**CZECH TECHNICAL UNIVERSITY IN PRAGUE**

**FACULTY OF BIOMEDICAL ENGINEERING**

**Department of Biomedical Technology**

# Cultured neurons as a platform for assessing psychoactive substances

Master thesis

Study programme: Biomedical and Clinical Informatics
Specialization: Nanotechnology

Author of the master thesis: Bc. Kateřina Nevšímalová
Supervisor of the master thesis: Ing. Václav Petrák, Ph.D.

**Kladno, May 2023**

# MASTER'S THESIS ASSIGNMENT

## I. PERSONAL AND STUDY DETAILS

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|---|---|---|---|
| Student's name: | **Nevšímalová Kateřina** | Personal ID number: | **482944** |
| Faculty: | **Faculty of Biomedical Engineering** | | |
| Department: | **dept** | | |
| Study program: | **Biomedicínská a klinická informatika** | | |

## II. MASTER'S THESIS DETAILS

Master's thesis title in English:

**Cultured neurons as a platform for assessing psychoactive substances**

Master's thesis title in Czech:

**Kultivované neurony jako platforma pro hodnocení psychoaktivních látek**

Guidelines:

The goal of this thesis project is to record and analyze the activity of neurns that have been stimulated by psychoactive substances. This will be done by recording the activity of neurons using microelectrode arrays. The following steps will be undertaken: 1) Based on a comprehensive literature review, explain the mechanism of action of various types of psychoactive substances on the nervous system. 2) Record the spontaneous activity of nerve cells and then the activity after stimulation with chosen psychoactive substances. 3) Analyze the recorded signals to determine the basic parameters of neural activity, including the time of spike occurrence, the presence of bursts, and the degree of network synchronization. 4) Quantitatively evaluate the statistical parameters of both spontaneous and stimulated nerve cell activity. Specifically, compare the spontaneous activity of individual cultures and evaluate changes before and after substance stimulation. 5) Assess the suitability of using nerve cells cultured on microelectrode arrays as a model system for studying psychoactive substances.

Bibliography / sources:

[1] Zwartsen A. et al, Hyperthermia exacerbates the acute effects of psychoactive substances on neuronal activity measured using microelectrode arrays (MEAs) in rat primary cortical cultures in vitro, Toxicology and Applied Pharmacology, ročník 397, číslo 115015, 2020
[2] Jones I.A. et al., The potential of microelectrode arrays and microelectronics for biomedical research and diagnostics, Analytical and Bioanalytical Chemistry, ročník 399, číslo 7, 2011

Name of master's thesis supervisor:

**Ing. Václav Petrák, Ph.D.**

Name of master's thesis consultant:

**RNDr. Jan Krůšek, CSc**

Date of master's thesis assignment: **14.02.2023**
Assignment valid until: **20.09.2024**

doc. Ing. Zoltán Szabó Ph.D.
Head of department

prof. MUDr. Jozef Rosina, Ph.D., MBA
Dean

**DECLARATION**

I hereby declare that I have completed this thesis with the topic “Cultured neurons as a platform for assessing psychoactive substances” independently, and that I have attached an exhaustive list of citations of the employed sources.

I do not have a compelling reason against the use of the thesis within the meaning of Section 60 of the Act No. 121/2000 Sb., on copyright, rights related to copyright and amending some laws (Copyright Act).

In Kladno 18.5.2023 Bc. Kateřina Nevšímalová

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**Master Thesis title:**
Cultured neurons as a platform for assessing psychoactive substances


## Abstract:

The aim of this study was to assess the effects of the neurostimulation substances MDMA and 25CN-NBOMe on the nervous system by stimulating a cultivated hippocampal neural line. We investigated whether the effects of these substances are dose-dependent, hence several different concentrations were tested. In vitro microelectrode arrays were used to measure electrical neural signals. Changes in neuronal activity were assessed based on seven electrophysiological parameters such as Mean Firing Rate (MFR), Inter Spike Interval (ISI), and others. After stimulating neurons with 1µM MDMA, we observed a significant change in MBR, which dropped almost to zero, while MFR and network synchronization did not change significantly. We observed a significant inhibitory effect when stimulating with 25CN-NBOMe at concentrations of 3 mg/L and 30 mg/L, at which both MFR and MBR dropped almost to zero. However, it is strange that this significant effect of reducing nerve activity was not caused by the 25CN-NBOMe stimulation at a concentration of 10 mg/L. To confirm these results, it would be necessary to repeat these experiments.



## Key words:

Neuronal activity, microelectrode array, new psychoactive substances

## Abstrakt:

Cílem této práce bylo posouzení vlivu neurostimulačních látek MDMA a 25CN-NBOMe na nervovou soustavu formou stimulace kultivované hipokampální linie neuronů. Zkoumali jsme, zda jsou účinky těchto látek dávkově závislé, proto bylo testováno několik různých koncentrací. Pro měření elektrických neurálních signálů byla použita in vitro mikroelektrodová pole. Změny neuronální aktivity neuronů byly posouzeny na základě sedmi elektrofyziologických parametrů jako např. Mean Firing Rate (MFR), Inter Spike Interval (ISI), a další. Po stimulaci neuronů 1µM MDMA jsme pozorovali významnou změnu v MBR, která klesla téměř na nulu, zatímto MFR a synchronizace sítě se statisticky významně nezměnily. Dále jsme pozorovali významný inhibiční účinek při stimulaci 25CN-NBOMe o koncentracích 3 mg/L a 30 mg/L, při kterých MFR i MBR klesly téměr na nulovou hodnotu. Je však zvláštní, že tento významný účinek redukce nervové aktivity nezpůsobila stimulace 25CN-NBOMe o koncentraci 10 mg/L. Pro potvrzení těchto výsledků by však bylo potřeba tyto experimenty zopakovat.

## Klíčová slova:

Neuronální aktivita, mikroelektrodové pole, nové psychoaktivní látky

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# List of symbols and abbreviations

| Symbol | Meaning |
|---|---|
| MDMA | 3,4-methylenedioxy-N-methylamphetamine |
| 25CN-NBOMe | 2,5-dimethoxy-4-(2-((2 methoxybenzyl)amino)ethyl)benzonitrile |
| MEA | Microelectrode array |
| NPS | New psychoactive substances |
| AP | Action Potential |
| RMP | Resting Membrane Potential |
| fMRI | functional Magnetic Resonance Imaging |
| EDU | Europol Drugs Unit |
| EMCDDA | Europan Monitoring Centre for Drugs and Drug Addiction |
| UN | United Nations |
| MFR | Mean Firing Rate |
| MBR | Mean Bursting Rate |
| BD | Burst Duration |
| ISI | Inter Spike Interval |
| IBI | Inter Burst Interval |
| SIB | Spikes Intra Burst |
| SIB/NS | Spikes Intra Burst/Network Spikes |

# 1 Introduction

New psychoactive substances NPS, often called by the popular term ‘legal highs’ or ‘designer drugs’ are a persistent and growing problem today. NPS is a group of substances that resemble illegal drugs in structure, properties, and/or their effects.

New psychoactive substances pose a major risk to public health. Not only because of the large number and easy availability on the market, or the attractiveness especially among the young generation, but mainly because of their large number and easy availability on the market, but mainly because of the lack of studies and information about the toxicity of these substances, their dosage, purity, and also about what are the side effects that we still do not know about. [1], [2]

Neuroscience offers various possibilities to investigate these effects on the nervous system. And one possibility is precisely in vitro neuronal cell culture, which provides another model for investigating neuronal activities and interactions of neurons in the human brain. Microelectrode arrays (MEAs) are small devices containing an array of usually several tens of microelectrodes enabling the *in vitro* measurement of the electrical activity of excitable cells, which include neurons. with the help of microelectrode arrays, we are able to analyze spontaneous activity and natural communication between neurons, as well as electrophysiological changes in activity as a reaction to a chemical, physical, or even mechanical stimuli. The use of MEAs in pharmacology and cytotoxicology is thus offered.

This study is motivated by today's situation with new psychoactive substances and the need to understand more and better the effects of psychoactive drugs. By focusing not only on MDMA (ecstasy) but also on 25CN-NBOMe in this work, I try to contribute to this complex topic and provide some basis or direction for further research in this area.

## 1.1 Aims and objectives

The main objective of this work is to utilize in vitro cultured neurons as a model for testing the effects of psychoactive drugs on the nervous system and subsequently assessing these effects. The psychoactive drugs under investigation were MDMA (also

known as ecstasy) and 25CN-NBOMe, which belongs to the group of phenethylamines of new psychoactive substances.

The sub-objectives leading to the fulfillment of the main task began with studying the mechanisms of action of psychoactive drugs on the nervous system. This involved studying the issues related to new psychoactive substances and understanding the technology of microelectrode arrays for recording electrical signals from excitable cells.

Furthermore, the execution of the actual experiment involved stimulating neurons and recording electrical signals. This step included the preparation of appropriate concentrated samples of individual stimulation substances and subsequently using microelectrode arrays to record spontaneous and stimulated neuronal activity.

Another sub-objective was the processing of these signals, which involved filtering, extracting electrophysiological events such as spikes, bursts, or network synchronization, and calculating key parameters that describe neuronal signals. These parameters include Mean Firing Rate (MFR), Mean Bursting Rate (MBR), and Inter Spike Interval (ISI), among others.

Based on these steps, the subsequent analysis of the results was conducted in terms of changes in individual parameters and overall trends of cellular electrical activity in response to the applied stimulants.

# 2 Overview of the current state of art

## 2.1 Neural Electrophysiology and Measurement Techniques

### Neuron structure

A neuron, or nerve cell, is the fundamental unit of nervous tissue. Neurons are highly specialized cells and mediate the reception, processing, and transmission of electrical signals, thus enabling intercellular communication. The basic structure of neurons, as depicted in *Figure 1*, includes the soma (the body of the neuron), dendritic processes and the axon. The soma contains the plasma membrane, nucleus, and other organelles. Dendrites, often branching extensively, are convergent paths that lead signals to the neuron (directly to the neuron soma). Dendrites are adapted to receive information from the axons of other neurons, and the degree of branching of the dendrites indicates the potential capacity of the neuron to process information. The axon, the divergent path of a neuron, is the fiber used to transmit information through the axon and then send it to another neuron or another cell. Most neurons have one axon, but some can have more. The degree of convergence (how many inputs the neuron receives) and divergence (how many target cells the information from the neuron is sent to) is determined by the structure of the neuron. [3], [4]

Axons often extend long distances from the neuron from which they originate, and unlike dendrites, they are usually not so branched. There are two types of axons: myelinated and unmyelinated. A myelinated axon, as shown in *Figure 2.1*, has three parts. Part of the initiation segment (axon initial segment, AIS), which is located at the exit of the axon from the neuron body, then the axon fiber itself, which is covered with a myelin sheath (formed from Schwann cells in peripheral nerves and from oligodendrocytes in central nerves) punctuated by notches of Ranvier, and finally the terminal segment, which serves to transmit the conducted information to another neuron or other cell. An action potential is formed in the AIS, which is further conducted by the axon, the structure of which helps to safely transmit information without attenuation, and the terminal segment then already enters the synaptic processes. [3]–[5]

Propagation of AP through a myelinated axon takes place in jumps, the so-called saltatory conduction. This is because myelin forms the electrical insulation of the fiber,

so the signal "jumps" these coated sections, thereby increasing the signal transmission speed and thus guaranteeing AP transmission over long distances at high speed, although the transmission speed depends on many other biophysical factors. [3], [4]

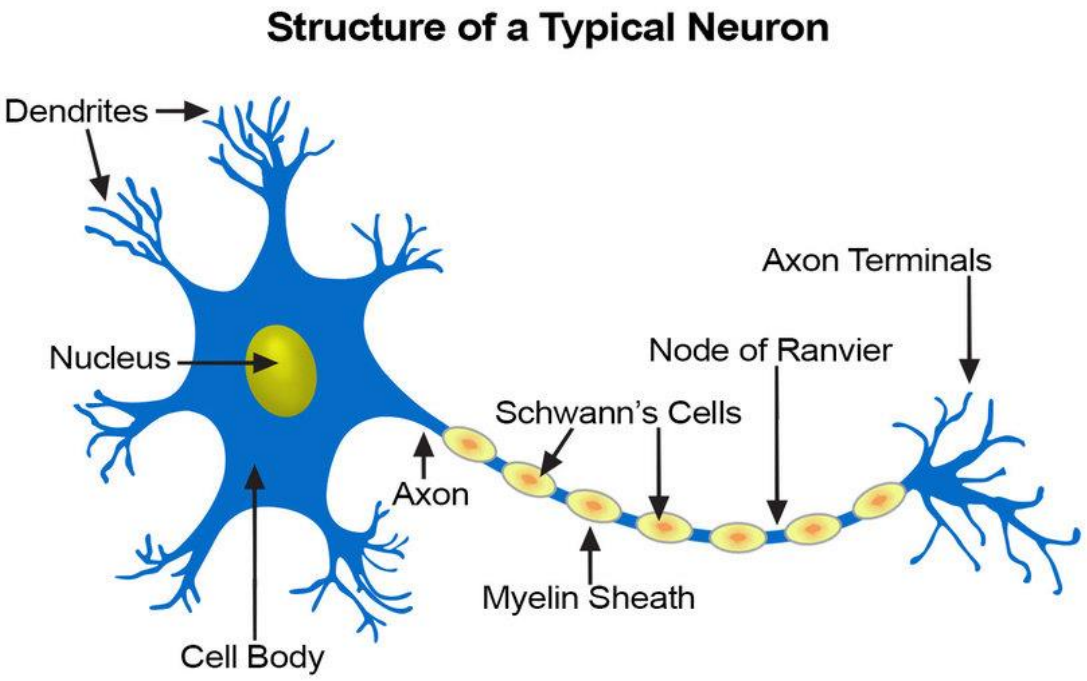


**Figure 2.1:** Simplified structure of a typical neural cell's main parts [6]

Neurons are organized into so-called neural circuits, which are sets of several nerve cells ensuring the processing of specific types of information. Neural circuits contain three groups of neurons. Afferent neurons, which have the task of transmitting information from the periphery to the brain, are also called sensory neurons. Another group is efferent neurons, or motor neurons, which, on the other hand, conduct signals from the brain to the periphery. The last group of nerve cells are interneurons, which ensure synaptic connections and the transmission of information between afferent and efferent neurons. [4]

## Neural electrophysiology

„Most neurons are distinguished by their specialization for long-distance electrical signaling and intercellular communication by means of synapses.“ [4]

Cellular membranes, not only of neurons, exhibit a certain value of electric potential. This is a difference in voltage inside and outside the cell, given by the uneven distribution of differently charged ions. Specifically, neurons exhibit a negative resting membrane potential, known as the resting membrane potential (RMP), which is around -

70mV. The permeability of ion channels is in equilibrium, with K+ ions predominating inside the cell and Na+ ions outside. [4], [7]

Neurons use several types of electrical signals to handle information, but in all cases, it involves a disturbance of the resting membrane potential. In sensory neurons, receptor potentials can be generated as a result of their activation. Another type of electrical signal is synaptic potentials, which mediate communication between neurons at their connections, i.e. synaptic contacts, where information is exchanged from the presynaptic neuron to the postsynaptic neuron across the synaptic cleft.

