University of Veterinary Medicine Vienna

Institute of Medical Biochemistry

(Head: Univ.-Prof. Dr.rer.nat. Florian Grebien)

Neurotoxic effects of cannabidiol on murine mesencephalic primary cells in combination with glycolytic inhibitor 2-deoxy-D-glucose and effects of ketogenic diet on the viability of primary cells *in vitro*

Bachelor Thesis

University of Veterinary Medicine Vienna

Submitted by

Lena Daxböck

Vienna, December 2020

Supervisor: Dipl.-Biol. Dr. rer.nat Rudolf Moldzio

Reviewer: Ao.Univ.-Prof. Dr.med.vet. Alois Strasser

Acknowledgement

I would first like to thank my supervisor, Dipl.-Biol. Dr. rer.nat Rudolf Moldzio, for giving me the opportunity to work on my bachelor thesis in his lab. You supported me excellently and always had an open ear when I had questions and when I needed some advice.

I would also like to acknowledge Christopher for his valuable guidance and endless patience throughout my practical work. You explained to me everything I needed to know and provided me with tools that will help me in the future.

I would also like to thank Barbara who heartily welcomed me and helped me out in scientific and emotional questions.

I wish to show my gratitude to all members of the department of Biomedical Sciences for a positive working atmosphere. I would particularly like to single out Natascha, I want to thank you for your encouraging words.

In addition, I would like to thank my parents for their loving support. Mum, you are always there for me, you give good advice and help me out when self-doubt seems to be overwhelming. Dad, even though you are not the most optimistic person I know, you have always believed in me and I know I can always count on you! Leon, my little brother, thank you for your patience and the happy distractions when I had to rest my mind. Our snowman was gorgeous. Finally, I want to thank Marko for being there for me, for calming me down when I felt bad and for constantly motivating me to not give up. You helped me more than you might think.

Table of Contents

1. Introduction

People have been aware of the physical, psychological and emotional benefits of phytocannabinoids, extracts of the annual herbaceous plant *Cannabis sativa*, for centuries.(Maroon & Bost, 2018) There is some evidence that extracts were used around 5,000 years ago in ancient China.(Zou & Ujendra Kumar, 2018) Generally, cannabinoids can be categorized into three main groups. The extracts from the plant *Cannabis sativa*, internally synthesized endocannabinoids and synthetic cannabinoids.(Chilakapati & Farris, 2014) Cannabidiol (CBD), a phytocannabinoid, has recently become an intensively researched substance for its potential therapeutic efficacy on neurological disorders, inflammation, addiction, epilepsy and cancer treatments.(Maccarrone, 2020; Maroon & Bost, 2018) Nevertheless, these findings are under debate. Scientist have presented controversial results of their investigations.(Maroon & Bost, 2018) Recently, there has been a strong sales growth of CBD products in Austria, promising that CBD provides the cure for multiple illnesses. Though, the effects of CBD have not yet been fully understood. Therefore CBD medication was proposed to remain unavailable without prescription as long as it has not been proven safe and sufficient for positive therapeutic effects.(Huestis et al., 2019)

CBD plays a role in recent cancer treatment research. It is known that cancer cells have a higher need of energy than normal cells. Most energy is required for tumor growth and immune system activation.(Fadaka et al., 2017) The increased usage of carbohydrates is well studied. Tumor cells reprogram the metabolism to an upregulated glycolysis to obtain ATP, which leads to a downregulated oxidative phosphorylation. However, recent studies argue that the mitochondria of cancer cells remain intact.(Park et al., 2020) This damage of respiratory mechanisms, especially in the mitochondria is also known as the Warburg effect.(Park et al., 2020) CBD is considered a potential anticancer agent i.a. by interfering with the mitochondrial energy metabolism. Mitochondrial activity in cancer cells is reduced due to the downregulation of oxidative phosphorylation, thus protecting the cancer cells against reactive oxygen species (ROS).(Harris & Johnson, 2019) It is assumed that CBD can cause an increased production of ROS in cancer cells, preventing tumor growth and metastasis.(Huestis et al., 2019; Maroon & Bost, 2018)

A ketogenic diet is a high in fat and low in carbohydrates diet. The application of a ketogenic diet is considered to support cancer therapy, altering the primary energy metabolism from glucose to ketone bodies.(Lin et al., 2020) A ketogenic diet targets the Warburg effect, as

glucose levels are highly reduced. While normal cells are able to change their metabolism by using ketone bodies as an alternative source of energy, some cancer types might not be able to adapt because of their downregulated mitochondria and therefore get damaged.(Weber et al., 2018)

One aim of this thesis is to ascertain if CBD in combination with inhibiting aerobic glycolysis causes more cytotoxicity in glioma and neuroblastoma cells than in murine mesencephalic primary cells. Furthermore, effects of a ketogenic diet were investigated on primary cells *in vitro*.

1.1. The endocannabinoid system

The endocannabinoid system is found in primitive species; therefore, it is considered a phylogenetically old part of the neuronal network. Phytocannabinoids act on the same receptors as endocannabinoids.(Burggren et al., 2019)

The endocannabinoid system includes cannabinoid receptors (CBRs), endocannabinoids, and enzymes responsible for the synthesis and degradation of the endocannabinoids. The endocannabinoid system plays an important role as a widespread neuromodulatory system involved in central nervous system development, synaptic plasticity, learning and other fundamental brain functions. Additionally, it is involved in the response to environmental factors such as psychological stress, drugs, cigarettes, alcohol, radiation, and hypoxia (Lu & Mackie, 2016; Torii et al., 2017) CB_1 and CB_2 are the main receptors of the complex endocannabinoid system (Fig.: 1).

