate the ischaemic insult in vitro and to quantify the resulting damage. This is achieved by translating the concept of blood flow interruption on an in vitro system, to do so an experimental procedure known as OGD (Oxygen Glucose Deprivation) it's used. By depriving the cell culture medium of oxygen and glucose, the cell is forced into anaerobic glycolysis, simulating the metabolic stress that causes ATP depletion and the subsequent impairment of the ionic channels that leads the process to the final excitotoxic glutamate overload.

Ischaemia-like condition can be achieved in vitro by replacing the normal O₂/CO₂ equilibrated medium with N₂/CO₂ equilibrated medium in absence of glucose and maintaining the cells in hypoxic chamber for a certain time interval (~ 6-12 hours). This condition can be easily achieved by degassing the glucose-free medium and pushing N₂ to saturation while the culture is inside the incubator. After a certain period is possible to simulate the reperfusion by restoring the medium and the incubation conditions [39].

Another method utilized to produce an excitotoxic model is the treatment of the cell culture with synthetic NMDA agonists (glutamate), the excess of these molecules over-activates NMDA receptors, causing a massive calcium influx. Overload of calcium ions is then translated in mitochondrial stress, leading to reactive oxygen species production [40] and it's also responsible of the abnormal release of glutamate. These three phenomena are part

of a positive feed-forward loop that rapidly brings the cell culture to an excitotoxic condition. Differently from OGD this technique models the excitotoxic cascade of ischaemia but without anoxia and glucose deficit, is so a more reductionist approach that leads to a more controlled cellular death but losing the overall clinical relevance of the OGD model.

To make a quantitative analysis on the ischaemic damage produced by the OGD or NMDA-treated it's possible to look at abnormal concentrations of enzymes like lactate dehydrogenase (LDH) and capsases 3/7. Those enzymes are markers for increased employment of anaerobic glycolysis (LDH) and cell death (capsases 3/7).

2.2. Micro Electrode Arrays

The measures described before are relative to severity of the cell death and cellular damage induced by OGD, so it's necessary to perform complementary measures that are instead indicative to the effect of the insult on the functional properties of the cultured neural network. Functional impairment can be measured through the electrophysiological signals produced by the culture, looking at how these signals are propagated and to their properties is fundamental to characterize the effect of both the damage and therapy [41].

Micro electrode arrays (MEAs) is the type of technology that better responds to the depicted experimental needs. MEA devices consists in a cell culture dish in which are embedded microscopic electrodes capable of sensing the electrophysiological signals produced from neurons or neural circuits [42], which can be cultured directly on the device.

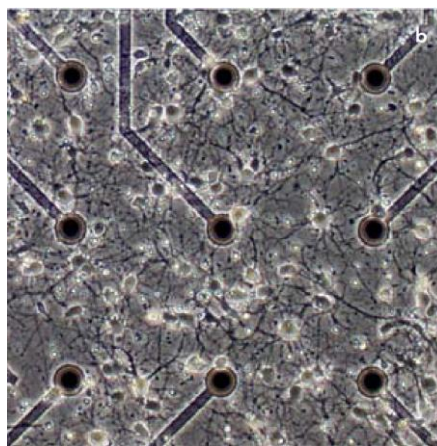


Figure 14. Dissociated hippocampal neurons are grown on an MEA support. Black dots are electrodes and straight lines are conductor lines. Interelectrode spacing: 200 μm . From [42].

MEA surfaces, in fact, can be coated with substances that enhances cell attachment and growth. Commonly those substances are adhesion proteins like laminin, collagen, polylysine or combination of laminin with other polymers. MEA structure originates by a glass wafer (transparent or opaque) used as substrate, the electrodes are made of non-toxic, corrosion-resistant materials such as platinum, gold, iridium or titanium nitride; they are insulated from each other and the culture medium using inorganic or organic materials like silicon oxide, silicon nitride, polyimide or epoxy resin. The size of the electrodes has to match a single neuron dimension, in general it can be in a range between few tens of micrometers of diameter (10-60 μm) and with 100-200 μm of distance one from the other.

Each electrode is connected with a contact pad through a thin conductor line so that an extracellular recording amplifier can amplify the signal picked by the electrode. Since there are multiple electrodes, instrumentation systems are also equipped with multichannel analogic and digital signal processors. Most commonly MEA are characterized by 60 planar electrodes but they exist in a wide range of models from high density MEA (519-4096 electrodes) to 3D tip shape MEA capable to record from different planes of the culture [43].

2.2.1. Neuro-electronic junction

To accurately interpret the extracellular field potentials recorded by the micro-electrode array, it is essential to understand the biophysical mechanisms underlying signal transduction. The physical coupling between neurons and electrodes is known as neuro-electronic junction [44], this complex interface is characterized by different phenomena:

- **Electrode-electrolyte interface:** when the electrode is placed into an ionic conductor i.e. an electrolyte (solution where charge is carried by ionic movement, such as culture medium), it will develop an electrified interface. Water dipoles and unsolvated ions orient themselves in the field within a thin layer at the electrode surface (inner Helmholtz plane) while solvated ions having bigger dimensions are concentrated on a second layer (outer Helmholtz plane), outside this region we have a diffusion layer (Gouy-Chapman) in which the amount of charge decreases exponentially with the distance from the electrode. These layers are electrically modelled as a series of three capacitors (one for each layer to describe the charge

distribution) in parallel with a resistance of “charge transfer”, which dictates the baseline impedance and thermal noise of the recording system.

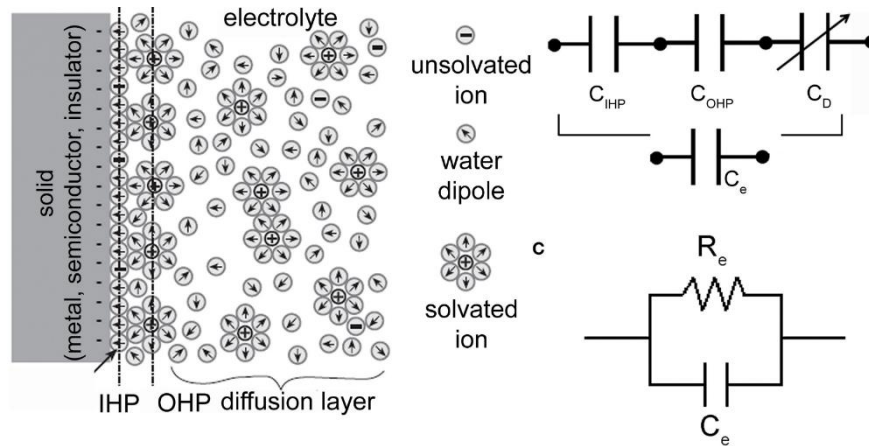


Figure 15. Schematic representation of the electrode-electrolyte interface. From [44].

- **Neuron-to-microtransducer junction:** this interface models the cleft between the neuron’s membrane and the microelectrode, it consists of an equivalent circuit which couples the biological membrane of the electrogenic cell to the recording stage, making possible to record the signal. In this circuit, the neuron's membrane acts as a current source [9]. The critical parameter governing the recorded signal amplitude is the sealing resistance (R_{seal}), parallel to the membrane surface, which represents the resistance of the narrow extracellular space between the cell and the electrode. It can be interpreted as how tight the neuron is attached to the microtransducer. A high sealing resistance indicates tighter cellular adhesion which prevents the ionic transmembrane currents from shunting into the bulk electrolyte, thereby generating a measurable voltage drop across the cleft. Another component: the spread resistance (R_{spread}) instead describes the distance microelectrode-neuron and a capacitor that consider the charge distribution from Helmholtz and diffused layers. microelectrode-neuron and a capacitor that consider the charge distribution from Helmholtz and diffused layers.

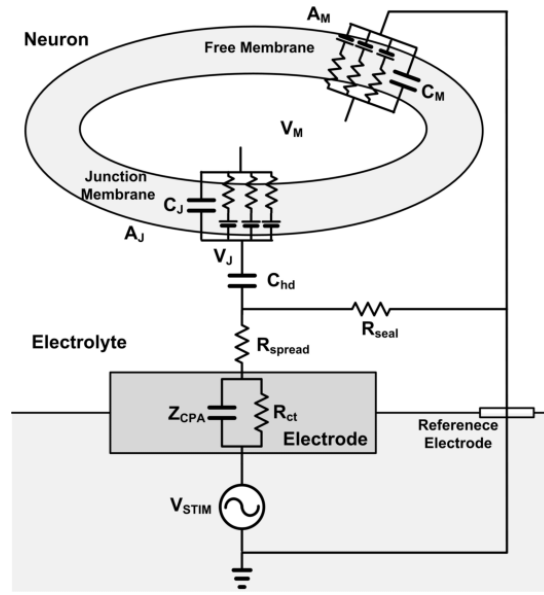


Figure 16. Schematic representation of neuron-electrode interface. From [44].

Ultimately, the quality of the electrophysiological recordings, specifically the Signal-to-Noise Ratio (SNR), is highly dependent on optimizing this physical interface. Crucially, the physical size of the electrode imposes a fundamental bioengineering trade-off: decreasing the electrode area enhances spatial resolution but simultaneously increases the electrical impedance at the electrode-electrolyte interface. Higher impedance proportionally increases thermal noise, thereby degrading the SNR. For this reason, modern MEA systems employ highly porous materials, such as Titanium Nitride (TiN) or carbon-nanotubes (CNTs), which maintain a small geometric footprint while drastically increasing the effective electrochemical surface area, ensuring both high spatial resolution and optimal SNR. Therefore, successful signal detection relies on a delicate bioengineering balance: minimizing the electrode impedance through these advanced surface coatings while simultaneously maximizing the R_{seal} by promoting robust neural adhesion over the microtransducer [44].

2.2.2. Signals observable by MEA recording

The circuitual modelling of the neuro-electronic junction allows to physically interpret the extracellular potentials recorded by the MEAs. The signals that collected using this type of technology are intrinsically filtered by a high pass filter (arising by the sealing resistance

and the capacitor of the interface), which act as a temporal derivative of the actual potential on the neuron's membrane. Consequentially what the system observes is not the absolute value of the membrane potential (unless the circuit operates in ideal conditions i.e. $R_{seal} \rightarrow \infty$, $C_d \rightarrow 0$), rather its rapid temporal variations.

These assumptions explain the different type of signals and waveforms observable during a MEAs acquisition. In the ideal condition in which a single neuron it's coupled with the electrode (single unit) the recorded action potential (spike) will exhibit a biphasic shape for the soma (a negative peak representing the sodium influx, followed by a positive rebound for potassium efflux) or a triphasic shape for propagating axonal waves (second derivative, associated to travelling waves).

However, in a more realistic scenario, the recorded signal it's almost never produced by a single neuron because of the dimensional gap of the electrode with respect to the single neuron, so in general the signals are characterizing multi-unit activity (2-3 neurons for electrode). Furthermore, axonal action potentials are rarely detected due to their intrinsically low amplitude (effect of the high local impedance), limited contact area, and the spatial averaging effect of the larger electrode surface. Under the light of these considerations, the predominantly observed waveforms are the effect of the linear superimpositions of the activity produced by neurons (often synaptically linked) near the electrode. This results in complex, highly asymmetric waves frequently appearing as large negative monophasic-like spikes interspaced by distorted biphasic or triphasic signals. In order to distinguish and isolate the individual neuronal sources from this superimposed multi-unit activity, computational signal processing procedures known as spike-sorting are fundamentally required.

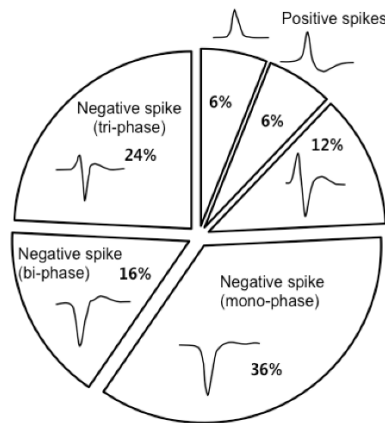


Figure 17. Categorized spike waveforms detected by flat type MEAs. From [42]

Beyond these physical constraints, there are also physiological reasons behind the great variability of the waveforms that characterize the MEA recorded neural activity. The exact profile and amplitude of the extracellular signals are heavily influenced by the local density of voltage-gated ionic channels near the recording site and by the developmental stage of the cultured neurons. As hiPSC-derived neurons mature, the increased expression of ion channels leads to faster depolarization kinetics, yielding much sharper and more pronounced extracellular peaks. Furthermore, neuronal maturation is accompanied by the formation of robust synaptic connections [45]. Therefore, MEA recordings serve as a crucial tool to assess the functional maturity of the in vitro model: a fully developed and healthy network is characterized not only by stable, high-amplitude spikes but, most importantly, by the emergence of synchronized, rhythmic firing patterns known as bursts and network bursts [46].

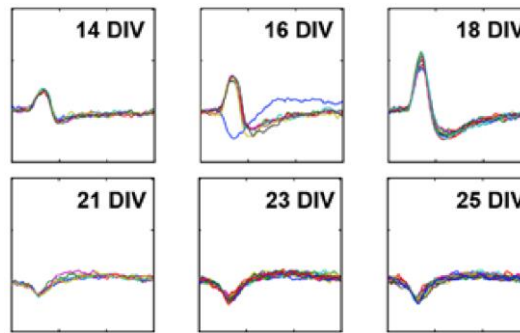


Figure 18. Tracking of the spike waveform changes as cultures age. From [42]

2.2.3. Analysis of MEA signals: from raw data to high-level features

Extracellular signals acquired from Micro-Electrode Arrays (MEAs) are difficult to manage, as they are simultaneously recorded from numerous microtransducers (typically 60 or more) at high sampling frequencies ($f_s = 10\text{--}25\text{ kHz}$). Furthermore, the morphology of the raw trace is influenced by a multitude of physical and biological factors, including the thermal noise of the electrode-electrolyte interface, slow electrochemical fluctuations, and sub-threshold synaptic events. To extract meaningful electrophysiological metrics, it is necessary to isolate the informative frequency bands and compress the continuous voltage recordings into an operational, low-dimensional data structure.

