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Impact of cannabidiol on mitochondrial activity modulation in N18TG2 cells

Bachelor's thesis

University of Veterinary Medicine Vienna

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1 Introduction

1.1 The endocannabinoid system

The endocannabinoid system (ESC) is an endogenous receptor-ligand system that is part of the nervous system. Its constituent parts are located all over the body. Besides several other functions in the central nervous system (CNS) as well as in the periphery, it serves as a communication medium between the brain and the body. Thus, it not only affects the brain but also other important systems like the immune, cardiovascular and respiratory system, neuronal development and bone formation, and the release and impact of hormones. Additionally, the energy metabolism and other cellular processes are modulated by the ECS. The levels of endocannabinoids, which are the endogenous ligands of the ECS, constantly adapt to environmental conditions to preserve homeostasis. Thereby, not only pathological perturbations like neuroinflammation or chronical stress, but also physiological processes like thinking, learning and sleeping are influenced (De Petrocellis & Di Marzo, 2009). The following chapter is going to describe the three parts of the ESC, including two main membrane receptors, at least two known endogenous ligands, and metabolic enzymes that catalyze the formation and metabolism of the endocannabinoids that are needed to achieve these functions (Lowe et al., 2021).

1.1.1 Receptors of the endocannabinoid system

There are two known G protein-coupled receptors that are a major part of the ESC, the cannabinoid receptors 1 and 2 (CB1R, CB2R). Even though the endocannabinoids and phytocannabinoids, which are both ligands of the ECS, are lipophilic, they target these receptors on the cellular surface to pass the cellular membrane. Additionally, other targets of the cannabinoids to enter the cell are discussed. The CB1 and CB2 receptors are wide-spread all over the body, to achieve many biological effects. They not only appear in the CNS but also in other organs, tissues, glands and even on immune cells. (Lutz, 2020; Maroon & Bost, 2018; Zou & Kumar, 2018).

CB1Rs are found all over the body, however, they are predominantly located in the CNS. Furthermore, CB1Rs can be found in the peripheral nervous system (PNS). All types of cells in the brain including neurons, astrocytes, oligodendrocytes and microglia can express them. In

contrast to brain regions like the central amygdala where CB1 receptors are very rare, other regions like the hippocampus, the neocortex, the basal ganglia and the cerebellum show high levels of CB1R. The latter are important for memory, emotion, pain and movement, which indicates the psychoactive effect of its agonists. They can be found in GABAergic and glutaminergic neurons as well as in other cells that are part of the neurotransmitter system implicating that they can have inhibitory and excitatory effects on the postsynaptic neurons (Lutz, 2020; Maroon & Bost, 2018; Zou & Kumar, 2018).

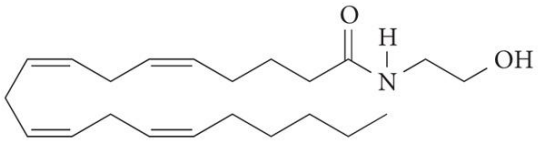
CB2Rs are found in peripheral tissues all over the body including the PNS. Similar to CB1R, they play a role in many biological processes in the cardiovascular system, the gastric and hepatic system and others. Most commonly, they are found in peripheral immune cells and in immune cells of the brain, the microglia, in which their main responsibility is to mediate the immunomodulatory impact of cannabinoids. Furthermore, they are found in neurons such as in glutamatergic neurons in the hippocampal region and dopaminergic neurons in the mesencephalon. Consequently, agonists of the CB2R can play a role in neurological activities like movement, cellular learning, pain regulation, drug addiction and neuroinflammation. Interestingly, the CB2R located in these regions are upregulated under pathological conditions like chronic pain, neuroinflammation or stroke (Lutz, 2020; Zou & Kumar, 2018).

1.1.2 Endocannabinoids

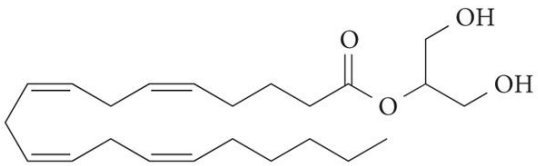
Endocannabinoids are small lipophilic mediators which are endogenous ligands of CB1R and CB2R. So far, arachidonoyl-ethanolamide (anandamide) and 2-arachidonoyl-glycerol (2-AG) are the best-studied substances that belong to this group (figure 1). While anandamide shows only weak binding to CB1R, 2-AG seems to have full agonistic properties to CB1R as well as to CB2R.

Both endocannabinoids, 2-AG and anandamide, are derivatives of the arachidonic acid and therefore belong to the eicosanoids. However, the syntheses and degradations of the molecules as well as the involved enzymes are rather different. They both have two different production pathways. Anandamide is made from N-acyl-phosphatidylethanolamines through the phosphodiesterase. For 2-AG, the hydrolysis of phosphatidylinositol by phospholipase C and diacylglycerol lipase via the intermediate product diacylglycerol is the major pathway. This synthesis takes place at the postsynaptic neuron and requires stimulation by a neurotransmitter.

The signal further runs retrograde and subsequently activates CB1R at the presynapse. This inhibits the release of neurotransmitters into the synaptic cleft. Depending on the cell type, different functions are triggered by this signal (Battista et al., 2012; Tsuboi et al., 2018).



Arachidonoyl ethanolamide



2-Arachidonoyl glycerol

Figure 1. Molecular structure of the endocannabinoids 2-arachidonoyl-glycerol and arachidonoyl-ethanolamid (anandamide) (Mechoulam et al., 2014)

1.1.3 *Cannabis sativa* and its phytocannabinoids

Phytocannabinoids are the herbal counterparts of the endocannabinoids and are obtained from *Cannabis sativa* (figure 2). It is a very versatile plant native to Central Asia. Not only the numerous phytocannabinoids, whose psychoactive effect were already recognized during ancient times, but also the use in cellulose fiber and wood fiber production made the plant very popular. Lately, its pharmacological application in many different medical fields is being discussed. *Cannabis sativa* can contain over 100 different phytocannabinoids, of which Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the best characterized (Andre et al., 2016).

The quantitative composition of the cannabinoids differs between plant species. THC is a major part of the drug-type *Cannabis sativa* also known as marijuana or drug-hemp, whereas CBD mostly occurs in fiber-hemp. Phytocannabinoids are harvested from the trichomes, which are part of the female florescence (figure 2). Like the endocannabinoids, they are ligands to the endocannabinoid receptors CB1 and CB2 with different affinities and impacts (Grassa et al., 2019).

Figure 2. Anatomy of *Cannabis sativa* with female flowers and trichomes (Glodowska, 2016)

1.1.4 Cannabidiol (CBD)

One of the best-studied phytocannabinoids obtained from *Cannabis* is CBD. Figure 3 outlines the structure of the CBD molecule. Besides the CB1R and CB2R receptors of the ECS, other cellular sites, e.g., the transient receptor potential vanilloid channel (TRPV), serotonin receptors (5-hydroxytryptamine receptors, 5-HT_{1A}), G protein-coupled receptor 55 (GPR55) and peroxisome proliferator-activated γ receptors (PPARγ), are targeted by CBD. At least 65 molecular targets for CBD are reported to contribute to its effects (Andre et al., 2016).

CBD has only weak affinity for the two endocannabinoid receptors, CB1 and CB2. Nonetheless, in CB1R binding, it has shown to act negatively allosteric and counteract the impact of THC and some endocannabinoids. This is discussed to be the reason why it can be considered as an antipsychotic, anti-epileptic and antidepressant drug and an agent relevant to the therapy of neurological diseases in the CNS and PNS without having the same side effects as THC (Laprairie et al., 2015). Additionally, CBD shows agonistic affinity to CB2R. Since CB2Rs are mainly responsible for modulating the immune cells and are extensively expressed on microglia cells, different neurological diseases involving Alzheimer's disease, multiple sclerosis, epilepsy and neuro inflammation, are possible targets for CBD-containing drugs due to its inhibitory action on microglia (Hamilton et al., 2021).

CBD shows higher affinity to TPRV channels, which are a major part of the nociceptive nervous system. Nonetheless, they can also be found in other parts of CNS, PNS and peripheral tissues. The activation of these channels through CBD is associated with anti-hyperanalgesic, anti-seizure and anti-inflammatory actions and a decrease in heart rate. Furthermore, the agonistic effect of CBD on 5-HT_{1A} indicates a possible use in mental disorders. According to different studies, CBD acts for example against depression, panic attacks, catalepsy, aggression and different autonomic stress responses (Britch et al., 2021; De Petrocellis et al., 2011). Additionally, both, the TPRV cannel as well as the 5-HT_{1A} receptor play a role in cancer control and mediate apoptosis and anti-proliferative effects. Controlling reactive oxygen species (ROS), ER stress and the immune response are Important parts of these effects of CBD (Seltzer et al., 2020).

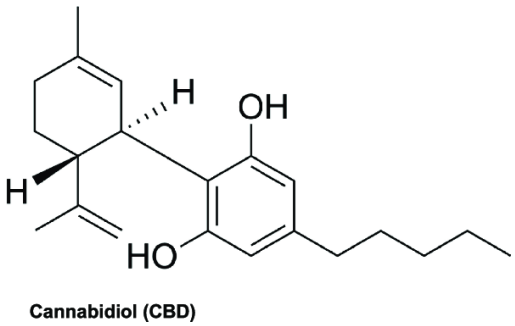


Figure 3. Molecular structure of CBD (Peyravian et al., 2020)

1.2 Mitochondria

For all cellular processes, cells need constant supply of energy. The first step in providing the cells with all the necessary nutrients, is the digestion of nutrients like lipids, polysaccharides or proteins, to make them biochemically available. In addition, the chemical bond energy is stored in molecules which can be used in other reactions. The latter is one of the major functions of mitochondria, which is described more in detail in the following chapter. Their appearance, shape and number, vary depending on the type of tissue where they are located. Depending on the energy requirements of the respective cells, the mitochondria may fuse or fission, resulting in changed morphology and number. The general structure of these organelles, however, is retained (figure 4). They consist of an outer and a folded inner cell membrane. Unlike the inner membrane, which forms a diffusion barrier for molecules to set up an electrochemically membrane potential, small uncharged molecules and ions can easily pass through the outer

membrane via diffusion or various channels. The inner membrane encloses the mitochondrial matrix and forms several cristae to enlarge its surface, since it is the site of the respiratory chain. Most of the enzymes needed for the mitochondrial function, as well as the mitochondrial DNA and ribosomes are stored in the matrix (Fontanesi & Krauss, 2015; Kühlbrandt, 2015).

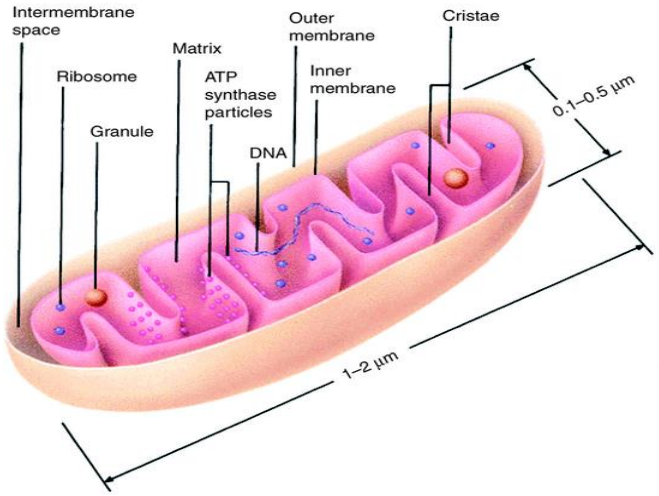


Figure 4. Mitochondrial structure and components (Bowler & Bowler, 2005)

The biggest amount of energy can be obtained from the metabolic pathway of glucose that can be separated into glycolysis, citric-cycle and the respiratory chain. Glucose is obtained via the enzymatic breakdown of polysaccharides during digestion. It is then taken up by cells to harvest the stored energy. During the whole process, food molecules are getting oxidized, gradually leading to the synthesis of carbon dioxide and water. Additionally, small doses of energy are released in the form of high energy electrons. They are taken up by electron transfer molecules like nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) to transfer them to the inner mitochondria membrane where the respiratory chain reactions take place. During this last step a major part of the cell's ATP is being produced (Alberts et al., 2015).

1.2.1 Production of energy

Glycolysis is the first step in the metabolic pathway of glucose. Its major goal is to break down the glucose that consists of six carbon atoms into two pyruvate molecules with three molecules of carbon each. Therefore, ten separate steps, each involving different enzymatic reactions, take

place. During this conversion, the sugar molecules are getting oxidized through removal of two electrons. These highly energetic molecules are transmitted to the electron transfer molecule NAD^+ creating two NADH per glucose molecule. Additionally, four molecules of ATP can be formed during the process. Considering the fact that two ATP molecules are used during the process start, there is a net output of two ATP and two NADH, which will be converted to ATP in further steps (Alberts et al., 2015).

To increase the amount of energy the cell can produce out of one glucose molecule, the glycolysis end-product pyruvate can be further degraded. This happens in the citric acid cycle, which takes place in the mitochondrial matrix. However, these reactions can only occur in aerobic milieu. If there is no oxygen available, the molecules of pyruvate are degraded by the lactate dehydrogenase to reproduce NAD^+ for further glycolysis cycles.

Before entering the citric cycle, pyruvate is transported into the mitochondria and decarboxylated to form acetyl-CoA. At this step, one molecule of NADH is generated. The molecule can enter the citric acid cycle in the form of acetyl-CoA, which has two atoms of carbon. The first step is to transfer the acetyl group from the acetyl-CoA to oxaloacetate to form citrate, a molecule consisting of six carbon atoms. During the next seven steps of the cycle, the two carbon atoms of the acetyl- group are oxidized gradually to regenerate oxaloacetate for further cycles. The energy that is released during the reactions is stored in the form of three NADH, one $FADH^+$ and one GTP molecule per cycle. Because each molecule of glycose gets degraded into two pyruvate molecules during glycolysis, this output is only half of the energy one can obtain from one molecule of glucose. While the NADH and the $FADH^+$ molecules transfer their high energy electrons to the inner mitochondria membrane for the respiratory chain, GTP can directly transfer its terminal phosphate group to ADP to produce one molecule of ATP (Alberts et al., 2015).