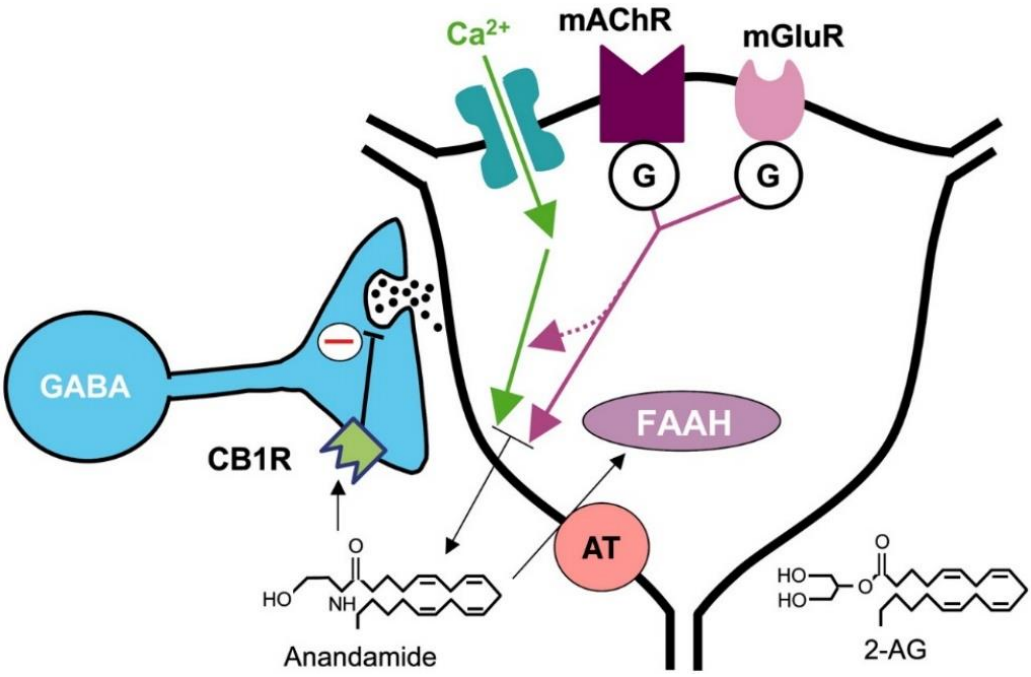

Figure 1. The neuronal endocannabinoid system. Increased intracellular calcium levels or activation of G protein-coupled neurotransmitter receptors, represented by the type I metabotropic glutamate receptor (mGluR) and the muscarinic acetylcholine receptor (mAChR) leads to synthesis of endocannabinoids such as 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide, AEA). The uptake of endocannabinoids into postsynaptic or presynaptic cells is mediated by anandamide transporters (AT). Fatty acid amide hydrolase (FAAH) is involved in synthesis and degradation.(Alger, 2004)

1.1.1. Cannabinoid receptors

CBRs belong to the G-protein coupled receptor family (GPCRs), primarily coupling to proteins of the $G_{i/o}$ classes.(Lu & Mackie, 2016) The biological effects of cannabinoids are mainly mediated by CB_1 and CB_2 receptors.(Wu, 2019) The activation of GPCRs inhibits adenylyl cyclases and certain voltage dependent Ca^{2+} channels, whereas several mitogen-activated protein kinases (MAPK) are activated. If activated, CB_1 and CB_2 receptors differ among their impact on cell structure and function.(Lu & Mackie, 2016)

CB_1 receptors are predominantly expressed in in the central and peripheral nervous system. The majority of CB_1 receptors are present on axon terminals playing a major role in inhibiting neurotransmitter release via a presynaptic mechanism.(Lambert, 2009) The high expression level of CB_1 receptors in brain tissue suggests participation in modulating memory, emotions, pain, and locomotion. Delta-9-tetrahydrocannabinol (Δ^9-THC), a molecule known to target CB_1 receptors, reduces nociception in animal studies. Acute, visceral, inflammatory, and chronic pain was shown to be reduced.(Maroon & Bost, 2018) CB_1 receptors of the central nervous system (CNSs) have shown an inhibition of glutamatergic, GABAergic, glycinergic, cholinergic, noradrenergic and serotonergic neurotransmission when activated. CB_1 receptor-mediated inhibition of adrenergic, cholinergic and sensory neuroeffector transmission has been shown in the peripheral nervous system.(Szabo & Schlicker, 2005)

CB_2 receptors are mainly located on the surface of immune cells, suggesting their importance in regulating the immune system.(Dariš et al., 2019) The rrole of the CB_2 receptors in several systems such as the cardiovascular system, respiration, the CNSs, the gastrointestinal tract, the liver and reproduction are studied.(Ye et al., 2019)

1.1.2. Endocannabinoids

Endocannabinoids are hydrophobic lipids acting as autocrine, paracrine and endocrine messengers, essential for bioregulation.(Navarrete et al., 2020) They are internally synthesized *de novo* and released, as they cannot be stored in vesicles due to their hydrophobicity.(Deutch, 2013) Endocannabinoids are also characterized as endogenous ligands with a short half-life. They bind and activate CB_1 and CB_2 receptors.(Piscitelli, 2015) Uniquely, they are able to diffuse from postsynaptic to presynaptic cells, known as retrograde transmission.(George T Griffing & Thai, 2019)

N-arachidonoylethanolamine (anandamide, AEA), and 2-arachidonoylglycerol (2-AG) are the two best-studied endocannabinoids. AEA and 2-AG are derivates of arachidonic acid, a

polyunsaturated fatty acid. Endocannabinoids are important for physiological, central and peripheral processes.(Correia-Sá, 2020) The synthesis requires several steps catalyzed by enzymes. AEA is produced in cell membranes by hydrolyzation of N-arachidonoyl-phosphatidylethanolamine with the help of the N-acylphosphatidylethanolamine, specific phospholipase D (NAPE-PLD).(Navarrete et al., 2020) AEA transporters are mainly located in neurons and glia. The fatty acid amide hydrolase (FAAH) is responsible for the inactivation of AEA. FAAH is found in intracellular membranes of post synapses and other organs. When AEA is inactivated, it is metabolized to arachidonic acid and ethanolamine.(Navarrete et al., 2020) 2-AG synthesizes is stimulated by depolarization of neuronal membranes or if Gq-coupled receptors (GPCRs) are activated. 2-AG is involved in prostaglandin synthesis as it provides arachidonic acid as an intermediate metabolite.(Navarrete et al., 2020) Furthermore, increased intracellular calcium concentrations stimulate the phospholipase C (PLC) for a higher metabolization of membrane phosphoinositides to diacylglycerol (DAG). Then the DAG lipase transforms DAG into 2-AG.(Deutch, 2013) Three more endocannabinoids are well studied, namely noladin ether, virodhamine and N-arachidonyl-dopamine. All five mentioned endocannabinoids have the same 19-C backbone in common, differing only in the constitution of the R-group.(George T Griffing & Thai, 2019)