Finally, there are action potentials (APs), electrical signals responsible for transmitting information through long neuronal axons. AP is also known as peaks or impulses, and its structure is shown in Figure 2.2. It is a specific electrical signal used to convey information from one neuron to another or to another cell over a longer distance using axons. This involves the temporary local reversal of the negative membrane potential due to ion flow through the membrane, and this deviation from the resting state travels further. As mentioned above, the AP travels down the axon fiber, and thus the information is propagated further. Nerve cells have the ability to selectively allow certain ions to pass through the membrane, and the AP is generated due to changes in permeability. [4], [7]

An action potential is only triggered if the input signal exceeds a certain threshold. If the signal does not reach the threshold, an action potential will not be generated and will not propagate further. In most cases, only local electrical disturbance occurs. The first phase of the AP is depolarization, initiated by the opening of the sodium channel, which allows Na+ ions to enter the cell. This raises the membrane potential, and once it reaches about +30mV, potassium channels open and, due to the concentration gradient, K+ ions begin to exit through the membrane. This phase is called repolarization, and towards the end of this phase, the membrane potential temporarily drops below the RMP, known as hyperpolarization. Then there is a return to RMP. The refractory phase is the period during which an action potential cannot be generated. This applies to completely polarized cells as described above. However, there are also partially polarized cells called pacemakers that can generate APs randomly without any stimulus. Such cells are important, for example, in the cardiac system. [4], [7]–[9]

Action potentials are all-or-none signals, which means that the size of the signal does not increase with an increasing stimulus that caused the AP. In other words, if the threshold for AP generation is reached, the signal will always be propagated with the same amplitude regardless of the size of the stimulus that caused it. This distinguishes APs from, for example, postsynaptic potentials. The duration of the APs is about 5 to 10 milliseconds for most of the nerve cells. [4], [9]

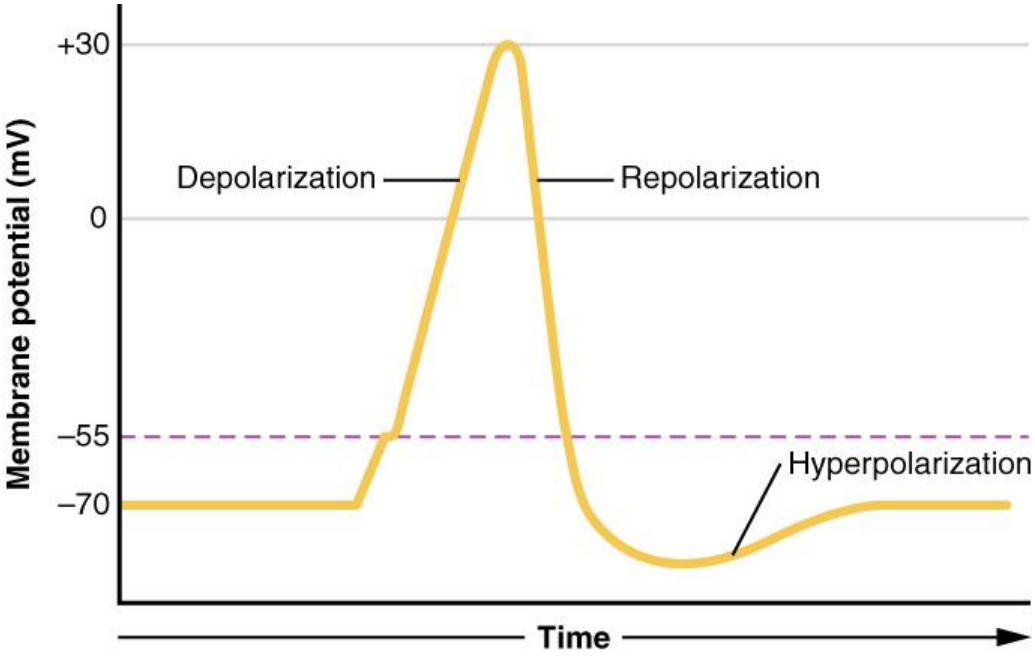


**Figure 2.2:** Graph of the neuronal action potential [7]

## Electrophysiological measurement techniques

Electrical signal measurement techniques of nerve cells can be generally divided into three groups based on their sensitivity and resolution: microscale, mesoscale, and macroscale methods.

**Patch Clamp** (microscale/nanoscale) is an electrophysiological technique used for studying biological membranes, individual ion channels within the membranes, and the ionic currents within them. Ion channels, as previously mentioned, are structures that enable specific movements of ions from the endoplasmic to the extracellular space and vice versa, and are important for cell signaling processes. Generally, this technique involves placing a glass microelectrode, also called a pipette, onto the cell membrane. The ion channels present in the membrane can then be measured and controlled. This measuring technique has several configurations that are used for different purposes. The most common mode is the Whole-Cell technique, where the microelectrode is attached to the cell membrane, which is disrupted, and the pipette enters the cytoplasm, which then

expands into the space of the pipette. Another option is the Cell-Attached technique, where there is no disruption of the cell membrane, and the microelectrode is only attached and held in place by gentle suction. This method is often used for measuring neuronal action potentials. The last two techniques stem from the previous two but involve working with a detached piece of the cell membrane. The Inside-Out configuration occurs when the Cell-Attached pipette displaces a piece of the membrane by tearing it off, leaving the outer side attached to the pipette. Finally, the Outside-Out method is created by withdrawing the pipette in the Whole-Cell configuration, which separates the membrane whose ends are then joined together to create a vesicle connected to the pipette space. [10]–[13]

**Electroencephalography** (macroscale), EEG for short, belongs to macroscopic electrophysiological methods, because, unlike patch-clamp recording the membrane potential of a single neuron, EEG records the electrical signals of an entire group of nerve cells. It is a non-invasive method where electrodes are placed on the surface of the head and measures the voltage difference between two points. Another difference from other electrophysiological methods is the origin of the signal. The source of the EEG signal is usually synaptic potentials, which are longer, not short action potentials. Macroscopic electrophysiological methods can include, for example, functional magnetic resonance imaging (fMRI). [13], [14]

**Microelectrode array** (mesoscale), MEA for short, is another method for measuring the electrical signals of nerve cells that allows the measurement of extracellular action potentials (EAP). This technique is described in more detail below in Chapter 2.2. [13]

## 2.2 Microelectrode Array (MEA) Technology

Microelectrode arrays are small biosensors used for sensing extracellular electrical signals of any electrogenic cells or sheets of tissue, such as neuronal cells and networks, retinal cells, or cardiac myocytes. As a bio electrophysiological tool, it has applications both in vitro and in vivo. The main advantage of MEA is the possibility of sensing signals from several places at the same time, so they allow studying the course of activity in entire cellular networks with high temporal and spatial resolution. Another great advantage is that when studying the activity or stimulation of cells in vitro, the cell culture deploys and cultivates directly on the surface of the microelectrode array. This allows long-term experiments to be carried out. The most common uses of the in vitro

microelectrode fields are in the areas of neurobiology (e.g. neuroregeneration, drug testing) and in the areas of cardiac electrophysiology (reg. measurement of conduction velocity). [15]–[17]

### *In vitro* MEAs

In vitro electrode arrays contain arrays of microelectrodes with dimensions approximately comparable to the dimensions of neuronal cells, typically with diameters of 10, 20 or 30 μm, and with different interelectrode distances. Individual microelectrode arrays basically differ in their electrode geometry, their number, the distances between them, and the materials used for individual components. Standard MEAs for in vitro studies they contain 60 electrodes, but MEAs with 120, 256, or even 519 or more electrodes are also produced. Electrodes are mostly made of titanium nitride (TiN), but there are also electrodes that are modified with, for example, colloidal gold or carbon nanotubes in order to reduce impedance. The electrodes are embedded in the substrate, which is typically glass for transparency and its use in light microscopy, but the substrate can also be an opaque printed circuit board (PCB). The device may also include an internal reference electrode (iR). Contact pads and tracks are usually made of TiN or transparent indium tin oxide (ITO). The surface of the MEA is covered with an electrically insulating layer, which is most often silicon nitride (SiN). The impedance of the electrodes for classic 60-electrode arrays is less than 100kΩ (the impedance increases with a larger diameter of the electrodes). The devices are highly stable, temperature compatible so they can be autoclaved, and have an excellent signal-to-noise ratio. Lifetime depends on the number of uses of the MEA and on the nature of substances and cells that come into contact with the MEA during testing. Commercial manufacturers of these devices are, for example, MultiChannel Systems (Reutlingen, Germany) or Alpha MED Scientific (Osaka, Japan). [15]–[18]

### Culturing neurons on *in vitro* MEAs

Neurons are cultured on planar-type microelectrode arrays. There is a direct growth of the neuronal network and its synaptic connections on the surface of the MEA. The direct development of the culture on the MEA gives rise to the configuration of neurons on the electrode, which is a specific connection that precisely allows us to measure the activity of neurons in the form of action potentials (AP). These recorded APs

can have their source in neurons up to 100 μm away, but most often they are signals from within 30 μm

around the recording electrode. The measured signals have the same speed as the actual transmembrane action potentials of neurons. On the one hand, there is the direct development of neurons on the surface MEA is an advantage and provides us with accurate measurements, on the other hand, long-term cultivation can affect measurements negatively. For example, it has been shown that in mature neuronal cultures glial cells sprout between the electrode and the neuron, making AP detection difficult and thus reducing the yield of this electrode. Before placing the cells on the microelectrode array, it is necessary to cover the surface of the MEA with some substances, such as polylysine or laminin, which will support the adhesion and growth of cells on the MEA. Neuron cultures seeded on MEA are usually covered with a chamber with a semipermeable membrane, often made of Teflon. Different membranes have different properties, but in general, it is always the main one in order to protect cells from contamination and desiccation. Therefore, membranes permeable to gases (O2, CO2) and impermeable to water are chosen. The cover with the membrane is placed on the ring and together they form a cell culture chamber. In the case of neural networks, the spontaneous activity of the network, i.e. communication between neurons, or a change in electrical activity as a result of mechanical, electrical, or chemical stimulus. [16]–[19]

## The use of *in vitro* MEAs in pharmacology

Microelectrode arrays are tools for the non-invasive study of both acute and long-term effects of various chemical substances, drugs, or toxins on biological tissue. Thanks to its multi-channel system, the dynamics of the effects of these substances can be observed in time and space, thus obtaining important information about the specificity of the effects of specific substances on specific cells. In recent years, the use of electrophysiological methods has become very popular in the field of pharmacology and medicine. And that's thanks to that a close relationship has been found between some diseases and disorders of bioelectrical mechanisms mediated by ion channels and pumps. Hence electrophysiology, molecular bioelectricity, the study of ion channels and pathways, are today areas of interest for pharmacologists, molecular biologists and other specialists. Use of MEA as a system for in vitro testing of chemical substances and pharmaceuticals, brings valuable information and guidelines for understanding the mechanisms of effects of these substances and for the development of new drugs. When

the composition and effects of a specific substance are known, by stimulation we study the structure of cells and their receptor representation. In this type of experiment, known stimulants (e.g. bicuculline) or various neurotransmitters are often tested. Conversely, when stimulated with an unknown chemical substance, we investigate the properties of this substance and the mechanisms of its effects on various known cell cultures. Here, MEA can be used to test illegal drugs or new psychoactive substances, just like in this work. [15], [19]–[21]

## 2.3 New Psychoactive Substances (NPS)

New psychoactive substances (NPS) are a global problem that is faced by a large number of countries around the world. Their occurrence and quantity are constantly increasing, and although there are several pieces of legislation that control these substances, the laws still lag behind the dynamic nature of the NPS market.

NPS are emerging as alternatives to illegal drugs. NPS are their chemical derivatives or other chemical substances that imitate the effect of illegal drugs (such as LSD, ecstasy, or others). Manufacturers and sellers of these substances are trying to circumvent the drug market. These are new, untested substances with an often unclear composition and mechanism of action.

### Classification and legal matters related to NPS

NPS categories are of two types. Effect Group Classification divides NPS according to the effect on the human nervous system, and Structural Group Classification divides NPS according to their chemical structure. It is the most commonly used UNODC Early Warning Advisory (EWA) approach on NPS. Below are the individual groups of NPS according to their effects with the percentage representation of their occurrence (examples of the groups according to the chemical structure to which they belong are given in parentheses). [22]–[24]

- **Stimulants** 36% (e.g. synthetic cathinones, phenethylamines, piperazines)
- **Synthetic cannabinoid receptor agonists** 30% (synthetic cannabinoids)
- **Classic hallucinogens** 15% (e.g. phenethylamines, lysergamides)
- **Synthetic opioids** 11% (e.g. fentanyl analogs, piperazines)
- **Sedatives/hypnotics** 4% (e.g. benzodiazepines)
- **Dissociatives** 3% (e.g. phencyclidine-type substances) [24]

The Early Warning Advisory of the United Nations Office on Drugs and Crime (EWA, UNODC) reports that over 1,150 substances from a total of 37 countries and territories have been reported worldwide as of 18/05/2022. The relative representation of individual states and NPS categories in the number of reports varies, however, the number of reports has remained more or less stable for the past 4 years, at over 500 reported substances per year. In its 2022 Annual Report, UNODC reported that 6.9 tons of new psychoactive substances were seized in 2020 (41,100 seizures). Of this, 5.1 tons (21,230 catches) went to the EU. The current drug market and its development are characterized there as more complex, characterized by the high availability of these substances. In this report, UNODC also expressed concern about the crossing of NPS markets with illegal drugs, for example, the production of fake medicines or cannabis preparations with a low-THC preparations. NPS is sold directly in illegal markets under the auspices of the illegal drug. This most often happens when illegal drugs are in short supply or completely absent from the market. This makes the risk for potential users even greater and the use of these substances more dangerous. [23], [25], [26]

The most important, still valid United Nations (UN) conventions on psychotropic and narcotic substances, conventions to which important laws, regulations or directives still refer today, i.e. anti-drug conventions are the *Single Convention on Narcotic Drugs* of 1961 and *Convention on Psychotropic Substances* of 1971. These conventions contain lists (Schedules) of psychotropic and narcotic substances, which are graded according to the level of risk. They also contain the measures and procedures that the contracting states are obliged to follow when encountering these substances, and by which they will bring them under legal control. The documents also provide for accelerated approval of changes to these lists that can be proposed by any of the contracting parties or the World Health Organization (WHO). These conventions became a major change in political and legal approaches to drugs in several countries of the world. [27]

As regards the legislation of these substances in Europe, in 1997 the Council of the European Union adopted the *'Joint Action on New Synthetic Drugs'*. This document summarizes the mechanisms and procedures to ensure the rapid and efficient exchange of information on new synthetic drugs between EU member states. These plans lead to the faster provision of the necessary measures in the field of new psychoactive substances (introduction of these substances under control). Three phases were defined in the fight against NPS: early warning, risk assessment, and control regulation. As part of the early

warning phase, the council agreed that each member state will inform the Europol Drugs Unit (EDU) of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) about the production, transport, or usage of NPS on their territory. Today, this agency is still functioning and is a very important institution of the European Union in the field of illegal drugs that supports EU and national policymaking. As part of this document, the EU Early Warning System on NPS (EWS) was established, which is a project to ensure awareness of NPS, run by the EMCDDA in cooperation with Europol. This article is no longer in force, its validity ended in 2005 when it was repealed by another council decision. [2], [28]–[30]

It was a document approved by the European Council in May 2005 named *'Council decision on the information exchange, risk-assessment and control of new psychoactive substances'*. Due to the definitions in this document a 'new psychoactive substance' means *"a new narcotic drug or a new psychotropic drug in pure form or in a preparation that has not been scheduled under the 1961 United Nations Single Convention on Narcotic Drugs or that has not been scheduled under the 1971 United Nations Convention on Psychotropic Substances, and that may pose a threat to public health comparable to the substances listed in these conventions."* [31] This definition od NPS is still valid today. This Council Decision supersedes the previous Joint Action of 1997, see above. Some procedures from the previous Joint Action are preserved here, e.g. the three-phase response strategy to NPS. But certain changes also appear. The change compared to this document is the expanded definition of new psychoactive substances, so this term includes a larger number of substances than before. Another difference is the new approach to regulating these substances, which is more efficient and ensures greater protection of member states against NPS. In essence, the European Union provides flexibility and speed in intervening against NPS (which already includes narcotic and psychotropic substances) appearing in the member states. Another benefit of this amendment is the guarantee of transparency, and therefore the provision of feedback from the EMCDDA and Europol on the functioning of the system. This decision also strengthened the EWS project created in the previous Joint Action. This article is also no longer in force, it was repealed by the Directive of the European Parliament and of the Council in 2017. [2], [31], [32]