For the last step of the glucose metabolism, the high energy electrons that were released during previous stages of the metabolic pathway, are transported to the inner mitochondria membrane by the electron carriers NADH and $FADH^+$. They function as fuel to the respiratory chain, where most of the chemical energy is harvested and stored as ATP. The respiratory chain consists of the four enzyme complexes NADH-ubiquinone oxidoreductase, succinate dehydrogenase, ubiquinol-cytochrome c oxidoreductase and cytochrome c oxidase and the ATP-synthase, that are shown in figure 5. Driven by the energy they get from the electrons, the main function

of the first, the third and the fourth complex, is to pump protons across the mitochondrial membrane into the intermembrane space to generate a membrane potential. To release the largest possible amount of energy, the energy level of the electrons decreases continuously as they travel along the chain. That assures that the energy can be used precisely. The affinity between complexes and electrons increases with every step to ensure an easy travelling along the chain. In the end, the low energy electrons react with molecular oxygen (O_2) to form water. Therefore, the respiratory chain is the only part of this metabolic pathway that needs oxygen in molecular form. The ATP synthase, which is the fifth complex of the respiratory chain, is finally responsible for converting the whole extracted energy to ATP. It is driven by the chemical gradient of H^+ through the membrane, which was produced by the first four complexes, and the resulting backflow of the protons. Four protons deliver enough energy to form one molecule of ATP (Alberts et al., 2015; Kühlbrandt, 2015; Radad et al., 2019).

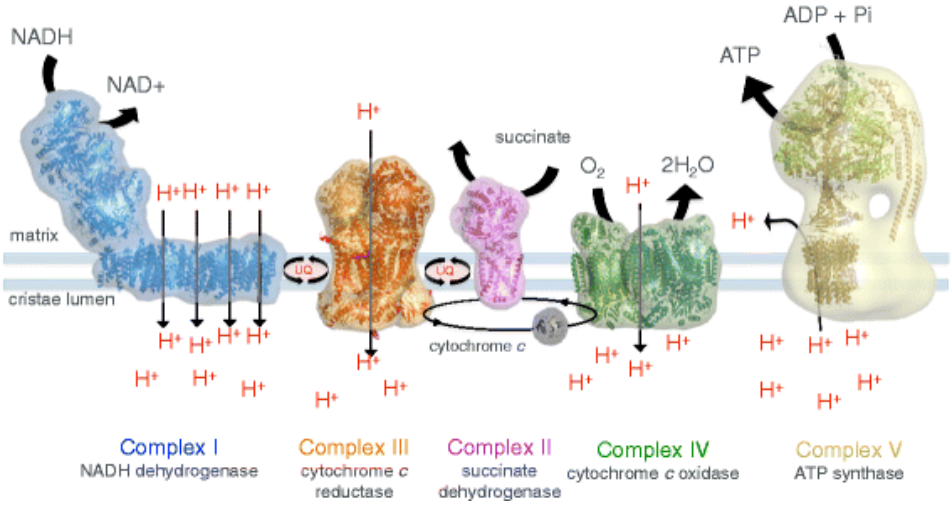


Figure 5. Complexes and function of the respiratory chain (Kühlbrandt, 2015)

Since the NADH is oxidized at the beginning of the respiratory chain, its electron travels through all the complexes. Thereby, 10 H^+ are pumped through the membrane delivering enough energy to form 2.5 ATPs. $FADH^+$ introduces its electron later to the chain and therefore results in only 1.5 ATPs. In total, the oxidative phosphorylation of one pyruvate molecule generates 10 ATPs. Considering the glycolysis, the pyruvate decarboxylation to acetyl-CoA and the fact, that every glucose molecule contributes for two pyruvate molecules, the net output of the whole glucose metabolism is 34 ATPs (Alberts et al., 2015).

1.3 Impact of CBD on mitochondrial function and possible implications

As described before, mitochondria are essential for cellular survival. They are not only responsible for energy supply and storage, but also regulate intracellular calcium levels (Ca^{2+}), which is an essential cation in cellular processes. Furthermore, cell metabolism and apoptosis are mediated by mitochondria through different pathways. The following examples summarize some influences, both positive and negative, that CBD exerts on cells (Chan & Duncan, 2021).

1.3.1 Effect of CBD on mitochondria

The voltage-dependent anion channel (VDAC) plays a role in sustaining cell metabolism and survival through the exchange of small ions like Ca^{2+} and metabolites, for instance ATP, across the outer mitochondrial membrane under physiological conditions. Additionally, it can intervene with pro- and anti-apoptotic factors to trigger pathways for cellular survival or death. High concentrations of CBD can directly interact with the VDAC1 resulting in a closure of the channel. While the conductance for important metabolites is inhibited, the small Ca^{2+} ions can still pass the channel resulting in an enhanced intramitochondrial Ca^{2+} concentration. Subsequently, the mitochondrial membrane potential (MMP) breaks down, leading to enhanced ROS production, swelling and death of the cell. This approach is discussed to be beneficial in treating cancer, however, it is only observable when using high concentration of CBD for treatment (Rimmerman et al., 2013; Ryan et al., 2009).

On the contrary, small concentrations of CBD have shown to exhibit neuroprotective and anticonvulsant properties, for instance through an interaction with the Na^{+}/Ca^{2+} -exchanger (NCX). Since its physiological action to pump Ca^{2+} out of the mitochondria can be reversed under pathological conditions, CBD restores its original function. As a result, e.g., epileptic seizures that would occur due to high intracellular Ca^{2+} levels, could be prevented. Additionally, the pro-apoptotic pathway triggered by Ca^{2+} is inhibited (Ryan et al., 2009).

1.3.2 Implication of CBD in neuroblastoma and other cancers cells

As described before, high concentrations of CBD can reduce cell viability through different mechanisms. Harming the mitochondria followed by the production of ROS and therefore a lack of energy is one of the characteristics of CBD. This leads to a possible application of CBD in cancer treatment (Rimmerman et al., 2013).

In general, cancer cells are mutated cells. Usually, these mutations concern the activation of oncogenes or the inactivation of tumor suppressor genes. Through different mechanisms, the affected cells can change in many ways. For example, they no longer react to apoptotic signals, which allows them to survive even though they can negatively affect the health and be harmful for the organism. Cancer cells grow independently to growth signals and proliferate unregulated. Additionally, they can invade surrounding tissue and spread throughout the body to form metastases. They can also induce angiogenesis to improve access to oxygen and nutrients that are necessary for large tumor formation in new environments (*What Is Cancer? - National Cancer Institute* (acessed 30.04.2022)).

Another fatal change that occurs through mutation is the alteration of the cellular metabolism. Under normal conditions, non-cancerous cells gain most of the ATP needed from the oxidation of pyruvate in the citric acid cycle or rather in the subsequent oxidative phosphorylation in the mitochondria, as described in the previous chapter. If the cells microenvironment lacks oxygen, they can undergo anaerobic glycolysis followed by lactate fermentation (Jang et al., 2013).

Lots, but not all cancer cell types, by contrast, can switch to glycolysis under hypoxic as well as under normoxic conditions. This variation of the glucose pathway is known as “Warburg effect” or aerobic glycolysis. It was discovered in 1926 by Otto Heinrich Warburg. Since the net output of glycolysis is only two molecules of ATP for every molecule of glucose, the survival of the cancerous cells massively depends on the glucose supply. Because cancer cells proliferate and grow quickly and need even more energy for these processes, more glucose is necessary. In general, cancers that are not able to use the “Warburg effect” are supposed to better respond to treatments like CBD. Using the “Warburg effect”, the cells can also survive for some time even if their mitochondria are inactivated or damaged. Due to this prolonged survival even with impaired mitochondria, researchers are able to observe other mitochondrial responses in cell cultures and animal models of cancer (Jang et al., 2013). Therefore, also the N18TG2 cells used in this study, which are capable of the Warburg effect, are suitable for studies on the effects of CDB in models of mitochondrial impairment. (Fisher et al., 2016; Park et al., 2008).

1.3.2.1 Neuroblastomata

Neuroplastomata are one example of cancers, in which CBD can be applied as a treatment. These are solid and malignant tumors that originate from the sympathetic nervous system and are therefore located extracranial. Since they derive from progenitor cells of the sympathetic nervous systems, this type of cancer is commonly found in children and infants. In animals, neuroblastomata can be found as well. The primary tumors are paraspinal in the abdomen, mediastinum, adrenal glands, neck, chest, and the pelvis region. Additionally, metastasis can be found in bones, bone marrow, liver, lymph nodes and on the skin. The symptoms vary depending on the locations of tumor and metastasis. They range from neurological impairment and abdominal distension to pain, fever and irritability. Neuroblastomata are heterogenous malignant which means that the prognosis for recovery differs between low-, intermediate- and high-risk diseases. These differences are for instance caused by variations in the cellular composition, the stage or the localization of the tumor and the age of the patient. While classical chemo- and radiotherapy works well in low- and intermediate-risk diseases, the survival rate of patients suffering from high- risk disease is less than 40 %. Therefore, the development of new therapies is mandatory (Fisher et al., 2016; Park et al., 2008).

1.3.3 Use of CBD in neurodegenerative diseases

Besides other pathogenic mechanisms like the accumulation of proteins, neurotoxic or neuroinflammatory events, mitochondria contribute to the pathogenesis of many neurodegenerative diseases such as Alzheimer's disease as well as Parkinson's disease. For instance, in the case of Alzheimer's disease, the involved protein aggregations lead to changes in the membrane permeability leading to Ca^{2+} influx. This causes mitochondrial damage, which further induces extensive production of ROS leading to cellular death. Through different cellular pathways, the oxidative stress can be reduced by only small concentrations of CBD. For instance, it prevents excessive ROS production via an inhibition of ROS-generating factors. This enhances mitochondrial function and cell survival. Moreover, neuroinflammation, which occurs in neurodegenerative diseases can be decreased. Especially pathways that follow PPARγ activation trigger this effect by preventing the expression of different cytokines and other pro-inflammatory factors (Seltzer et al., 2020).

1.4 Modulation of mitochondrial activity

Since functional disorder of mitochondria plays a role in the pathogenesis of several diseases, like neurodegenerative diseases, this chapter deals with the simulation of mitochondrial dysfunction. Therefore, different substances can be applied. The modulation of the mitochondrial activity not only provides a chance to study these diseases but also allows to obtain information about the mode and site of action of drugs added to the system. Therefore, different parts of a pathway are shut down or inhibited subsequently to study every step of a physiological or pathophysiological process. The tested drug can be co-applied to modulators or inhibitors of the cascade to study its effect on the particular part of the system. Concerning the mitochondrial respiratory chain, carbonylcyanide-3-chlorophenylhydrazone, rotenone and oligomycin are three substances of choice to manipulate this function as described in the following chapter.

1.4.1 Uncoupling of the oxidative phosphorylation through CCCP

The chemical uncoupler carbonylcyanide-3-chlorophenylhydrazone (CCCP) has a harmful effect on the mitochondria without affecting the single enzymes of the respiratory chain or the ATP-synthesis directly. Its mode of action is to uncouple the transport of electrons across the respiratory chain complexes from the ATP-synthesis. More precisely, it prevents the development of the membrane potential that is normally formed when H^+- ions are pumped out of the mitochondrial matrix into the intermembrane space by the complexes 1, 3 and 4. The operating mechanism behind this activity relies on proton leakage. Like the majority of chemical uncouplers, CCCP, whose molecular structure is shown in figure 6, is a lipophilic weak acid that can easily diffuse across the inner mitochondrial membrane. Thus, it can take up a proton in the intermembrane space and diffuse to the matrix of the mitochondrion. After deprotonating, it is situated again to the intermembrane space to repeat this procedure. The membrane potential, which is built up by H^+ protons across the inner mitochondrial membrane, cannot be sustained leading to a stop of the ATP synthesis. The energy that was obtained from glycolysis and the citric cycle and stored in the form of high energy electrons cannot be used for ATP formation resulting in an lack of energy for cell survival (Lou et al., 2007; Zhang et al., 2016).

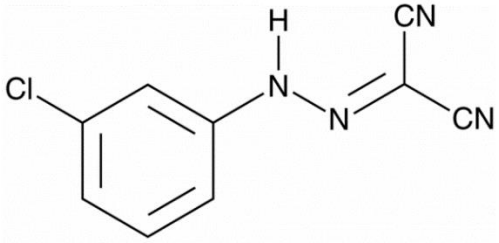


Figure 6. Molecular structure of the uncoupler carbonylcyanide-3-chlorophenylhydrazone (CCCP) (*CCCP | CAS 555-60-2 | Cayman Chemical | Biomol.De*, n.d.)

1.4.2 Inhibition of complex 1 through rotenone

Rotenone is an inhibitor of the mitochondrial complex 1. In some parts of the world, like the USA, Canada, Africa and Norway it is still used as a piscicide in fishing industry, for instance to eliminate diseased animals or invasive species. Although the usage of rotenone as pesticide, herbicide and insecticide is banned in the European Union, the USA and Canada, some countries still use it as plant protectant in agriculture (Radad et al., 2019). However, rotenone not only harms animals and plants but is also toxic to humans. Naturally, rotenone occurs in tropical and subtropical plant species like *Lonchocarpus, Munduela, Derris* and *Tephrosia*, from which it can be extracted. Figure 7 shows the chemical structure of the rotenone molecule. Due to its lipophilicity, it can pass the cell membrane easily without the need of any transporters and impair the function of the mitochondria. (Radad et al., 2019).

As reported before, complex 1, the NADH-ubiquinone oxidoreductase, is the first enzyme of the mitochondrial respiratory chain and additionally the one with the most complex structure. Its function is to reduce the NADH to NAD^+ and pass the gained electron to ubiquinone. Rotenone blocks the transfer of the electrons between the iron-sulfur center of complex 1 and ubiquinone, resulting in a blockade of the oxidative phosphorylation. Without oxidative phosphorylation, cells are no longer able to produce the amount of ATP necessary for survival. Additionally, some electrons are able to reduce oxygen that is no longer needed at complex 4 resulting in the formation of superoxide radicals. These can lead to oxidative stress, and also cell degeneration (Heinz et al., 2017).

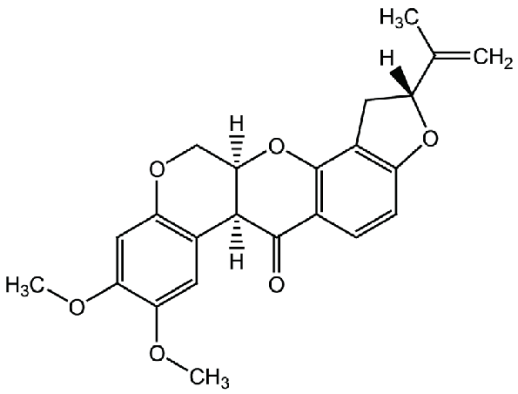


Figure 7. Molecular structure of the complex 1 inhibitor rotenone (Mpumi et al., 2016)

1.4.3 Inhibition of ATP-synthase through oligomycin

Another modulator of the mitochondrial activity is oligomycin (figure 8). It is an antibiotic synthesized by *Streptomyces*. Similar to the other substances described before, it harms the mitochondria through affecting the respiration, especially the ATP synthesis. More precisely, it binds to a polypeptide in the part of the ATP synthase that is embedded in the inner membrane of the mitochondria, called F_O-subunit. Thereby, the protons cannot follow their gradient into the mitochondrial matrix and no ATP can be synthesized any longer. Since the proton gradient becomes too high through this blockage of the back flow, also the first four complexes are inhibited consequently (Al Maruf et al., 2014).