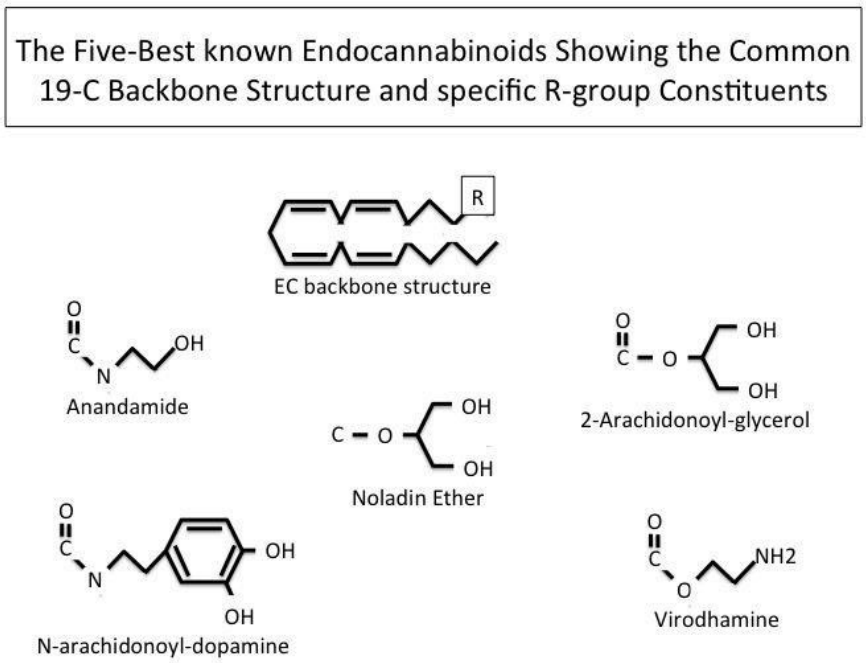

Figure 2. Overview of the five-best known endocannabinoids(George T Griffing & Thai, 2019)

1.1.3. Phytocannabinoids

Phytocannabinoids are secondary metabolites of the plant *Cannabis sativa*. These herbal cannabinoids can be taken up by smoking, vaporizing, oral ingestion, intravenous injection, sublingual absorption, or rectal suppository. More than 500 chemical constituents have been found in *Cannabis*, of which around 104 are referred to as phytocannabinoids.(Lafaye et al., 2017) Several subtypes can be further distinguished.(Fisar, 2008) However, all of them show the same terpenophenolic C-21 backbone. The most studied constituents are the psychoactive Δ^9-tetrahydrocannabinol (Δ^9-THC) and the non-psychoactive cannabidiol (CBD) (Fig.: 3). THC is a partial agonist for the CB_1 and the CB_2 receptors, with a higher affinity for CB_1. THC is referred to have observable effects on behavior, nociception, and appetite. Furthermore anti-inflammatory, antitumor, and antiemetic properties have been reported.(Poyatos et al., 2020) On the other hand, many side effects have been observed ranging from anxiety to immunosuppression.(Andre et al., 2016)

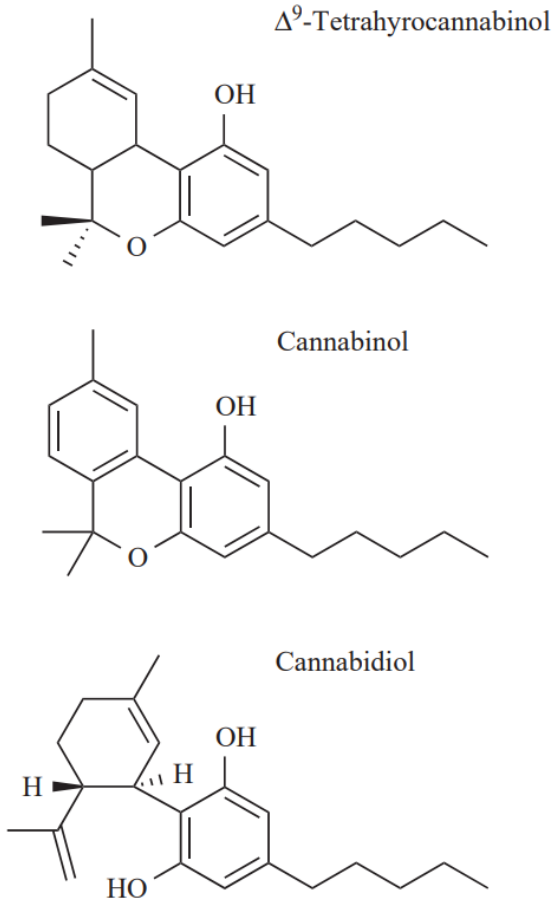

Figure 3. Structure of the isomer delta tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD)(Fisar, 2008)

1.1.4. Cannabidiol

CBD is a cannabis-derived compound, which in contrast to THC, does not act psychoactive. It has become a molecule of great interest due to its promising therapeutic potential, especially for brain disorders. By now, more than 65 molecular targets have been found including the G-protein receptor protein 55 (GPR55), transient receptor potential (TRP) channels, and cytochrome P450s.(Elsaid & Le Foll, 2020) Unlike THC, CBD shows a low affinity to the cannabinoid receptors CB_1 and CB_2, although recent studies have shown the negative allosteric activity of CBD on CB_1.(Poyatos et al., 2020) Recently, CBD has been a well-researched molecule due to its ability to interact with many different molecular targets. CBD seems to be a promising candidate for several treatments such as anti-tumor therapies. The efficacy as cancer treatment is already demonstrated in animal models for several cancer types such as breast neoplasm, glioma, glioblastoma, and leukemia.(Alharris et al., 2019; Elsaid & Le Foll, 2020) Alharris et al. showed in their studies that CBD induces apoptosis in neuroblastoma cells, one of the most aggressive cancers in infants and young children, through activation of cannabinoid receptors. Especially, CBD induced activation of the 5-HT2A serotonin receptor and the transient receptor potential cation channel subfamily V member 1 (TRPV1 or vanilloid receptor 1) were shown to play an important role in mediating apoptosis and autophagy and preventing cancer growth of gliomas and prostate cancer.(Alharris et al., 2019)