Due to the increasing appearance of new NPS on the market, the EU was forced to react by issuing an updated Directive and Regulation regarding the control of NPS within the

*Official Journal of the European Union*. The difference between a Directive and a regulation is that the regulation is legally binding and directly effective in all EU member states and must be immediately implemented into the laws of the country. On the other hand, the Directive indicates the goals that member countries should achieve, but the way of implementing these rules into laws is chosen by the country itself. Both of these pieces of legislation replace the 2005 document and are still in force today. These documents include a stronger EU EWS (from the previous 2005 document) and an accelerated risk assessment procedure. They will maintain the three-phase process of responding to NPS and, in addition, speed up and make these processes more efficient, for example by shortening deadlines. In accordance with the Directive, the inclusion of NPS in the definition of "drug" at the European level is also within the framework of reducing the availability of NPS, their sale, and the overall spread of the dangers they bring. [33], [34]

Another important international organization founded by the UN in 1997 is the Union Nations Office on Drugs and Crime (UNODC). It specializes in the fight against crime, terrorism, corruption, and illegal drugs. Within the framework of UNOCD, a project called UNODC Early Warning Advisory (EWA) was created in 2013. It was created for the purpose of monitoring the occurring NPS, and their analysis and also for the purpose of informing the representatives of the member states about the events in this issue and also for their support. [35], [36]

UNODC and EMCDDA have been working closely since the Memorandum of Understanding signing between MCDDA and UNDCP in Vienna in 1998. This signed the collaboration between the two organizations in order to join forces against the common enemy - the drug market. [37]

## Specific groups of new psychoactive substances and their effects

The most represented category on the market now is synthetic cannabinoids and cathinones. Synthetic cannabinoids (SCs, SCBs) are man-made substances with structural differences, that act as Synthetic Cannabinoid Receptor Agonists (SCRA). This includes e.g. APINACA, JWH-018, and others, SCBs have similar structural properties to delta-9-tetrahydrocannabinol (9-THC), a well-known hallucinogen found in cannabis. This allows it to bind to the cannabinoid receptors (not just those) and produce similar effects on humans as THC, focusing on the endocannabinoid system. SCs have stronger and more toxic effects than natural cannabinoids. It is currently the largest NPS group

monitored by the EMCDDA. These preparations represent a major threat to public health, especially with the increasing trend of associating the use of SCs with severe intoxications and deaths. Young people are the most at-risk group. Preparations containing SCs are often cheaper than preparations containing THC, are easily available, and often have attractive packaging and pleasant names (e.g. K2, Spice, etc.). A common form of SCs use is smoking (cigarettes, pipes, hookahs). However, there are also products for oral use in the form of powders, tablets, or herbal diffusions. The largest producers of SCs are in China and the most common form is powder. After being transported to Europe, they are most often completed by dissolving them (e.g. using acetone) and mixing them with plant bases such as lemon balm, mint, or thyme. These mixtures are further dried, packaged, and sold through the dark web or traditional brick-and-mortar stores. [24], [38]–[41]

# 3 Methods and Materials

## 3.1 Cell Culture

### 3.1.1 Cell origin

For the purposes of our work, we used cell cultures of hippocampal neurons from neonatal Wistar rats. We obtained cell cultures of neurons already developed and ready for the experiment from the Institute of Physiology of the Czech Academy of Sciences (CAS), where the cell lines were seeded and cultured.

In most cases, neuroscientists use primary cells for the formation of neuronal cultures, and these cells are often hippocampal neurons. This is because hippocampal neurons, unlike other central nervous system neurons, are relatively simple. In addition, they have been studied for a long time and today there are extensive databases containing various types of hippocampal neurons. Another reason is the excellent ability of the pyramidal cells of the hippocampus to form dendrites and rich synaptic connections, which is very important in the formation of a neural network. [42]

### 3.1.2 Cultivation

The performed hippocampal neuron culture procedure combines the scientific protocol of Burgallosi et al. 2012 [43] and the cultivation procedure of Krůšek et al. 2019 [44]. Neurons were isolated from Wistar rats on the first day after birth. They were cultured directly on the surfaces of the microelectrode arrays in Neurobasal-A medium supplemented with Glutamax, which supplies cells with glutamine, and B27, growth-promoting supplements. A light microscopy image of hippocampal cells on MEA is below (*Image 3.1*). Prior to seeding cells, MEA surfaces were coated with poly-L-lysine. Glial pads were not used due to concerns about impaired contact between neurons and electrodes (not confirmed). On the third day *in vitro* (3 DIV), glial growth was suspended for 24 hours using cytosine arabinoside (AraC), as glial cells and their proliferation in neuronal culture are undesirable. During the entire cultivation period, the cell cultures were kept in an incubator at a constant air humidity, a temperature of 37°C, and 5% CO2, covered with an MEA-MEM Teflon membrane (MultiChannelSystems, Reutlingen, Germany), protecting the cells from contamination and drying.

The cultivation period lasted 13, 14, or 21 days. A few days before the planned experiment, the condition of the neurons was checked by recording the neuronal activity with a recording device. Multiple neuronal cultures were required for testing, so MEAs were sterilized and cleaned with tergazyme between cultures. [42]–[44]

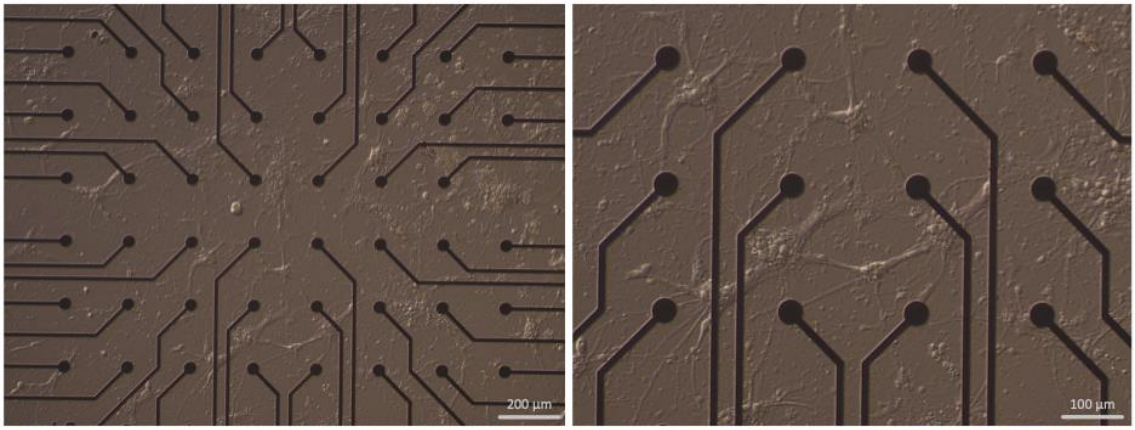


**Image 3.1:** Light microscope image of hippocampal neurons on a microelectrode array at two different magnifications

## 3.2 Chemical Substances and NPS Used

Stimulating substances were obtained from the Forensic Laboratory of Biologically Active Substances of the University of Chemistry and Technology (UCT, Prague). A handover protocol was drawn up between the University of Chemistry and Technology and the Faculty of Biomedical Engineering CTU in Prague on the received substances and their exact quantity. We took over all stimulants in powder form.

### MDMA

MDMA, widely known as ecstasy or "the party drug", is a well-known and widely used illegal drug. It is a very popular and frequently used psychedelic substance, especially among younger generations. It has specific acute effects on humans, which include feelings of euphoria, increased empathy, and heightened sensory perception.

One of the main mechanisms of MDMA's action is its affinity to the brain's serotonin receptors (5HT1A, 5HT2A). It thus causes the release of the serotonin neurotransmitter, responsible for mood modulation, as well as dopamine and norepinephrine. Some studies also report the impact of MDMA on neurodegeneration in the brain. MDMA acts as a neurotoxin for the nerve cells and the nerve tissue is damaged by free radicals.

It's important to mention that even though the interest in MDMA on the illegal drug market is enormous, and it's generally known that this substance is widely used by young people today, there are still discrepancies and inconsistencies among studies regarding the specific mechanisms of this substance's action. [45], [46]

## 25CN-NBOMe

Another substance we were investigating was 2,5-dimethoxy-4-(2-((2 methoxybenzyl)amino)ethyl)benzonitrile, which belongs to the extensive family of N-2-methoxybenzyl (NBOMe) classified as new psychoactive substances. These substances are found on the illegal drug market mostly as alternatives to LSD due to similar hallucinogenic effects (but more stimulating), often under the name "Smiles" or "N-Bombs". 25CN-NBOMe is a 2C-CN substitution derivative of phenethylamine (PEA). They generally belong to the 2C or 2C-X family of substances, a term coined by Shulgin to refer to psychedelic phenethylamines that contain one amino group in position 2, two methoxy groups and another third functional group described as "X" in their benzene ring. Shulgin was also the first to prepare most of these substances (eg 2C-I, 2C-B, etc.) and described their properties and effects in the book PiHKAL (Shulgin and Shulgin, 1991). [47]–[50]

This substance, together with other related compounds, such as 25I-NBOMe or 25B-NBOMe, act as an agonist of serotonin receptors 5-$HT_{2A}$ and have comparable effects. It was found that binding the N-group to 2C-X compounds (N-benzylation) leads to an increased affinity specifically for the 5-$HT_{2A}$ receptor (and others, e.g. 5-$HT_{2C}$) and to an increase in the ability to specifically interact with this receptor subtype compared to others subtypes. And since it is assumed that, in general, substances with agonistic properties towards the 5-$HT_{2A}$ receptor through this mechanism significantly influence the individual manifestations of the psychedelic effects of drugs from the group of classical psychedelics on humans, and that some of these agonists can influence and support neuroplasticity, these substances can be an interesting aspect of therapeutic use and potentially much more dangerous for recreational users of these drugs compared to classic psychedelics. [47], [51]–[54]

NBOMe substances have been appearing on illegal markets since 2010, as already mentioned, with the purpose of replacing LSD. They are often sold in the form of powder, pills, liquid, or absorbent paper and are used in a variety of ways, including smoking,

intravenously, etc. They can cause unwanted auditory and visual hallucinations, feelings of fear and anxiety, or seizures. Several cases of intoxication and death have already been reported and associated with substances of this class. The most represented group of NBOM compounds on the drug market are 25I-NBOMe, 25C-NBOMe, and 25B-NBOMe. [47]–[50], [54]

NBOMe substances have been found to have neurotoxic and cardiotoxic effects. For example, one study by (Peng Xu et al., 2019) demonstrated more than 50-fold greater efficacy in reducing the viability of 25C-NBOMe in SH-SY5Y cell lines (a human neuroblastoma cell line) than methamphetamine at the same cell dose in vitro. [55]

Another study (Šíchová K. et al., 2022), investigating 25CN-NBOMe in terms of pharmacokinetics, thermoregulation, systemic toxicity and behavioral effects tested in Wistar rats determined toxicity category 3 with a lethal dose of 300mg/kg according to the Acute Toxicity Testing Guidelines for Chemical Substances 423 Organization for Economic Co-operation and Development (OECD), i.e. a compound of moderate level of toxicity. The study also found the ability of 25CN-NBOMe to easily cross the blood-brain barrier, which was confirmed by the detection of higher concentrations of the substance in the brain than in the blood serum. The time when it was still possible to detect some concentration of the substance in the serum and brain was up to 8 hours. In behavioral testing of mice, they also observed increasing anxiety and worsening locomotor abilities with increasing doses of 25CN-NBOMe. In contrast to hyperthermic reactions in humans to 25CN-NBOMe, hypothermia was more likely to occur in test rats. [47], [50], [56]

## 3.3 Recordings

### 3.3.1 Recording system

To measure the electrical activity of the neuronal culture, we used the recording system MEA2100-System (MultiChannel Systems, Reutlingen, Germany), consisting of several main components: a microelectrode array, a filter amplifier, a main unit, an external source, and a thermostatic unit.

#### Microelectrode array

We used a standard sixty-electrode MEA for in vitro applications, type 60MEA200/30iR-Ti-gr (MultiChannel Systems, Reutlingen, Germany). Titanium nitride (TiN) electrodes are embedded in a transparent glass substrate for the possibility of

applying light microscopy to a biological sample on the MEA surface. The electrode arrangement is in the shape of an 8x8 grid with missing corners. The electrodes in the grid are numbered with a double number, where the first digit indicates a column and the second digit a row. The diameter of the electrodes is 30 µm and the inter-electrode distance is 200 µm. The MEA contains one internal reference electrode (iR) also located in a glass substrate, i.e. of recording channels is only 59. The reference non-recording electrode number is 15. The material of contact pads and tracks is also titanium nitride. A layer of silicon nitride (SiN) is used for electrical insulation on the surface of the MEA. The ring forming the cell culture dish on the surface of the MEA is 6 mm high and made of glass. To protect the sterile cell line, we covered the ring with an MEA-MEM chamber with a semipermeable Teflon membrane (fluorinated ethylene-propylene), which is permeable to $CO_2$ and $O_2$ and impermeable to water. *Image 3.2* shows the microelectrode array 60MEA200/30iR-Ti-gr (MultiChannel Systems, Reutlingen, Germany), we used for our work. [18]

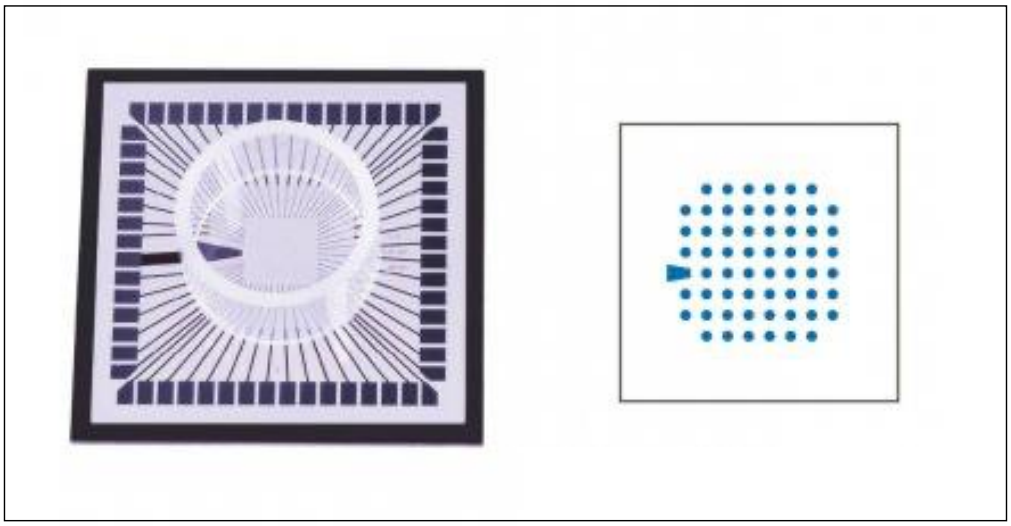

**Image 3.2:** Microelectrode field of 60MEA200/30iR-Ti-gr type on the left. A diagram of the MEA field with the marking of the reference electrode on the right. [18]

### Central unit

The main USB-MEA60 unit for data collection contains an A/D converter (to convert the analog signal to digital). This unit has its own power supply.

### Filter amplifier

The Filter Amplifier FA60 SBC provides signal filtering and amplification, as the electrical signals detected from the extracellular space of cells are very small.

### External power source

The PS40W external power supply is used to power the main unit.

### Thermostatic unit

The TC01 temperature controller maintains a constant temperature of the substrate, thus providing an ideal temperature environment for cells.

### Software

MC-Rack is a software that enables recording of signals, their visualization in real time and fast and simple processing of signals.

## 3.3.2 Experimental procedure

First, we prepared the stimulating substances stock solutions. Since all the stimulants were received in powder form, we created the stock solution by mixing the powder with the ECS+ salt solution. The stock solution was more concentrated than the required concentration for dosing. We investigated four various concentrations of each stimulant when each stimulant had different required dosing concentrations. We filtered the stock solutions through a sterile filter with a pore size of 0.22 µm (Millipore Express PES Membrane, Millex-GP).