Another mode of action of oligomycin is mediated by the help of the oligomycin-sensitivity conferring protein (OSCP). It is located on top of the second subunit of the ATP synthase, the F_1-subunit as shown in figure 8. This soluble part of the enzyme catalyzes the ATP synthase itself. The OSCP protein connects both subunits F_1 and F_O via other enzymes. Although oligomycin does not directly bind to OSCP, it can destroy this connection of the subunits, which leads to a decrease in the activity of the ATP synthase (Antoniel et al., 2014; Senior, 1979).

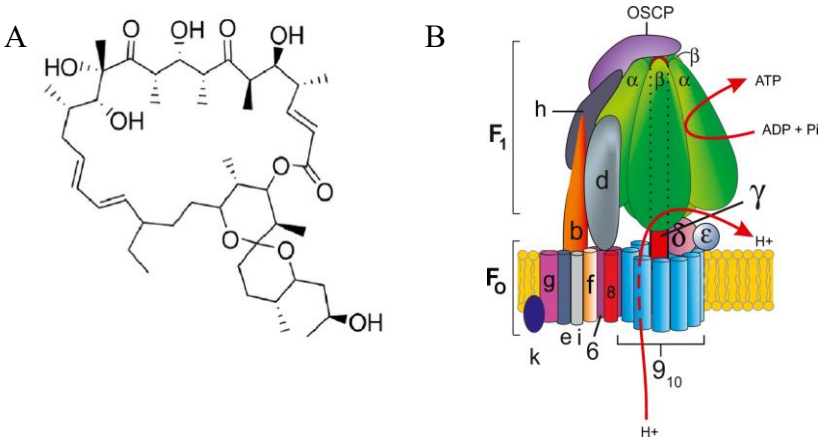


Figure 8. A) Molecular structure oligomycin, an inhibitor of the ATP synthase (Bakar et al., 2014)
B) F_1 and F_O subunits of the ATP synthase and the proteins they consist of (Artika, 2019)

1.5 Aims of this study

Following the hypothesis, that CBD affects mitochondrial activity, this thesis was designed to shed light on the acute effects of cannabidiol on neuroblastoma cells. Mitochondria play a major role in both, cancer cells as well as some neurodegenerative diseases. To study the impact of CBD on the mitochondria, and to model neurodegenerative pathogenesis, the mitochondria were modulated through mitochondrial toxins. Thereby, it should be determined whether CBD acts on cultures affected by these substances to show damaging or protecting properties. Additionally, it was aimed to study on which mitochondrial site CBD may act: on complex I in a model of superoxide formation, on the proton gradient formation or on the ATP synthase.

To study the viability of the mitochondria, JC-1-assays to measure the mitochondrial membrane potential and OxoPlates® to determine the cellular oxygen consumption were the two parameters of choice. This thesis was split into two parts starting with pretests to optimize the used methods and determine the concentrations of the mitochondrial toxins that are most efficient on our cells. Afterwards, the cannabidiol-experiments were performed by applying the previously improved methods.

To conclude, through answering the following questions, the aims stated above should be clarified:

(1) What concentrations of the mitochondrial toxins CCCP, rotenone and oligomycin show the highest impact on the N18TG2 cells and can be used in further experiments?

(2) What impact does CBD have on the mitochondrial membrane potential and the oxygen consumption of the neuroblastoma cells?
(3) Does CBD show any influence on CCCP- or rotenone-treated cultures to mitigate or reinforce their effects?
(4) Is it possible to detect the site of action of CBD through modulation with CCCP and rotenone?

2 Material & Methods

2.1 Material

2.1.1 Cells

N18TG2 murine neuroblastoma-cells, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany

2.1.2 Chemicals

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), abcam, GB
B27 supplement minus AO, Invitrogen, UK
Cannabidiol, THC Pharm, Germany
Carbonyl cyanide-3-chlorophenylhydrazoe, Sigma-Aldrich, Germany
Colourless Dulbecco's Modified Eagle's Medium (DMEM)- high glucose, Sigma-Aldrich, Germany
Dimethyl sulfoxide (DMSO), Merck, Germany
Dulbecco's Modified Eagle's Medium (DMEM)- high glucose, Sigma-Aldrich, Germany
Dulbecco's Phosphate Buffered Saline (DPBS) 10x, Thermo Fisher Scientific, USA
Dulbecco's Phosphate Buffered Saline (DPBS) Thermo Fisher Scientific, USA
Foetal bovine serum, Sigma-Aldrich, Germany
L-Glutamine, Sigma-Aldrich, Germany
Natriumsulfit wasserfrei, Merk, Germany
Oligomycin from *Streptomyces*, Sigma-Aldrich, Germany
Paraffin oil viscid puriss, Sigma-Aldrich, Gemany
Poly-D-lysine hydrobromide (PDL), Sigma-Aldrich, Germany
Rotenone, Sigma-Aldrich, Germany
Sodium Pyruvate solution, Sigma-Aldrich, Germany
Trypan blue, Sigma-Aldrich, Germany
Δ9-Tetrahydrocannabinol, Weltapotheke, Austria

2.1.3 Equipments

30mL PP Universal Container, Thermo Fisher Scientific, USA

96-well black plates, Thermo Fisher Scientific, USA

Big squid [white], Ika, Germany

Cell Culture Flasks 50 cm^2, Greiner bio-one, Germany

Cell Culture Flasks 75 cm^2, Greiner bio-one, Germany

Cellstar® Tube 15 mL, Greiner bio-one, Germany

Cellstar® Tube 50 mL, Greiner bio-one, Germany

Centrifuge, Beckmann Coulter, Germany

Combitips advanced®, Eppendorf, Germany

Counting chamber Neubauer improved, Bartelt, Austria

Eppendorf research plus pipette, Eppendorf, Germany

Eppendorf Tubes® 5 mL, Eppendorf, Germany

epT.I.P.S.® - Pipettenspitzen, Eppendorf, Germany

Examination gloves, Hartmann, Germany

Incubator, Cell Xpert, Eppendorf, Germany

Incubator, NAPCO Model 5410, Germany

Lamin Air Holten, Bartelt, Austria

Laminar Flow HERAsafe® , Kendro Laboratory Products, Germany

Microplate Reader Spark®, Tecan, Switzerland

Microscope, Nikon Diaphot 300, Japan

Multimode Plate Reader EnSpire® 2300, PerkinElmer, USA

Multipette® M4, Eppendorf, Germany

OP96U OxoPlates®, PreSens Precision Sensing GmbH, Germany

Pipette tips, Greiner bio-one, Germany

Pipetts, Eppendorf, Germany

Pipetts, Gilson, USA

Pipetus® standard, Hirschmann Laborgeräte, Germany

Reaction Tubes 1mL PP, Greiner bio-one, Germany

Reaction Tubes 2mL PP, Greiner bio-one, Germany

Reagent reservoir Dual Solution®, VWR, USA

Vortex 1, IKA, Germany
Water bath, GFL, Germany

2.2 Methods

2.2.1 Cultivation of N18TG2 neuroblastoma cells

The N18TG2 cells that were used for all experiments, are murine neuroblastoma cells and were acquired from the "German collection of Microorganisms and Cell Cultures". For cultivation, 75 cm^2 cell culture flasks with a maximum volume of 15 mL were used. In addition, small 25 cm^2 flasks were kept as a backup. The cells were grown in N18 medium whose composition is presented in table 1. To avoid overgrowing, the cells were split 1:10 every two to three days. A split-ratio of 1:2 was applied on Monday for usage of the cells on Tuesday to guarantee a constant growth rate. For splitting the cells, we first collected them followed by replacing eighter 90 % or 50 % of the cell suspension with fresh medium.

Table 1. Composition of N18 cell culture medium

N18 medium		
volume	**stock concentration**	**ingredients**
44 mL	/	DMEM (Sigma-Aldrich, Germany)
5 mL	/	fetal bovine serum (Sigma-Aldrich, Germany)
1 mL	100 mM	sodium pyruvate (Sigma-Aldrich, Germany)
1 mL	2 mM	L-Glutamine (Sigma-Aldrich, Germany)

2.2.2 Counting cells for creating cell suspensions

To create the cell suspensions containing the right number of cells for the experiments mentioned below, the cells had to be counted. Therefore, they were detached from the cell culture flasks and rinsed in either the medium in the flask for the OxoPlates® or in treatment medium without serum and phenol red to seed them out for JC-1-assays. From these cell suspensions, 20 µL were taken for counting. At first, they were diluted 1:5 with phosphate buffered saline (PBS) to obtain a smaller denseness of the cells. For staining the cells, 100 µL of the dye trypan blue were added. It is used to differentiate dead and vital cells. The mode of action behind this application is that the dye is not able to pass intact cell membranes. Therefore, living cells

remain unstained. If the cell degenerates, the membrane is permeable, and the cytoplasm is stained blue. For further counting the cells, around 10 µL of this solution was transferred into a hemocytometer (Neubauer improved, Bartelt, Austria). The cells in the four intended squares of the chamber were counted. The average of the obtained number was taken and further multiplied by 1,000 times the dilution factor to obtain the cells/mL. The dilution factor composed of the two dilutions with PBS (1:5) and trypan blue (1:2) resulting in the factor 10. For calculating the amount of cell suspension (in mL), needed to produce 1 mL of the final dilution, the desired amount of cells/mL was divided by the actual amount of cell/mL in the counted suspension.

2.2.3 Cannabidiol treatment for JC-1 and OxoPlates®

For the experiments concerning the effect of cannabidiol on modulated mitochondria, cells were pretreated four hours before the actual measurement started. Therefore, a 25 mM CBD stock was diluted 1:25 using PBS. To treat the cells that were later used in the OxoPlate® experiments, 150 µL of this dilution was added directly into the cell culture medium in one of the cell culture flasks creating a final concentration of 10 µM. For the treatment of the cells that were seeded out into the 96-well plate for the JC-1 assay, the CBD stock was diluted 1:250 with PBS using two intermediate steps. Through adding 15 µL of this solution to each well of the 96-well plate used for the assay, the same final concentration of 10 µM was established. Also in this experiment, the treatment was directly added to the treatment medium that was still in the wells. Since the CBD was dissolved in the organic solvent dimethyl sulfoxide (DMSO) to create the stock solution, the vehicle control wells were also treated with DMSO in a concentration similar to the cannabidiol treatment (0.04 %). Although the cells had to be left in the CBD-treated medium for the duration of four hours, they were washed down and counted for the OxoPlates® during this period of time. Also, the JC-1 dye could be added during the last 30 minutes of the incubation time. In the pretests, to obtain the concentrations of the applied mitochondrial modulators that are most efficient, the cells were used without any pretreatment.

2.2.4 Measuring the membrane potential applying JC-1-assay

For measuring changes in the mitochondrial membrane potential, the dye 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) was used. It consists of cationic, lipophilic

monomers. Within healthy mitochondria that possess a normal membrane potential, the dye can diffuse into the mitochondrial matrix and form dimers that emit red fluorescence (~590 nm). With hyperpolarization, more JC-1 can diffuse from the cytoplasm into the mitochondrial matrix to form polymers, followed by an increase of the red fluorescence signal. If the membrane potential decreases due to depolarization, the JC-1 monomers diffuse back to the cytosol (figure 9). This is an indicator for unhealthy mitochondria. Due to a decreasing concentration of dye particles in the mitochondria, the JC-1-polymers disaggregate leading to a shift to green fluorescence (530 nm) (*JC-1 - Mitochondrial Membrane Potential Assay Kit (Ab113850) | Abcam* (acesses 10.05.2022). For analyzing the data, the red/green ratio of the fluorescence signals has to be obtained. An increase of this ratio indicates a rise in membrane potential, whereas a decrease signals mitochondrial depolarization.

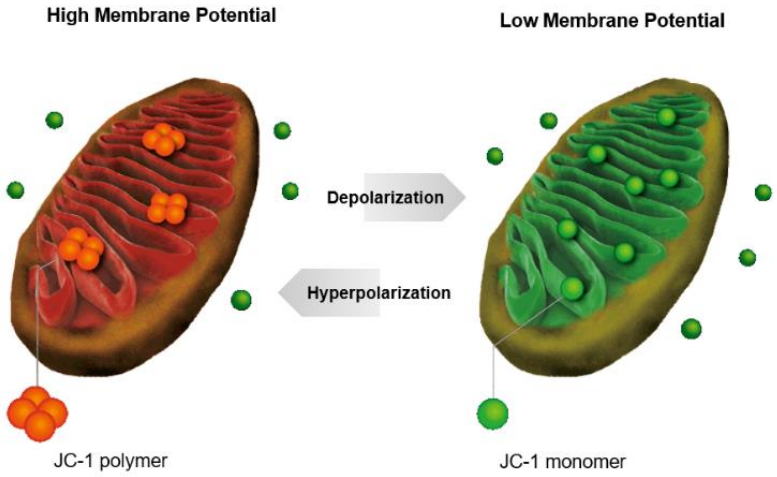


Figure 9. Changes in membrane potential leading to fluorescence shifts of JC-1 signals (*JC-1 MitoMP Detection Kit - Dojindo Molecular Technologies, Inc.* (acessed 03.06.2022).

Since the JC-1-assay relies on fluorescence, black 96-well microtiter plates were used to enable the measurement of each well independently without interferences. The day before the measurement, the cells were cultivated in poly-D-lysin-precoated plates, using 150 µL of cell suspension with a concentration of 400,000 cells/mL per well. For collecting the cells, as well as for the cell suspension, treatment medium without serum and phenol red was used (table 2). The plates were precoated at least one hour, but mostly the day before the experiments with

50 µL of poly-D-lysin (100 µg/mL) per well and kept in the incubator at 37 °C. Before using it, the plate had to be washed twice with PBS.