1.1.5. Effects of CBD on the brain

CBD shows a low affinity to both CB_1 and CB_2 receptors, which makes it a non-psychotropic drug. However, it was observed that CBD can act as an antagonist to CB_1 in the mouse vas deferens and brain tissues *in vitro*.(Seltzer et al., 2020) It is considered that CBD has only partial efficacy on CB_2.(Burggren et al., 2019)

Further, modulation of intracellular calcium levels and the potential to lower the release of pro-inflammatory cytokines have been discovered. Considering these anti-inflammatory effects, CBD is a potential modulator for nociception and inflammation.(Millán-Guerrero & Isais-Millán, 2019) As CBD is known to have multiple targets it can modulate the activity of excitatory and inhibitory signaling pathways, especially in the prefrontal cortex and the basal ganglia. The detailed mechanisms are still not completely understood, while there is substantial evidence that CBD helps regulating the release of glutamate and γ-aminobutyric acid (GABA).(Pretzsch et al., 2019)

There are studies about the benefits of CBD pointing out its great potential as a therapeutic agent, however, there are also studies showing contrary effects. Therefore, medicinal use of CBD needs to be further investigated and well-controlled clinical studies are necessary.(Millán-Guerrero & Isais-Millán, 2019)

1.2. Cell metabolism

Cell growth is the result of coupling between substrate catabolism and several metabolic processes required for biomass formation and maintenance processes.(Rigoulet et al., 2020) The energy metabolism is a very complex system including various pathways, enzymes and chemical reactions. Glycolysis, the Krebs cycle, and oxidative phosphorylation are the three main parts of cell metabolism consisting of many chemical steps each. In the end, adenosine triphosphate (ATP), the primary currency of energy, and other carrier molecules are synthesized.(Alberts & Johnson, 2015)

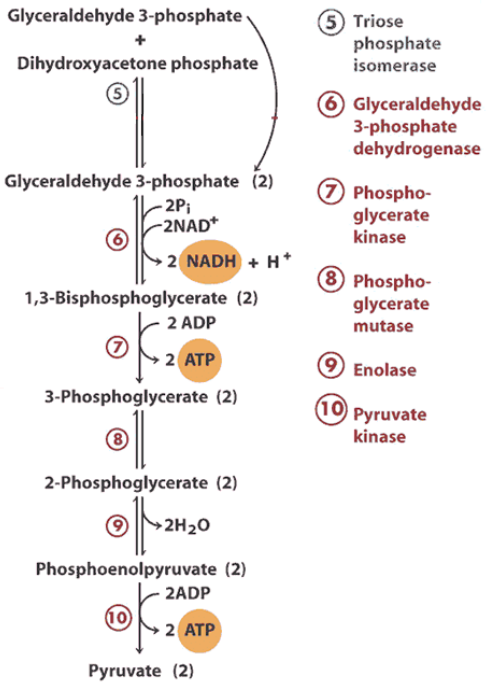

Figure 4. Overview of glycolysis pathway. First, glucose is metabolized into three-carbon sugar phosphates and then further to pyruvate(Redalen, 2006)

Glucose ($C_6H_{12}O_6$) is one of the main sources of energy for cells. Therefore, the uptake of carbohydrates is essential for mammalian cells to maintain their biological function. Glucose,

an aldohexose, is metabolized as follows: First, during glycolysis, located in the cytosol, one molecule glucose is catabolized into two molecules of pyruvate, ATP and nicotinamide adenine dinucleotide (NADH) (Fig.: 4). Second, the enzyme CoA transforms pyruvate into acetyl-CoA. Following that, it enters the tricarboxylic acid cycle (TCA), where most of the carbon compounds are oxidized, generating guanosine triphosphate (GTP) or ATP (depending on the cell type), three molecules of NADH and one flavin adenine dinucleotide ($FADH_2$) molecule per cycle (Fig.: 5).(Alberts & Johnson, 2015)