The experimental procedure diagram for the recording part is illustrated in *Figure 3.1*.

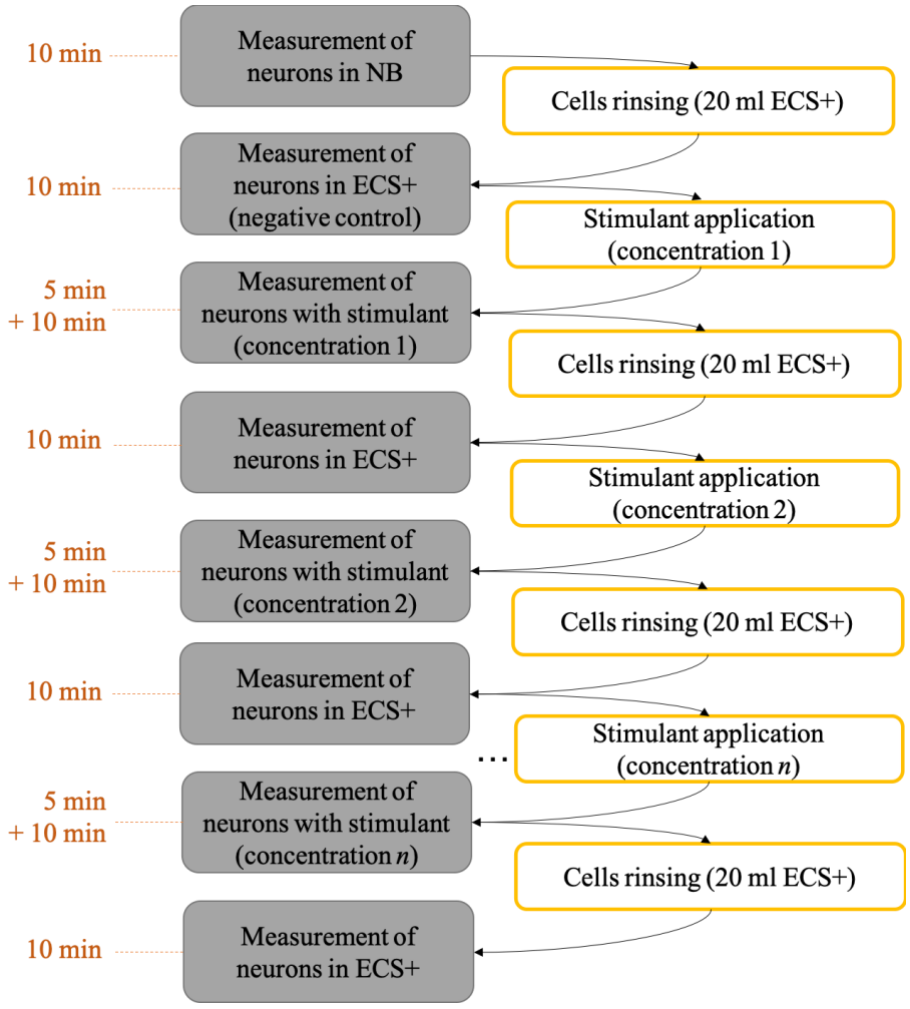


**Figure 3.1:** Diagram of individual steps in the experimental procedure for the recording of neural network stimulating

The first step in the experimental procedure was to measure cells in a neurobasal medium (NB). We removed the microelectrode array with neurons from the cell incubator and placed it into a measurement system with regulated temperature (see Chapter 3.3.1). After all channels (amplifiers) were running, we covered the cells with the metal cover and started a ten-minute recording of cellular activity in the neurobasal medium. This measurement serves more as a control for the state and condition of the neuronal network. All recordings were recorded at a sampling rate of 25 kHz.

We then stopped the recording and opened the Teflon membrane to perform cell washing and substance application directly in the recording system. This was done to eliminate unnecessary manipulation of the cells and to avoid mechanical irritation that could affect the cells. Also, to capture the immediate reaction of neurons to the applied stimulant in the recording and to cell rinsing could be done directly in the recording system.

We then gradually and slowly rinsed the cells with twenty milliliters of ECS+ salt solution. Using magnets, we placed the ends of two suction tubes and started suctioning and pumping at the same time to maintain the same volume of fluid in the chamber, about 500 µl (the speed of suction and pumping was the same). The suction tube led to a glass flask, where the sucked fluid was drained, and the pumping tube was connected to a flask filled with 20 ml of ECS+ solution. This system of suction tubes fixed by magnets to the recording system directly during the experiment is shown below in *Image 3.3.*

After washing, we started a ten-minute measurement of cells in pure ECS+. This recording serves as a negative control for us.

We then stopped the recording, removed the corresponding volume of the solution from the chamber, mixed it with the stock solution of the stimulating substance in such a ratio that the resulting concentration after application back to the cells was the one we desired, and at the same time, the volume of the solution in the chamber was approximately maintained. We performed this mixing for faster mixing of the substance in the solution after application to the cells. Then we opened the metal lid and pipetted this mixed solution into the cells. We closed the lid and started the ten minutes recording after about 3 minutes.

We then proceeded to rinse the cells with another twenty milliliters of ECS+, and we measured the cells in this solution (10 minutes) in the same way as in the previous washing. In this case, we cannot call this step a negative control, as we do not know the washability of the stimulating substances and, therefore, we do not know if any amounts of the substance remained in the solution or if the substance had already been incorporated into some neuronal structures.

The next steps are the same as the previous two and are repeated (stimulant application, rinsing). The concentration of applied stimulants gradually increases. For both testing substances, four different concentrations were applied.

The last step was to rinse neurons with 20 ml of ECS+ and record the cells in ECS+ for the final 10 minutes. With this step, we aimed to determine the reversibility of the culture to its original activity, to its original state.

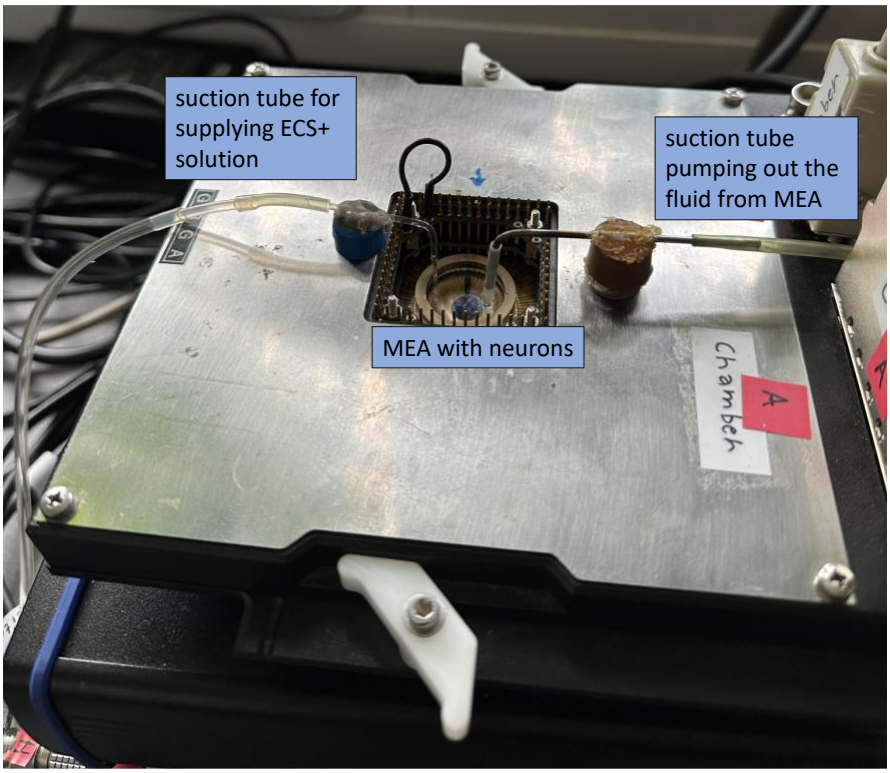


**Image 3.3:** Suction tubes fixed by magnets to the recording system directly during the experiment. The system serves to wash cells with ECS+ saline between neuronal stimulations.

## 3.4 Signal Processing

After obtaining raw data from MEA, I performed preprocessing, including data conversion and signal filtering. Subsequently, I conducted signal analysis, involving the determination of spike detection threshold, spike and burst detection, synchronization assessment, and extraction of relevant electrophysiological parameters. I only included active channels showing neuronal activity in the analysis. Postprocessing involved data visualization and interpretation of the obtained findings. *Figure 3.2*. shows the data processing procedure from the signal recording to the interpretation of the results.

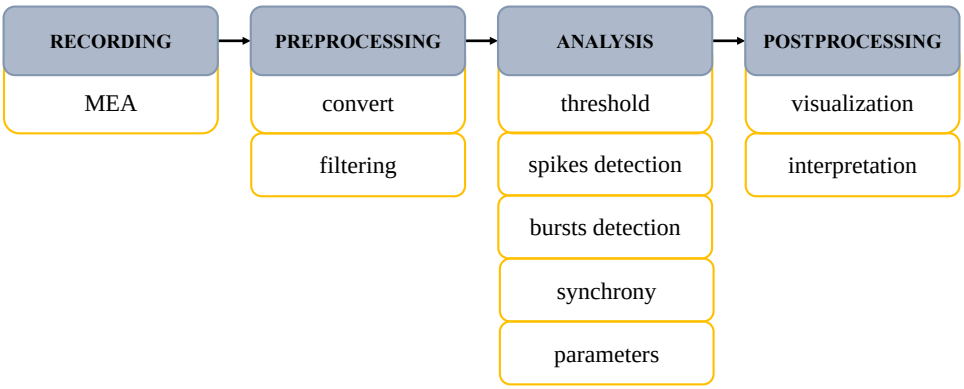


**Figure 3.2:** Data processing from their acquisition to the interpretation of the results

### 3.4.1 Preprocessing

#### Conversion

We obtained the raw data in the data format of .mcd (MultiChannel Devices). I converted all files to text files using the MC_Datatool program (MultiChannel Systems, Reutlingen, Germany), because of the incompatibility of the .mcd format with other programs. In these text files, the first column always contained time information in milliseconds, and all other columns contained voltage values recorded for individual electrodes.

#### Filtering

I performed the filtering and subsequently all the data analysis and results visualizations (see Chapters 3.4.2 and 3.4.3) with scripts in the Matlab 9.11.0.1769968 (R2021b) programming environment created by me.

I used a second-order Butterworth digital bandpass filter with 300 Hz and 6 kHz cut-off frequencies to process the data. This filter effectively removed the low-frequency drift component (using a high-pass filter at 300 Hz) and attenuated high-frequency noise (using a low-pass filter at 6 kHz) that could interfere with the analysis. This allowed me to focus on the frequency range of interest containing relevant electrophysiological information. *Figure 3.3* shows the raw signal obtained from the measurement and the same signal after filtering.

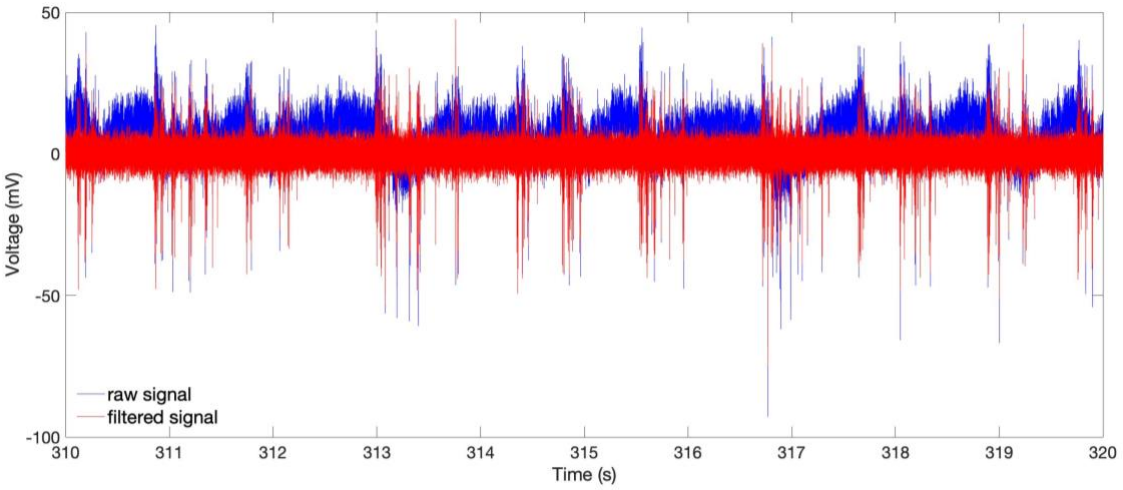


**Figure 3.3:** Visualization of the raw signal (blue color) and the filtered signal (red color).

### 3.4.2 Analysis

#### Threshold

To determine the threshold for spike detection, an estimation of the background noise level was necessary. The method for threshold determination is based on calculating the average noise level or estimating the mean value. A sliding window of 1 second was applied to the signal, and for each segment, the mean value of the absolute signal amplitude within that segment was computed according to *Formula 1).* The threshold was then calculated according to *Formula 2).*

$$noise\_estimate = mean(abs(segment)) \quad 1)$$

$$threshold = M * noise\_estimate \quad 2)$$

The multiplier *M* was determined based on the noise level. The optimal multiplier *M* was obtained by plotting several potential options directly on the signal plot. Such a plot is shown in *Figure 3.4*, where the filtered signal is plotted with horizontal lines representing different thresholds.

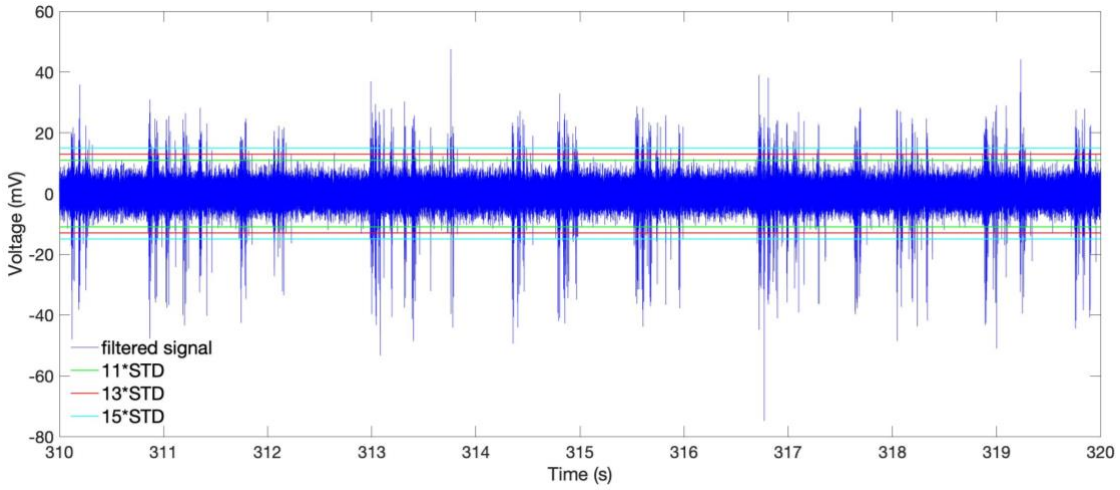


**Figure 3.4:** Filtered signal plot with horizontal lines representing individual threshold values (11*STD, 13*STD, 15*STD)

#### Spikes detection

Spikes represent individual action potentials and therefore the activity of neurons. Spikes were detected based on a predetermined threshold. To ensure the detection of spikes of both polarities, both positive and negative thresholds were included in the detection process. After establishing the thresholds from the noise estimate, I searched for action potential peaks as local minima using the *findpeaks* function. The

threshold was defined as the minimum peak height, and I also incorporated a refractory period as the minimum spacing between detected peaks, which I set to one millisecond. The refractory period, also known as the refractory phase, is the time during which a neuron is unable to generate another action potential. After identifying spikes of positive and negative polarities, these spikes were merged and filtered based on the minimum distance between them to exclude duplicate detection of the same spike in both polarities. *Figure 3.5* shows a filtered signal with a threshold line and detected spikes.