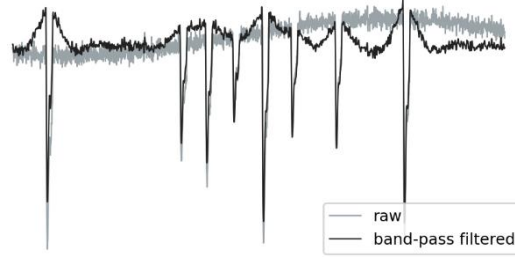


Figure 19. Raw MEAs voltage trace before and after filtering.

The first pre-processing stage applied to raw MEA signals consists of digital band-pass filtering. This operation removes both the low-frequency component ($< 100\text{--}300\text{ Hz}$), which is predominantly constituted by Local Field Potentials (LFPs) and slow baseline drifts, and the high-frequency component ($> 3.5\text{ kHz}$), which is dominated by electronic and thermal noise. Filtering is conventionally performed offline using zero-phase digital Butterworth filters (typically of 2nd to 4th order), which process the signal in both forward and reverse directions to eliminate non-linear phase shifts, thereby preserving the exact temporal and morphological characteristics of the extracellular waveforms.

Once the informative content representing Multi-Unit Activity (MUA) or single-unit activity $x(t)$ has been isolated, the next step is to identify the exact timestamps at which action potentials (spikes) occur and aggregate them into a discrete stochastic point process known as a spike train. By discretizing the total recording duration T into small temporal frames Δt (matching the sampling period $1/f_s$), the continuous trace of a generic electrode i is converted into a sparse binary sequence $s_i(t)$:

$$s_i(t) = \sum_{k=1}^{N_i} \delta(t - t_k^{(i)})$$

where N_i is the total number of spikes detected on channel i , $t_k^{(i)}$ represents the timestamp of the k -th spike, and $\delta(\cdot)$ is the Dirac delta function (which in discrete time assigns the value 1 to frames containing a spike and 0 otherwise). This binarization compresses the data by several orders of magnitude while retaining the full temporal coding of the neuronal population.

To achieve this conversion, a computational procedure known as spike detection is required. Several algorithmic approaches have been proposed in the literature. Traditional

techniques rely on hard thresholding, which fixes a static voltage threshold Th , typically defined as the mean of the filtered signal μ plus a multiple k of the background noise standard deviation σ , and flags a spike whenever the signal crosses this level:

$$Th = \mu + k\sigma$$

$$\sigma = \sqrt{\frac{1}{M-1} \sum_{n=1}^M (x_{\text{noise}}[n] - \mu)^2}$$

where $x_{\text{noise}}[n]$ is a manually selected, spike-free baseline segment of M samples. However, simple threshold-crossing methods are inherently affected by temporal jitter, because the instant at which the waveform crosses the threshold does not coincide with the true peak of the action potential, varying as a function of spike amplitude and slope. Subsequent methods introduced contiguous, non-overlapping time windows within which the local peak-to-peak excursion ($\max - \min$) is compared against a differential threshold, as implemented in the Spike Detection Differential Threshold (SDDT or PDDT) algorithm; nevertheless, rigid windowing can split biphasic waveforms falling across window boundaries. More sophisticated techniques employ energy operators, such as the Non-Linear Energy Operator (NEO), or time-frequency decompositions via Continuous and Discrete Wavelet Transforms (CWT/DWT), although these approaches entail substantially higher computational costs.

To combine high computational efficiency with sub-millisecond timestamp accuracy, the Precision Timing Spike Detection (PTSD) algorithm, developed by Maccione et al. [47] and integrated into the SPYCODE [48] software, used in this study to obtain network features, operates via an event-driven search governed by three biophysically grounded parameters: the differential threshold $Th = k\sigma$, the Peak Lifetime Period (PLP), and the Refractory Period (RP). Rather than slicing the signal into rigid windows, PTSD first identifies all local Relative Maxima and Minima (RMM) by detecting sign inversions in the discrete first-order difference of the filtered trace ($\Delta x[n] = x[n] - x[n-1]$). For each candidate local extremum at index n_1 , the algorithm opens a forward look-ahead window of duration equal to the PLP (typically 2 ms, matching the expected biophysical duration of an extracellular spike) to locate the most prominent RMM of opposite polarity at index n_2 . Instead of comparing a single peak to zero, PTSD evaluates the local peak-to-peak excursion against the differential threshold:

$$\Delta V_{\text{RMM}} = |x[n_1] - x[n_2]| > k\sigma$$

$$0 < \frac{n_2 - n_1}{f_s} \leq \text{PLP}$$

If this condition is satisfied, the event is validated as a spike and its exact timestamp is assigned to the peak of maximal absolute amplitude. Immediately following the assigned timestamp, the algorithm enforces a dead-time window equal to the RP (typically 1-2 ms), during which no further RMMs are evaluated, thereby mimicking the absolute refractory period of voltage-gated sodium channels and preventing multiphasic repolarization rebounds from generating false double detections.

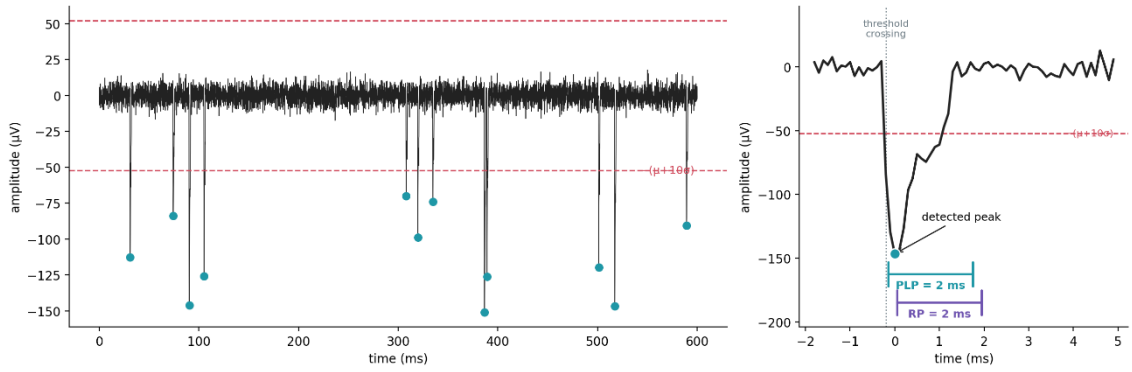


Figure 20. Visualization of PTSD algorithm logic.

Extracting spike trains for each channel of the MEA enables the computation of first-level, single-channel features. The basic excitability of channel i is quantified by its Firing Rate (FR_i), defined as the total number of detected spikes N_i divided by the recording duration T :

$$\text{FR}_i = \frac{N_i}{T}$$

Channels exhibiting $\text{FR}_i \geq 0.1$ spikes/s are classified as Active Electrodes (AE). By partitioning the timeline into short discrete bins of width Δt_{bin} (e.g., 10 ms), one obtains the Instantaneous Firing Rate (IFR_i), expressed as the spike count per bin. Furthermore, the temporal structure of firing on channel i is captured by the Inter-Spike Interval (ISI), which measures the time elapsed between two consecutive spikes:

$$\text{ISI}_k^{(i)} = t_{k+1}^{(i)} - t_k^{(i)}$$

Constructing the probability density histogram of the base-10 logarithm of the ISIs ($\log_{10}(\text{ISI})$) provides a powerful tool to characterize bursting activity (high-frequency spike packets separated by quiescent periods). In mature in vitro neuronal networks, the logarithmic ISI histogram (logISIH) exhibits a characteristic bimodal distribution: a first peak at short time scales corresponding to tightly packed intra-burst spikes, and a second peak at longer time scales reflecting inter-burst pauses.

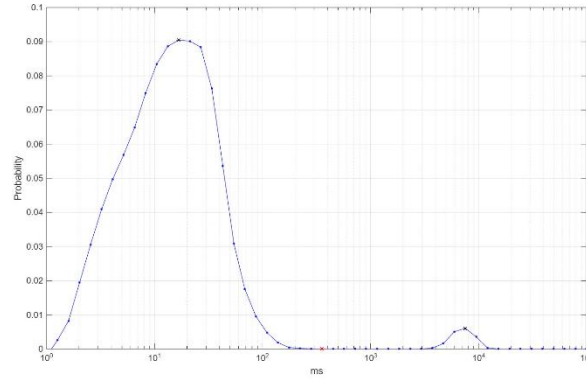


Figure 21. Log-ISI bimodal distribution with ISI_{th} (red dot).

By automatically locating the local minimum (valley) between these two peaks, SPYCODE's self-adapting algorithm (Burst Detection v2-PA) extracts a channel-specific threshold ISI_{th} that discriminates intra-burst spikes ($\text{ISI} \leq \text{ISI}_{th}$) from isolated spikes ($\text{ISI} > \text{ISI}_{th}$). Sequences of at least 5 consecutive spikes with intervals below ISI_{th} on channels with a bursting rate ≥ 0.4 bursts/min are labelled as bursts. This partitioning simultaneously isolates the fraction of Random Spikes (RS), the percentage of stochastic spikes occurring outside bursts, and defines the single-channel Inter-Burst Interval (IBI) between consecutive bursts.

These local descriptors are subsequently integrated across all active electrodes to derive high-level, network-scale features indicative of collective dynamics. Averaging the individual firing rates across all N_{AE} active electrodes yields the global Mean Firing Rate (MFR):

$$\text{MFR} = \frac{1}{N_{AE}} \sum_{i=1}^{N_{AE}} \text{FR}_i$$

Similarly, single channel burst trains are merged into a single cumulative burst event train by projecting the onset timestamps (the initial spike $t_{\text{onset},m}$) of every burst detected across the entire array onto a common timeline. Measuring the time distance between consecutive burst onsets across any pair of electrodes defines the Inter-Burst Event Interval (IBEI):

$$\text{IBEI}_m = t_{\text{onset},m+1} - t_{\text{onset},m}$$

Analogously to the single-channel logISIH, binning the IBEI values on a logarithmic scale (logIBeIH) reveals a bimodal (or multimodal) profile: the first peak at short intervals captures the rapid cascade of electrodes recruiting one another during a synchronous population event, a Network Burst (NB), whereas the peak at longer intervals reflects the global silent periods between consecutive population discharges. From the local minimum separating these modes, an adaptive threshold (maxIBeI) is extracted to group single-channel bursts into candidate Network Bursts, which are confirmed if at least a minimum percentage of active electrodes (typically 20%) participates in the event. Once the N_{NB} Network Bursts are identified, with start and end times $[t_{\text{start},j}, t_{\text{end},j}]$, global synchronization is summarized by the Network Burst Rate (NBR), the mean Network Burst Duration (NBD), and the Network Inter-Burst Interval (NIBI):

$$\text{NBR} = \frac{N_{\text{NB}}}{T_{\text{min}}}$$

$$\text{NBD} = \frac{1}{N_{\text{NB}}} \sum_{j=1}^{N_{\text{NB}}} (t_{\text{end},j} - t_{\text{start},j})$$

$$\text{NIBI}_j = t_{\text{start},j+1} - t_{\text{start},j}$$

Furthermore, by aligning and averaging the array-wide Instantaneous Firing Rate ($\Delta t_{\text{bin}} = 10$ ms) across all detected Network Bursts, SPYCODE computes the Spike Time Histogram (STH) profile (expressed in spikes/bin), which characterizes the average temporal envelope of population recruitment. Fitting exponential functions to the rising

and falling phases of the mean STH curve from its baseline to its peak (STH amplitude) yields two kinetic time constants:

$$I_{\text{rise}}(t) \propto 1 - e^{-t/\tau_{\text{rise}}}$$

$$I_{\text{decay}}(t) \propto e^{-t/\tau_{\text{decay}}}$$

where the rise time constant (τ_{rise} , Rise Tau) quantifies the speed of excitatory synaptic recruitment at burst initiation, and the decay time constant (τ_{decay} , Decay Tau) reflects the kinetics of synaptic depression and inhibitory feedback terminating the synchronous discharge.

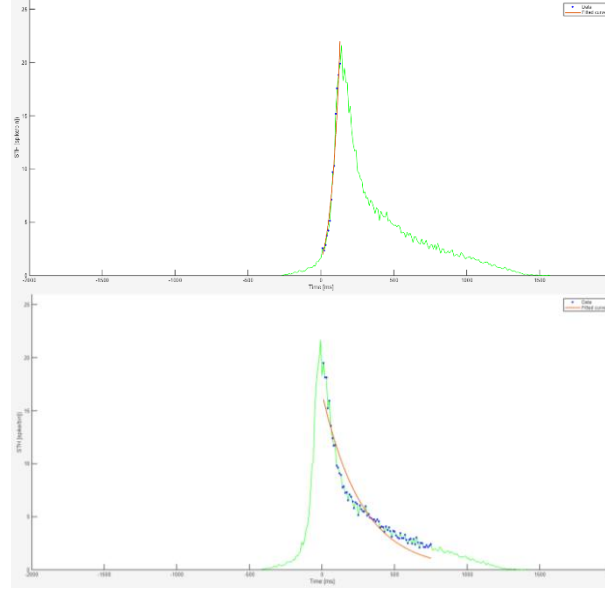


Figure 22. STH rise/decay exponential fitting.

Beyond firing and bursting statistics, the topological organization of the network can be investigated in terms of functional and effective connectivity, which quantifies the statistical interdependence or information transfer between recording sites by comparing spike trains across all electrode pairs (x, y) . Three principal methodologies, ordered by increasing mathematical complexity and directional specificity, are commonly employed. Cross-Correlation (CC): The classical linear approach for estimating functional connectivity measures the probability of observing a spike in target train y at a time lag τ relative to a spike in reference train x . Given the binned spike trains $x(t_s)$ and $y(t_s)$ over M time bins, and denoting by N_x and N_y the total number of spikes recorded in train x and

train y respectively, the symmetric normalized cross-correlation function $C_{xy}(\tau)$ (or evaluated at $\tau = 0$ for zero-lag synchrony) is computed:

$$C_{xy}(\tau) = \frac{1}{\sqrt{N_x N_y}} \sum_{s=1}^M x(t_s) y(t_s - \tau)$$

While computationally fast, standard zero-lag Cross-Correlation is symmetric ($C_{xy}(0) = C_{yx}(0)$) and purely undirected; even when evaluated across time lags τ ($C_{xy}(\tau) = C_{yx}(-\tau)$), it captures only linear co-activations and cannot distinguish direct synaptic transmission from common drive originating from a third shared neuronal population.