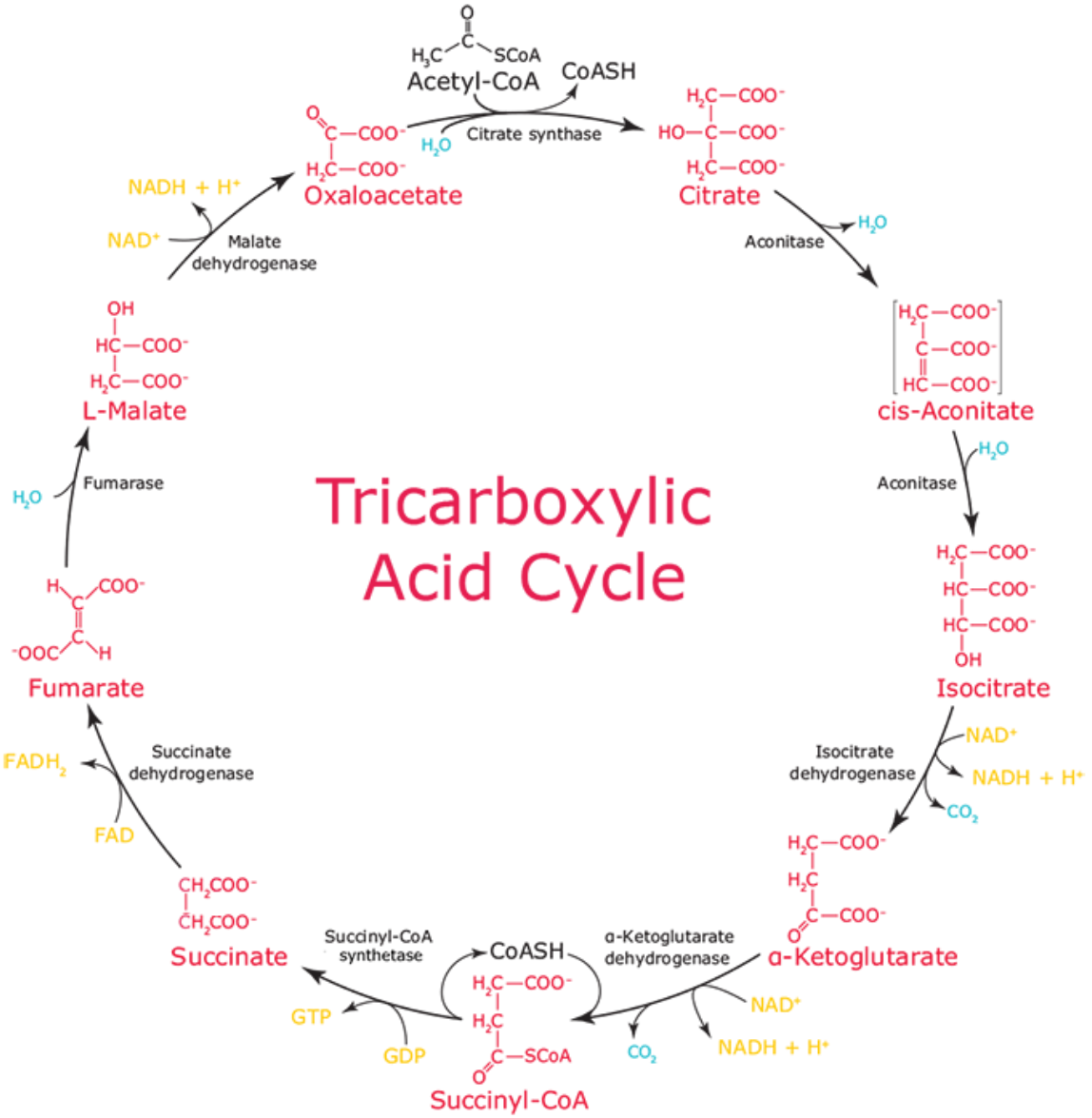

Figure 5. Overview of the TCA: In eukaryotes, it occurs in the mitochondrial matrix and begins with the transformation of pyruvate into acetyl-CoA. In a series of chemical reactions acetyl-CoA is oxidized to oxaloacetate. The outcome of the TCA is three NADH, one $FADH_2$, and one GTP.(The Krebs Cycle — Harnessing Chemical Energy for Cellular Respiration, 2007)

Finally, the electron carriers NADH and $FADH_2$ are transported to the inner mitochondrial membrane, where the mitochondrial oxidative phosphorylation is located. Molecular oxygen (O_2) serves as oxidizing agent by accepting the electrons. The electron transport chain consists of five complexes (I, II, III, IV and the ATP synthase) that produce a proton gradient across the membrane. The proton electrochemical potential provides the essential energy used by the ATP synthase to phosphorylate to synthesize ATP (Fig.: 6).(Alberts & Johnson, 2015; Rigoulet et al., 2020)

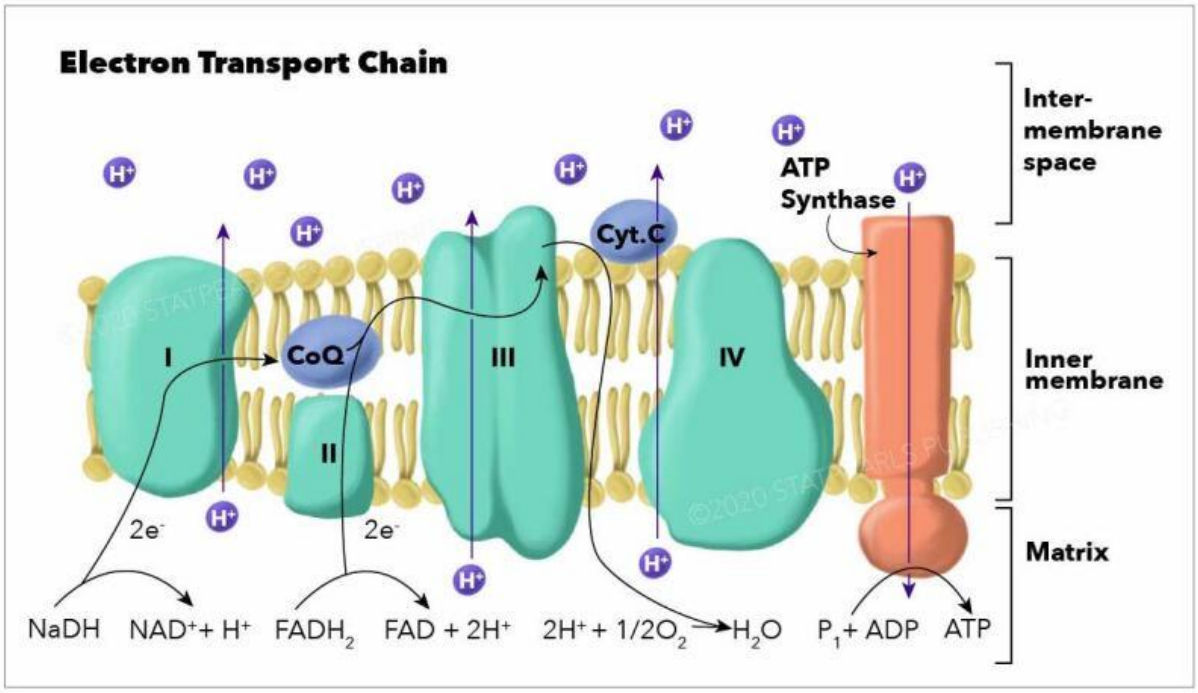

Figure 6. Oxidative phosphorylation: Overview of the electron-transport chain, embedded in the inner mitochondrial membrane. It consists of NADH dehydrogenase (I), succinate dehydrogenase (II), cytochrome-c reductase (III), and cytochrome-c oxidase (IV). Mobile electron carriers such as coenzyme Q and cytochrome-c are also involved. The electron transport chain ends with the phosphorylating ATP synthase.(Ahmad et al., 2020)