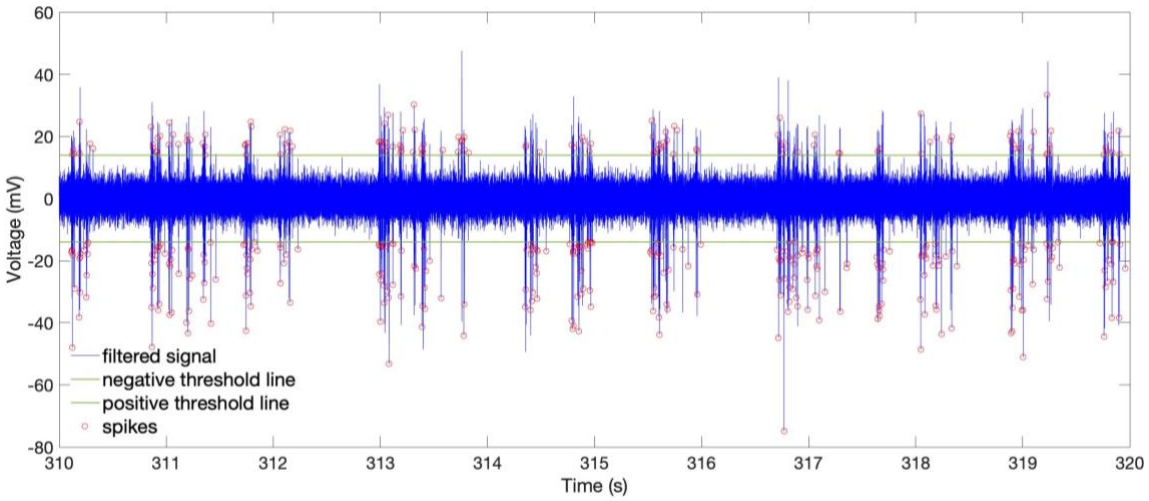


**Figure 3.5:** Representation of filtered signal with threshold lines (positive and negative polarity) and detected spike peaks

## Burst detection

Bursts are short segments with a recurring occurrence of spikes. Bursts can be detected based on several criteria. In my script, I detected bursts based on three conditions. The minimum number of spikes in a burst was set to 10. I also set the maximum distance between spikes within a burst, known as the inter-spike interval (ISI), to 100 ms. Lastly, I defined the minimum duration of a burst as 30 ms. These criteria serve to identify and detect significant bursts in the analyzed signal. An example of a detected burst is shown below in the following *Figure 3.6.*

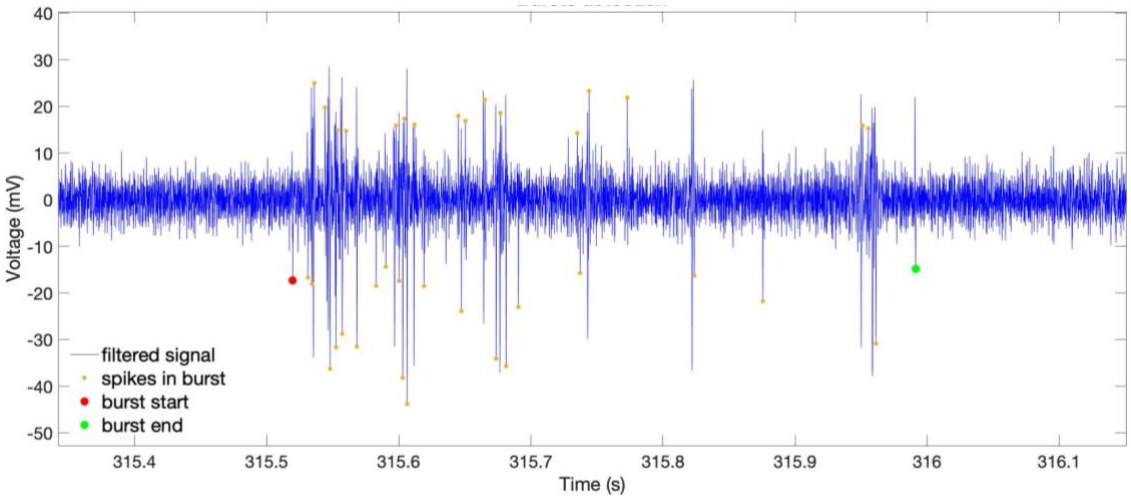


**Figure 3.6:** Example of a detected burst, where the red dot indicates the start of the burst, the green dot indicates the end of the burst, and the small yellow dots indicate the spikes inside the burst.

## Synchronization

To determine the synchronization of a cultured neuronal network, I used the sliding window method. The window length was set to approximately the same size as an action potential (AP) to ensure that it captures each spike. This window moves through individual channels over the entire time range of measurement (10 minutes) and detects spikes. A binary recording of 1 and 0 is assigned to each time window based on the presence or absence of a spike. For each time window, the synchronization measure is determined by summing these numbers, and then the overall average synchronization of all windows is calculated. The degree of synchronization is expressed as a percentage and indicates the average proportion of synchronously detected spikes to all detected spikes.

Following *Figure 3.7* shows an example of graphical visualization of neuronal network synchronization. From the plot we can also get approximate information about the overall activity of the neurons and the distribution of spikes for each recording eletrode over time.

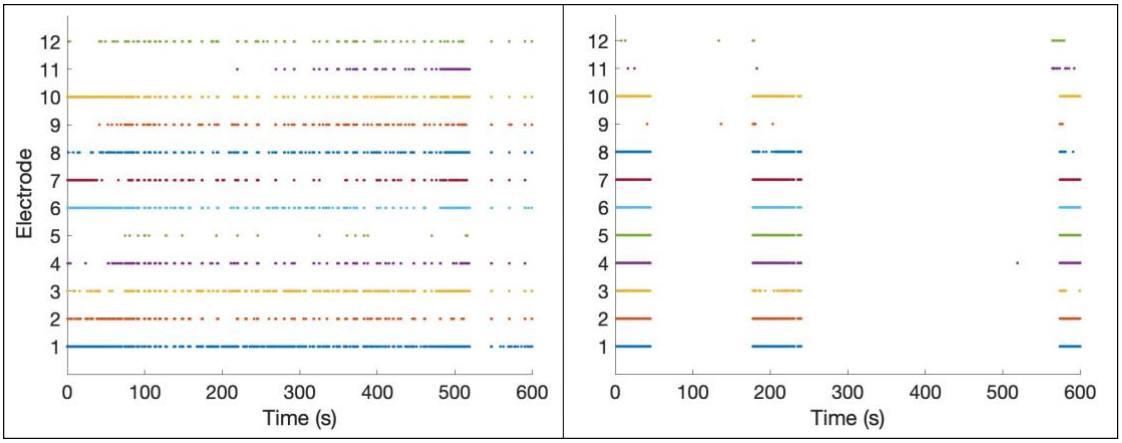


**Figure 3.7:** Figure of two examples of raster plots from two different measurements showing the temporal distribution of spikes on individual electrodes. These plots a visual assessment of the degree of synchronization of the neural network.

## Parameters

MFR (Mean Firing Rate) is the average spike frequency in units of spikes/s.

MBR (Mean Bursting Rate) is the average burst frequency in units of bursts/min.

BD (Burst Duration) is the average burst duration in units of seconds.

ISI (Inter Spike Interval) is the average

IBI (Inter Burst Interval) is the average time interval between two bursts (their initial spikes are taken). The basic unit is second.

SIB (Spikes Intra Bursts) is the average of number of spikes in a burst.

SIB/NS (Spikes Intra Burst/Network Spikes) is percentage of spikes in bursts to all spike

# 4 Results

This chapter presents the results of processing electrical signals from neuronal cultures obtained using measurements on a microelectrode array (MEA). My analysis focused on monitoring changes in neuronal activities following the application of neurostimulants (see *Chapter 3.2* above). To identify these changes, I extracted basic electrophysiological features such as spikes, bursts, and synchronization, and calculated parameters (see Chapter 3.4.2) that characterize neuronal activity. Calculated parameters are then used to evaluate changes in neuronal activities.

This chapter describes changes in the electrical activity of neurons during its development (*Chapter 4.*1), after MDMA stimulation (*Chapter 4.2*), and changes after 25CN-NBOMe stimulation (*Chapter 4.3*). Results are reported using median and interquartile range (IQR) unless otherwise stated in the text.

## 4.1 Development of neuronal culture over time

*Figure 4.1* shows the changes in electrophysiological parameters of MBR and ISI during the development of a culture intended for testing MDMA.

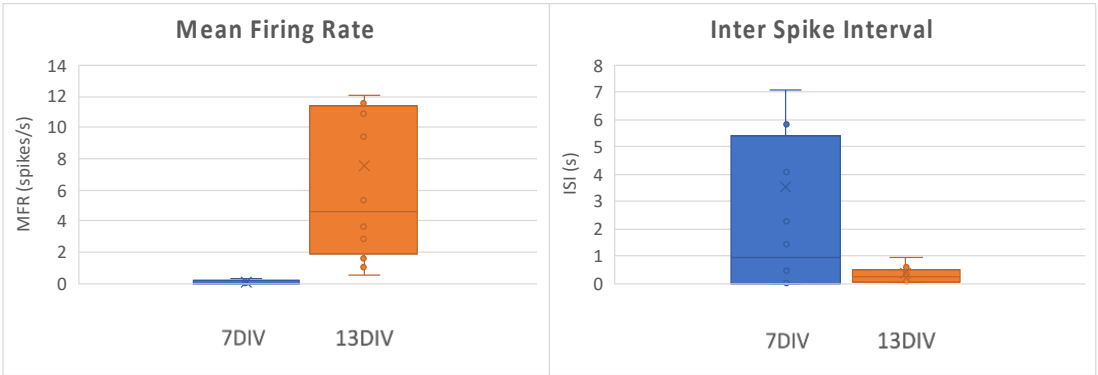


**Figure 4.1: (Left)** Change in Mean Firing Rate values of the cell culture prepared for MDMA testing on the seventh day in vitro (7DIV) and the thirteenth day in vitro (13DIV), which corresponds to the day of measurement. **(Right)** Change in Inter Spike Interval values of the same culture.

Median MFR 7DIV is 0,04 spikes/s (IQR 0,13 spikes/s). This value increased to 4,6 spikes/s (IQR 8,5 spikes/s) by 13DIV. The median ISI of 7DIV is 1 s (IQR 4,5 s). This value decreased to 0.2 s (IQR 0,3 s) by 13DIV.

In *Figure 4.2*, the change in neuronal network activity is visible in the cultured neurons for the MDMA experiment on the seventh day in vitro (7DIV) and the thirteenth day in vitro (13DIV).

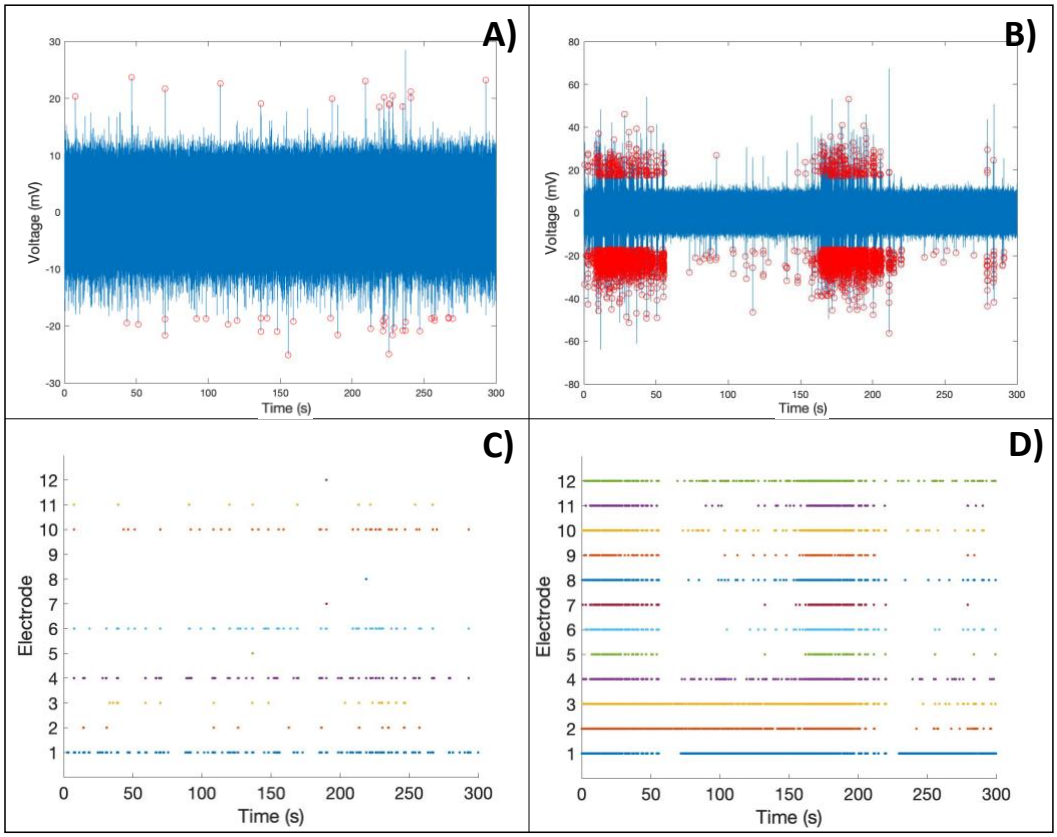


**Figure 4.2: (top)** Electrical signal of neurons for the experiment with MDMA **A)** seventh day in vitro (7DIV), **B)** on the thirteenth day in vitro (13DIV), the day of measurement. **(bottom)** Raster plot showing the occurrence of spikes in time (and synchronization) for twelve measuring electrodes **C)** on the seventh day in vitro, **D)** on the thirteenth day in vitro (13DIV), the day of measurement.

The average network synchronization value was 7DIV 20,1% (STD 16%). 13DIV increased to 82% (STD 38,4%).

The following two *Figures 4.3* and *4.4* show the same things as *Figures 4.1* and *4.2*, only for the cell line intended for testing 25CN-NBOMe.

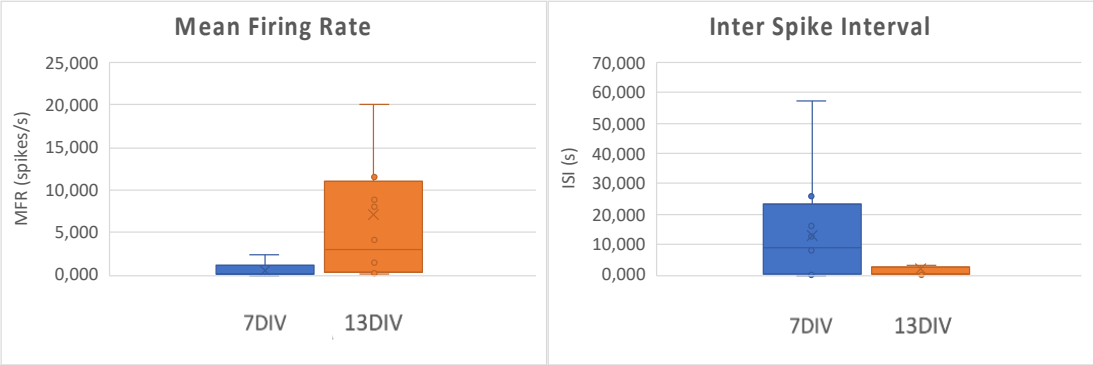


**Figure 4.3: (Left)** Change in Mean Firing Rate values of the cell culture prepared for 25CN-NBOMe testing on the seventh day in vitro (7DIV) and the thirteenth day in vitro (13DIV), which corresponds to the day of measurement. **(Right)** Change in Inter Spike Interval values of the same culture.