Table 2. Composition of treatment medium for JC-1-assays

Treatment medium		
volume	**stock concentration**	**ingredients**
48 mL	/	DMEM without phenol red (Sigma-Aldrich, Germany)
1 mL	/	B27 supplement (Sigma-Aldrich, Germany)
1 mL	100 mM	sodium pyruvate (Sigma-Aldrich, Germany)
1 mL	2 mM	glutamine (Sigma-Aldrich, Germany)

After 24 hours, a JC-1-stock (1 mg/mL) was diluted 1:50 (40 µL JC-1-stock + 460 µL DMSO + 1.5 mL warm PBS). Every well was treated with 15 µL of the solution resulting in a final concentration of around 3 µM JC-1. For this and all further steps, PBS containing D-glucose (1 g/L) and pyruvate (36 mg/L) was used. After an incubation time of 30 minutes at 37 °C, the supernatants were discarded, and the plate was washed twice with 100 µL warm PBS and then left in another 100 µL PBS for 15 minutes at 37 °C for the pretests. For the experiments including cannabidiol-treatment, only 90 µL PBS were used to cover the cells. This adjustment of the volume of PBS was done to ensure the volume of the mitochondrial treatment solutions to be exactly 1/10 of the well volume to obtain the right final concentrations (see below).

Subsequently, the plate was measured with a Spark plate reader. The green fluorescence was measured first at 535 nm, followed by the detection of the red fluorescence at 590 nm using an excitation wavelength of 280 nm, respectively. After the first measurement as reference, the cells were treated with CCCP, rotenone or oligomycin. Since these substances were dissolved in DMSO, control solutions containing the same concentration of DMSO (C_{CCCP}, C_R, C_O) were created. To obtain the concentrations of the mitochondrial inhibitors or DMSO, which are presents in table 3, in the respective wells, 11 µL treatment solution was added to each well in the pretests. In the cannabidiol-experiments, 10 µL CCCP (1 µM final concentration), rotenone (160 nM final concentration) and the DMSO control were added to the CBD-pretreated cells. The pretreatment of these cells was performed four hours before the first measurement like

described in chapter 2.2.2. After the modulation with the respective neuronal toxins, six more measurements were performed after 1, 3, 5, 10, 15 and 20 minutes. All steps were performed in darkness to avoid destruction of the JC-1-dye.

Table 3. Final concentrations of CCCP, rotenone and oligomycin treatments in primary tests

	CCCP [µM]	Rotenone [nM]	Oligomycin [µM]
1	100	160	3
2	50	80	2
3	10	40	1
4	5	20	0.5
5	1	10	0.25
6	0.5	5	0.1
7	0.25	2.5	ctl (DMSO)
8	ctl (DMSO)	ctl (DMSO)	ctl (DMSO)

To obtain the changes of the membrane potential, the ratio of the red and green fluorescence at every timepoint and of every well was calculated. As presented in figure 10, the wells of the plate were measured repeatedly.

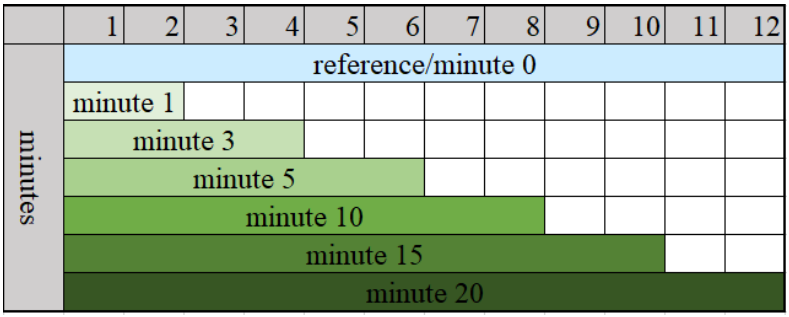


Figure 10. Scheme of reading the JC-1 assay. The columns represent the respective columns of the used 96-well plate, whereas the rows show the timepoints of measurement after treatment.

Since it is well known that the JC-1 dye is sensitive to light this could result in a bleaching effect, which influences the measurement. Especially the monomers that emit green fluorescence get bleached by light easily. Because it is unknown how much effect the bleaching has on the measurements, a bleaching factor for every well and measurement was calculated.

Because the mitochondrial membrane potential in the control cells was not altered by treatment and should be stable over the whole experiment, these wells were taken to calculate the factor. For every column of the plate and for every measurement, the red/green ratio of the control well was divided by the red/green ratio of the same well in the next measurement (formula 1).

$$\text{bleaching factors } (1,2,3,\ldots) = \frac{\frac{\text{red}}{\text{green}}\text{timepoint 0}}{\frac{\text{red}}{\text{green}}\text{timepoint 1}}, \frac{\frac{\text{red}}{\text{green}}\text{timepoint 1}}{\frac{\text{red}}{\text{green}}\text{timepoint 2}}, \frac{\frac{\text{red}}{\text{green}}\text{timepoint 2}}{\frac{\text{red}}{\text{green}}\text{timepoint 3}}, \ldots$$

Formula 1. Calculation of bleaching factor using the example of factors 1–3.

These factors were further taken into account for the calculations of the “real” red/green ratios without any bleaching effects. In the end, the red/green ratios from every well were multiplied by the respective bleaching factor of the column. For every measurement, the respective factor and additionally the ones of previous measurements were used (formula 2).

$$\text{"real" data} = \frac{\text{red}}{\text{green}}\text{ratio} * \text{factor measurement 1} * \text{factor measurement 2}$$

Formula 2. Calculation of the "real" red/green ratio using a well of the second measurement as an example.

2.2.5 Measuring the oxygen consumption rate (OxoPlates®)

Through modulation of mitochondrial function, the cellular consumption of oxygen can be altered. Therefore, it can serve as a parameter for mitochondrial integrity and energy metabolism. To determine the oxygen partial pressure in % of air saturation at several timepoints after treatment, OxoPlates® can be applied. This system uses an optical sensor that is positioned into the bottom of each well of a 96-well plate. The sensor, which is a thin polymer film, comprises of an indicator dye and a reference dye (figure 11). The reference dye emits fluorescence signals that are independent to the oxygen level and serves as internal quantity control for the detection-fluid in each well. The indicator dye, however, emits phosphorescence whose intensity depends on the amount of oxygen present in the well. To detect these two signals, the plate was measured in an EnSpire® Multimode Plate Reader using two different filter pairs for the indicator and the reference signal. The excitation filters for both measurements were 540 nm. The indicator was measured at 650 nm, while the emission of the reference was 590 nm (Bulkeley et al., 2021).

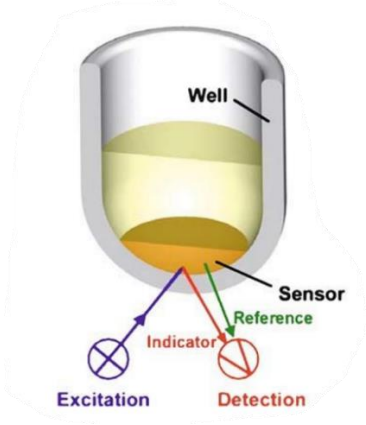


Figure 11. OxoPlate®-well with the sensor and the direction of measurement (Schäferling & Duerkop, 2008)

For the experiments concerning the effect of cannabidiol on modulated mitochondria, the used cells were pretreated four hours before the actual measurement started, like described in chapter 2.2.3. One cell culture flask was treated with CBD, another one with DMSO as a vehicle control. CBD was used creating a final concentration of 10 µM in the flasks. In the pretests to obtain the concentrations of the applied mitochondrial modulators that are most efficient, the cells were used without any pretreatment.

For preparing the plate, 15 µL of the desired concentrations of CCCP, rotenone or oligomycin and the DMSO controls were added to the respective wells in the OxoPlate®. For these solutions and all further steps, PBS containing D-glucose (1 g/L) and pyruvate (36 mg/L) was used. While all three substances were used in the primary tests using the in-well concentrations shown in table 3, only CCCP (1 µM final concentration) and rotenone (160 nM final concentration) were used in the experiments with cannabidiol pretreatment. Subsequently, the cells were collected from the flasks and counted to create a cell suspension containing 4,000,000 cells/mL (see chapter 2.2.2). While centrifuging them at 650 x g for 5 minutes, air-saturated PBS (see below) (100 µL in the pretests, 85 µL in the cannabidiol-experiments) was added to each well. The cell pellets containing 4,000,000 cells/mL were then dissolved in 1 mL air-saturated PBS to replace the cell-culture medium and added to the OxoPlate® (50 µL/well). To avoid exchange of molecular oxygen between the samples and the air, each well was overlaid by 100 µL of paraffin oil. After these steps the plate was ready to be read. The whole time, the plate had to be protected from light to avoid destruction of the sensor.

To calculate the oxygen partial pressure of each well, a calibration with two standards (cal 0, cal 100) was performed. For cal 100, air-saturated PBS (+ glucose & pyruvate) was used. To make sure that it is saturated, it was placed on the stirrer overnight or for at least 2 hours. As cal 0 solution, double-distilled water was supersaturated with Na_2SO_3 to make it oxygen-free. For this purpose, Na_2SO_3 was added to the water until insoluble crystals were seen as an indicator of complete saturation.

Quadruplicates were performed, using 150 µL of the respective calibration standards per well. Like the sample wells, also the standards were overlaid by 100 µL of paraffin oil.

Finally, the partial oxygen pressure (pO_2) had to be calculated. Therefore, the ratio I_R between the indicator and reference signal ($I_R = \frac{I_{indicator}}{I_{reference}}$) was determined for each well. The averages of the four wells for cal 100 and cal 0 were taken to get the calibration constants k_0 and k_{100}. Finally, all needed data was collected to proceed the final calculation of the partial oxygen pressure using the following formula:

$$pO_2 = 100 \cdot \left(\frac{k_0}{I_R} - 1\right) / \left(\frac{k_0}{k_{100}} - 1\right)$$

Formula 3. Calculation of the partial oxygen pressure for each well of the OxoPlate®

2.2.6 Statistical analysis

To be able to create meaningful statistical analysis, at least six independent experiments in the pretests and five for the cannabidiol-experiments were performed. Thereby, at least duplicates were measured for each condition. Since the data is not normally distributed, non-parametric tests were applied to evaluate statistical significance of the data. For interpreting the results of the pretests, the Kruskal-Wallis (H)-test (*), followed by a Chi-squared-test was used. To compare the different independent sample groups in the cannabidiol-experiments, the Mann-Whitney U-test (#) was executed. For all the tests, an α-cutoff of 5 % was used, meaning that all contrasts with p-values < 0.05 were interpreted as statistically significant. The diagrams were created using the means and the standard error of mean (S.E.M) of the datapoints. Whereas the statistical analysis was performed using StatView, the final graphs were created in Python.

3 Results

3.1 Pretests for optimizing the methods (JC-1, OxoPlates®)

Besides testing the sensitivity and usefulness of JC-1 and the OxoPlates®, the pretests were performed to determine an optimized concentration of the neuronal toxins CCCP, rotenone and oligomycin. These were used in following experiments to modulate the mitochondrial function of the cells. Therefore, the concentrations of these substances that trigger the highest effects on our N18TG2 cells were determined. All pretests were performed in cooperation with my fellow student Lea Klein.

3.1.1 Effect of CCCP on N18TG2-cells

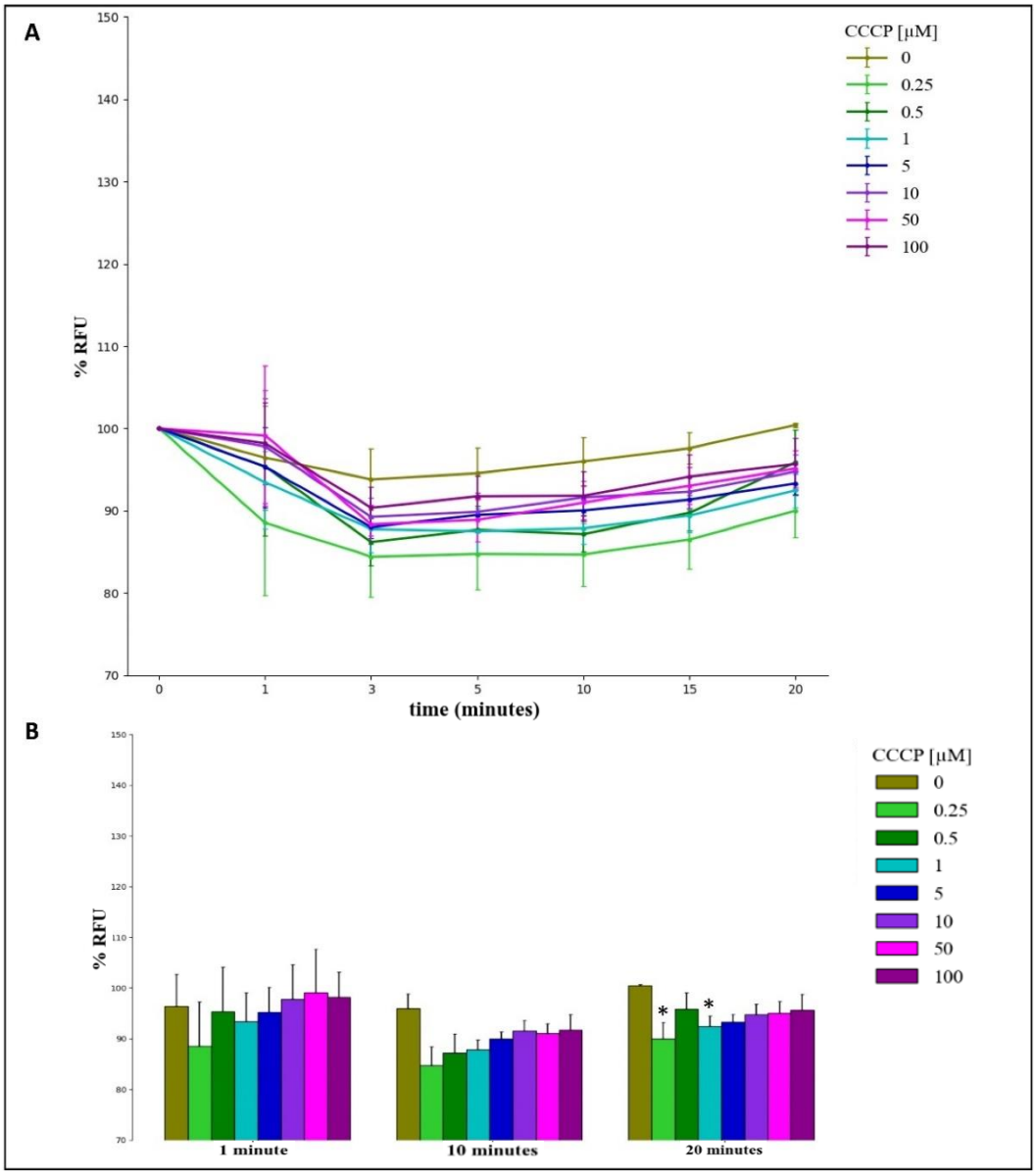


Figure 12. Effect of CCCP on the MMP of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 6 independent experiments. **A)** Depolarizing effect of different CCCP concentrations in regard to the first measurement (0 minutes). **B)** Significance of 0.25 µM and 1 µM CCCP compared to the control determined by the Kruskal-Wallis-test (*).