Transfer Entropy (TE): Rooted in Shannon's information theory and Wiener-Granger causality, Transfer Entropy is an asymmetric, directional, and model-free measure that captures both linear and non-linear causal interactions [49]. Rather than measuring simple co-occurrence, $TE_{X \rightarrow Y}$ quantifies the amount of information (in bits) that the past state of the source channel $x_{n-1}^{(k)}$ provides about the current state of the target channel y_n , beyond what is already predicted by the target's own past history $y_{n-1}^{(l)}$:

$$TE_{X \rightarrow Y} = \sum P(y_n, y_{n-1}^{(l)}, x_{n-1}^{(k)}) \log_2 \frac{P(y_n | y_{n-1}^{(l)}, x_{n-1}^{(k)})}{P(y_n | y_{n-1}^{(l)})}$$

Because $TE_{X \rightarrow Y} \neq TE_{Y \rightarrow X}$, Transfer Entropy yields a directed graph of information flow across the array.

Generalized Transfer Entropy (GTE): In in vitro cultures dominated by highly synchronous Network Bursts, standard Transfer Entropy can be confounded by global population discharges, during which almost all electrodes fire simultaneously, generating spurious all-to-all connectivity estimates. Generalized Transfer Entropy (introduced by Stetter et al. [50]) overcomes this limitation by extending TE in two ways: first, it includes instantaneous (same-bin) interactions alongside lagged ones to account for fast synaptic propagation within the time bin; second, and most importantly, it conditions the probability distributions on the global network firing level $G_n = \sum_i x_i[n]$ being below a dynamical threshold g_{th} (thereby restricting the causal inference to inter-burst or moderate-activity regimes):

$$GTE_{X \rightarrow Y} = \sum P(y_n, y_{n-1}^{(l)}, x_n^{(k)} | G_n < g_{th}) \log_2 \frac{P(y_n | y_{n-1}^{(l)}, x_n^{(k)}, G_n < g_{th})}{P(y_n | y_{n-1}^{(l)}, G_n < g_{th})}$$

This conditioning filters out global bursting artefacts and isolates true effective synaptic connectivity.

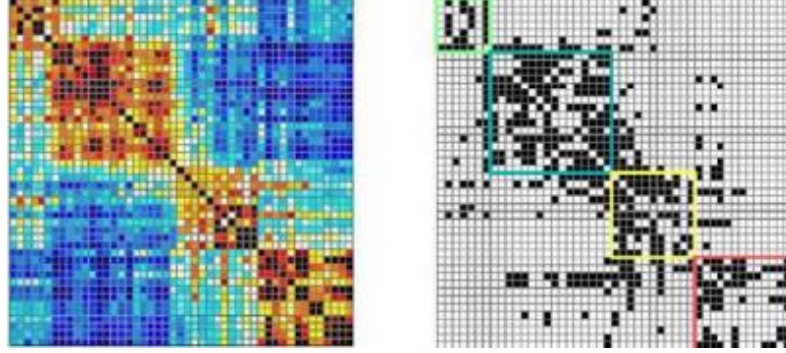


Figure 23. Weighted adjacency matrix (left) thresholding produces binary adjacency matrix (right).

All three connectivity computations yield an $N \times N$ connectivity matrix W (where N is the number of electrodes and diagonal self-connections $W_{ii} = 0$ are excluded). By applying a statistical threshold θ (e.g., $W_{ij} > 0.2$) to retain only significant functional links, W is converted into a binary or weighted adjacency matrix A representing a topological graph, from which global graph-theoretical metrics, such as Mean Connection Strength and Network Density, are extracted:

$$\text{Strength} = \frac{1}{N(N-1)} \sum_{i \neq j} |W_{ij}|$$

$$\text{Density} = \frac{1}{N(N-1)} \sum_{i \neq j} \mathbb{I}(|W_{ij}| > \theta)$$

where $\mathbb{I}(\cdot)$ is the indicator function.

Taken together, this hierarchical suite of single-channel, population-burst, and graph-connectivity features provides a comprehensive mathematical framework to monitor not only basal neuronal excitability, but also network synchronization and the functional synaptic rearrangements induced by ischemic injury and electroceutical stimulation.

2.2.4. MEA as an active instrument: therapeutic stimulation

Besides its sensing and recording capabilities, MEA technology also offers the possibility to electrically stimulate the culture by switching the microelectrodes from passive sensors

to active actuators. In this way, it is possible to inject specific current patterns into the neural network and simultaneously observe the stimulation's effects, effectively transforming the MEA into a closed-loop platform capable of both monitoring and modulating neuronal activity [51]. This feature is fundamental to assessing the preliminary phases of electroceutical-based therapies.

The fundamental mechanism underlying electrical stimulation is the modulation of the neuronal membrane potential through an artificially generated extracellular electric field. By injecting a controlled current pulse, a displacement current is induced across the cell membrane; if this local depolarization reaches the activation threshold of voltage-gated sodium channels, an action potential is artificially elicited. However, to ensure the long-term viability of the biological culture and to preserve the physical integrity of the microelectrodes, stimulation protocols must strictly employ biphasic, charge-balanced pulses. This configuration typically consists of a cathodic phase followed immediately by an anodic phase of equal charge area. This precise balance is crucial to prevent the accumulation of residual electrical charge, avoiding irreversible Faradaic reactions that could lead to local pH shifts, generation of toxic electrochemical byproducts, and rapid electrode corrosion.

In the specific context of *in vitro* ischemic modelling, electrical stimulation is leveraged not merely to trigger random network firing, but to therapeutically reshape the synaptic weights of the cultured network. While High-Frequency Stimulation (HFS) is traditionally associated with Long-Term Potentiation (LTP), the mitigation of ischemic damage in this experimental paradigm relies on the induction of Long-Term Depression (LTD) via Low-Frequency Stimulation (LFS) protocols (e.g., 1 Hz) [52] .

The rationale behind this specific electroceutical approach is deeply rooted in the pathophysiology of stroke. As previously established, an ischemic insult triggers a massive, uncontrolled release of glutamate, culminating in lethal NMDA-mediated excitotoxicity and pathological network hyper-activation. By delivering a targeted LTD protocol, the neural network is driven to actively reduce its synaptic efficacy, a process biologically mediated by the internalization of post-synaptic AMPA receptors. This strategic down regulation of overall network excitability could be a powerful neuroprotective mechanism. By dampening the lethal excitatory drive, the LTD stimulation may effectively act on the

network, reducing its metabolic demand and protecting the surviving neuronal population from post-ischemic energy exhaustion and subsequent apoptosis.

2.3. Fluorescence imaging

While electrophysiological recordings are a powerful tool to study the functional activity of neuronal networks and to establish preliminary ground-truth, these measurements are intrinsically limited by their spatial resolution and the complexity of the acquired signals. As discussed in the previous sections, MEA recordings often represent the linear superimposition of multiple adjacent events (Multi-Unit Activity), masking the behaviour of individual cells. To ensure robust experimental findings, it is necessary to integrate complementary analytical techniques capable of providing distinct insights into the underlying biological processes.

For this reason, fluorescence imaging is a widely adopted methodology to significantly enhance the resolution and specificity of network analyses. This technique offers the versatility to label specific membrane channels, detect apoptotic molecules, or continuously track intracellular calcium dynamics. Thanks to its high molecular specificity, fluorescence spectroscopy enables the direct visualization of single-cell dynamics and the precise identification of information transmission pathways across the network, effectively bypassing the spatial limitations of a standard 60-electrode array [53].

Ultimately, the multimodal integration of these diverse measurements enables mechanistic inferences, allowing for the formulation of cross-validated, data-driven answers to the scientific questions regarding the validation of the *in vitro* ischemic model and the evaluation of treatment efficacy.

2.3.1. Confocal laser microscopy and fluorescent probes

Fluorescence images are obtained by acquiring photons emitted by a fluorescent probe. Fluorescent probes are molecular biosensors specifically engineered to bind biological targets. They are generally composed of two domains: an optical reporting domain (the fluorophore) and a recognition element. The first provides the photophysical capabilities, while the second is responsible for target binding. The recognition mechanism varies

depending on the experimental requirements: it can rely directly on physicochemical affinity (e.g., antibodies or intercalating agents), on enzymatic activation (fluorogenic substrates), or, for more complex dynamic measurements like Ca^{2+} tracking, on highly selective chemical chelators.

Light is emitted by fluorophores when irradiated by a light source at a specific wavelength. The absorption of the energy carried by incident photons causes the fluorophore's electrons to "jump" from a ground energy state to a higher and unstable excited state (Jablonski diagram). This energetic promotion lasts for a few nanoseconds, after which the electrons return to their initial energetic level, releasing the residual energy in the form of a newly emitted photon with a longer wavelength (Stokes shift) [54]. The wavelength difference between absorbed and emitted photons allows for the implementation of dichroic mirrors and emission filters to optically separate the intense excitation light from the weak emission signal originating from the biological sample, ensuring the isolation of the signal of interest.

To acquire and translate the emitted photons into quantitative data, it is possible to employ fluorescence microscopy techniques such as the Confocal Laser Scanning Microscope (CLSM). Differently from standard epifluorescence systems, confocal devices allow for superior spatial resolution through an excitation strictly localized in space, using point-by-point laser illumination coupled with a pinhole placed at the confocal plane of the light detector (photomultiplier). In this optical configuration, only the light collected exactly from the focal plane is measured, significantly enhancing lateral and axial resolution. The advantage of receiving only the in-focus light is that signals originating from above and below the focal plane are physically filtered out. This makes it possible to cleanly distinguish different optical sections of the specimen (optical sectioning), thereby obtaining highly defined cellular profiles and enabling accurate 3D reconstructions [55].

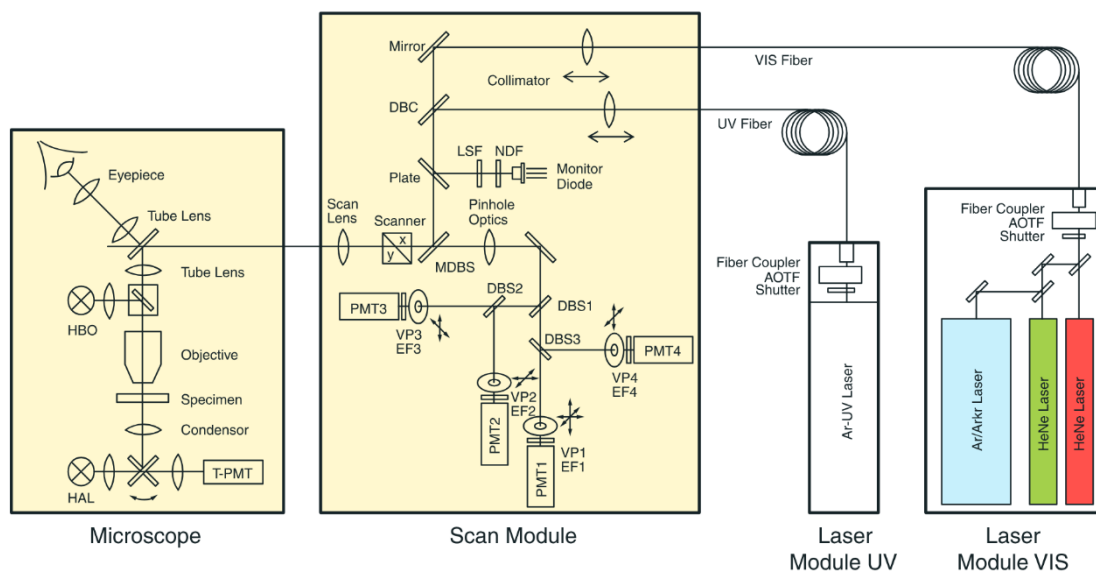


Figure 25. The optical diagram (optical path) of the Zeiss LSM510 is schematically shown in the figure. AOTF: acusto optical tunable filter; DBC: dichroic beam combiner; DBS: dichroic beam splitter; EF: emission filter; HAL: halogen lamp; HBO: mercury vapor short-arc lamp; LSF: line selection filter; MDBS: main dichroic beam splitter; NDF: neutral density filter; VP: variable pinhole; PMT: photomultiplier, T-PMT: transmission-photomultiplier. From [55].

The availability of diverse fluorophores with distinct target specificities and excitation wavelengths makes the CLSM an intrinsic multiplexing system, allowing the parallel acquisition of multiple biological signals. Within the experimental framework of this thesis, fluorescence imaging was utilized across four primary analytical axes:

- **Structural and morphological analysis:** Nuclear markers such as DAPI (which intercalates into adenine-thymine-rich regions of DNA) were utilized to strictly localize cell bodies. This structural imaging is fundamental to quantify cellular density, map network topology, and identify individual cells as Regions of Interest (ROIs).
- **Synaptic plasticity and receptor trafficking:** To validate the molecular mechanisms underlying the neuroprotective electrical stimulation (LTD), specific immunofluorescence assays targeting AMPA receptors were performed. By quantifying the density and localization of these specific glutamate receptors, it is possible to directly assess the efficacy of the stimulation protocol in inducing synaptic down-regulation, thereby lowering network excitability.

- Pathology and viability assessment: To quantify the extent of the damage induced by the ischemic model (e.g., NMDA-mediated excitotoxicity or OGD), apoptosis-sensitive fluorophores were employed. Specifically, fluorescent probes targeting Caspase-3/7 activation allowed for the direct visualization and quantitative assessment of the percentage of neurons actively undergoing programmed cell death following the excitotoxic insult.
- Functional monitoring (Live-Cell Calcium Imaging): Moving beyond static endpoint measures, the real-time computation of the network was recorded using fluorescent ionic trackers to observe intracellular calcium fluctuations in living cells. This technique acts as an optical transducer of electrophysiological events, monitoring both physiological network bursts and pathological calcium overload with sub-cellular resolution.