Median MFR 7DIV is 0,03 spikes/s (IQR 0,39 spikes/s), 13DIV 3 spikes/s (IQR 9 spikes/s). The median ISI of 7DIV is 9 s (IQR 18,2 s), 13DIV 0.4 s (IQR 1,8 s).

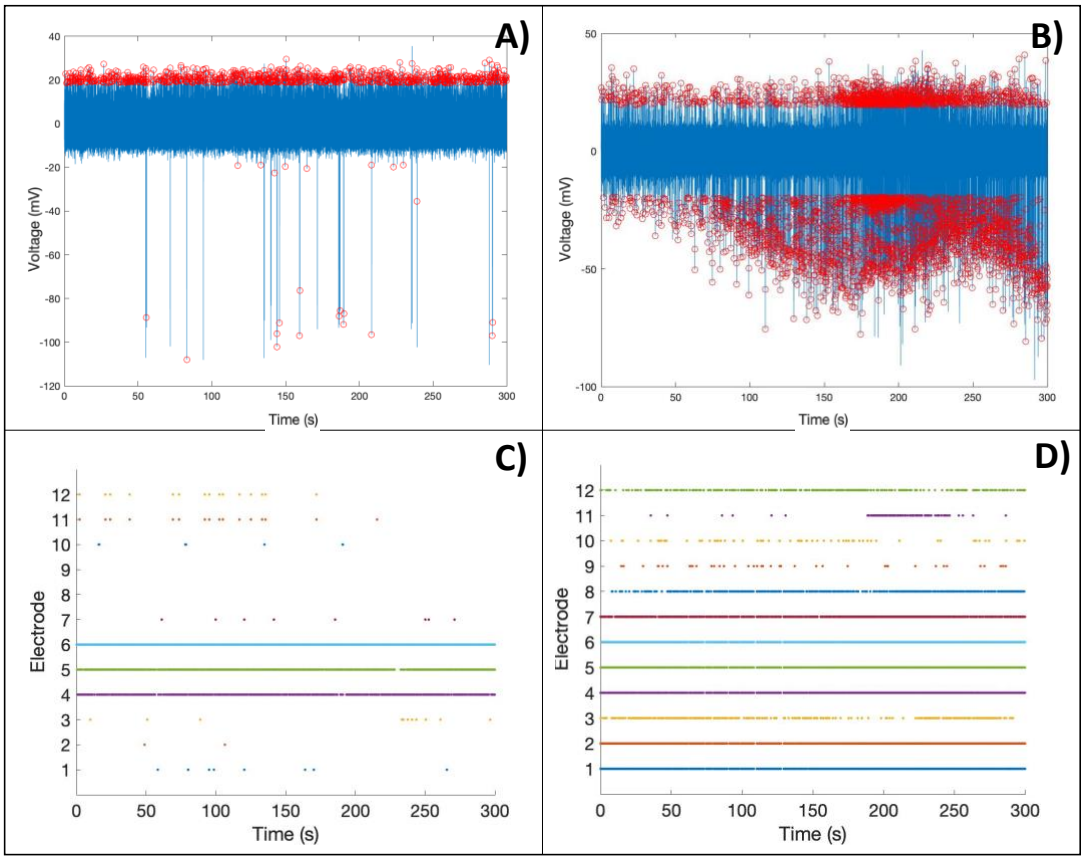


**Figure 4.4: (top)** Electrical signal of neurons for the experiment with 25CN-NBOMe **A)** seventh day in vitro (7DIV), **B)** on the thirteenth day in vitro (13DIV), the day of measurement. **(bottom)** Raster plot showing the occurrence of spikes in time (and synchronization) for twelve measuring electrodes **C)** on the seventh day in vitro, **D)** on the thirteenth day in vitro (13DIV), the day of measurement.

The average network synchronization value was 7DIV 4% (STD 19,5%). 13DIV increased to 63, 6% (STD 48,1%).

## 4.2 Application of MDMA

To stimulate neural cell cultures with MDMA, we used concentrations of 1 µM, 3 µM, 5 µM, and 10 µM. The hippocampal cell culture on which this substance was tested was thirteen days old from deployment (13 DIV). To calculate the threshold for detecting spikes, I used a multiple of 7 STDs for every channel for these measurements (see *Chapter 3.4.2*), which seemed to be optimal for the purposes of my work.

For this measurement, I used MC-Rack software (MultiChannel Systems, Reutlingen, Germany) to select 12 representative active electrodes, from which I obtained and analyzed electrical signals of the neuronal culture.

*Figure 4.5* shows three plots (for three electrodes) representing the activity of neural spikes over time at the top. In this case, the random value on the y-axis does not carry any information, it is just for the overlap elimination. On the bottom, there are signals represented in normal scales (time on the x-axis and voltage on the y-axis) for three different electrodes.

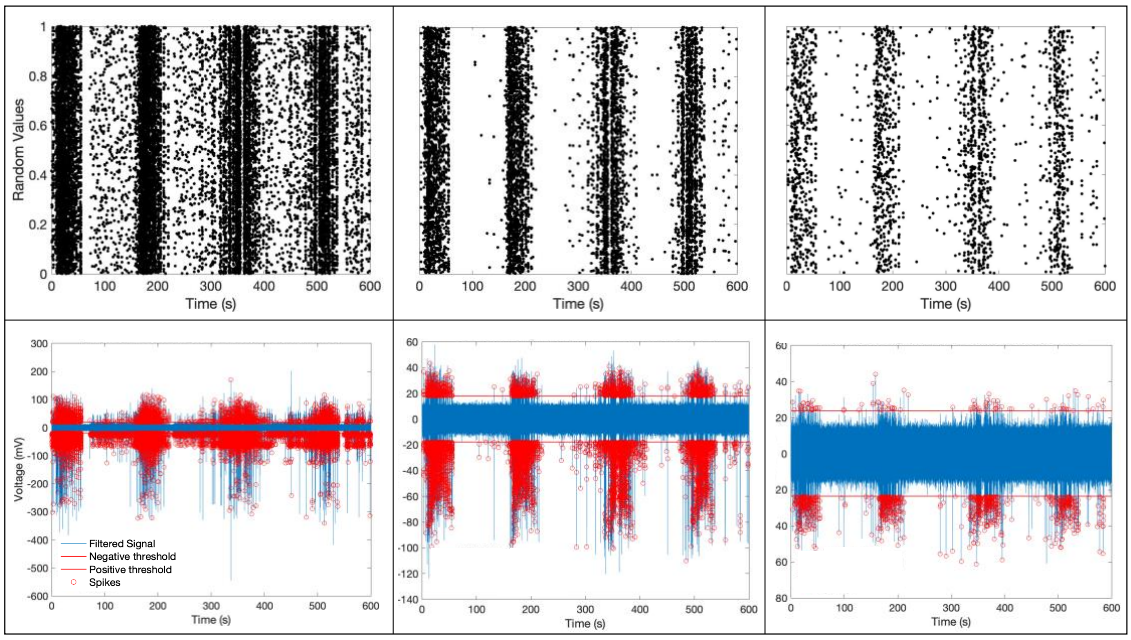


**Figure 4.5: (top)** Synchronized periodic activity of neurons recorded by three electrodes with varying noise levels recorded during the pre-stimulation measurement in the Neurobasal medium. **(bottom)** Extracted spikes (extracellular action potentials). The y-axis represents random numbers to prevent spikes from overlapping.

*Figure 4.6* shows the synchronization of each measurement. The chart shows a slight fluctuation during individual measurements. The highest degree of synchronization reached an average value of 82% (STD 38.5%) and was recorded during the first cell control in Neurobasal. The lowest degree of synchronization was achieved during the control measurement in ECS+ (average 58.3%, STD 49.3%). A trend of a slight decrease in synchronization can be seen after the application of MDMA, and a slight increase after each subsequent ECS+ rinse. However, these fluctuations are not statistically significant.

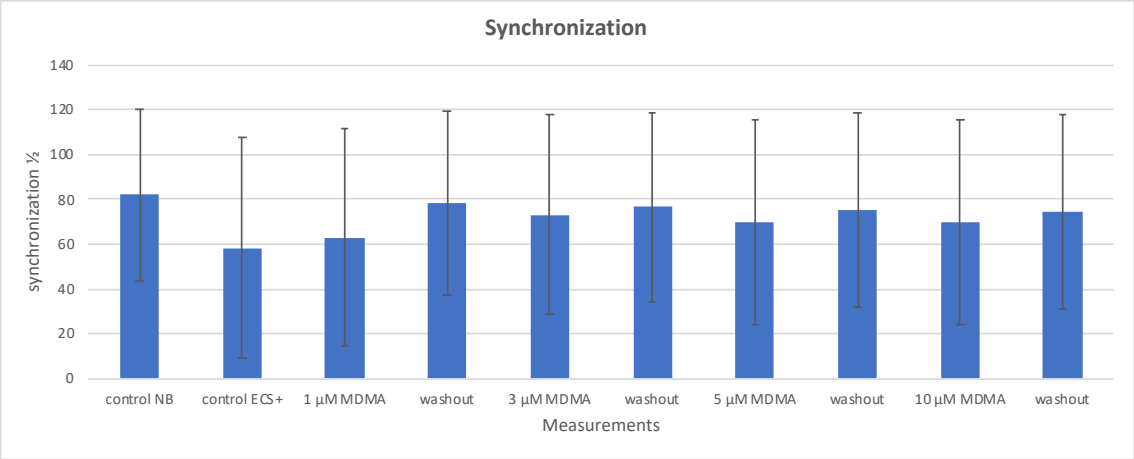


**Figure 4.6:** Neural network synchronization during measurements (experiment with MDMA). Each column represents the average synchronization for each measurement, with error bars indicating the standard deviation.

In the following raster plots for four different measurements shown in *Figure 4.7*, the degree of network synchronization is visually represented.

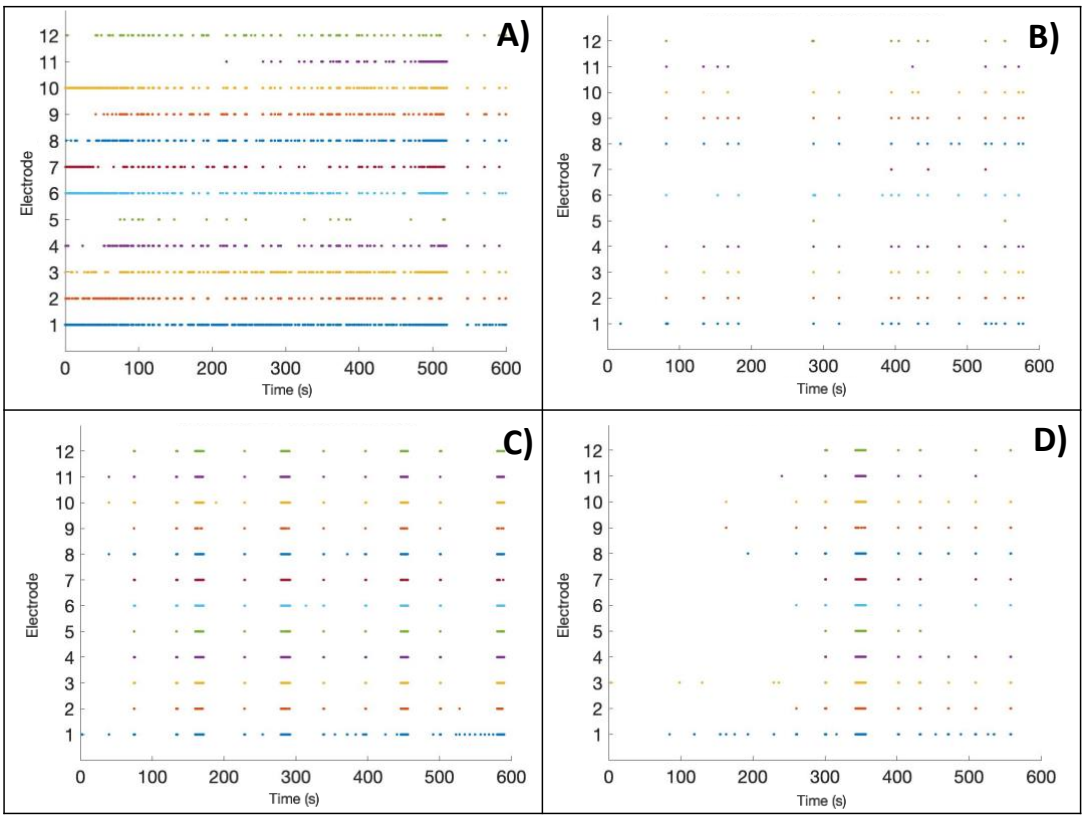


**Figure 4.7:** Raster plots showing the time distributions of spikes for individual electrodes across four different measurements, visually representing the degree of network synchronization. **A)** Raster plot of network synchronization during the control measurement in the ECS+. **B)** Measurement after the application of 1µM MDMA. **C)** Measurement after the application of the 10µM MDMA. **D)** Measurement after cell washout of ECS+ following the last application od 30µM MDMA. The x-axis is time and the y-axis is individual electrodes.

In the plot, it is visible that synchronization persists in all four measurements. However, the plots indicate a reduced firing frequency, for example, in the measurement after the application of 1µM MDMA compared to the negative control measurement in Neurobasal.

The following *Figure 4.8* summarizes the obtained result values of electrophysiological parameters during each measurement phase.

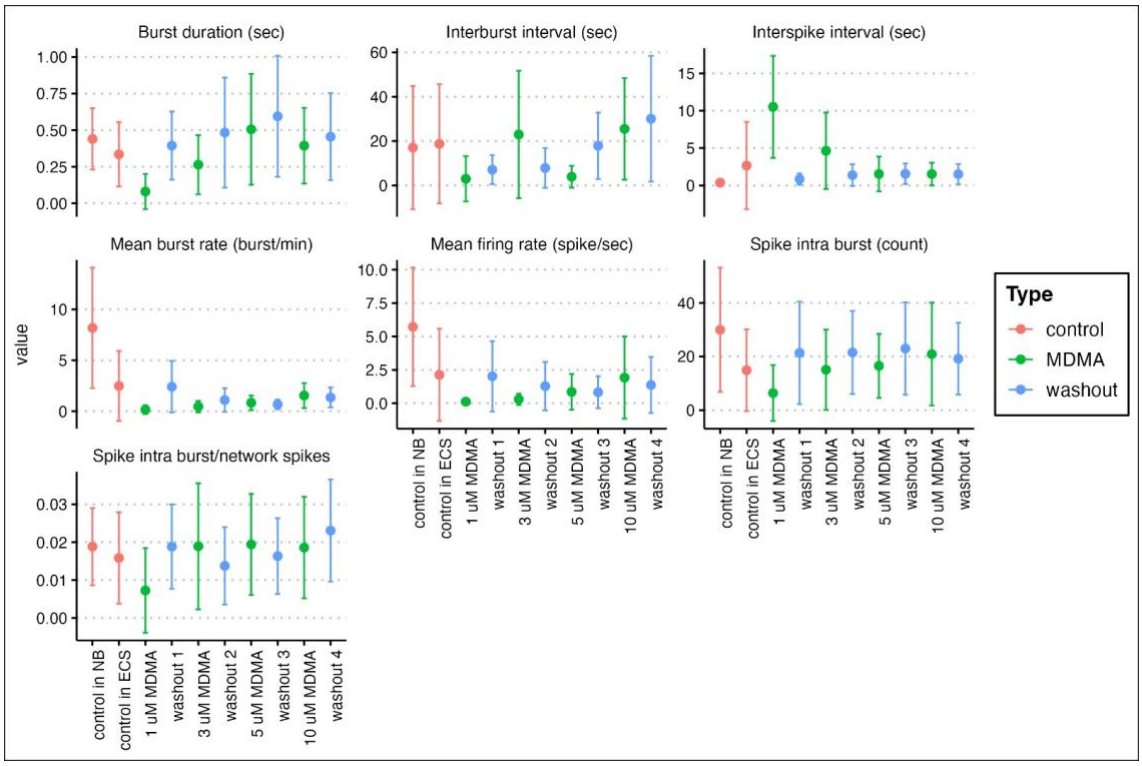


**Figure 4.8:** The resulting values of all electrophysiological parameters obtained during the measurement (experiment with MDMA)

The highest values of Mean Firing Rate (MFR) were observed during the control measurement of cells in the Neurobasal medium with a median of 4.6 spikes/s (IQR 8.5 spikes/s). The neural culture reached the lowest MFR values after the application of 1µM MDMA (median 0.09 spikes/s, IQR 0.07 spikes/s). No statistically significant change in MFR was observed during the measurements compared to the negative control (pure ECS+).