All tested concentrations of CCCP showed a tendency of depolarizing the mitochondrial membrane potential (figure 12 A). The concentrations of 0.25 µM and 1 µM triggered statistically significant effects 15 and 20 minutes after the treatment with a decrease of the membrane potential of about 10.43 % and 7.98 % compared to the control (figure 12 B).

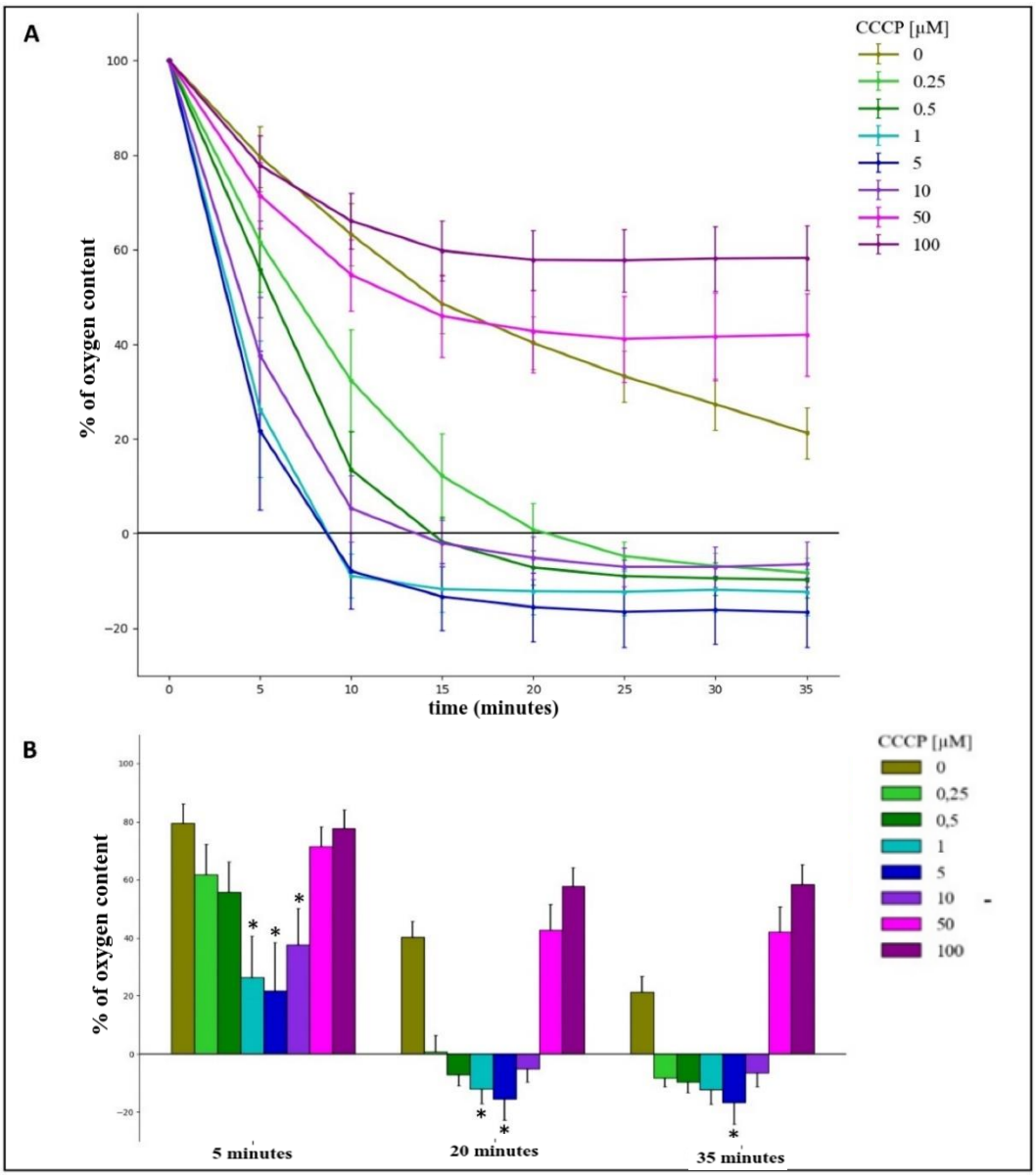


Figure 13. Effect of CCCP on the oxygen consumption of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 6 independent experiments. **A)** Acceleration of the oxygen consumption by CCCP. **B)** Significance of 1 µM, 5 µM and 10 µM CCCP compared to the control determined by the Kruskal-Wallis-test (*).

The oxygen consumption of the cells was only significantly increased by lower concentrations of CCCP (1 µM, 5 µM and 10 µM, figure 13 A). Thereby, the oxygen contents of the measured wells dropped below the basal line (0 %) after some time. Compared to the control, the 5 µM and 1 µM solutions provided the highest decrease of the oxygen partial pressure of about 72.17 % and 71.16 % within the first 10 minutes of the measurement (figure 13 B), respectively.

The impact of higher concentrations of CCCP (50 µM, 100 µM) was not significant, however, provided a tendency of modulating the oxygen consumption.

3.1.2 Effect of rotenone on N18TG2-cells

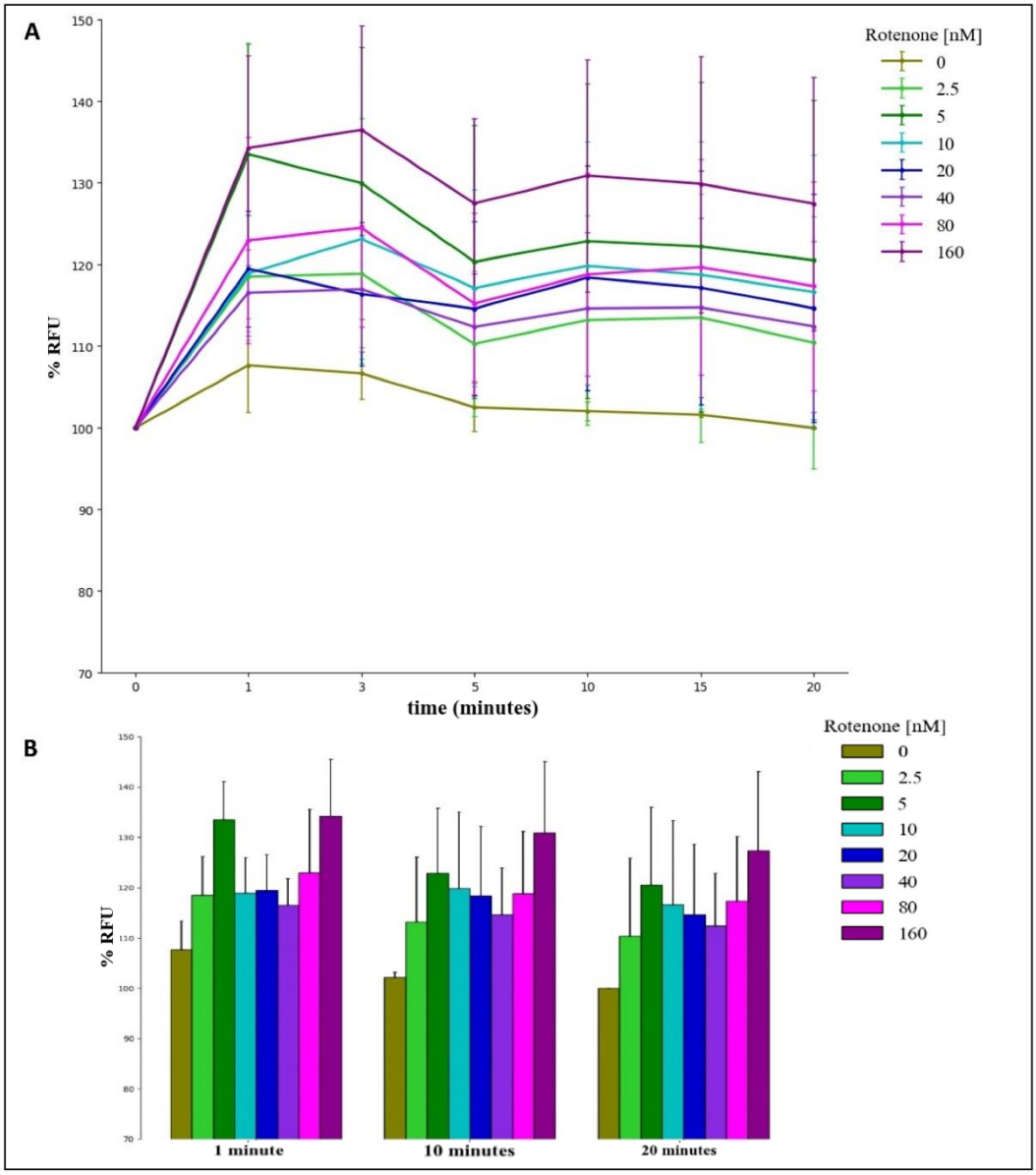


Figure 14. Effect of rotenone on the MMP of the N18TG2 cells. Data represent the mean +/- S.E.M. of n =3 independent experiments. **A)** Tendency of different rotenone concentrations to hyperpolarize the MMP in regard to the first measurement (0 minutes). **B)** No significant effects of rotenone on the MMP compared to the control could be determined by the Kruskal-Wallis-test.

The inhibition of the respiratory chain through rotenone caused a marginally hyperpolarization of the mitochondrial membrane potential (figure 14). Although the concentration of 160 nm

rotenone seems to trigger the highest effect, none of the observed changes are statistically significant and there was no indication for concentration dependent reactions.

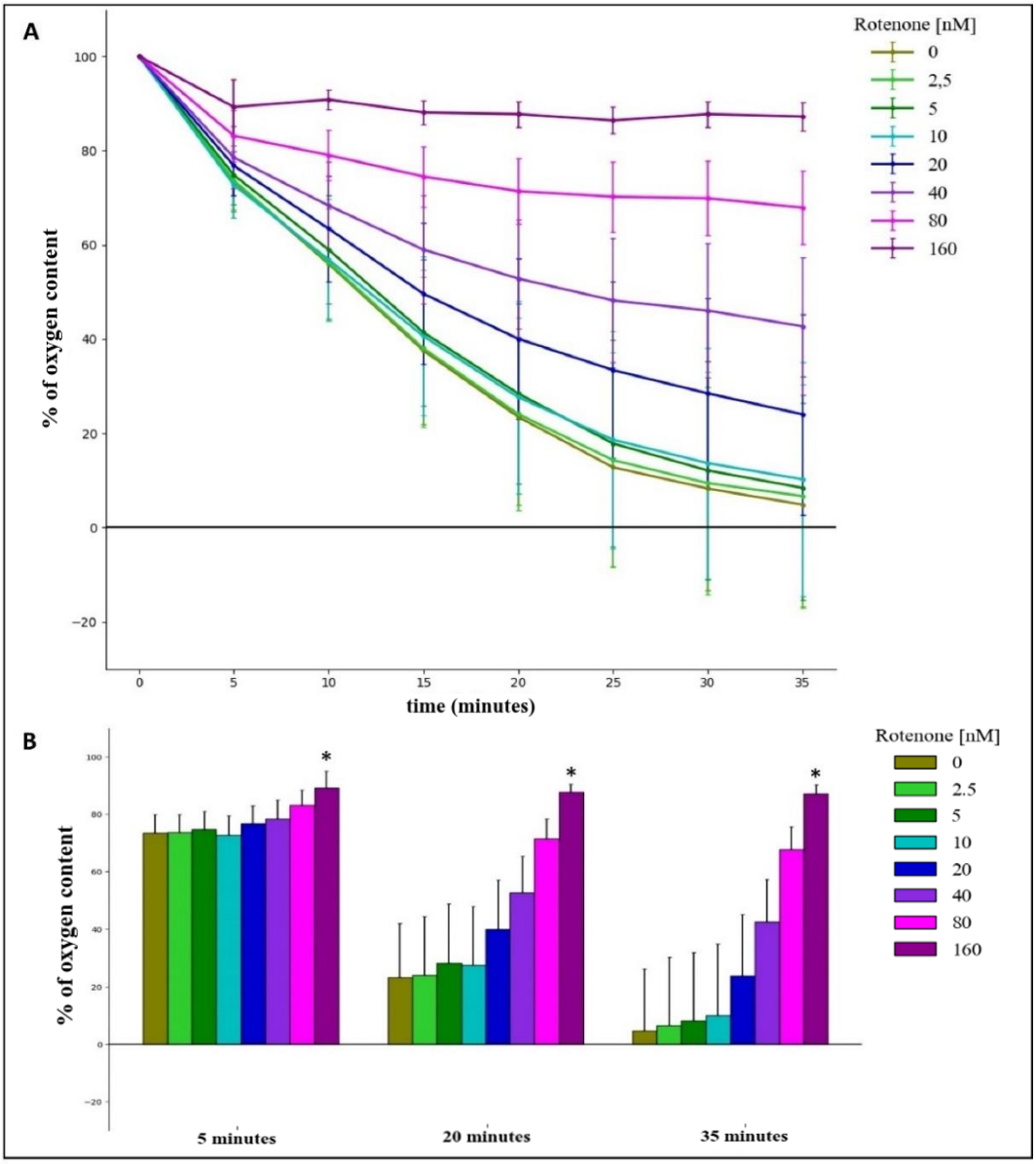


Figure 15 Effect of rotenone on the Oxygen consumption of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 5 independent experiments. **A)** Inhibition of the oxygen consumption by different concentrations of rotenone. **B)** Significance of 160 nM rotenone compared to the control determined by the Kruskal-Wallis-test (*).

The impact of rotenone on reducing the oxygen consumption was concentration dependent (figure 15). Although the concentrations of 20 nM and 40 nM had a toxic effect, only the 160 mM concentration inhibited the oxygen consumption of the cells significantly. This leads to a

stabilized partial oxygen pressure in the wells significantly at around 87.7 % starting 10 minutes after the treatment. For lower concentrations of rotenone (2.5–10 nM) no effect was observed.