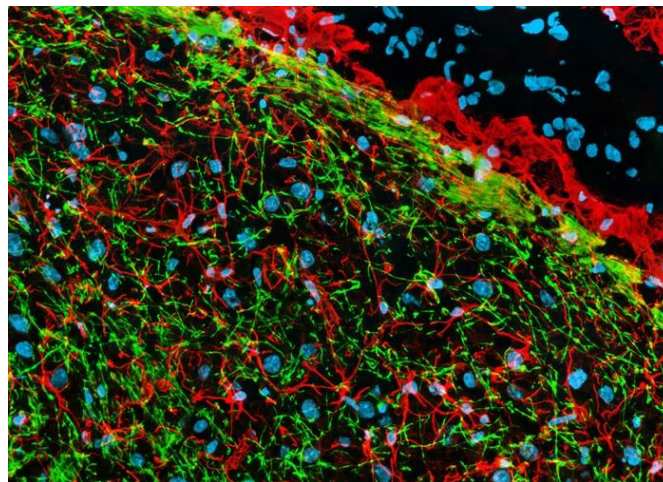


Figure 26. Example of multichannel fluorescence image (cortical neurons derived from rat). (courtesy of Prof. G. Shaw, EnCor Biotechnology Inc.).

2.3.2. Acquisition and interpretation of calcium signals

Intracellular Calcium signals are a complex phenomenon due to their ubiquity as a second messenger. In fact, calcium it's involved in a wide range of neuronal events with different effects and time dynamics, ranging from highly localized, sub-millisecond microdomain transients that trigger neurotransmitter release, to minutes-hours dynamics such as gene transcription, apoptosis or synaptic plasticity. Intracellular calcium levels express a great variability, going from a minimum of 100 nM to a maximum that can be 100 times higher.

Regulation of these concentration it's managed by buffering organelles such as endoplasmic reticulum and mitochondria.

To optically capture these versatile dynamics, two main class of fluorescent biosensors are currently employed: synthetic chemical chelators (e.g. Fura-2, Fluo-4) and Genetically Encoded Calcium Indicators (GECIs) [56]. While GECIs offer the advantage of cell-type specific targeting through genetic expression, chemical dyes like Fura-2 are preferred for their high signal to noise ratio, superior loading uniformity in in vitro networks and for their suitability for absolute quantification.

Unlike electrophysiological MEA recordings, which directly sense the membrane potential variation as an explicit signal of a spike production, calcium imaging provides an indirect measure of neuronal spiking activity. The fluorescence measured through the microscope it's not an absolute measure of voltage, but, rather a signal proportional to the concentration of free intracellular Ca^{2+} bound to the fluorophore. Furthermore, raw fluorescence is dependent on experimental artefacts such as uneven distribution of the dye and photobleaching. To achieve a normalized quantification of the signal it's necessary to obtain the fractional change of the fluorescence with respect to the baseline. Alternatively a more robust approach to the measure it's to produce ratiometric measures by using dyes excitable with two wavelength like Fura-2 and calculating the ratio of the two fluorescence of emission obtaining an intrinsically normalized quantification that yields a signal strictly proportional to the underlying calcium concentration while cancelling out concentration and illumination artifacts.

Extract information from the raw optical recordings (time-lapse image stacks) requires a rigorous computation pipeline.

The initial step involves pre-processing and spatial segmentation to identify individual neuronal soma and define it as region of interest (ROIs). For each ROI and for each frame, the spatial average of the pixels inside the ROI it's computed in order to extract a fluorescent trace, in this way the high-dimensional video data are compressed into one dimensional time series [57].

Interpreting and inferring spiking activity from these traces it's a fundamental biophysical challenge due to the temporal discrepancy that exist between fast sodium/potassium signals (spike) and slower calcium transients. Calcium influx and clearance are characterized by a

fast peak and a slow exponential decaying profile, acting as a biological low pass filter with respect to the spike signal. When a neuron for example fires a burst of action potentials, the calcium transients are summed and overlapped, masking the underlying discrete spike train. Therefore, the observed calcium trace can be modelled as a mathematical convolution of the spike train with the exponential calcium impulse response of the biosensor.

To infer the underlying spike train and thereby produce signals with a functional outcome it's so necessary to resolve an inverse problem of deconvolution. Modern computational approaches employ blind-sparse deconvolution methods such as constrained non-negative matrix factorization (CNMF) and fast non-negative deconvolution methods (OASIS) [58]. By enforcing “non negativity” (since neurons can't fire negative spikes or produce negative fluorescence signals) and sparsity (spikes are discrete and isolate events), these algorithms successfully deconvolves the low-pass filtered calcium signals, providing an accurate estimation of the spike train that produced the observed fluorescent trace, allowing for a precise characterization of the network functionality.

2.3.3. The Evolution of Cellular Segmentation: From Manual Annotation to Deep Learning

Once fluorescence signals are acquired, they must be assigned to specific Regions of Interest (ROIs), which generally correspond to individual cell somata. The primary objective is to discriminate valid cellular signals from background noise, or to distinguish between different cellular phenotypes (e.g., healthy versus tumoral cells). The computational procedure of extracting ROIs from biological images, such as those obtained via fluorescence microscopy, is known in the literature as cellular segmentation.

In recent years, cellular segmentation has acquired continuously growing importance, not only for experimental measurements in research but also for histology-based diagnostic practices. Consequently, the necessity for a precise, accurate, and standardized methodology has driven a rapid evolution of segmentation techniques [59].

Historically, segmentation was primarily performed manually, relying entirely on the knowledge and experience of the scientist to visually recognize the cells of interest and annotate their contours using software such as ImageJ/Fiji. Although the human eye provides a highly precise discriminative tool, this approach suffers from severe limitations.

It introduces significant intra- and inter-operator variability, compromising data reproducibility. Furthermore, beyond human subjectivity, the manual evaluation of every single cell is an extremely time-consuming task. Today, both research and clinical datasets frequently comprise thousands or even millions of cells. While such large sample sizes significantly increase the statistical robustness of the extracted data, they strictly demand fully automated and reproducible methodologies [60].

With the advent of modern computer vision and image processing, new techniques based on pixel-wise analysis to infer morphological features have vastly expanded the possibilities for cellular segmentation. Among the multitude of traditional techniques, the most prominent include thresholding, edge detection, region-based methods, and clustering [59]. Despite improving efficiency and objectivity, the performance of these heuristic methods often degrades significantly when applied to complex medical images characterized by high noise levels, non-uniform illumination, or ambiguous and overlapping boundaries.

In recent years, deep learning has completely revolutionized the field of cellular segmentation, offering the capability to handle complex, high-dimensional data by enabling automatic feature extraction and achieving unprecedented accuracy. Deep learning models are deep artificial neural networks fundamentally composed of an input layer, multiple hidden layers, and an output layer. Various architectures have become dominant, with Convolutional Neural Networks (CNNs) serving as the foundation for the vast majority of modern segmentation models.

Convolutional Neural Networks represent the successful integration of deep learning and advanced image processing technologies. Their conceptual predecessor is the neocognitron proposed by Fukushima, which was heavily inspired by Hubel and Wiesel's hierarchical receptive field model of the mammalian visual cortex. Mirroring the biological mechanisms of the nervous system's visual processing, Convolutional Neural Networks consist of a cascade of layers designed to progressively extract morphological features from the image. The core architecture is built upon three main components: convolutional layers, pooling layers, and fully connected layers. When an input image is presented to a Convolutional Neural Network, it is first processed by multiple convolutional layers. These layers apply a series of trainable convolutional filters (analogous to Gabor or steerable filters) to perform a multidimensional extraction of edges, patterns, and textures, ultimately

generating a highly complex feature map of the image. The data then passes through pooling layers, where the feature map is compressed via a spatial down-sampling procedure. This step is essential both to reduce computational complexity and to foster translation invariance, leading to more abstract and high-level feature representations. Finally, the network flattens the spatial feature map and routes it through fully connected layers, where the learned features are mathematically combined to make a high-level decision, typically generating a single categorical classification label for the entire image.

While traditional Convolutional Neural Networks were conceptualized for global image classification, cellular segmentation requires dense, pixel-wise labeling (i.e., every single pixel must be assigned to a specific object or to the background). This challenge was elegantly resolved by replacing the fully connected layers of standard Convolutional Neural Networks with up-sampling convolutional layers, giving rise to Fully Convolutional Networks (FCNs).

Building upon the Fully Convolutional Networks paradigm, Ronneberger et al. developed the U-Net architecture specifically for biomedical image segmentation. Since its introduction, U-Net has become the de facto standard in the field and serves as the foundational backbone for the Cellpose-SAM model utilized in this study [61]. The U-Net architecture features a symmetric U-shaped design: a contracting path (encoder) on the left for context and feature extraction via convolutions and pooling, and an expanding path (decoder) on the right that restores the original spatial resolution using up-convolutions. Its exceptional suitability for biomedical imaging stems from the use of "skip connections", which directly merge high-resolution, low-level spatial features from the encoder with complex, high-level semantic features from the decoder, yielding a highly accurate, pixel-perfect segmentation map.

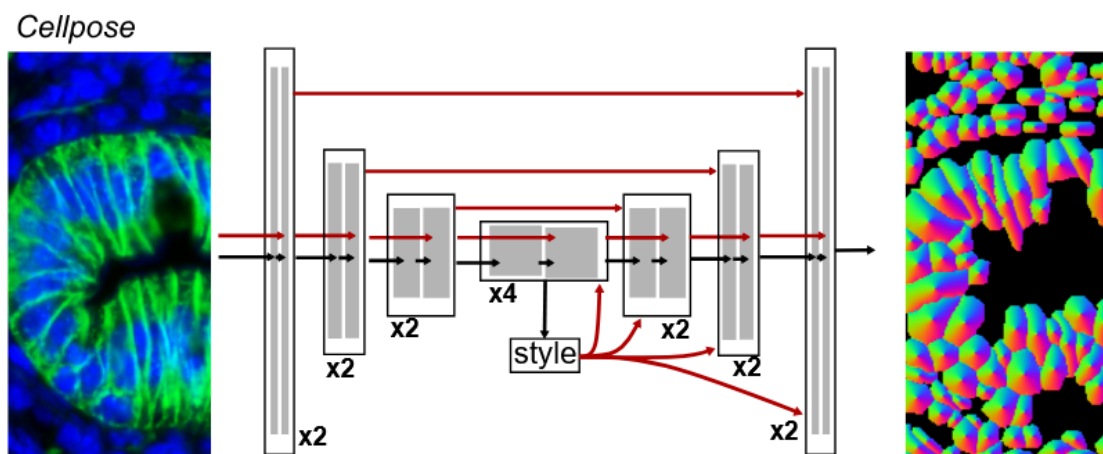


Figure 27. Schematic of the U-net backbone used in the original Cellpose model with skip connections and global style vector. Shown on the right is the representation of the flow vectors that Cellpose predicts as an intermediate to mask reconstruction. From [61].

Despite their unparalleled performance, deep learning methods also suffer from inherent limitations based on their intrinsic nature. Firstly, these models require massive, accurately annotated training datasets, a significant bottleneck in the biomedical field where expert annotation is scarce. To mitigate this, strategies like data augmentation (artificially expanding the dataset via rotations, flipping, and scaling) are heavily employed. Secondly, deep learning algorithms demand immense computational power; training and running these large networks efficiently is nearly impossible unless the workstation is equipped with high-performance Graphics Processing Units (GPUs). Finally, interpretability remains a critical issue. While traditional methods produce transparent, rule-based outputs, deep learning models are frequently perceived as "black boxes". Although these complex networks extract intricate and abstract features, their internal decision-making processes remain largely opaque to the human operator.

Chapter 3

Materials and methods

3 Cell cultures

3.1 hiPSC culture and glutamatergic neuronal differentiation

Human induced pluripotent stem cell-derived neuronal cultures were generated using a previously characterized hiPSC line genetically modified to obtain homogeneous populations of excitatory glutamatergic neurons through the forced expression of the transcription factor Neurogenin-2 (Ngn2). The hiPSC line was received in frozen vials from Dr. Frega, University of Twente. Glutamatergic neurons were derived from control line 1 (C1; healthy 30-year-old female donor, Ngn2), originally generated from fibroblasts and reprogrammed via episomal reprogramming [38].

hiPSCs were thawed and maintained in E8 Flex medium (Thermo Fisher Scientific) supplemented with E8 supplement (2%, Thermo Fisher Scientific), penicillin/streptomycin (1%, Sigma-Aldrich), G418 (50 µg/ml, Sigma-Aldrich), and puromycin (0.5 µg/ml, Sigma-Aldrich). Glutamatergic cortical layer 2/3-like neurons were generated by doxycycline-induced overexpression of mouse Ngn2. Neuronal differentiation was initiated by treatment with doxycycline (4 µg/ml, Sigma-Aldrich), as previously described. Cultures were maintained under standard incubator conditions at 37 °C, 5.5% CO₂, and 95% humidity. Half of the culture medium was refreshed every 2 days.

After neuronal induction, cultures were maintained until the appropriate stage of maturation before being used for MEA-based electrophysiological recordings, LTD induction, and oxygen-glucose deprivation experiments.

3.2 Neuronal culture plating and maintenance on MEAs

Neuronal networks were plated on microelectrode array (MEA) devices following the protocol established for hiPSC-derived cultures. Polydimethylsiloxane (PDMS) rings (6 mm inner diameter, ≈ 1 mm height; base: curing agent 10:1, cured at 80 °C for 15 min) were used to confine the culture over the active electrode area without generating patterned networks. The day before plating, the rings were positioned on the MEA surface; the

devices were then sterilised (120 °C, 3 h) and coated overnight with poly-L-ornithine (50 µg/ml, Sigma-Aldrich) and human laminin (20 µg/ml, BioLamina).

At day in vitro (DIV) 0, hiPSC-derived glutamatergic neurons were detached with Accutase (Sigma-Aldrich) and plated on the pre-coated devices at a density of 1200 cells/mm². Rat astrocytes were co-plated to support neuronal survival, growth and functional maturation. During the first week, cultures were maintained in Neurobasal medium (Thermo Fisher Scientific) supplemented with B27 (2%), penicillin/streptomycin (1%), GlutaMAX (1%), human BDNF (10 ng/ml) and NT-3 (10 ng/ml), plus doxycycline (4 µg/ml). Fetal bovine serum (2%) was added at DIV7 to support the astrocytic component, and doxycycline was withdrawn at DIV14. Half of the medium was refreshed every two days until the experimental time point.