1.3. Metabolism in cancer cells

Cancer is a result from a series of molecular events that fundamentally alter the normal properties of cells, leading to uncontrolled cell division.(Adjiri, 2016) There are more than a hundred different cancer types that differ among their aberrant properties.(Alberts & Johnson, 2015) However, hallmarks of cancer cells can be observed, including loss of sensitivity to anti-growth signals, to programmed cell death or apoptosis. They can become immortal and display altered metabolic properties, higher mutation rates. Cancer cells succeed to avoid immune

destruction and are able to attract blood vessels. Furthermore, they can invade tissues and become metastatic.(Adjiri, 2016). An altered tumor metabolism and reduced mitochondrial activity provides the energy needed for cancer growth, migration, invasion, and tumor metastasis. However, many metabolic pathways and mechanisms of this complex and dynamic system are still not understood or have not even been discovered yet. Nevertheless, these metabolic changes are an important part of cellular transformation and provide a key target for new anti-cancer drugs.(DeBerardinis & Chandel, 2020; Frezza, 2020)

In 1924, Otto Heinrich Warburg showed that cancer cells, even if enough oxygen is available, have an increased uptake of glucose and higher rates of lactate secretion. These facts indicate an increased aerobic glycolysis compared to normal cells. Instead of further using the glucose substrates for oxidative phosphorylation in the mitochondria, glucose is metabolized to lactate. Nevertheless, suppression of pyruvate oxidation does not necessarily mean the total loss of oxidative metabolism.(DeBerardinis & Chandel, 2020) Retaining mitochondrial respiration gives the cancer cells the possibility to synthesize oncometabolites. However, a reduction seems to protect cancer cells as the mitochondrial production of ROS consequently is decreased.(DeBerardinis & Chandel, 2020; Harris & Johnson, 2019) Even if cancer metabolism and the role the Warburg effect plays is not yet completely understood, inhibiting glycolytic pathways with substances such as 2-deoxy-d-glucose (2-DG) is investigated as a promising adjuvant anticancer therapy.(Aerobic Glycolysis and the Warburg Effect, 2012)

1.4. Effects of CBD on mitochondria

Cannabidiol binds to several molecular targets such as CB_1 and CB_2 receptors, as well as ion channels located in the plasma membrane. Recent studies have shown that CBD, as a highly lipophilic compound that directly interacts with mitochondria without involving CB_1 and CB_2 receptors, GPR55 receptors, nor even Ca^{2+} permeable channels in plasma membrane. Intracellular Ca^{2+}, mainly stored in the endoplasmatic reticulum (ER), needs to serve as reservoir for the rapid CBD-induced change in the intracellular Ca^{2+} homeostasis.(Olivas-Aguirre et al., 2019)

Increased levels of cytosolic free Ca^{2+} and consequently increased intramitochondrial Ca^{2+} levels were measured in cells treated with CBD.(Olivas-Aguirre et al., 2019) Calcium plays an essential role for cells since intracellular Ca^{2+} signals determine the destiny of a cell, modulating cell survival or death. Another crucial factor for cell vitality is the mitochondrial

transmembrane potential (ΔΨm), indicating the mitochondrial permeability transition. Long-lasting changes of ΔΨm can cause unwanted loss of cell viability and induce pathologies.(Zorova et al., 2018) If the mitochondrial outer membrane is abnormally permeable, apoptotic pathways can be activated. Too high levels of Ca^{2+} directly promote the loss of the mitochondrial transmembrane potential. Several factors modulating cell death are then then released, such as cytochrome-C (Cyt-C). Cyt-C in the cytosol mediates the formation of apoptosomes and further activates the initiator caspase 9 consequently activating executioner caspases driving apoptosis.(Olivas-Aguirre et al., 2019)

Another molecular target of CBD is the voltage-dependent anion channel (VDAC). It is a multifunctional channel, found in the mitochondrial outer membrane, mediating metabolic cross-talk between the mitochondria and the rest of the cell.(Rimmerman et al., 2013; Shoshan-Barmatz et al., 2015) The direct interaction between VDAC and CBD results in a fixed open state of the channel. Consequently, a high influx of Ca^{2+} through the outer mitochondrial membrane, causes an intramitochondrial Ca^{2+}-overload (Fig.: 7).(Olivas-Aguirre et al., 2019) VDAC channels display ion-selectivity depending on the voltage across the membrane. Voltage change leads to a reduced conductance of the channel that then becomes impermeable to large molecules such as ADP and ATP.(Rimmerman et al., 2013; Shoshan-Barmatz et al., 2015) Cancer cells show a higher expression of VDAC and a use of chemotherapy seems to trigger an upregulation of VDAC expression. This might be one of the reasons why cancer cells are more negatively affected by CBD, which makes CBD a very promising anti-cancer agent. Generally, healthy cells seemed to be less sensitive to CBD.(Olivas-Aguirre et al., 2019)