3.1.3 Effect of oligomycin on N18TG2-cells

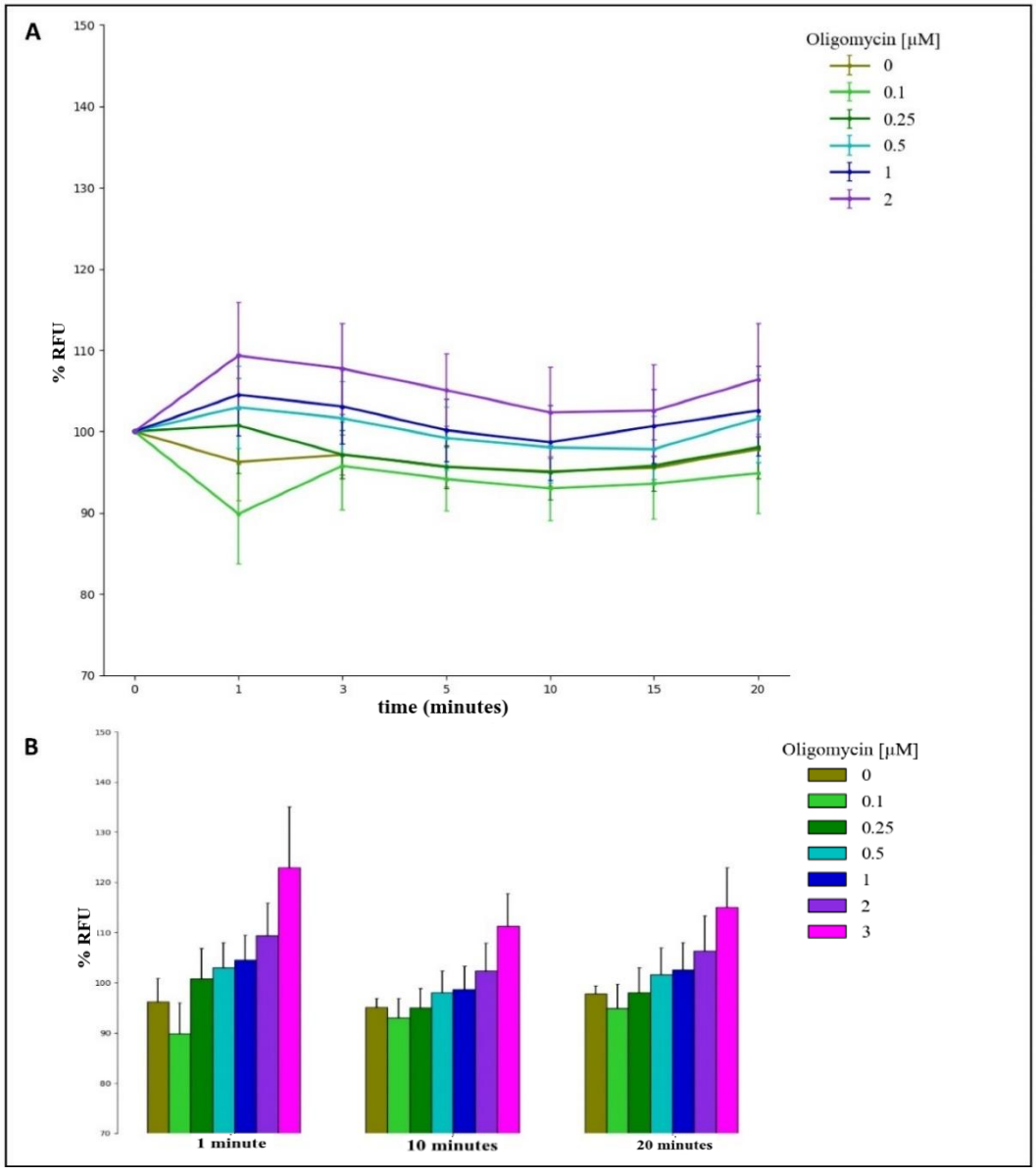


Figure 16. Effect of oligomycin on the MMP of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 6 independent experiments. **A)** Effects of different oligomycin concentrations on the MMP in regard to the first measurement (0 minutes). **B)** No significant effects of oligomycin on the MMP compared to the control could be determined by the Kruskal-Wallis-test.

In JC-1 experiments, oligomycin showed no significant impact on the mitochondrial membrane potential (figure 16). Nonetheless, the concentration of 3 µM had a tendency of hyperpolarizing

the membrane potential leading to an increase of the red/green ratio of about 15.15 % on average. Smaller concentrations (2–0.1 µM) did not show variations over 10 % compared to control.

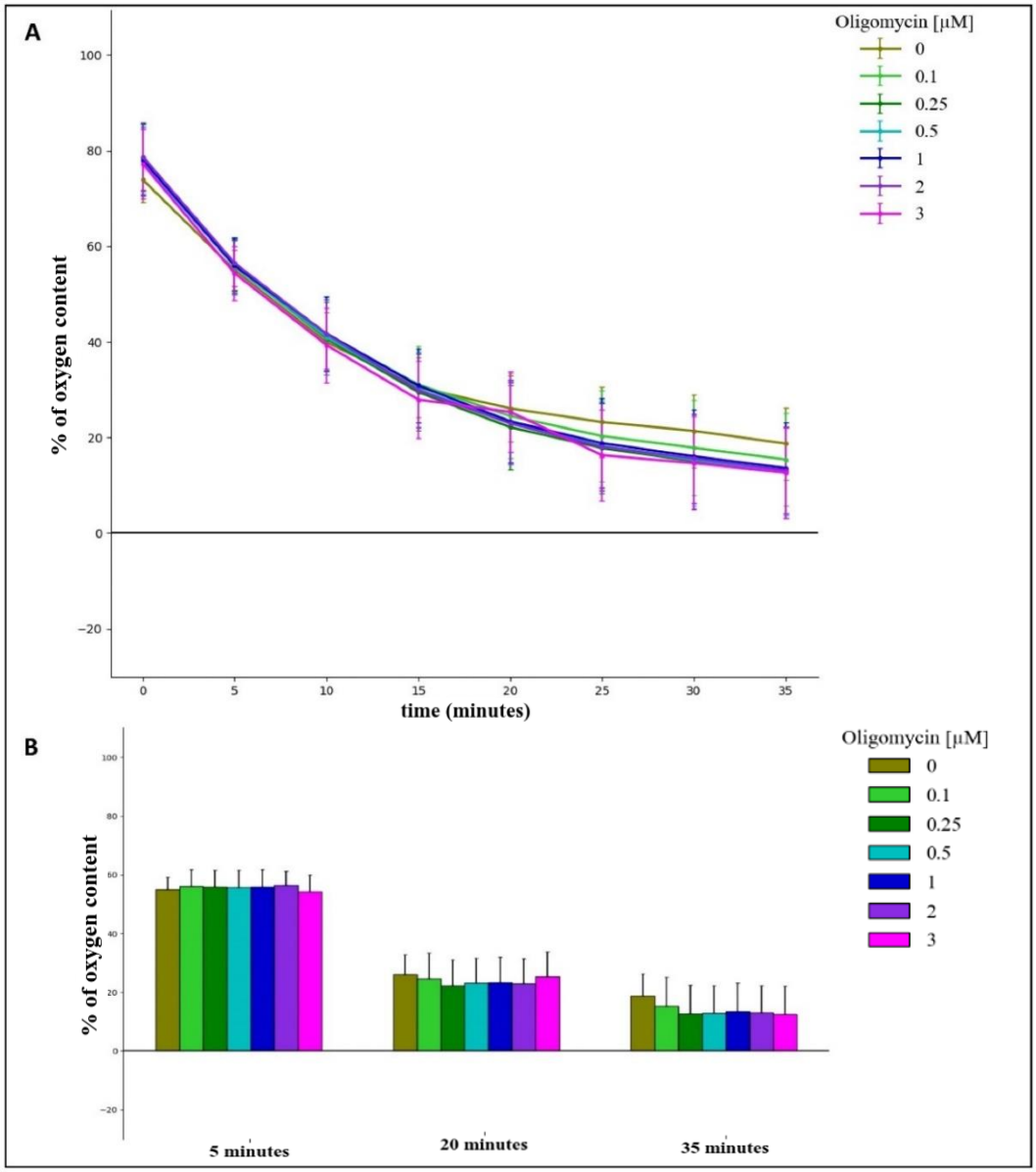


Figure 17. Effect of oligomycin on the oxygen consumption of the N18TG2 cells. Data represent the mean +/- S.E.M. of n =8 independent experiments. **A)** Effect of different oligomycin concentrations on the oxygen consumption. **B)** No significant effects of oligomycin on the oxygen consumption compared to the control could be determined by the Kruskal-Wallis-test.

Since the graphs from all used oligomycin concentrations, ranging from 0.1–3 µM, follow the shape of the control line nearly congruently (figure 17), no statistical effect of oligomycin on the oxygen consumption of the cells can be observed.

3.2 Effects of CBD on oxygen consumption rate and mitochondrial membrane potential after mitochondrial modulation

As the second and major part of this thesis, I focused on testing the effects of CBD on the mitochondria by applying JC-1 assays and OxoPlates®. Additionally, the impact of CBD on CCCP-, or rotenone-modulated cells was investigated. Therefore, cells were treated either with CBD and one of the two mitochondrial toxins or just with the cannabinoid, creating four different and independent sample groups (+/- 10 µM CBD, +/- 1 µM CCCP/rotenone). That way the effects of the modulators and CBD together as well as independently could be studied. The concentrations of the modulators were obtained from the pretests.

3.2.1 Effect of CBD on the membrane potential of the cells

A comparison of the membrane potentials of cells only treated with CBD and completely untreated cells revealed that CBD had a significant hyperpolarizing effect on the cells (increase of the red/green ratio by 17.78 %). For further observations, all membrane potentials measured at the beginning of the JC-1 assays (timepoint 0) were defined as 100 % to facilitate the comparison of the effects through CCCP and rotenone treatments.

3.2.2 Effect of CBD on cells with modulated mitochondria through CCCP

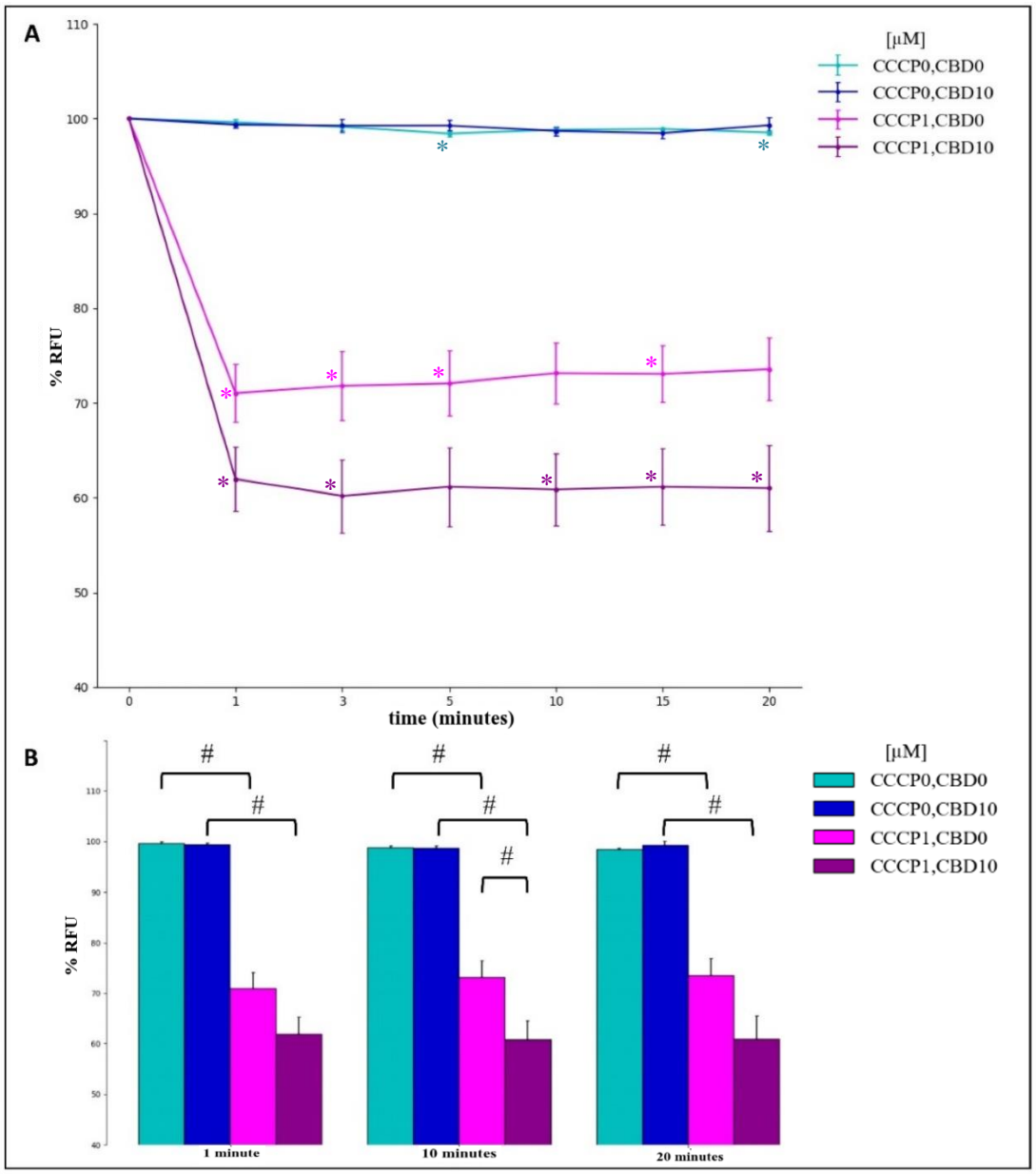


Figure 18. Effect of CCCP and CBD on the MMP of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 5 independent experiments. **A)** Effects of 1 µM CCCP and 10 µM CBD on the MMP compared to control. The (*) indicate significant differences compared to the respective 0-minute timepoint (Kruskal-Wallis-test). **B)** Significant impacts of 1 µM CCCP and 1 µM CCCP + 10 µM CBD on the MMP compared to the respective controls (Mann-Whitney-*U*-test (#)).

Starting 1 minute after treatment, 1 µM CCCP significantly depolarized the mitochondrial membrane (figure 18 A). Compared to the vehicle control, a decrease of 28.6 % relative fluorescent units (RFU) was observed. Together with CBD, the effect of CCCP was increased

leading to a further significant depolarization of about 12.29 % RFU, 10 minutes after CCCP was added (figure 18 B). The unmodulated control cells were not altered through CBD over the period of the experiment.