Recordings were performed on commercial microelectrode arrays (Multi Channel Systems, MCS, Reutlingen, Germany) consisting of 64 titanium nitride (TiN) electrodes arranged in an 8 × 8 grid on a glass substrate. Each electrode was 30 µm in diameter, with an inter-electrode spacing of 200 µm; 59 electrodes were used for recording and one served as the reference. Signals were acquired with the MEA2100 recording system (MCS) and the MC_Rack software at a sampling rate of 10 kHz. After a 10-min acclimatation outside the incubator, spontaneous activity was recorded under stable conditions (37 °C, 5% CO₂) [45], [51].

4. Experimental protocol

4.1. Oxygen-glucose deprivation

Ischaemia-like injury was reproduced in vitro by oxygen–glucose deprivation (OGD) [39]. Cultures grown on MEA devices were exposed to OGD for 4 h in glucose-free, oxygen-deprived conditions. The MEA was kept on a thermal plate to hold the culture at 37 °C; the hypoxic environment was obtained by sealing a plexiglass box onto the plate and saturating it with nitrogen, injected through a dedicated inlet fitted with a HEPA filter and connected to a nitrogen cylinder via a flexible tube. A Petri dish filled with sterile water was placed inside the box to preserve humidity. After the insult and the reperfusion period of 1 hour (simulated by restoring the standard glucose-containing medium and incubation conditions) the cell cultures underwent electrical stimulation and after 2 hours of stabilization the experimental endpoints have been extracted.

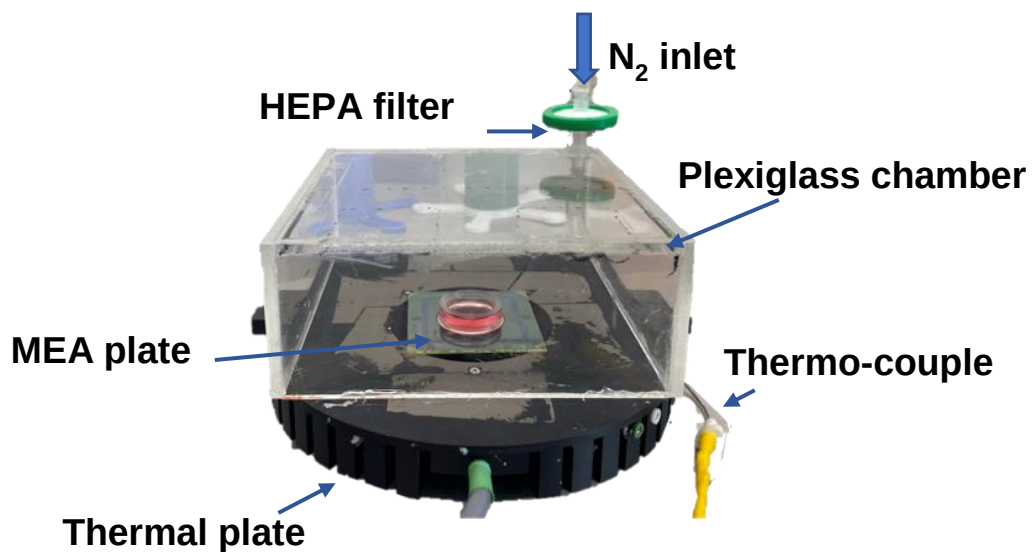


Figure 28. OGD hypoxic chamber used in the study.

4.2. Electrical stimulation

Electrical stimulation was delivered through the MEA to induce a long-term depression (LTD)-like reduction of network activity. Before the insult, spontaneous activity was recorded to establish baseline firing and bursting; electrodes showing stable spontaneous

spiking and bursting were selected as stimulation sites, ensuring their functional engagement within the network. LTD was induced with biphasic (240 μ s was the duration for each phase), charge-balanced pulses delivered at 2 V, 1 Hz, for 15 min through all the 60 electrodes [51]. Spontaneous activity was recorded again after stimulation to quantify changes in firing rate, bursting and overall network dynamics. Unstimulated cultures maintained under identical conditions served as controls.

4.3. Experimental design

The experiment was carried out in three independent sessions, on separate days and starting from distinct cell preparations. Each session included plates of both conditions, following a randomised block design that allows the between-session variability to be estimated and removed without confounding it with the treatment effect. The composition of the sessions by which we extracted immunofluorescence data is reported in Table 1.

Session	CTR (OGD)	TRT (OGD + ES)	Total plates
Day 1	2	2	4
Day 2	2	1	3
Day 3	2	3	5
Total	6	6	12

Table 1. Composition of the immunofluorescence experimental sessions. Each session contains both conditions; replication within session is unbalanced.

Composition of experimental session for electrophysiological data is instead described in the Table 2.

Session	CTR (OGD)	TRT (OGD + ES)	Total plates
Day 1	1	1	2
Day 2	2	2	4
Day 3	2	2	4
Total	5	5	10

Table 2. Composition of MEAs recordings experimental sessions. Each session contains both conditions.

5. Immunofluorescence imaging

Apoptotic cell death was assessed by fluorescence imaging of activated caspase 3/7. After OGD and the respective treatment, MEAs were incubated with the CellEvent Caspase-3/7 Detection Reagent (Thermo Fisher Scientific), used at a 1:100 dilution from the stock with an incubation period of 45 min; the reagent is a fluorogenic substrate that becomes fluorescent upon cleavage by activated caspase 3/7, selectively labelling apoptotic cells. Cell nuclei were counterstained with DAPI.

Images were acquired on an Olympus FV4000 laser-scanning confocal microscope with a UPLXAPO 4× dry objective (NA 0.16, working distance 13 mm), as 16-bit, 2048 × 2048-pixel single-plane images (pixel size 1.554 μm; field of view $\approx 3.18 \times 3.18$ mm), using a Galvano scanner, a 143 μm pinhole and 4-frame Kalman averaging. The caspase channel (Orange dye; 594 nm excitation, ≈ 619 nm emission) and the DAPI channel were acquired sequentially to minimise spectral crosstalk. Acquisition parameters were kept constant across sessions, so that intensity measurements remain comparable between days.

6. Data analysis pipeline

The data analysis pursued two complementary readouts of the response to the ischaemic insult: an imaging readout, quantifying the amount, the spatial organisation and the intensity of cell death, and an electrophysiological readout, quantifying the functional state of the neuronal network. The results were then compared between the control (CTR, OGD) and the treated (TRT, OGD + electrical stimulation) groups. Two principles are shared by both readouts and motivate many of the choices described below: the experimental unit is the single MEA culture, so every quantity is reduced to one value per culture; and, since the three recording sessions differ in culture age and preparation batch, each session is treated as a block, so that batch-related variability cannot be confounded with the treatment effect.

The fluorescence measurements were analysed along the following pipeline (Section 7):

- **DAPI channel – nuclear segmentation:** nuclei were segmented with the Cellpose-SAM deep-learning model, which separates touching nuclei robustly and automatically, avoiding the arbitrariness of a manual threshold on a densely packed channel (Section 7.1).

- **Caspase-3/7 channel – apoptotic-foci detection:** the caspase signal was reduced to objective apoptotic foci by intensity thresholding followed by an Analyze Particles step: the thresholding separates the genuine signal from the background, while the particle analysis, constrained in object size and circularity, keeps only plausible foci, discarding noise (Section 7.1).
- **Cell-death characterization:** three complementary descriptors were derived from these objects (Sections 7.2-7.3): the apoptotic fraction (how many cells die), as the ratio of apoptotic foci to nuclei; the spatial organisation of the dying cells, quantified with the Clark–Evans index, normalized for density and edge effects so that a mere change in the number of dying cells does not appear as a change in their arrangement; and the per-cell caspase intensity (how strongly are each dying cell signals), measured within a perinuclear virtual soma rather than over the whole image, so that signal strength is not confounded with the number of cells that die.
- **Correlation analysis:** the caspase intensity was further tested for a monotonic dependence on the number of cells in culture and on the distance between apoptotic foci, by means of the Spearman correlation coefficient (Section 6.3).

The electrophysiological measurements were analysed through the following pipeline (Section 7):

- **Raw signal – spike detection:** the raw MEA signals were band-pass filtered and spikes were detected with the PTSD algorithm, which preserves the precise timing of each event.
- **Burst and network-burst detection:** spikes were organised into bursts and network bursts through the logarithmic inter-spike-interval and inter-burst-event-interval distributions.
- **Feature extraction:** eight network features were extracted for each culture, both before (Pre) and after (Post) the insult (Section 8.1; Table 3).
- **Normalization:** the features were first log-transformed, because firing and bursting metrics are approximately log-normal and the logarithm makes the feature variation between the Pre and Post conditions symmetric and comparable; they were then standardized feature by feature (z-score), because the eight metrics differ widely in

scale and variability and, without this step, the most variable ones would dominate any multivariate measure.

- **Multivariate analysis:** instead of testing the eight features one by one, the whole Pre→Post change was summarised by a single multivariate distance, the displacement. This aggregation avoids the loss of power entailed by correcting many separate tests, integrates evidence that is individually weak but coherent across features, and remains stable in the present regime, in which the number of features approaches the number of cultures and classical multivariate tools (MANOVA, Mahalanobis distance) would be ill-conditioned or not estimable.

All between-group comparisons, whether on a single feature, on an imaging descriptor, or on the multivariate displacement, were assessed with the same exact, session-stratified permutation test (Section 9), which is valid without assumptions on the distribution of the data and, by permuting the condition labels only within sessions, respects the block structure of the design. When the eight features were examined individually, the p-values were additionally corrected for multiple comparisons with the Benjamini–Hochberg false-discovery-rate procedure, whereas the displacement, being a single test, required no such correction. The software used at each step is reported in Section 10.

7. Image analysis

7.1. Segmentation and pre-processing

Image analysis combined an assisted segmentation stage in Fiji/ImageJ with a set of custom Python scripts. The nuclei were segmented from the DAPI channel with the Cellpose deep-learning model [61], which returns one region of interest (ROI) per cell. The caspase-3/7 channel was instead processed in Fiji to isolate the apoptotic foci: starting from the raw image, an intensity threshold set to the mean plus three standard deviations ($\mu + 3\sigma$) turns it into a binary mask that separates signal from background; the `Analyze Particles` command is then run on this mask, keeping the objects between 20 and 300 μm^2 with circularity between 0-1, with the `include holes` option active, so that every caspase-positive region is retained as a focus; finally `Clear Outside` removes everything lying outside those ROIs, leaving a clean image with the apoptotic signal only. The nuclei ROIs

and the apoptotic-focus ROIs are exported as ImageJ ROI sets and read back into Python through the `roi_read` function of the `roifile` library, where all the quantification takes place.

7.2. Apoptotic fraction and spatial spreading

The extent and the spatial organisation of cell death are quantified by the `apoptosis.py` script, which receives three inputs per plate: the caspase image, the ROI set of the nuclei and the ROI set of the apoptotic foci.

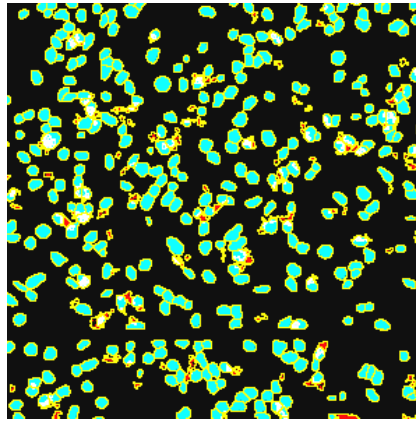


Figure 29. DAPI and Caspase 3/7 signals merged, ROIs boundaries highlighted in yellow.

The primary readout is the apoptotic fraction of the plate, defined simply as the number of apoptotic foci over the number of nuclei,

$$\% \text{apoptosis} = \frac{N_{\text{apop}}}{N_{\text{DAPI}}} \times 100$$

The spatial arrangement of the dying cells is then summarised by the Clark-Evans aggregation index [62], computed by the `clark_evans` function on the focus centroids (converted to micrometres through the pixel size, 1.5537 μm).

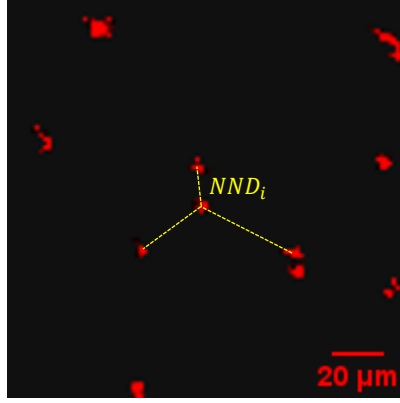


Figure 30. *NND measure visualization.*

The index contrasts the observed mean distance between each focus and its nearest neighbour with the distance one would expect if the same foci were scattered at random over the field,

$$R = \frac{NND_{obs}}{NND_{exp}}, \quad NND_{obs} = \frac{1}{N_{apop}} \sum_{i=1}^{N_{apop}} NND_i$$

Since points close to the image border have, on average, fewer nearby neighbours, the expected distance is corrected for edge effects following Donnelly correction [67],

$$NND_{exp} = 0.5 \sqrt{\frac{A}{N_{apop}}} + \left(0.0514 + \frac{0.041}{\sqrt{N_{apop}}} \right) \frac{P}{N_{apop}}$$

where A and P are the area and the perimeter of the image field and N_{apop} the number of foci. An index R below one denotes clustering, a value around one a random layout, and above one a regular dispersion. This density normalisation is what makes the measure meaningful in a treatment study: the raw nearest-neighbour distance grows on its own as the foci become fewer, so a treatment that merely reduced the number of dying cells would spuriously appear to disperse them; dividing by the density-matched expectation removes this artefact and isolates the genuine change in clustering.

7.3 Caspase-intensity analysis

The intensity of the caspase signal is analysed separately, by the `caspases_intensity.py` script, and is deliberately measured around each nucleus rather than over the whole image, because a whole-image average would confound the strength of the signal with the number of cells that are dying. For every nucleus the `analyze_image.py` function draws a perinuclear disc of 12 μm radius around the DAPI centroid, a “virtual soma” and averages the caspase intensity over the signal (non-zero set of pixels S_i) pixels inside it; as the images are already background-subtracted, a nucleus is classified as apoptotic when its disc contains any signal. Only the apoptotic nuclei enter the measurement, so that the quantity reflects the intensity per dying cell, independently of how many cells die.

$$I_i = \frac{1}{|S_i|} \sum_{p \in S_i} \text{Intensity}(p)$$
$$I_{\text{global}} = \frac{1}{N_{\text{apop}}} \sum_{i=1}^{N_{\text{apop}}} I_i$$

Because the perinuclear disks have a 12 μm radius, two nuclei closer than about 20 μm share pixels, so the caspase intensity attributed to a cell can depend on how densely the dying cells are packed; out-of-focus light in dense regions may contribute in the same direction.