The neuronal culture reached the highest values of Mean Bursting Rate (MBR) during the control measurement in the Neurobasal medium (median 8.4 bursts/min, IQR 10.3 bursts/min). A statistically significant change compared to the negative control in ECS+ was observed in the MBR values after the application of 1µM MDMA (p-value 0.0105), with a median of zero (IQR 0.1 bursts/min).

Changes in the Burst Duration (BD) parameter compared to the negative control were not statistically significant in any of the measurements (p-value < 0.05). The highest median value of BD, 0.4 s (IQR 0.4 s), was observed in neuronal activity in ECS+ after washing out the 10 µM concentration of MDMA. Conversely, the lowest BD value with a median of zero (IQR 0.2 s) was measured after the application of 10 µM MDMA.

The highest median value of the Inter Spike Interval (ISI), 9.5 s (IQR 20.5 s), was measured after the application of 1 µM MDMA. The lowest ISI value was observed in the control measurement in the Neurobasal medium, with a median of 0.2 s (IQR 0.3 s). No statistically significant changes were confirmed in this parameter.

The highest mean value of the Inter Burst Interval (IBI), 23.3 s (IQR 20.4 s), was observed in cells after the application of 10 µM MDMA. The lowest IBI values were exhibited by the neuronal culture following the application of 1 µM MDMA. In this case, the median IBI value was zero, and this change compared to the negative control was statistically significant (p-value 0.00009). No significant changes in this parameter were observed in the other measurements.

The parameter Spikes Intra Burst (SIB) reached its highest median value of 22.5 (IQR 23.1) during the measurement of neurons in ECS+ after washing with 3 µM MDMA. However, this difference was not statistically significant. A statistically significant change in the SIB parameter compared to the negative control was confirmed in the measurement after the application of 1 µM MDMA (p-value 0.00389), where the mean value was zero (IQR 12).

The highest values of the Spikes Intra Bursts (SIB) parameter, with a median of 0.03 (IQR 0.02), were observed in the culture after washing with the highest concentration of MDMA (10 µM). Conversely, cells treated with 1 µM MDMA exhibited the lowest value of SIB/NS (zero median, IQR 0.02). No statistically significant changes in this parameter were observed in any of the measurements compared to the negative control ($p < 0.05$).

## 4.3 Application of 25CN-NBOMe

To stimulate neural cells with 25CN-NBOMe, we used concentrations of 1 mg/L, 3 mg/L, 10 mg/L, and 30 mg/L. Concentration units are given in mg/L in this case, as the estimate of tested concentrations for this substance is based on an article that tested this substance in vivo and reported concentrations units of mg/kg. [48]

For these measurements, I also chose a multiple of 7*STD for calculating the threshold for individual channels. I also used the same software to select 12 active electrodes that would represent the cell culture for the purposes of our study. The following *Figure 4.9* shows the time distributions of spikes from three different electrodes obtained during the control measurement in NB at the top. These plots again represent

time values on the x-axis and auxiliary random numbers on the y-axis to separate the spikes and enhance visualization. And the signals from three different measuring electrodes with detected spikes on the bottom. Both were obtained during the control measurement of neurons in the Neurobasal medium. In contrast to the previous cell culture used in the MDMA experiment, it is evident that the activity of these neurons has a different nature. In this case, the electrical activity is much more consistent and sustained throughout the entire time horizon.

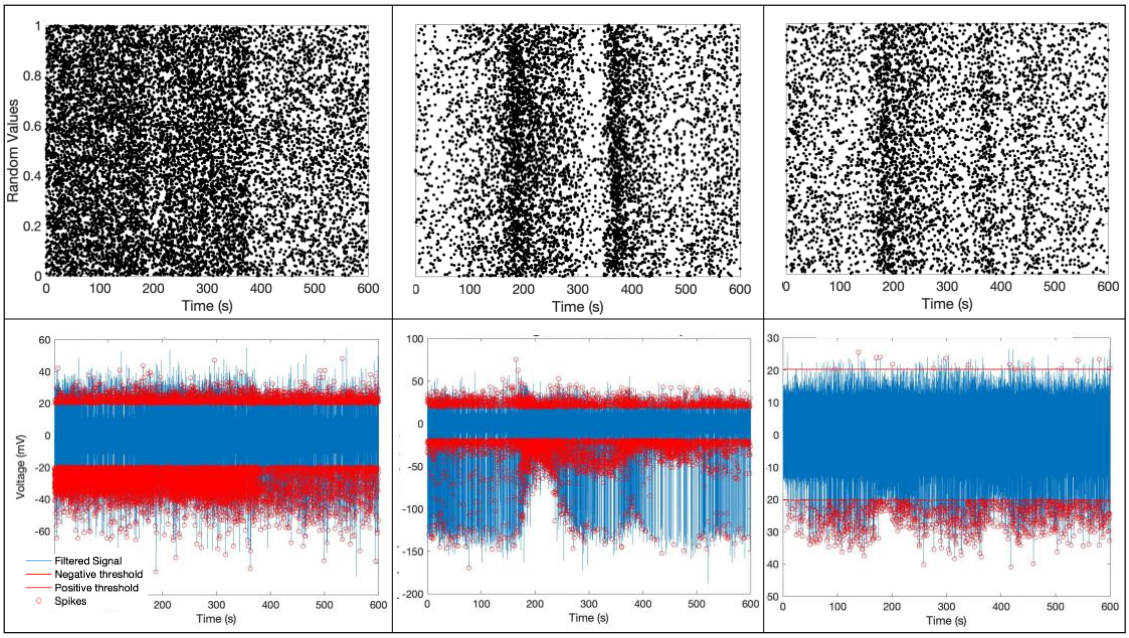


**Figure 4.9: (top)** Synchronized periodic activity of neurons recorded by three electrodes with varying noise levels recorded during the pre-stimulation measurement in the Neurobasal medium. **(bottom)** Extracted spikes (extracellular action potentials). The y-axis represents random numbers to prevent spikes from overlapping.

The following *Figure 4.10* illustrates the average network synchronization rates for each measurement. The highest synchronization rates were achieved in cells after the application of 10 mg/L 25CN-NBOMe. In this measurement, the average network synchronization was 76.6% (STD 42.2%). Conversely, zero synchronization was observed in cells after the application of 3 mg/L 25CN-NBOMe and 30 mg/L 25CN-NBOMe. These changes are statistically significant compared to the measured synchronization in the negative control (ECS+). Other changes in synchronization rates are not statistically significant. Note that the synchronization values for those concentrations are missing in the plot. I did not include them in the plot, because of their zero values.

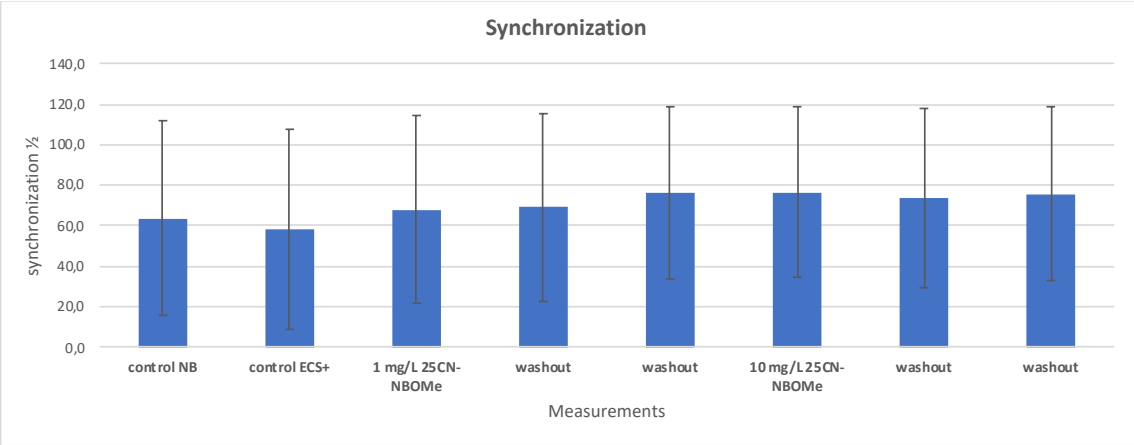


**Figure 4.10:** Neural network synchronization during measurements (experiment with 25CN-NBOMe). Each column represents the average synchronization for each measurement, with error bars indicating the standard deviation. Zero synchronization values for concentrations 3 mg/L and 30 mg/L of 25CN-NBOMe are not shown here.

The following raster plots in *Figure 4.11* provide a visual representation of network synchronization for four different measurements in the form of temporal distributions of spikes for individual electrodes.

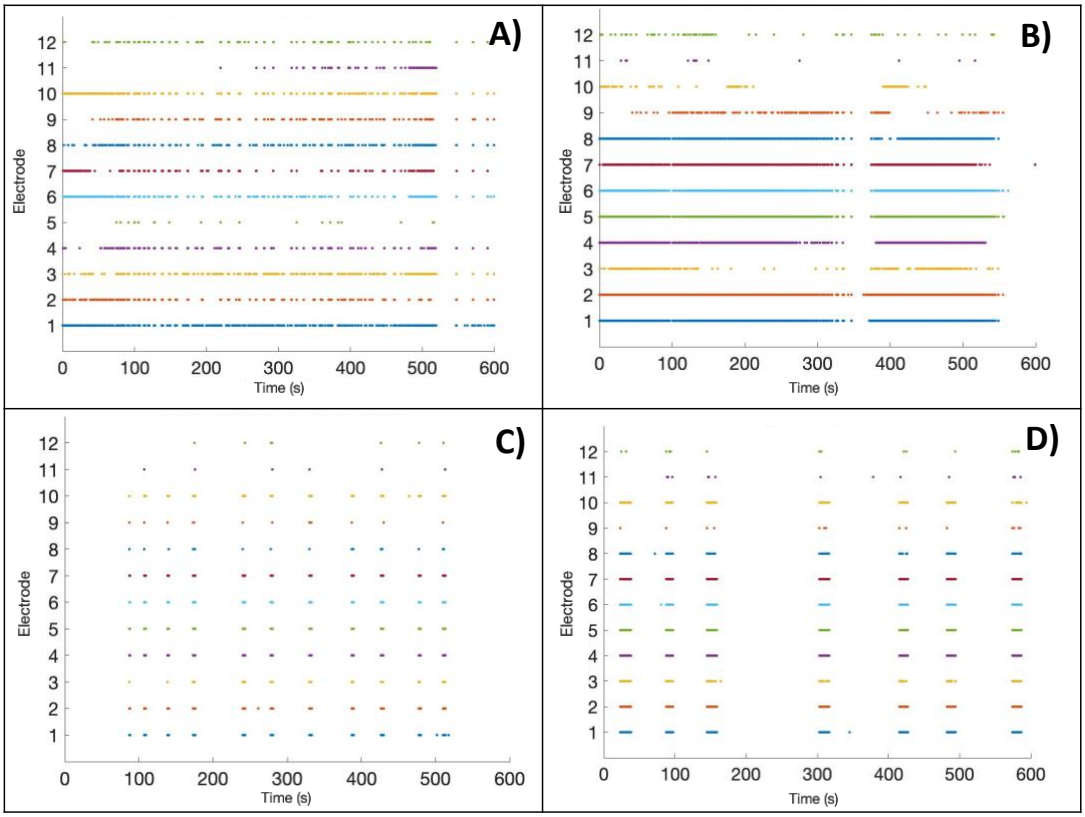


**Figure 4.11:** Raster plots show the time distributions of spikes for individual electrodes across four different measurements, visually representing the degree of network synchronization. **A)** Raster plot of network synchronization during the control

measurement in the ECS+. **B)** Measurement after the application of the 1 mg/L of 25CN-NBOMe. **C)** Measurement after the application of 10 mg/L of 25CN-NBOMe. **D)** Measurement after cell washout of ECS+ following the last application. The x-axis is time and the y-axis is individual electrodes.

From the plot of synchronizations, it can be observed, for example, an increase in inter-spike intervals (ISI) in cells after the application of 1 mg/L 25CN-NBOMe. compared to the negative control, and conversely, a subsequent decrease in ISI in the measurement of activity after the application of FEA 10 mg/L 25CN-NBOMe.

The following *Figure 4.12* summarizes the obtained result values of electrophysiological parameters during each measurement phase. The designation "FEA" in this graph means the abbreviation for phenylethylamine, i.e. for 25CN-NNBOMe.

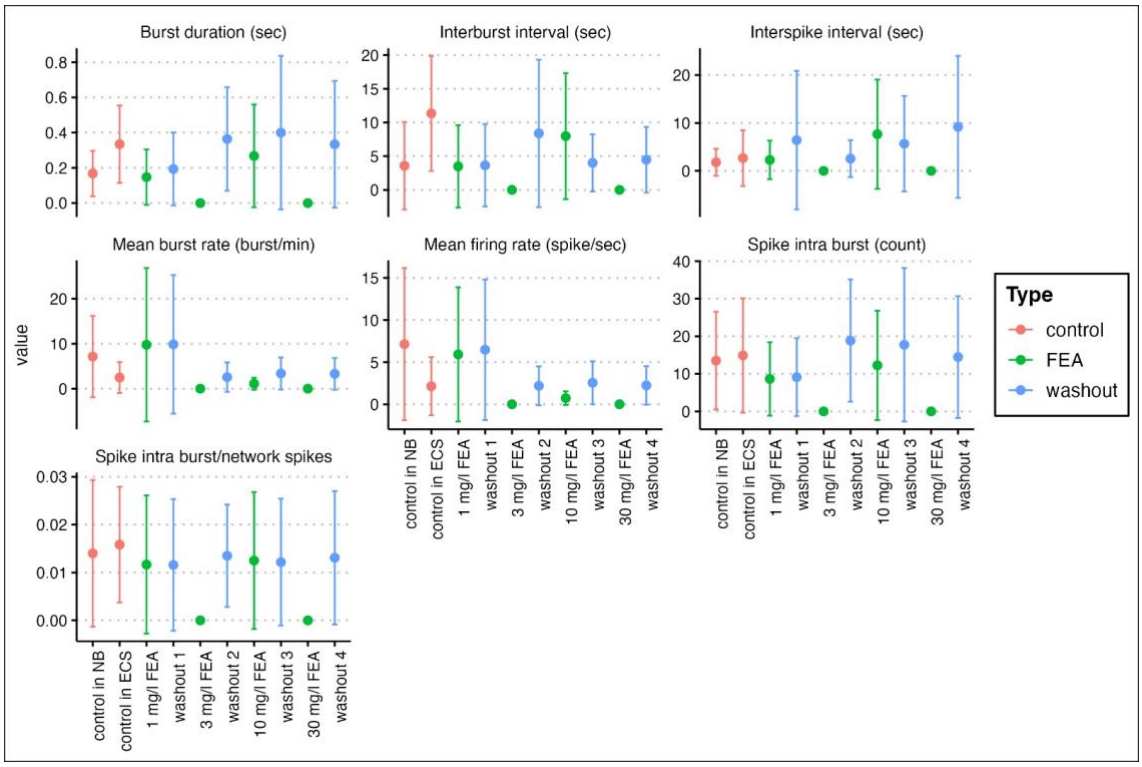


**Figure 4.12:** The resulting values of all electrophysiological parameters obtained during the measurement (experiment with 25CN-NBOMe)

The highest mean value of MFR, 3.1 spikes/s (IQR 9.3 spikes/s), was observed in neurons during the measurement in ECS+ after washing with 1 mg/L of 25CN-NBOMe. Statistically significant changes with zero medians compared to the negative control were observed after the application of concentrations of 3 mg/L and 30 mg/L of 25CN-

NBOMe. In the overall distribution of MFR parameter changes for all measurements, these are the only significant changes.