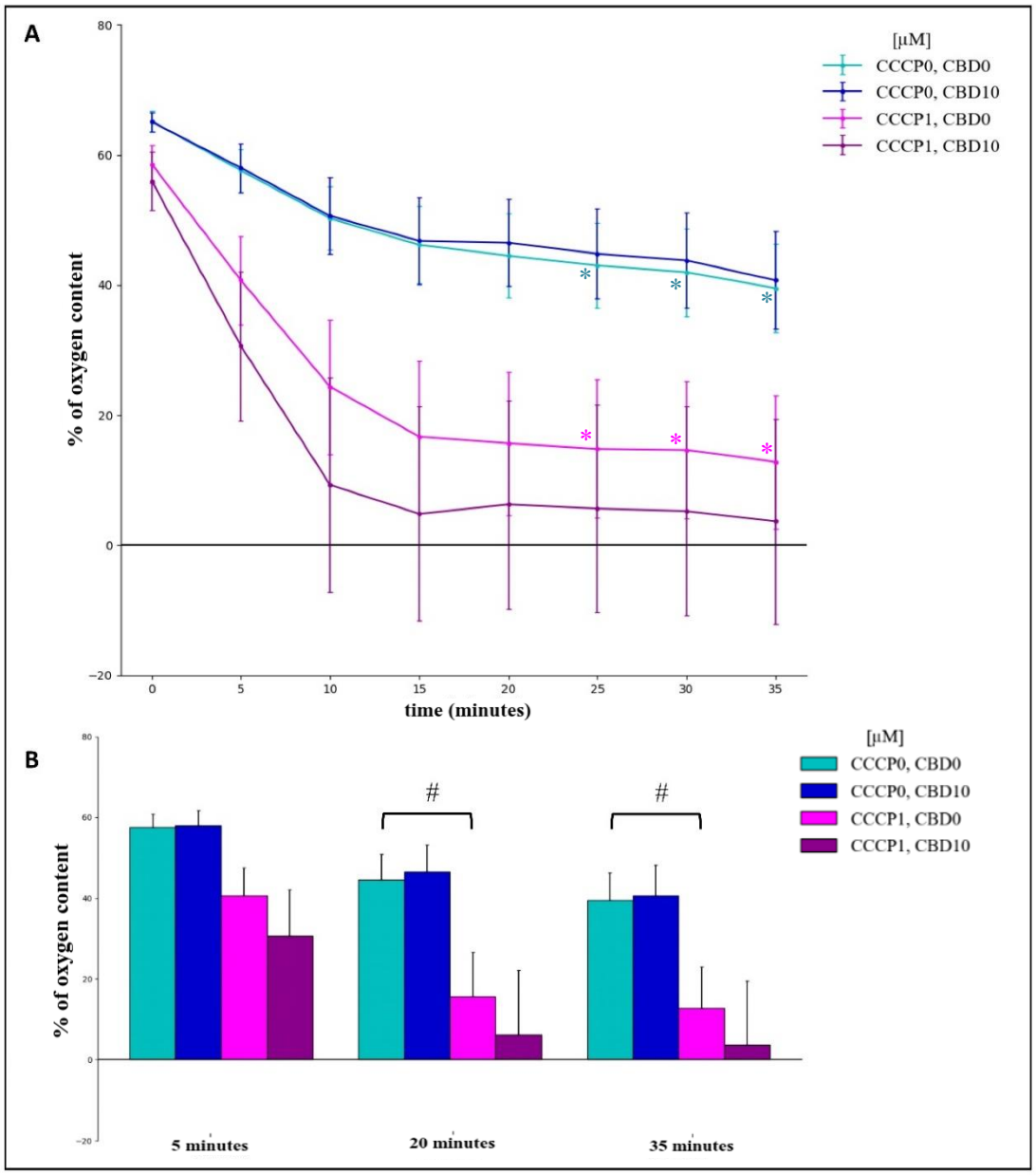


Figure 19. Effect of CCCP and CBD on the oxygen consumption of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 5 independent experiments. **A)** Acceleration of the oxygen consumption by 1 µM CCCP and 10 µM CBD. Significant impacts compared to the 0-minute timepoint (*, Kruskal-Wallis-test) **B)** Significant impact of 1 µM CCCP on the oxygen consumption compared to control determined by the Mann-Whitney-*U*-test (#).

Like in the pretests, CCCP increased the oxygen consumption of the cells significantly (figure 19), showing the maximum change in oxygen partial pressure (29.49 %) at the 15-minute

timepoint. While the CBD treatment had no effect on untreated controls, it appeared to have a tendency of increasing the oxygen consumption of CCCP + CBD treated cells compared to CCCP controls.

3.2.3 Effect of CBD on cells with modulated mitochondria through rotenone

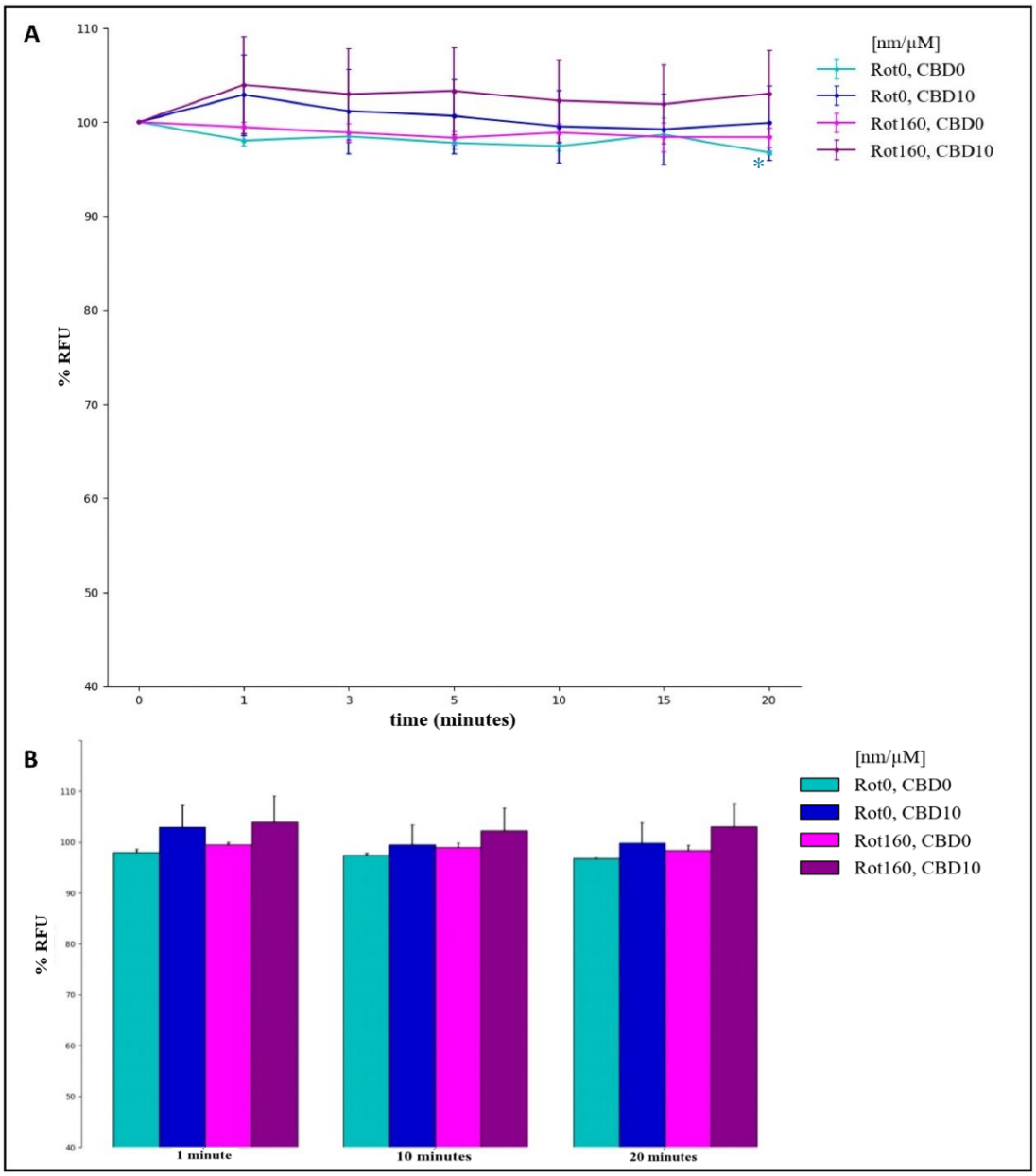


Figure 20. Effect of rotenone and CBD on the MMP of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 5 independent experiments. **A)** Effects of 160 nM Rotenone and 10 µM CBD on the MMP compared to control. Significant impact compared to the 0-minute timepoint (*, Kruskal-Wallis-test) **B)** No significant effects of rotenone or CBD on the MMP could be determined by the Mann-Whitney-*U*-test (#).

Similar to the pretests, the impacts of rotenone and CBD was not observed during the measurement of the membrane potential after the treatment (figure 20). The drugs seem to have an effect on the membrane potential during this experiment neither together, nor independently.

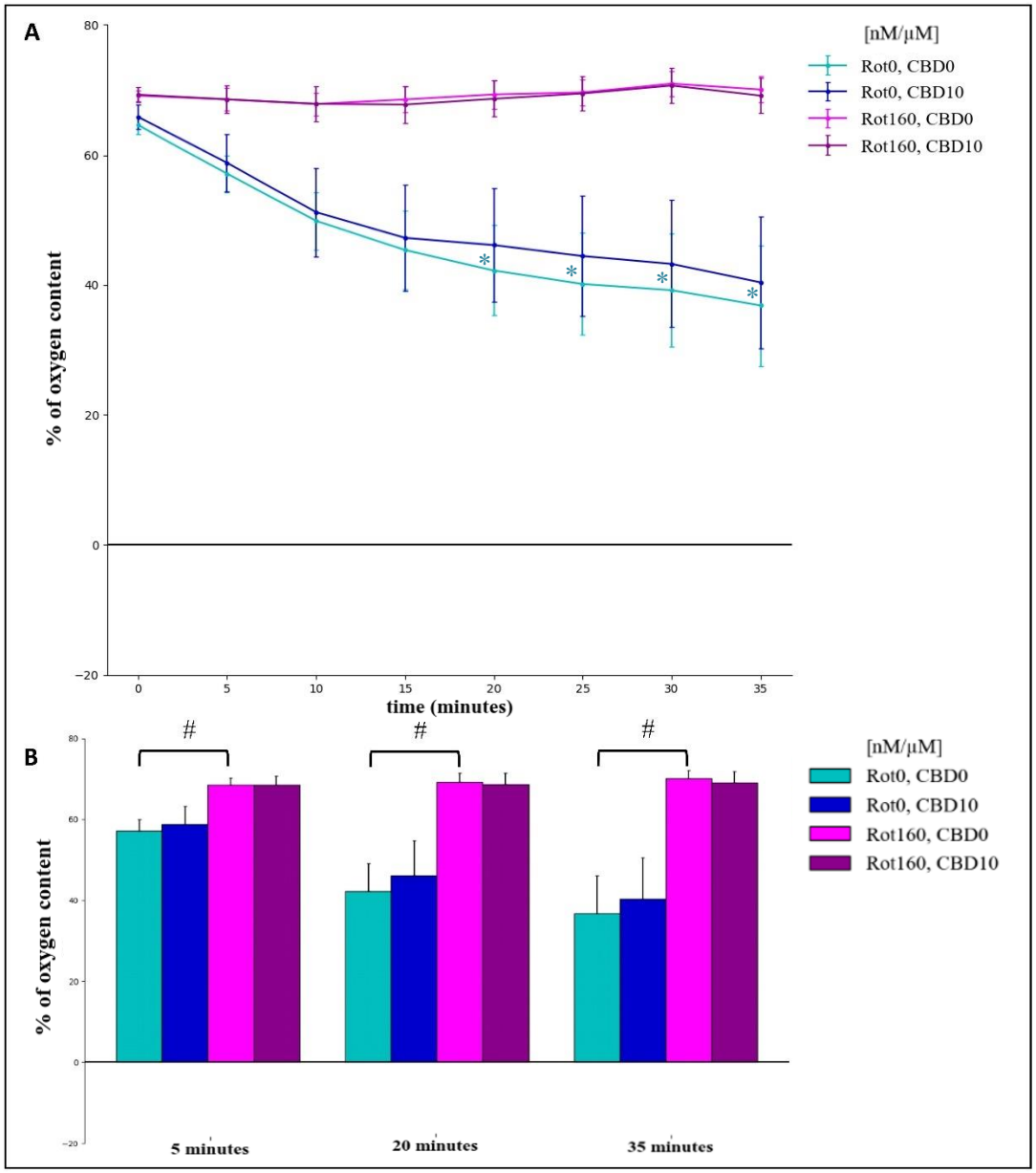


Figure 21. Effect of rotenone and CBD on the oxygen consumption of the N18TG2 cells. Data represent the mean +/- S.E.M. of n = 5 independent experiments. **A)** Effects of 160 nM rotenone and 10 µM CBD on the oxygen consumption compared to control. Significant impact compared to the 0-minute timepoint (*, Kruskal-Wallis-test). **B)** No significant effects of rotenone on the oxygen consumption could be determined by the Mann-Whitney-*U*-test (#).

Whereas the partial oxygen pressure in the controls, which were not treated with rotenone, decrease over time, the oxygen in the treated wells remains stable at around 69.25 % (figure 21).

This distinction to control was significant from the beginning. Although CBD slightly decreased the oxygen consumption of unmodulated cells, the cannabidiol treatment did not trigger significant effects on the control, or the rotenone treated cells.

4 Discussion

4.1 Method optimization using CCCP, rotenone and oligomycin

The following section discusses the pretests to optimize the methods. During this process, these methods were tested and the concentrations of the mitochondrial toxins chosen for further experiments were determined. Since biological systems such as cells react differently to changes in their environment, the relatively high standard errors observed in the experiments were expected.

4.1.1 Determination of an optimized CCCP concentration for the cannabidiol-experiments

In accordance with the literature, small concentrations of CCCP (0.25 µM, 1 µM) significantly depolarized the MMP due to its ability to transport protons across the inner mitochondrial membrane. Therefore the sustainability of the MMP is prevented (Fedyaeva et al., 2014). Since the complexes of the respiratory chain are not affected by CCCP, they try to compensate the loss of the MMP through enhanced activity. This results in an increased oxygen consumption, as observed in the OxoPlate® experiments. Also this finding correlates with previous studies (Pilatus et al., 2001).

Treatment with small CCCP concentrations (1 µM, 5 µM) lead to a significant decrease of the partial oxygen pressure below the basal line (0 %). This phenomenon could be explained by the definition of the basal line by a Na_2SO_3 solution. Even though this solution was oversaturated, it is not guaranteed that it was completely oxygen-free.

The ability of higher CCCP concentrations to reduce the cellular respiration is connected to reduced viability of the cells (Shen et al., 2003).

A comparison between the two experiments highlights, that lower concentrations of CCCP appear to have a greater effect on the cells. Since 1 µM CCCP triggered significant effects on the MMP as well as on the oxygen consumption, we decided to further proceed with this concentration.