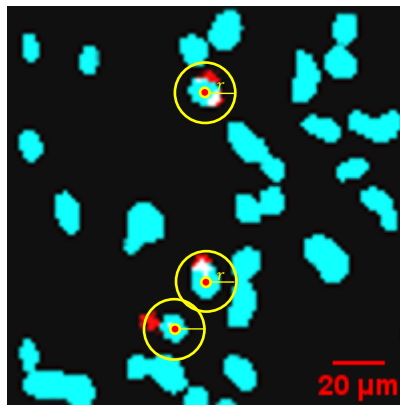


Figure 31. Illustration of single-cell caspase 3/7 intensity measure.

Monotonic dependence between two per-cell or per-plate variables was quantified with the Spearman rank correlation coefficient ρ , computed with `scipy.stats.spearmanr`

Spearman's ρ is the Pearson correlation applied to the ranks of the data rather than to their raw values; it therefore measures whether one variable tends to increase (or decrease) with the other, without assuming a linear relationship or a specific distribution, and is robust to outliers. Denoting by d_i the difference between the ranks of the two variables for the i -th observation and by n the number of observations, in the absence of ties ρ is given by:

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}$$

with ρ ranging from -1 (perfect monotonic decrease) through 0 (no monotonic association) to $+1$ (perfect monotonic increase). Two dependencies were assessed. First, the per-nucleus caspase intensity was correlated with the distance to the nearest apoptotic nucleus (NND) to check for a distance-dependent optical bias. Second, the per-plate mean intensity was correlated with the total number of DAPI nuclei per plate, used as a proxy for culture density, to test whether cell confluency affected the measured signal through altered penetration and dosing of the fluorogenic reagent; this second correlation was computed on all plates and, separately, within the CTR and TRT groups.

8. Electrophysiological data

Data analyses were performed using in-house code developed in MATLAB (The MathWorks, Natick, MA, USA) and SpyCode [48]. Briefly, data were filtered with passband 100 Hz (2^o order) – 3.5 kHz (4^o order) Butterworth filter. The spike detection was performed with the precision time spike detection (PTSD) algorithm. The noise threshold for individual spike detection was set equal to $\mu + 10 \sigma$, where μ and σ are the mean and the standard deviation of the signal, respectively. Each electrode was considered active (AE) if its firing rate was higher than 0.1 spikes/s (spk/s). The network mean firing rate (MFR, i.e., the number of spikes in the unit time) was computed by averaging the firing rates of each active channel.

Bursts were detected based on the distribution of logarithmic neuronal inter-spike interval (ISI) setting minimum number of spikes belonging to the event equal to 5 and a bursting rate threshold equal to 0.4 bursts/min. Network burst detection was computed with the distribution of the inter-burst-event interval, setting 20% as the minimum number of electrodes participating to the network event. We computed the network bursting

rate (NBR), i.e., the number of network events per minute) and the network burst duration (NBD). Finally, we computed the instantaneous firing rate profile by considering temporal windows of 10 ms and counting the number of spikes occurring in each bin in the whole network. We averaged the instantaneous firing rate profile across all detected network bursts, obtaining the spike time histogram (STH) profile, from which we extracted the peak amplitude (STH in spikes/bin), and by exponential fitting of STH profile rise/decay phases the time constants τ_{rise} , τ_{decay} .

All metrics were extracted for each culture, with values computed for the "Pre" and "Post" phases.

8.1. Network Features Analysis

For the electrophysiological data the experimental unit was, as above, the single MEA plate. Eight features describing network activity were extracted for each plate before (Pre) and after (Post) the ischaemic protocol: the number of active electrodes (AE), the mean firing rate (MFR), the fraction of random spikes occurring outside network bursts (RS), the network burst rate (NBR) and network burst duration (NBD), the peak amplitude of the network spike-time histogram (STH amplitude), and the rise and decay time constants of the network burst profile (Rise Tau and Decay Tau). Data were available for five control plates (CTR, OGD) and five treated plates (TRT, OGD + electrical stimulation) recorded across the three independent experimental sessions.

Feature	Definition	Unit	Family
AE	number of active electrodes (firing rate > 0.1 spk/s)	%	spiking
MFR	mean firing rate averaged over active electrodes	spikes/s	spiking
RS	percentage of random spikes (spikes outside bursts)	%	spiking
NBR	network burst rate	bursts/min	bursting
NBD	network burst duration	s	bursting
STH amplitude	peak of the network-burst-averaged firing profile	spikes/bin	bursting

Rise τ	time constant of the exponential fit to the STH rising phase	ms	bursting
Decay τ	time constant of the exponential fit to the STH decaying phase	ms	bursting

Table 3. Network features extracted from the MEA recordings, grouped into spiking/activity and bursting-dynamics families.

These eight features describe two complementary aspects of network behaviour. The spiking/activity features quantify how much and how widely the network fires: AE measures the spatial extent of the activity (how many sites are recruited), MFR the average firing intensity of the active sites, and RS the fraction of spikes occurring outside organized bursts, i.e. the asynchronous background activity. A reduction of AE or MFR after the insult therefore indicates a loss of active sites or of firing intensity, whereas an increase of RS reflects a shift towards a less organized, more random firing.

The bursting-dynamics features describe how the network organizes its synchronous events: NBR measures how frequently network bursts occur and NBD how long each event lasts, the STH amplitude captures the peak intensity of the averaged burst, and the Rise and Decay time constants describe its temporal shape, i.e. how quickly the burst builds up and subsides. Shorter or less frequent bursts, a lower STH amplitude or a faster decay thus denote a weakening or acceleration of the synchronous regime, whereas the opposite changes denote its strengthening.

Because the working hypothesis was that electrical stimulation keeps the network close to its own baseline rather than driving it in a particular direction, the outcome was the magnitude of the Pre→Post departure, i.e. a per-plate, per-feature dissimilarity. The dissimilarity measure used was the base-2 log fold-change, which is symmetric and stable on ratio-scaled data.

$$D = \log_2 \left(\frac{\text{Post}}{\text{Pre}} \right)$$

From definition, smaller value denotes a state closer to baseline, so the hypothesis of a stimulation-induced normalization corresponds to smaller dissimilarities in TRT than in CTR.

Each feature was compared between conditions with the same session-stratified permutation test described below, applied here to the dissimilarity and evaluated one-sided in the pre-specified direction (H_1 : TRT < CTR). The statistic was the CTR – TRT difference averaged within each session and then across sessions, and the null distribution was obtained by exact enumeration of all relabelling of the condition within each session (72 permutations), so the reported p-values are exact. Because eight features were tested, p-values were additionally adjusted for multiple comparisons using the Benjamini–Hochberg false-discovery-rate procedure, and both raw and adjusted values are reported. No plate was excluded: the logarithmic transform already compresses extreme ratios and removing a highly dissimilar plate would have biased the comparison in favour of the hypothesis.

To assess whether the global network state was preserved, each plate was represented as a point in an eight-dimensional feature space. Each log-transformed feature was standardized (z-score) using the mean and standard deviation computed jointly over all plate and time-point values, so that no feature dominated the distance because of its scale. For every plate the Euclidean distance between its standardized Post and Pre vectors summarized the overall Pre→Post displacement:

,

$$d_i = \sqrt{\sum_j \left(z_j(\text{Post}_i) - z_j(\text{Pre}_i) \right)^2}$$

computed in logarithmic space (equivalent to the root-sum-of-squares of the standard-deviation-scaled $\log_2$ fold-changes). The per-plate distances were compared between conditions with the one-sided session-stratified permutation test. This single multivariate test summarizes the whole feature set in one comparison and therefore does not incur a multiple-comparison penalty.

Two properties make this measure reasonable. First, because the same per-feature mean and standard deviation are used for the Pre and the Post state, the mean cancels in the difference and each feature contributes to the distance exactly as its log fold-change scaled by its own variability, $\log_2(\text{Post}_j/\text{Pre}_j)/\sigma_j$; the standardization therefore only balances the features and does not displace the states. Second, the Euclidean (L2) norm expresses the overall change as the root-sum-of-squares of these standardized per-feature changes, so

that features moving in the same direction add up coherently; Equivalently, the displacement is a diagonal Mahalanobis distance: each feature is scaled by its own variability, while inter-feature correlations are not modelled. This is a deliberate choice not an assumption of independence, since some features are correlated (e.g. NBD and NBR) made because the full covariance matrix is ill-conditioned with number of samples comparable to the number of features; correlated features therefore contribute somewhat redundantly, a limitation accepted in exchange for stability and order-invariance.

The overall analytical strategy follows established practice. The base-2 logarithmic transformation is motivated by the approximately log-normal distribution of neuronal firing and bursting metrics, which makes the log fold-changes approximately symmetric and stabilizes their variance. The use of exact, distribution-free permutation tests rather than parametric tests, whose asymptotic normality assumptions are not warranted at this sample size follows the classical randomisation principle [63], while the restriction of the relabelling to within-session blocks implements the exchangeability-block formulation of permutation inference. Finally, summarising the eight features into a single standardized Euclidean distance and assessing it by permutation places the multivariate analysis within the distance-based PERMANOVA framework; this is appropriate precisely when the number of variables approaches or exceeds the number of samples, a regime in which classical MANOVA and principal-component analysis are unstable or not estimable. The idea of representing population activity as a vector in a multidimensional space, and of reasoning geometrically about it, dates back to the population-vector framework [64] and underlies modern neural state-space analyses; the displacement used here applies the same geometric intuition of distance between states to network-level MEA features rather than to single-unit firing, and to a Pre→Post comparison rather than to stimulus decoding. To rule out a regression-to-the-mean artefact, the baseline (Pre) values were compared between conditions for each feature with a two-sided session-stratified permutation test; no significant baseline difference was detected, confirming that the two groups started from comparable states.

It should be noted that the limited number of cultures constrains the statistical power of the analysis: the feature-by-feature tests are therefore regarded as exploratory, and the primary inference is the aggregated multivariate comparison.

9. Statistical analysis

All comparisons between the control and the treated condition rest on the same principle. The experimental unit is the MEA plate, so the cell-level values are first aggregated to a single value per plate. Because the plates were prepared over three independent sessions and each session contains both conditions (Table 1, Table 2), the comparison uses a stratified permutation test [63], [65], [66], that treats the experimental day as a stratum.

The test is implemented in `stratified_permutation_test`, and its statistic computed by `stat_effect` is the difference between the two conditions averaged within each day and then across days,

$$T_{\text{obs}} = \frac{1}{G} \sum_{g=1}^G (\bar{x}_g^{\text{CTR}} - \bar{x}_g^{\text{TRT}})$$

where G is the number of days; subtracting within the block removes the session effect, exactly as a blocking factor would. Under the null hypothesis the condition label is exchangeable among the plates of the same day, so the null distribution is built by reshuffling the CTR/TRT labels within each day while keeping the per-day group sizes fixed.

The validity of the procedure rests on a single assumption, exchangeability: under the null hypothesis the condition labels of the cultures recorded in the same session are interchangeable, which the within-session permutation scheme guarantees by construction. Because all admissible relabellings are enumerated, the resulting p-value is exact and makes no assumption on the shape of the underlying distributions, a property that is particularly valuable at the present sample size, where the asymptotic approximations underlying parametric tests, of which the permutation test is the exact counterpart, are not warranted.

$$H_0: \frac{1}{G} \sum_{g=1}^G (\mu_g^{\text{TRT}} - \mu_g^{\text{CTR}}) = 0$$

The obtained p-value is the fraction of permutations whose statistic is at least as extreme as the observed one,

$$p = \frac{\#\{k: T_k^* \geq T_{\text{obs}}\}}{N}$$

$$p = \frac{\#\{k: |T_k^*| \geq |T_{\text{obs}}|\}}{N}$$

The significance threshold was 0.05 for both two-sided and one-sided tests.

10. Software and computational environment

The analysis relied on three environments, each dedicated to a specific part of the workflow. The electrophysiological processing: band-pass filtering, PTSD spike detection, burst and network-burst detection, and extraction of the eight network features was carried out in MATLAB (The MathWorks, Natick, MA, USA) with the SpyCode package. The caspase-channel pre-processing (thresholding and the Analyze Particles step) and the export of the nuclei and apoptotic-foci ROI sets were performed in Fiji/ImageJ, while the nuclei of the DAPI channel were segmented with the Cellpose-SAM deep-learning model. All the subsequent image quantification and statistical analyses were implemented in Python (version 3.11).

On the imaging side, the confocal images were read into Python with tifffile and the ImageJ ROI sets of the nuclei and of the apoptotic foci were imported with roifile; the regions of interest were then represented as geometric polygons through shapely, whose validity routines were used to repair malformed contours, and rasterised into pixel masks with Pillow (PIL). The perinuclear “virtual soma” disc used for the per-cell caspase-intensity measure was drawn with scikit-image, and the nearest-neighbour distances underlying the Clark–Evans index, together with the other distance computations, were obtained with the spatial-distance routines of SciPy; the Spearman rank correlation was likewise computed with SciPy.

On the electrophysiological side, the extracted feature tables were read from the spreadsheet with openpyxl, and all the numerical operations the apoptotic fraction, the per-cell caspase intensity, the logarithmic and z-score transforms of the features and the Euclidean displacement were carried out with NumPy, while pandas was used to organise and aggregate the per-cell and per-plate data. The exact, session-stratified permutation test was implemented in-house with NumPy, using the standard-library itertools and math

modules to enumerate the admissible relabellings. Finally, all the figures were produced with Matplotlib and Seaborn.

Chapter 4

Results

The results presented in this chapter focus on the possibility of preserving cellular viability after an ischaemic insult simulated with the OGD protocol. Glutamatergic neurons, obtained by differentiation of hiPSCs and cultured on MEAs, were exposed to oxygen–glucose deprivation in an anoxic chamber with glucose-free medium at controlled temperature for 4 hours; reperfusion was then simulated by 1.5 h of incubation under standard culture conditions. The TRT samples subsequently received low-frequency electrical stimulation for 15 minutes. The experimental endpoints were assessed after a further resting period of 2 h, required for the synaptic rearrangement and for incubation with the fluorescent markers. The procedure was repeated across three independent experimental sessions. The main endpoints are the apoptotic fraction, the spatial distribution of cell death and the overall network electrophysiological activity described by multivariate features analysis; the caspase signal intensity and the singular electrophysiological features are reported as secondary and less robust measure but useful for the discussion section.