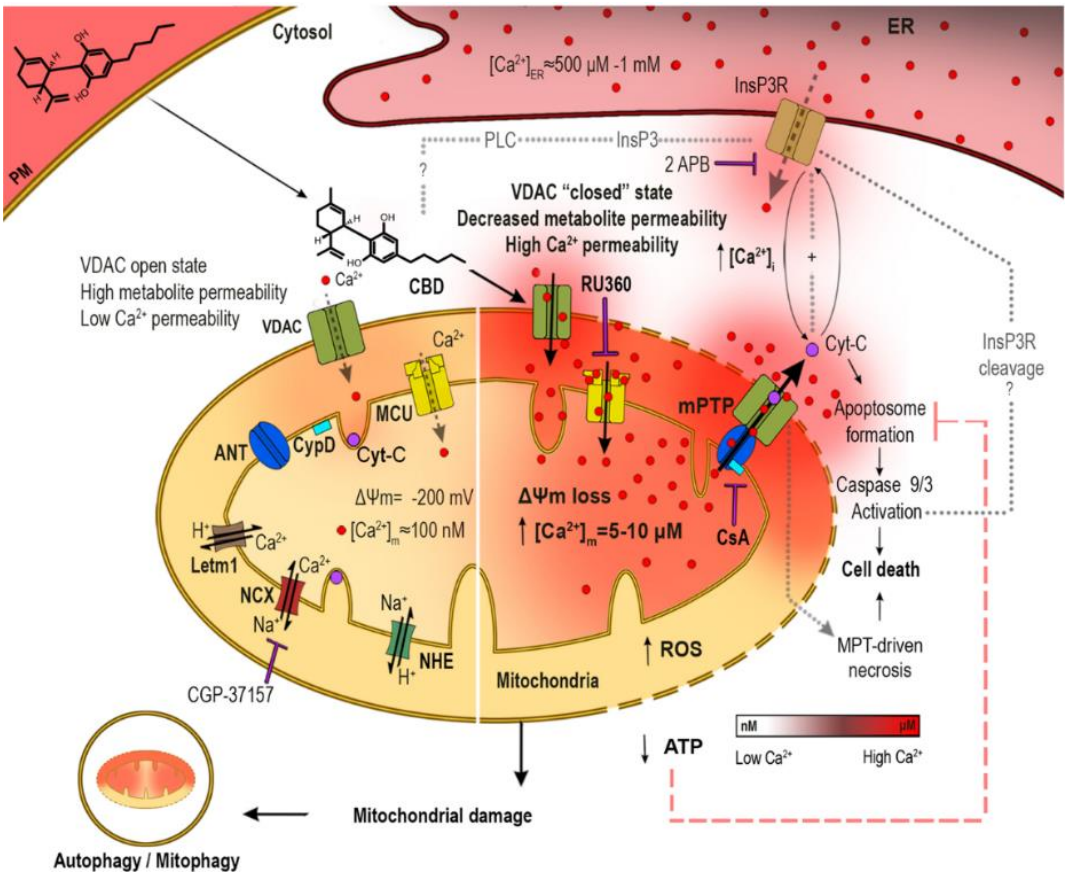

Figure 7. Scheme of the effects of CBD in acute lymphoblastic leukemia. High concentrations of CBD disturbed the Ca^{2+} homeostasis causing intramitochondrial Ca^{2+} uptake through VDACs that became permeable leading to the loss of the mitochondrial transmembrane potential (ΔΨm) and further consequences such as apoptosis.(Olivas-Aguirre et al., 2019)

1.6. Effects of 2-deoxy-D-glucose

2-deoxy-d-glucose (2-DG) is a structural analog of glucose, which has in contrast to glucose, no oxygen atom on the C2 atom. 2-DG is discussed as a therapeutic anti-cancer agent due to its inhibitory effect on cellular glucose metabolism.(Zhang et al., 2006) In cells, 2-DG is phosphorylated by hexokinases and transformed into 2-deoxy-glucose-6-phosphat (2-DG-P). In this phosphorylated state, 2-DG-P causes allosteric and competitive inhibition of phosphohexose isomerase.(Voss et al., 2018) Due to low levels of intracellular phosphatase in cancer cells, 2-DG-P accumulates and cannot be further metabolized. Consequently glucose metabolism is inhibited.(Aft et al., 2002)

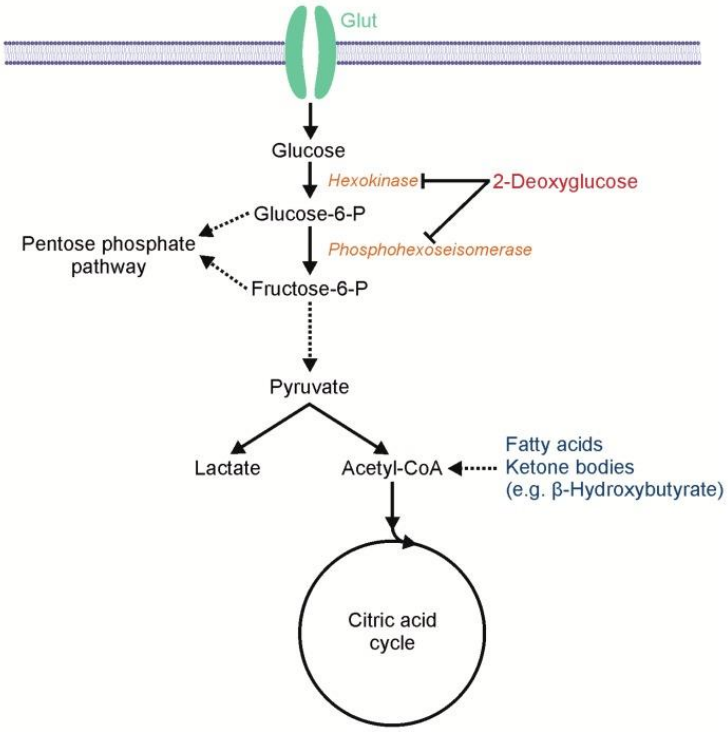

Figure 8. Scheme of how 2-DG affects glycolysis. 2-DG-P blocks further metabolization and inhibits the enzymes: hexokinase and phosphohexoseisomerase. blue: Ketones (f.i. BHB) and fatty acids interact with acetyl coenzyme A (Acetyl-CoA) and enable the Krebs cycle and further metabolic pathways.(Voss et al., 2018)

It has been proven that treating glioblastoma cells with 2-DG *in vitro* reduces glucose uptake and lactate production, increases mitochondrial ROS release, activates AMP-activated protein kinases (AMPK) and leads to induced autophagy.(Voss et al., 2018) AMPK directly phosphorylates several enzymes and is involved in transcriptional control of metabolism. Its role as a central regulator results in direct phosphorylation of transcription factors, co-activators, and co-repressors.(*AMPK Signaling*, 2020)