The only statistically significant changes in the parameter of Mean Bursting Rate (MBR) among the measurements are the zero MBR values in the measurement after the application of 10 mg/L and 10 mg/L of 25CN-NBOMe again. Other MBR values do not significantly differ ($p < 0,05$), with the highest values observed in the control measurement in the Neurobasal medium (median 3 bursts/min, IQR 9 bursts/min).

The highest median Burst Duration (BD) value of 0.5 s (IQR 0.6 s) was measured in neurons in ECS+ after the washout of 3 mg/L of 25CN-NBOMe. The smallest, again zero BD values, were also measured after the applications of 3 mg/L and 30 mg/L of 25CN-NBOMe, representing the only statistically significant changes in this parameter across all measurements.

The highest Inter Spike Interval (ISI) values were achieved in the neuronal culture after the application of 10 mg/L of 25CN-NBOMe. In this measurement, ISI has the highest median value but also the highest variability (median 3 s, IQR 24.4 s). In the measurements following the application of 3 mg/L and 30 mg/L 25CN-NBOMe, zero values are observed again.

The highest Inter Burst Interval (IBI) value was observed in the negative control measurement in ECS+ (median 15.5 s, IQR 15.2 s). The subsequent change in another measurement compared to this negative control, with a median IBI value of 0.5 s (IQR 3.8 s), is statistically significant. Other significant deviations are again observed in the measurements following the application of 3 mg/L and 30 mg/L of 25CN-NBOMe. The remaining median IBI values do not significantly differ from the negative control.

The Spikes Intra Burst (SIB) parameter reached the highest values when measuring cells in ECS+ after washout of 3 mg/L 25CN-NBOMe (median 20, IQR 29.4). However, the greatest variability is observed when measuring after the application of 3 mg/kg 25CN-NBOMe. The two zero values are also reflected here (3 mg/L and 30 mg/L).

The highest value of the ratio parameter Spikes Intra Burst/Network Spikes (SIB/NS), was observed in the measurement of neuronal activity in ECS+ after the 3 mg/L of 25CN-NBOMe washout (median 0.02, IQR 0.02). Once again, the only statistically significant changes are observed in the measurements following the application of 3 mg/L and 30 mg/L of 25CN-NBOMe.

# 5 Discussion

In the context of my thesis, I focused on processing electrical signals from cultured hippocampal cell cultures and evaluating changes in their activity based on stimulation with psychoactive substances MDMA (also known as ecstasy) and 25CN-NBOMe, classified as new psychoactive substances. The tested cell cultures, despite being the same type of cells, having the same deployment and cultivation conditions, and being the same age on the day of measurement (13DIV), displayed different behaviors and patterns of spontaneous activity, which is not unusual, each culture is unique. However, this is a complication for the subsequent evaluation of the effects of psychopharmaceuticals.

First, it was necessary to pre-process the signals obtained using microelectrode arrays. For processing these signals, I created scripts in the Matlab programming environment.

After selecting active electrodes that will represent the tested cell culture for the purposes of my work and converting data into a format compatible with Matlab (.txt) using MC Rack and MC_Datatool software (MultiChannel Systems, Reutlingen, Germany), I filtered the data. I used a digital filter with parameters and frequency ranges commonly used for filtering these types of signals. A 2nd order bandpass Butterworth FIR filter with frequency bands 300-6000Hz. [57]

For subsequent spike detection, I calculated the appropriate threshold for the signals. I chose the threshold to safely avoid the detection of false positive spikes from noise. The threshold was automatically calculated using a sliding window from the local mean value of noise (more of an estimate of the mean value) of each measuring channel, and spikes were detected as local minima (or maxima).

For spike detection, I used both positive and negative thresholds to capture spikes of both polarities, and I introduced a refractory phase to avoid double detection.

The threshold method for spike detection is a commonly used approach. The disadvantage of this static method compared to setting an adaptive threshold or detecting spikes based on their shape, can be the loss of some data or, conversely, the detection of false data. However, for the purposes of my work, this method is suitable and sufficient.

For burst detection, I set several conditions, such as the minimum duration of the burst, the minimum number of spikes within the burst, and the maximum inter-spike distance.

I calculated network synchronization, which is characterized by the synchronous generation of spikes by several neurons at a given time, using a sliding window of the size of approximately one action potential, which scanned the measuring channels and recorded common peaks.

The electrophysiological parameters that I extracted from the measured data and then used to assess changes in neuronal activity were Mean Firing Rate (MFR), Mean Bursting Rate (MBR), Burst Duration (BD), Inter Spike Interval (ISI), Inter Burst Interval (IBI), Spikes Intra Burst (SIB) and Spikes Intra Burst/Network Spikes (SIB/NS). These parameters were used to describe the electrical signals and assess changes.

Since the data does not follow a normal distribution, I used the Kruskal-Wallis test to assess the statistical significance of changes in individual values. The significance level was set at 0.05.

For the interpretation of results, due to the diversity of my data, I decided to use the median and interquartile range (IQR). These metrics are less sensitive to outliers.

## 5.1 Development of neuronal culture over time

In this chapter, we tracked the time development of neuronal network activity over time. We expected increasing spike parameters. From the obtained parameters Mean Firing Rate (MFR), Inter Spike Interval (ISI) values, and plots of the time distribution of the signals and spikes, neurons demonstrated an increasing frequency of spikes and decreasing interval between spikes with the age of the cell line. Also, the synchronization was increasing with the age of culture. These are expected results indicating normal growth of the neuronal line.

## 5.2 Application of MDMA

MDMA (ecstasy) is a stimulant substance frequently found in the illegal drug market. Despite the high risk and confirmed cases of intoxication, it is very popular and purchased primarily for its effects, which include mood enhancement and increased empathy. [46]

My goal was to analyze the influence of MDMA on the neuronal system and to determine whether the effect of this stimulant is dose-dependent. Therefore, we applied MDMA with various increasing concentrations (1 µM, 3 µM, 5 µM, and 10 µM) and washed the cells between individual applications. Spontaneous activity of the tested neural network for this purpose had a specific periodic course. (see *Figure 4.5 Chapter 4.2*).

Based on the knowledge from the literature, we expected a decrease in neural activity with MDMA. The study by L. Hondebrink et al (2016) reported a decrease in neural activity after MDMA exposure with an IC50 value of approximately 100 µM in *in vitro* testing and measurements on microelectrode arrays. [45]

As expected, the decrease in neuronal activity in our experiment is also noticeable, but more so on bursts rather than spikes. The influence of MDMA on neuronal activity was evident in the form of a decrease in the Mean Firing Rate (MFR). With each successive washing after the application of individual concentrations of MDMA, the average MFR slightly increased and then decreased slightly again after the stimulation with MDMA. However, after the application of the last concentration (10 µM), the MFR slightly increased again. It should be noted, however, that these changes were not statistically significant according to statistical tests. The Mean Burst Rate (MBR) had a similar pattern as the MFR. However, the first applied concentration of 1µM MDMA statistically significantly decreased the MBR almost to zero. This was followed by the same trend of fluctuation as with MFR. The interval between bursts (Inter Burst Interval, IBI) also statistically significantly dropped to zero after the application of 1µM MDMA, as no burst occurred in the culture during this measurement. Towards the end of the measurement (at the last application and washing), it tended to increase again.

The duration of bursts had rather a stationary or slightly increasing character, but statistically remained unchanged (except the 1µM concentration again). The distance between spikes (Inter Spike Interval, ISI) sharply increased (though not statistically significant) after the addition of 1µM MDMA and then tended to "settle" between the individual applications of other concentrations and washing. The Spike in Burst (SIB) value, except for the application of 1µM MDMA, where the SIB was zero, did not significantly change during the measurement. And the relative value of SIB/NS also did not, with a slightly increasing character.

The synchronization of the neural network did not change significantly during the application of individual concentrations of MDMA.

It should be noted that the first washing of neurons with the salt solution (ECS+) also caused a change in parameters. Therefore, it is necessary to consider the possibility that this change of environment also contributes to the influence on the network activity and may interfere with the effects of MDMA.

Regarding washability, the trends in some parameter changes across measurements suggest that cell activity tends to return to its "normal" activity after washing out individual concentrations of MDMA. The effect of MDMA seems to be washable, but this is not statistically proven and it's not possible to determine to what extent. The question remains whether this would be the case with longer exposure of this substance to the cell culture.

The increase in MFR and MBR after the application of 10µM MDMA may indicate that the neuronal culture has already adapted to these changes and is starting to stabilize into its "original" form. The rapid decrease in MBR upon the addition of 1µM MDMA could mean just a sudden response of neurons to a change in the environment. But it may also represent a reduction in neuronal activity through a direct effect of MDMA, which is consistent with the literature. It would probably be necessary to test for longer and let the substance incubate in the culture for a longer time.

As MDMA acts on the release of several neurotransmitters, such as serotonin, the reduction of network activity could be caused by the depletion of these neurotransmitters. Furthermore, there could be toxic damage to neurons due to the formation of free radicals, but this is not very likely given the course of neural activity during the increase of applied concentrations.

We found that the application of MDMA in our case did not lead to significant changes in the indicated spike electrophysiological parameters, but the application of 1uM MDMA led to a significant decrease in the burst parameters.

## 5.3 Application of 25CN-NBOMe

Another substance whose effects on neuronal culture were examined was 25CN-NBOMe, a new psychoactive substance, specifically in the phenethylamine group. We again tested the substance at several concentrations in the same regimen as before to

determine whether its effects are dose-dependent. We applied one concentration, rinsed the cells with ECS+, and applied a higher concentration. In this way, we tested four concentrations, 1 mg/L, 3 mg/L, 10 mg/L, and 30 mg/L. The activity of the cell culture was more evenly timed than with the cells in the previous MDMA testing. The spikes were much more evenly distributed (see *Figure 4.9 Chapter 4.3*).

The study of Šíchová K. et al (2022) investigating the behavioral effects of 25CN-NBOMe in rats observed reduced locomotor activity at concentrations of 0.2mg/kg and 1mg/kg. The influence of this substance on the activity of nerve cells *in vitro* has not yet been studied, but since 25CN-NBOMe acts as a selective agonist of the 5-HT2A membrane receptor, it is evident that by binding, it can affect the permeability of ion channels and thus the excitability of cells. From the mentioned study, we could assume that 25CN-NBOMe could have an inhibitory effect on the electrical activity of neurons. [50]

The most significant change in the obtained electrophysiological parameters came after the application of the second and fourth concentrations of 25CN-NBOMe (3 mg/L and 30 mg/L). Here all parameters fell almost to zero value, as spikes and bursts in these measurements almost completely disappeared. This is a statistically significant change, but the question is what a longer recording would do with these results after applying 3 mg/L 25CN-NBOMe and 30 mg/kg 25CN-NBOMe. Upon washout of these concentrations, both spike and burst activity reappeared. I assume that the effect of 25CN-NBOMe might me partially reversible under these experimental conditions.

The synchronization of the network gradually increased slightly (except for zero values in applications 3 mg/L NBOMe and 30 mg/L 25CN-NBOMe). The gradual, more periodically behaving activity of neurons was also evident from individual plots of electrical signals. However, these changes in synchronization were not confirmed as statistically significant. The frequency of bursts (MBR) does not change significantly, except for the application of the 2nd and 4th concentrations of 25CN-NBOMe, where the MBR is zero.

The distance between spikes slightly decreased after the application of 1 mg/kg 25CN-NBOMe but increased after the application of 10 mg/L 25CN-NBOMe (changes were not statistically significant). The ISI values after rinsing 25CN-NBOMe are almost the same. The distance of bursts decreased after the application of the first concentration

of 25CN-NBOMe, but after a significant change in almost no cell activity after the application of 3 mg/L 25CN-NBOMe, the average IBI increased, and it didn't change much afterward. The duration of bursts (BD) did not change significantly during the measurements (except for zero values after applications 3 mg/L 25CN-NBOMe and 30 mg/L 25CN-NBOMe), but their values after applications and washouts were lower than in the negative control measurements. The SIB parameter always slightly decreased after the application of 25CN-NBO and increased after the rinsing of this substance with ECS+.

It should be noted again, that the first washing of neurons with the salt solution (ECS+) also caused a change in parameters. Therefore, it is necessary to consider the possibility that this change of environment also contributes to the influence on the network activity and may interfere with the effects of 25CN-NBOMe.

It seems that the application of 25CN-NBOMe to nerve culture caused higher, but statistically insignificant, synchronization. Also, the application of two key concentrations of 3 mg/L and 30 mg/L of 25CN-NBOMe caused a significant suppression of neural activity visible both in the spike electrophysiological parameters and in the burst ones. However, it is strange that the concentration of 10 mg/L 25CN-NBOMe did not cause such a rapid inhibition in parameters. As I said before, it would be necessary to measure over a longer time horizon. It is possible that periodic bursts of spikes appear in the signals, only with long pauses between them, which we could not catch within a ten-minute time window. To confirm or refute this assumption and overall for a more specific description of the effects of 25CN-NBOMe, further research would be needed.

In the scope of this work, we had another psychoactive substance available for testing, which was deschloroketamine (DCK). However, the hippocampal culture prepared for this experiment was not in sufficient condition on the day of the experiment (it was already the third week in vitro). We proceeded with the experiment, but during the signal processing, I evaluated that such low activity is not sufficient for our purposes, and the potential results of stimulation with this substance would be inconclusive in this case.

# 6 Conclusions

In this study, I used cultured neuronal cells to investigate the effects of psychoactive substances, MDMA and 25CN-NBOMe, on the nervous system, and assess these effects through electrophysiological parameters.

We measured the activities of hippocampal cell cultures (both spontaneous and stimulated) in the form of electrical signals using in vitro microelectrode arrays (MEA). During the experiments, we stimulated the neuronal cultures with psychoactive substances and recorded changes in electrical activity and explored whether these effects are dose-dependent. We therefore tested various concentrations of the neurostimulants with intermediate washout periods from the cells. The psychoactive substances tested in this study were MDMA (also known as ecstasy) and 25CN-NBOMe, which belongs to the group of new psychoactive substances within the phenethylamine class.

I processed the electrical signals and detected basic electrophysiological features such as spikes, bursts, and network synchronization, quantified the neuronal activity using seven calculated parameters, including Mean Firing Rate (MFR), Mean Bursting Rate (MBR), Inter Spike Interval (ISI), and others. These parameters were used to compare the signals between individual measurements.

During the stimulation of cells with MDMA, I observed a statistically significant decrease in the number and frequency of bursts when applying 1µM MDMA to the cells. Network synchronization did not show significant changes at any of the applied concentrations. In the other parameters, no statistically significant changes related to MDMA were observed. The neuronal activity tended to return to its pre-stimulation activity pattern during the negative control after washout. This could suggest partial reversibility of MDMA effect.

Substance 25CN-NBOMe significantly inhibited effect on the neuronal culture activity at concentrations of 3 mg/kg and 30 mg/kg of 2CN-NBOMe. The record shows no bursts and almost no spikes during these periods. Interestingly, the cellular culture after applying 10 mg/kg did not exhibit the same significant suppression of activity. Network synchronization slightly increased during the application of increasing concentrations of 25CN-NBOMe, but these changes were not confirmed to be statistically significant.

As the limitations of my work, I see the use of limited concentrations of neurostimulants, which it would be interesting to expand to higher ones. Also, in the fact that the experiments were not repeated, based on which the results obtained could not be verified by multiple measurements.

Although the effects of MDMA and 25CN-NBOMe on neuronal cultures from my results are not entirely clear and the results obtained would need to be validated by further research, this study outlines a direction of future investigations on the same topic and potential expected outcomes. It would be beneficial to focus on longer time intervals for recording electrical signals and longer exposure times of these stimulants to neurons in cell media.

# List of Literature


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