11. Cell death

To determine whether activity-dependent network depression promotes neuronal rescue after ischemic-like injury, human iPSC-derived neuronal cultures were exposed to OGD and subsequently subjected to patterned electrical stimulation through the MEA. Apoptotic cell death was assessed by fluorescence imaging of activated caspase 3/7, while DAPI staining was used to identify cell nuclei. Representative images showed a marked reduction in caspase-3/7-positive signal in electrically stimulated cultures compared with OGD controls.

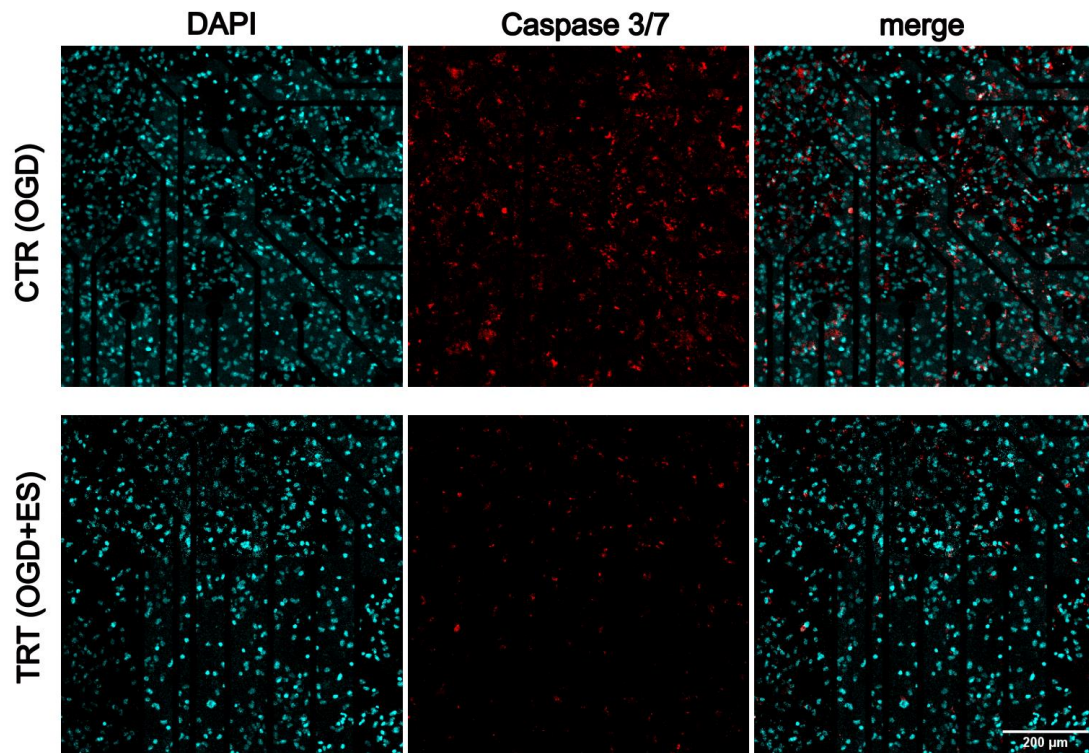


Figure 32. CTR and TRT individual signals, then merged.

Quantification of caspase-3/7-positive cells revealed that post-OGD electrical stimulation significantly decreased the percentage of apoptotic cells. OGD control cultures displayed a mean apoptotic fraction of approximately $7.4 \pm 3.6\%$, whereas cultures receiving electrical stimulation after OGD showed a reduced apoptotic fraction of approximately $2.4 \pm 1.2\%$ (Fig. 33; $*p = 0.017$). Thus, MEA stimulation produced an approximately threefold reduction in apoptosis after the ischemic-like insult had occurred.

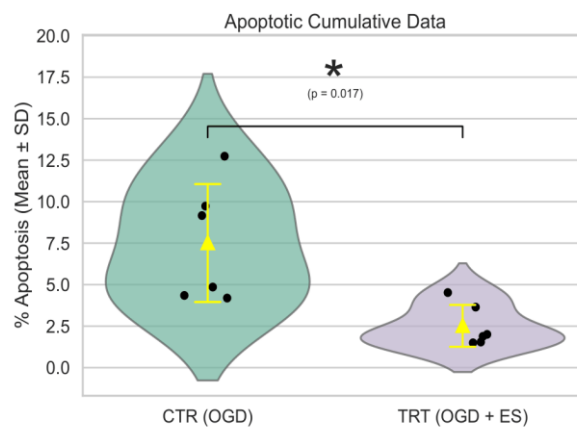


Figure 33. Results plot for apoptotic fraction

12. Spatial distribution of apoptosis

We next examined the spatial distribution of caspase-3/7-positive cells by Clark-Evans aggregation index. The mean of the R index between apoptotic cells decreased from approximately 0.84 ± 0.032 in OGD controls to 0.77 ± 0.068 in electrically stimulated cultures (Fig. 34; * $p = 0.044$). The lower aggregation index indicates that the apoptotic cells remaining after stimulation were more tightly clustered relative to a random distribution at the same density. Together with the reduced overall apoptotic fraction (Section 9), this shows that electrical stimulation lowers the total amount of cell death while the residual apoptotic events remain spatially concentrated.

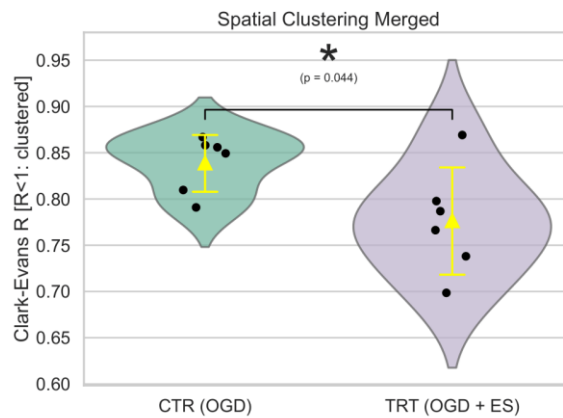


Figure 34. Results plot for spatial distribution of apoptosis.

13. Caspase 3/7 fluorescence intensity

To further characterise the apoptotic phenomenon, we analysed the intensity of the caspase 3/7 fluorescent signal, restricted to the apoptotic nuclei so that it reflects the average signal of a dying cell rather than the amount of cell death. As this intensity is not a direct measure of caspase 3/7 concentration and is sensitive to technical factors, it is treated as a complementary and less robust endpoint, used to support the conclusions discussed in the following sections. The mean per-cell intensity was higher in the treated cultures than in the OGD controls (* $p = 0.033$).

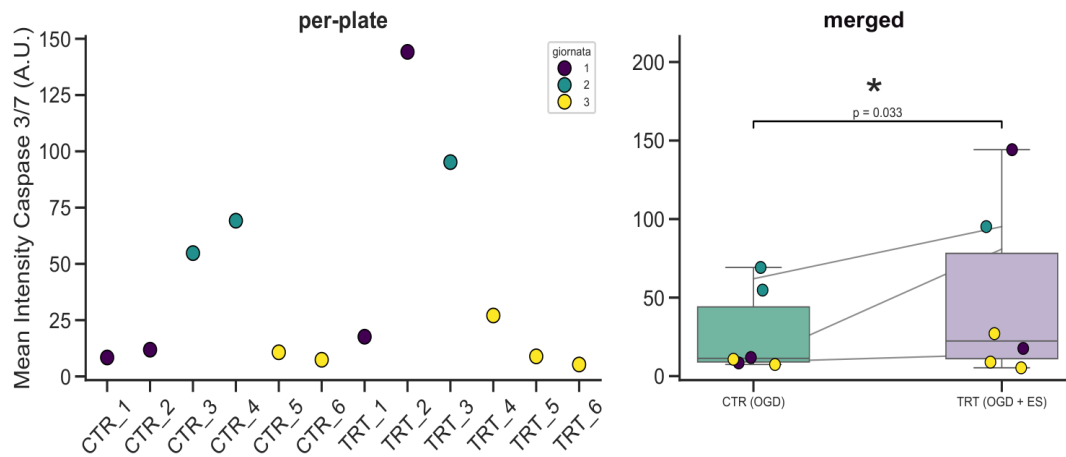


Figure 35. On the left average intensities per plate, on right global results plot for caspase 3/7 average intensity.

This difference was not attributable to the closer spatial packing of apoptotic cells in the treated group: the per-nucleus intensity showed no dependence on the distance to the nearest apoptotic nucleus (Spearman $\rho = -0.04$).

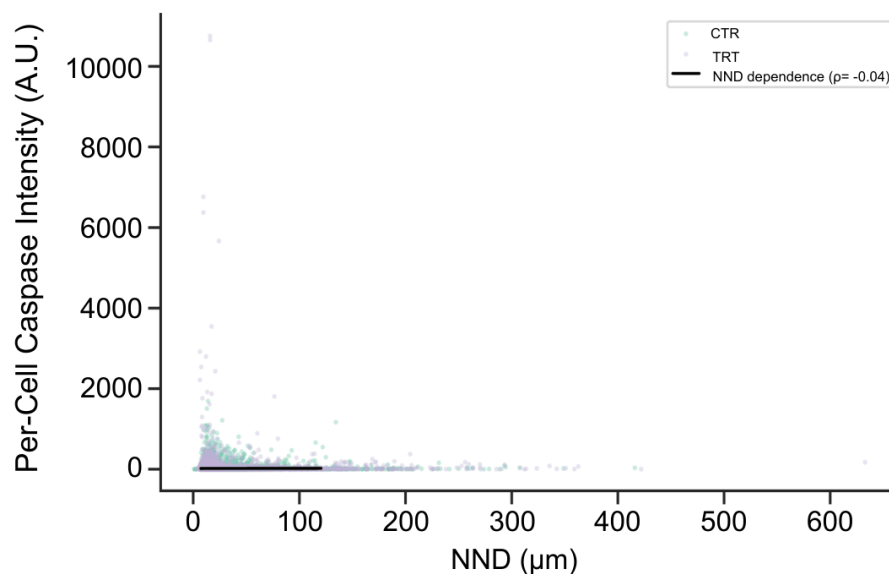


Figure 36. Scatter plot of the Spearman correlation analysis for mean cellular distance/ fluorescence intensity dependency.

We then examined whether the intensity was affected by the overall cell density, which can alter the penetration and effective dosing of the fluorogenic reagent. The per-plate mean intensity was negatively correlated with the number of nuclei per plate (Spearman $\rho = -0.54$, $p = 0.071$), a trend of consistent sign within both the CTR and TRT groups ($\rho = -0.54$ each). This indicates that part of the measured intensity reflects a density-dependent technical effect, further supporting a cautious, complementary interpretation of this endpoint

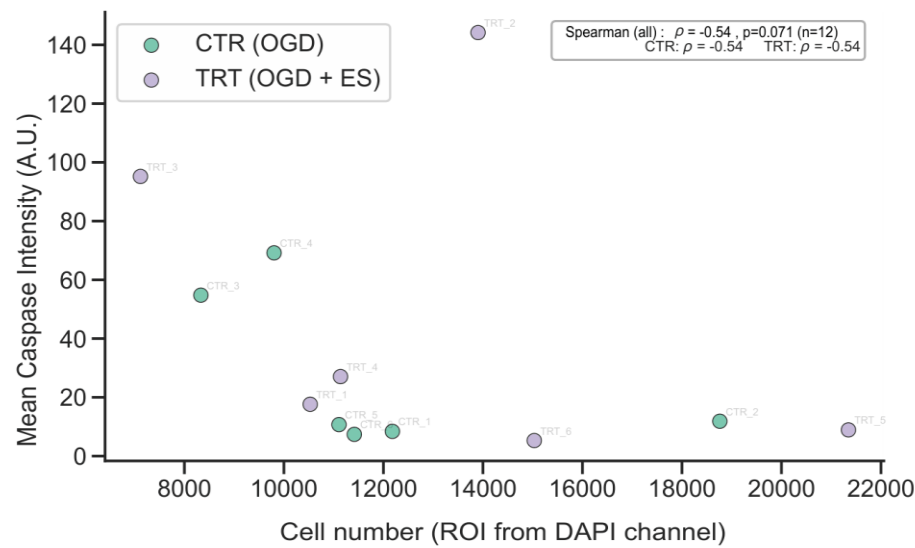


Figure 37. Spearman correlation analysis for cell-number/ fluorescence intensity dependency.

14. Electrophysiological features analysis

Raw features extracted from network activity exhibited high variance and several outliers (Fig. 38). Expressing their variation from baseline as base-2 log fold-changes yielded more symmetrical and compact distributions (Fig. 39).

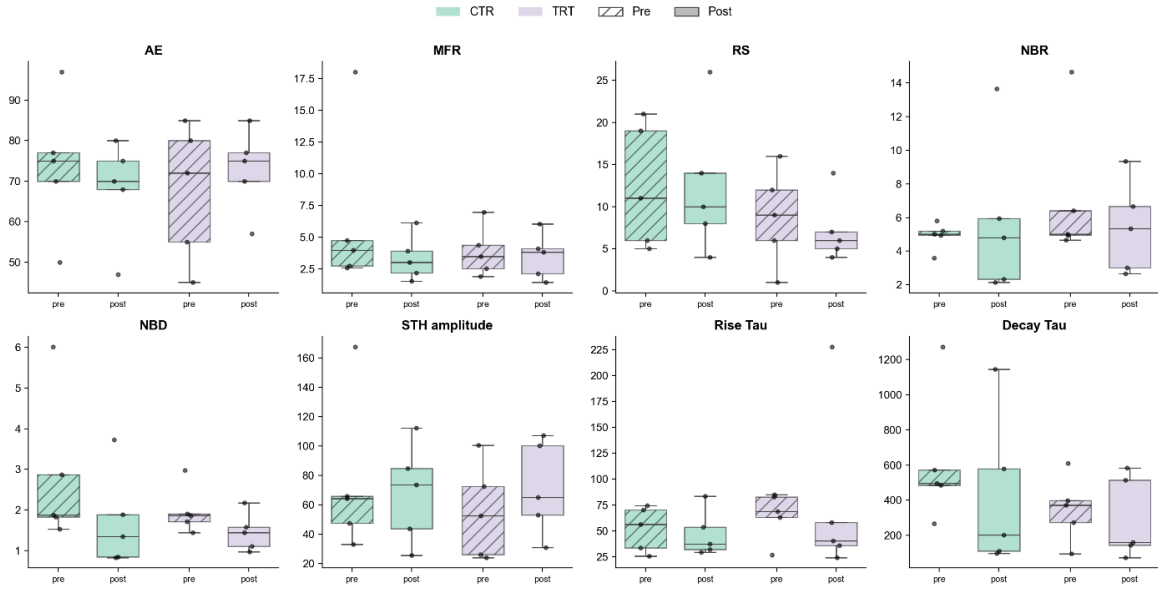


Figure 38. Raw network features.