4.1.2 Determination of an optimized rotenone concentration for the cannabidiol-experiments

Since rotenone inhibits the respiratory chain at the first complex, the cells cannot continue with oxidative phosphorylation and no molecular oxygen is needed anymore. In our study, the administration of 160 nM of rotenone inhibited the uptake of oxygen of the N18TG2 cells significantly and almost completely. This was in accordance with Telford et al. (2009), who reported a dose dependent inhibiton of rotenone on the oxygen consumption of neurons. Since this effect could be observed five minutes after the treatment, the inhibitory effect of rotenone on complex 1 seems to be immediate, which was also outlined by Telford et al. (2009).

In the JC-1 experiments, insignificant tendencies of hyperpolarizing the MMP, with 160 nM Rotenone having the highest impact, were observed. These findings are contradictory to literature, where a depolarization of the MMP through the damaging effects of rotenone, like ROS production, was examined. However, in contrast to our method of measuring the acute effects of rotenone, other studies applied incubation times of 15 minutes to 48 hours. (Isenberg & Klaunig, 2000; Moon et al., 2005). This leads to the conclusion that the damaging effects of rotenone on the MMP do not appear acute, but rather depend on secondary reactions of the cells.

Since 160 µM rotenone showed the highest effects, we decided to further proceed with this concentration for the CBD experiments.

4.1.3 Determination of an optimized oligomycin concentration for the cannabidiol-experiments

The JC-1 experiments show unsignificant hyperpolarizing activities of the MMP through oligomycin. These results correspond to literature, where the hyperpolarization of the MMP was linked to the direct interference of oligomycin with the ATP-synthase, resulting in a reduced H^+ reflux (Perry et al., 2011; Zimmermann et al., 2017).

Although the subsequent high proton gradient was reported to further lead to the stop of cellular respiration (Al Maruf et al., 2014), no effect of oligomycin on the oxygen consumption rate was discovered by the OxoPlate® experiments.

Since it is impossible, without being too speculative, to explain why oligomycin did not affect the mitochondria of our cells, we decided to not proceed with this substance in the cannabidiol experiments.

4.2 Impact of CBD on neuroblastoma cells

The following experiments were designed to ascertain whether CBD influences neuroblastoma cells. Additionally, it was investigated if the modulation of the mitochondria through the uncoupler CCCP or the ATP synthase inhibitor rotenone has an impact on these effects or if CBD may have the ability to counteract the damaging effects of these toxins. All these influences and interdependences could shed light on the mechanism behind the effects of CBD, for instance the site of its action.

4.2.1 Effect of CBD alone on neuroblastoma cells

The treatment with 10 µM CBD for four hours did hyperpolarize the MMP by 17.78 % compared to control. This could be seen before treatment with CCCP or rotenone (timepoint 0) in the JC-1 experiments. Since in the further course of the JC-1 experiments only the changes triggered by the mitochondrial toxins were observed, it was not expected to see further impacts of CBD alone. On contrary, Rimmerman et al. (2013) reported depolarizing effects of CBD. Nonetheless, it was reported, that the hyperpolarization of the MMP can result from impeding of the mitochondria and can lead to depolarization which is mostly accompanied by apoptosis (Iijima, 2006).

In contrast to previous studies of the institute, in which 10 µM CBD had a reducing effect on mitochondrial activity after four hours of treatment (Unterberger et al., 2016), the OxoPlate® experiments did not confirm these results.

4.2.2 Effect of CBD on CCCP treated neuroblastoma cells

To determine the impact of CBD on cells whose mitochondrial respiratory chain was uncoupled by CCCP, four sample groups (+/- CBD, +/- CCCP) were prepared.

Both experiments, JC-1 and with OxoPlates®, confirmed the significant deleterious effect of CCCP on the mitochondrial activity and the MMP that was described in the literature, as outlined in chapter 4.1.1. (Fedyaeva et al., 2014).

The treatment with CBD appeared to enhance the uncoupling effects of CCCP. This resulted in a further significant depolarization of the MMP compared to the CCCP control. Additionally, CBD led to insignificant acceleration of the oxygen uptake. Since the pattern of oxygen consumption of CBD + CCCP treated cells followed that of CCCP control cells, no protective effect against CCCP could be observed. This result is contradictory to a study conducted by Ryan et al., (2009), that reported neuroprotective effects of CBD against carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), which is also an uncoupler of the mitochondrial respiration. CBD alone showed no effect on the oxygen consumption and therefore an acute uncoupling effect of CBD can be excluded.

Concluding it can be implicated that the action of CBD is coupled to the effect of CCCP or vice versa. A voltage dependent binding site of CBD that is activated trough a depolarizing MMP to enhance the impact of CBD as well as a direct interaction between the two substances could be possible explanations. It has to be stated that since we do not know exactly where CBD binds and acts on the mitochondria, all these suggestions are still speculative. More research is needed to explain the observed effects of CBD.

4.2.3 Effect of CBD on rotenone treated neuroblastoma cells

As reported by Radad et al. (2019), rotenone induces effects that are similar to the symptoms of neurodegenerative diseases and therefore serves as a model substance. These experiments aimed to investigate the protecting effects of CBD on rotenone treated cells as well as the CBDs mode of action.

The harmful effect of rotenone could only be proven in the OxoPlate® experiments. As described in chapter 4.1.2., the lack of impacts of rotenone on the MMP could be caused by the treatment of the cells that was only performed acutely in contrast to previous studies (Isenberg & Klaunig, 2000; Moon et al., 2005).

Although Duvigneau et al. (2020) and Atalay et al. (2020) reportet protecting and antioxidative impacts of CBD on rotenone-induced alterations, no significant effect of CBD on rotenone-treated cells could be seen in this study.

These results suggest that the protective effects that CBD might have, are not acute. Furthermore, a direct interference between CBD and rotenone is -based on the obtained data- not likely.

4.3 Discussion about the JC-1 method

Especially the data collected with the JC-1 method showed a lot of variances and no significant effects most of the time. Thus, the observed tendencies could be only a result of background noise. This could also explain the lack of effects by rotenone on the MMP.

One explanation for the strong deviations of the data could be the photobleaching of the JC-1 dyes during the measurements (Nicholls & Ward, 2000). Although this was compensated by the calculation of a bleaching factor, like described in chapter 2.2.4, further studies are required to determine, if this is type of calculation is sustainable. Since the problem of photobleaching occurs in all fluorescence dyes, it might be an useful idea to modify the method of measurement. For instance, the reduction of the time of light exposure or the light intensity could be beneficial (Perry et al., 2011).

5 Conclusion and outlook of this study

This study aimed to elucidate whether CBD has damaging effects or protects against mitochondrial stress, to investigate possible therapeutical applications. The following paragraph takes up the questions stated in the introduction in order to summarize the answers obtained during the study.

(1) What concentrations of the mitochondrial toxins CCCP, rotenone and oligomycin show the highest impact on the N18TG2 cells and can be used in further experiments?
   - Due to the results obtained from the pretests, the concentrations of 1 µM CCCP and 160 nM rotenone were determined for the further cannabidiol experiments. Since oligomycin did not show significant impacts on the cells, this substance was not used in the CBD experiments.

(2) What impact does CBD have on the mitochondrial membrane potential and the oxygen consumption of the neuroblastoma cells?
   - The treatment with only CBD for four hours had a hyperpolarizing effect on the MMP. The OxoPlate® experiments could not reveal an acute impact of CBD on the oxygen consumption of the neuroblastoma cells.

(3) Does CBD show any influence on CCCP- or rotenone-treated cultures to mitigate or reinforce their effects?
   - In the present study, no acute protecting effect of CBD against rotenone or CCCP induced damages could be observed. On contrary, the negative impacts of the uncoupler CCCP were enhanced through CBD treatment.

(4) Is it possible to detect the site of action of CBD through modulation with CCCP and rotenone?
   - The enhanced depolarizing effect due to the CBD + CCCP treatment suggests either an acceleration of the negative effect of CBD by uncoupling or a direct interaction between the two substances. From the rotenone experiments, no indication on the mode of action of CBD can be taken.

To confirm or reject all the hypotheses taken above, further studies are needed. For instance, to evaluate the hyperpolarizing effect of CBD, it would be useful to look at the long-term effects

of this substance. Such an approach would also allow for testing of the effects of rotenone on the MMP. However, after long-term treatment it cannot be distinguished between acute effects that are directly caused by the substance and effects resulting from the cellular response. One approach to solve this problem would be to directly look at isolated mitochondria which was for instance also done by Vergun et al., (2003). To test the effects of uncouplers other than CCCP on CBD could provide more information about the reinforcement of the latter trough the uncoupling mechanism.

In summary, this study shows that it is not easy to determine the mode and site of action of CBD. Therefore, we assume that CBD acts through different binding sites to trigger different responses in cells.

6 Summary

Cannabidiol is one of more than 100 different phytocannabinoids that are derived from the plant *Cannabis sativa*. Together with the endogenous endocannabinoids, it acts on the endocannabinoidsystem, which contributes to the control of vital systems such as the immune, cardiovascular and respiratory system and to neuromodulation. However, how cannabidiol exerts its effects is only partially understood. Together with the cannabinoid receptors 1 & 2, at least 65 molecular sites are possible targets for cannabidiol.

In previous studies, cannabidiol has been reported to have both neuroprotective but also toxic properties. Therefore, it is controversially discussed as a therapeutic against neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease on the one hand, and to fight cancer on the other hand. Since mitochondria contribute to the pathogenesis of both kinds of disorders, an effect of cannabidiol on these organelles is suspected.

The aim of this study was to investigate the acute impact of cannabidiol on the mitochondria, as well as a possible neuroprotective effect against mitochondrial stress. This was modeled by carbonylcyanide-m-chlorophenylhydrazone, an uncoupler of the mitochondrial respiratory chain, and rotenone, a complex 1 inhibitor. Possible interactions of cannabidiol with these mitochondrial toxins should provide information about its site of action. As parameters to monitor the cellular viability after the modulation and treatment, JC-1 assays were used to observe mitochondrial membrane potential whereas OxoPlates® were used to measure the cellular oxygen consumption.

While cannabidiol alone showed a hyperpolarizing effect on the mitochondrial membrane potential, it had no impact on the cellular oxygen consumption. Moreover, a protective property against the mitochondrial toxin-induced stress was not observed. On the contrary, a significant enhancement of the damaging effect of carbonylcyanide-m-chlorophenylhydrazone by cannabidiol was observed, indicating a direct influence of cannabidiol on the mitochondria.

7 Zusammenfassung

Cannabidiol ist eines von mindestens 100 verschiedenen Phytocannabinoiden, welche aus der Pflanze *Cannabis sativa* gewonnen werden. Zusammen mit den körpereigenen Endocannabinoiden wirkt es auf das Endocannabinoidsystem, welches zur Steuerung lebenswichtiger Systeme, wie dem Immun-, Herz-Kreislauf- und Atmungssystem und zur Neuromodulation beiträgt. Wie jedoch Cannabidiol seine Wirkung entfaltet, ist nur teilweise verstanden. Die Cannabinoid Rezeptoren 1 & 2 gehören zu mindestens 65 anderen möglichen molekularen Zielen dieser Substanz in der Zelle.

In vorherigen Studien wurden Cannabidiol sowohl neuroprotektive als auch toxische Eigenschaften nachgesagt. Dies führt zur kontroversen Diskussion der Substanz auf der einen Seite als Therapeutikum gegen neurodegenerative Erkrankungen wie beispielsweise Morbus Alzheimer oder Morbus Parkinson und auf der anderen Seite zur Bekämpfung von Krebs. Da Mitochondrien zur Pathogenese beider Arten von Störungen beitragen, wird eine Wirkung von Cannabidiol auf die Mitochondrien vermutet.

Das Ziel dieser Arbeit war es, den akuten Effekt von Cannabidiol auf die Mitochondrien, sowie eine mögliche neuroprotektive Wirkung gegen mitochondrialen Stress zu untersuchen. Dieser wurde durch Carbonylcyanid-m-chlorophenylhydrazon, einem Entkoppler der mitochondrialen Atmungskette, und Rotenon, einem Komplex 1 Inhibitor, gemodelt. Mögliche Wechselwirkungen von Cannabidiol mit den mitochondrialen Giften sollten Aufschluss über dessen Wirkort geben. Als Parameter für die Lebensfähigkeit der Zellen nach Modulation und Behandlung wurden JC-1 Versuche zur Überprüfung des mitochondrialen Membranpotentials und OxoPlates® zur Messung des Sauerstoffverbrauchs der Zellen eingesetzt. Cannabidiol alleine zeigte eine hyperpolarisierende Wirkung auf das mitochondriale Membranpotential hatte jedoch keinen Einfluss auf den Sauerstoffverbrauch der Zellen. Auch eine protektive Eigenschaft gegen den durch mitochondriale Toxine verursachten Stress konnte nicht beobachtet werden. Im Gegenteil dazu wurde eine signifikante Verstärkung des schädigenden Effekts von Carbonylcyanid-m-chlorophenylhydrazon durch Cannabidiol festgestellt, welche auf eine direkte Wirkung von Cannabidiol auf die Mitochondrien hindeutet.

8 Abbreviations

2-AG 2-Arachidonyl-glycerol
5-HT_{1A} ..... 5-hydroxytryptamine receptors
cal 0 ..... calibration 0
cal 100 ..... calibration 100
CB1R ..... cannabinoid receptor 1
CB2R ..... cannabinoid receptor 2
CBD ..... Cannabidiol
CCCP carbonylcyanide-3-chlorophenylhydrazone
CNS ..... central nervous system
DMSO ..... Dimethyl sulfoxide
DPBS ..... Dulbecco's Phosphate Buffered Saline
ESC ..... Endocannabinoid system
FAD ..... flavin adenine dinucleotide
FCCP ..... carbonylcyanide-p-trifluoromethoxyphenylhydrazone
GPR55 ..... G protein-coupled receptor 55
JC-1 ..... tetraethylbenzimidazolylcarbocyanine iodide
MMP ..... mitochondrial membrane potential
NAD+ ..... nicotinamide adenine dinucleotide
NCX ..... Na+/Ca2+ -exchanger
OSCP ..... oligomycin-sensitivity conferring protein
PBS ..... phosphor buffered saline, phosphate buffered saline
PDL ..... poly-D-lysine
PNS ..... peripheral nervous system
pO_2 ..... partial oxygen pressure
PPARγ ..... Peroxisome proliferator-activated γ receptors
RFU ..... relative fluorescence units
ROS ..... reactive oxygen species
S.E.M ..... standard error of mean
THC Δ9-tetrahydrocannabinol

TRPV ..... transient receptor potential vanilloid channel
VDAC ..... voltage-Dependent Anion Channel

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10 Figures and tables