When evaluated individually on these transformed features, univariate comparisons between the CTR and TRT groups did not reach statistical significance after multiple-testing correction, highlighting the need for a larger sample size to resolve single-parameter differences.

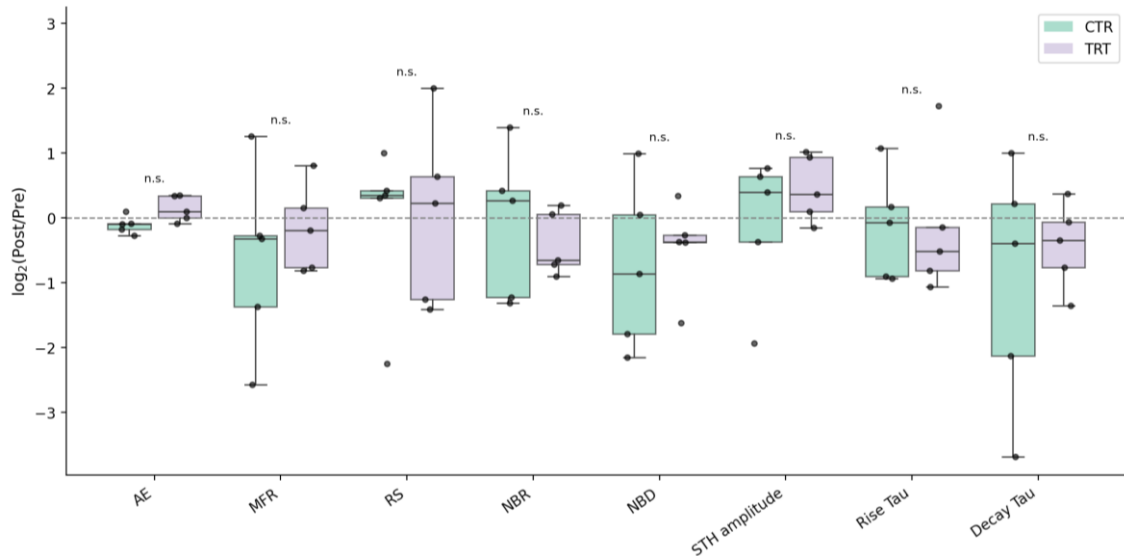


Figure 39. Network features expressed as log fold-change.

Conversely, the multivariate analysis which condenses the information of all eight features into a single overall Pre-to-Post displacement vector length for each plate revealed a statistically significant difference between groups, showing a marked reduction in the absolute Pre-to-Post distance in the TRT group compared with the CTR group (* $p = 0.042$, Fig. 40). Despite the limited number of samples, this metric indicates that electrical stimulation promotes a global stabilization of network activity around its pre-insult baseline, supporting the hypothesis of a neuroprotective effect induced by the LTD stimulation protocol.

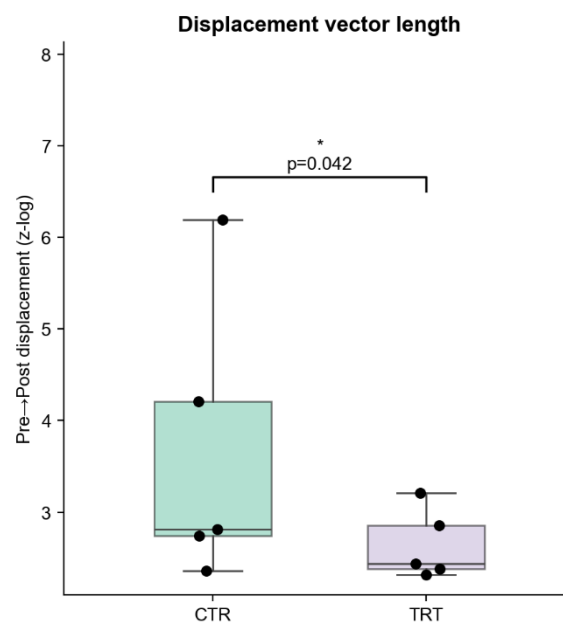


Figure 40. Results for the displacement vector, global distance between Pre and Post conditions.

Chapter 5

Discussion

15. Interpretation of the results

Taken together, the immunofluorescence and electrophysiological findings indicate promising potential for treating ischemic-like neuronal injury by electrically inducing synaptic neuroprotection. The apoptotic fraction measurements demonstrate a clear protective effect (approximately a threefold reduction in cell death), which could be further characterized by exploring longer OGD exposures. Furthermore, the spatial distribution analysis (Clark-Evans index) indicates a confinement of apoptotic spreading across the network: because the residual apoptotic cells in the TRT group exhibit a lower R value (higher clustering), cell death remains restricted to localized foci rather than propagating throughout the culture. In particular, by inspecting the fluorescence images merged with the inverted brightfield channel (Fig. 41), it is possible to visually appreciate a marked reduction of apoptotic signals in proximity of the microelectrodes sites.

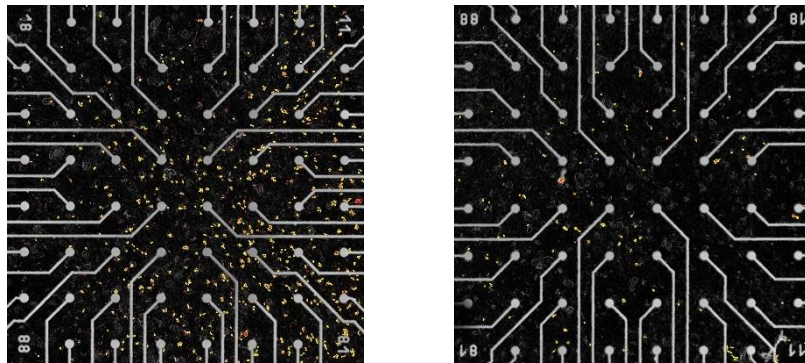


Figure 41. Caspase 3/7 ROIs highlighted on inverse of brightfield channel (CTR on the left).

Caspase 3/7 average fluorescence intensity, presented as a secondary measure, was analysed to discuss both biological and technical hypotheses. The differences between the intensities of the two groups could rely on distinct mechanisms. The first mechanism investigated was whether per-cell intensity could be influenced by cellular superimposition; by performing a Spearman correlation to verify a monotonic dependence

between single-cell caspase 3/7 intensity and nearest-neighbour distance (NND), we obtained virtually zero dependence between these two variables ($\rho = -0.04$, Figure 36). Instead, we observed a clear negative correlation trend between the number of cells in the culture and the mean intensity of the caspase signal ($\rho = -0.54$, Fig. 37); this interpretation is consistent with the presence of outliers, since some samples presented significant differences in cellular density. The second hypothesis to interpret this result is that cultures with reduced apoptosis allow the fluorescent probes to concentrate within the few cells in an apoptotic condition (such as sample TRT_2, Fig. 35 and Fig. 37). A last speculative hypothesis on this result is that, by inducing synaptic depression, impaired cells cannot diffuse the damage to the network, consequently undergoing a more localized and intense apoptotic death.

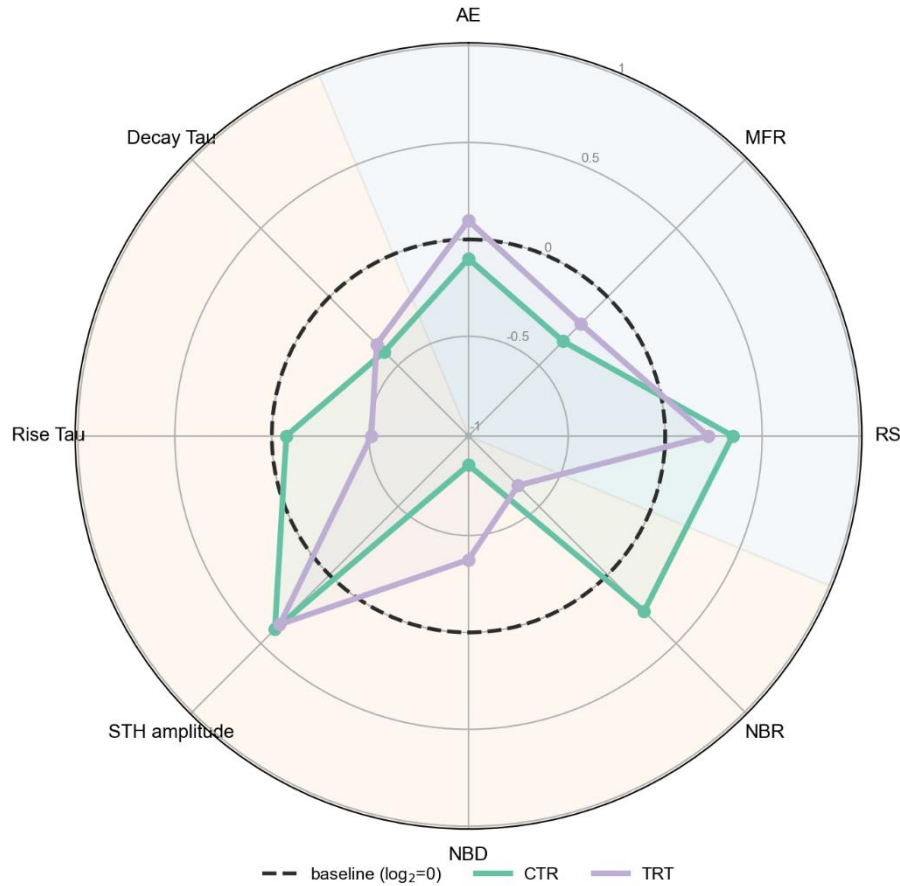


Figure 42. Radar plot of the features expressed as log fold-change with respect to the basal condition (Pre=Post).

Electrophysiological features offered complementary information to the fluorescence results, strengthening our observations regarding excitotoxicity assessment and the

mitigation of its effects. Looking at the culture behaviour described by the features, parameters such as NBD, NBR, and τ_{decay} show important differences in the network activity of the two groups after the ischaemic-like insult: CTR expresses a wider shift of its activity towards more frequent and chaotic network-bursting dynamics (with an increase in median NBR accompanied by a marked reduction in NBD and τ_{decay}) compared with TRT. The features also capture the general effects of OGD on the cultures: we find a reduced MFR for both groups, an augmented asynchronous activity (increase in RS, more marked in CTR), and an increase in unit recruitment inside the average network burst peak (STH amplitude). Finally, parameters such as AE channels and τ_{rise} leave space for further consideration. AE channels show an opposite trend between the two groups, with a reduction for CTR and an increase for TRT; this opposite behaviour can be interpreted by considering that TRT cultures, having higher cell viability despite the injury, preserve active units, whereas the reduction in CTR is clearly explained by its decreased cellular viability. Instead, τ_{rise} shows a decrease in both conditions; keeping in mind that τ_{rise} values are very short (a few milliseconds), we do not consider this a contradiction to the network-bursting considerations made above, since these variations have a negligible impact on the effective behaviour of the network.

Overall, these considerations are consistent with our biological hypothesis, providing positive feedback on the study while keeping its limitations clearly in mind.

16. Limitations

A primary, intrinsic limitation of this work lies in the use of an in vitro model: while two-dimensional neuronal cultures allow for highly controlled testing of electroceutical protocols without systemic confounding factors, they represent a substantial simplification compared with the cytoarchitectural, vascular, and glial complexity of an in vivo brain. Beyond this general constraint, the limitations of the study can be grouped into two main categories: reproducibility and statistical robustness. Regarding reproducibility, future iterations require a rigorous physical characterization of the custom OGD chamber, specifically monitoring the temporal dynamics of nitrogen saturation and measuring the actual dissolved oxygen tension within the culture medium. A second technical factor requiring tighter control is the standardization of effective cell plating density across preparations, which directly influences fluorophore penetration and per-cell dosing.

Regarding statistical robustness, the main limitation is the relatively small number of independent experimental sessions ($N = 3$, with 12 plates for imaging and 10 for electrophysiology), which limits statistical power for univariate electrophysiological comparisons. Additionally, the experimental design would benefit from the inclusion of a normoxic (non-OGD) sham control group to quantify the absolute baseline-to-insult shift, as well as the exploration of longer OGD durations to amplify the dynamic range of the apoptotic readout.

Chapter 6

Conclusions

This study, conducted on in vitro networks of human iPSC-derived glutamatergic neurons, demonstrates that delivering a low-frequency, LTD-inducing electrical stimulation protocol after an ischemic-like insult exerts a significant neuroprotective effect against the excitotoxic cascade, reducing overall apoptotic cell death by approximately threefold, confining its spatial propagation, and preserving global electrophysiological network dynamics closer to baseline. Immediate future developments of this work will focus on integrating functional and effective connectivity analyses both optically, via immunofluorescence quantification of post-synaptic AMPA receptors and PSD-95 puncta, and through electrophysiological measures, by reconstructing Pre-to-Post connectivity graphs from MEA recordings. Furthermore, biochemical assays such as Lactate Dehydrogenase (LDH) release will be integrated to independently quantify metabolic and necrotic damage following OGD. In a broader perspective, these findings lay the groundwork for validating low-frequency neuromodulation in more complex three-dimensional organoids and in vivo stroke models, where translating targeted electrical depression into a clinically viable intervention represents a hard bioengineering and neurosurgical challenge.

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