Many cancer types such as glioblastomas rely on glycolysis, which makes them sensitive for substances that inhibit glycolysis. Therefore, 2-DG is studied as a crucial factor to fight cancer.(Voss et al., 2018)

1.7. Ketogenic diet

Ketogenic diets are classified into four different types. All of them have in common a drastic reduction of the intake of carbohydrates. The consumed carbohydrates in this dietary nutrition should not be more than 50 grams per day. Therefore, the amount of proteins and especially lipids must be increased. Four types of ketogenic diet have been developed:(Shilpa & Mohan, 2018)

1. Standard ketogenic diet (SKD): Here the uptake of fat should cover around 70 % of the diet, whereas protein should account for 20 % and only 10 % of carbohydrates respectively.
2. High-protein ketogenic diet (HPKD): In contrast to the SKD, in this diet the consumption of carbohydrates is even more reduced and replaced by an increased protein uptake. Only five per cent of the diet can come from carbohydrates, 35 % from proteins and 60 % fat.
3. Cyclical ketogenic diet (CKD): In this model, the ketogenic diet need not be performed every day. A ketogenic period is followed by a short period of time where a higher uptake of carbohydrates is permitted.
4. Targeted ketogenic diet (TKD): In this diet, more carbohydrates can be eaten, but they must be combined with a hard physical workout.(Shilpa & Mohan, 2018)

The SKD is the most widely approved one and the HPKD also is used frequently. The CKD and the TKD, though, are not well researched yet.(Shilpa & Mohan, 2018) The following analysis is exclusively based on the SKD.

How do ketogenic diets function? The restriction of carbohydrates leads to lack of glucose. After three to four days, the glucose reservoirs are not capable of providing enough glucose for the CNS and normal fat oxidation. This condition forces the body to find an alternative way for energy production. The solution are ketone bodies. There are two types of ketones: acetoacetate and β-hydroxybutyrate, both synthesized in the liver. To synthesize ketone bodies, fat must be broken down. This metabolic switch must not be confused with the pathological ketoacidosis.(Shilpa & Mohan, 2018) Pathologically high serum and urine concentrations of ketone bodies is associated with a ketoacidosis, a metabolic state, which can be dangerous if not recognized and treated early.(Ghimire & Dhamoon, 2020)

1.8. Effects of a ketogenic diet on cancer

Considering cancer cell metabolism and the principles of a ketogenic diet, it seems promising to study the effects of a ketogenic diet as an adjuvant cancer therapy. A ketogenic diet reduces glucose availability and ketones instead are metabolized as an alternative source of energy. However, up to 60–80 % of cancers display the Warburg effect and therefore are not able to adapt their metabolism.(Panhans et al., 2020) The crucial consequence for these cancers might be energy starvation.(Weber et al., 2018) It is also discussed that insulin and the insulin-like growth factor promote cancer growth. Therefore, reducing the blood glucose level with the help of a ketogenic diet might help. Unfortunately, a few cases of pro-tumor effects have been reported, too. The efficacy depends on the cancer type and specific genetic mutations.(Weber et al., 2018)

1.9. Cell culture

Cell culture is a well-established laboratory method to study fundamental scientific issues. Cells are grown and cultivated under controlled conditions in diverse cell culture dishes such as petri dishes, 69-well plates, or other formats. Cell culture is a well-used tool due to its homogeneity and the advantage that data can be generated fast and reproduced easily. The possibility to grow eukaryotic and prokaryotic cells *in vitro* provides many different applications such as investigating basic cell biology, studying tumor biology, or testing substances for their potential effects as novel drugs. In industry, cultivating hybrid cell cultures on a broad-scale is a well-established method for the production of biopharmaceuticals.(Segeritz & Vallier, 2017)

The most crucial problem about cell culture is maintenance of sterile conditions. If there is no microorganism-free microenvironment, the success of a cell culture is questioned. Bacteria, viruses, parasites, and fungi are the common pathogenic microorganisms contaminating cell culture. In most cases, contamination means an irreversible damage for the whole cell culture. Therefore, the culture medium itself must be sterile and the medium change needs to be performed under sterile conditions, too. Antibiotics can be added to the culture medium to prevent bacterial contamination but several labs desist from the usage of antibiotics as it might interfere in the cells and therefore influence the experimental results.(Segeritz & Vallier, 2017)

The desired pH, CO_2 and O_2 levels and temperature depend heavily on the cells. There are three major cell types: primary cells, transformed cells and self-renewal cells.(Segeritz & Vallier, 2017)

1.9.1. Primary cell culture

If cells are directly isolated from a living organism such as animals or humans, they are referred to as primary cells. Mostly these cells are finite, meaning they cannot proliferate limitlessly. The advantages of primary cell cultures are, for instance, the possibility to investigate biochemical pathways or to identify inter- and intra-cellular mechanisms. Primary cell cultures provide an alternative to animal testing.(Segeritz & Vallier, 2017)

1.9.2. Primary culture of murine neuronal cells

Cultivation of neuronal cells is particularly challenging as mature neurons do not undergo cell division. Animal tissues *in vivo* are composed of several different cell types that then can be found in the derived multicellular culture composed of neurons, astrocytes, and microglia.(Gordon et al., 2013; Goshi et al., 2020) In the present study, mesencephalic cultures were used. They contain dopaminergic neurons, around 1 % of the total cells, which are important for the control of multiple brain functions.(Gaven et al., 2014) Most dopaminergic neurons are located in the ventral part of mesencephalon.(Chinta & Andersen, 2005) They are easily detectable by anti-tyrosine-hydroxylase immunocytochemistry.(Radad et al., 2018) Additionally, this staining allows morphological examinations. Apart from that, dopaminergic neurons appear to exhibit increased sensitivity to oxidative stress.(Guo et al., 2018) We assume that CBD inhibits the mitochondrial activity. Therefore, dopaminergic neurons seem to be a suitable model for our investigations on the effects of CBD.

1.10. Aims of the study

In my study, the effects of CBD/2-DG and BHB on primary cells have been investigated. This study is a complementary work to a previously performed study at the institute.(Svatunek, 2020) In this study, experiments were done with U-87 MG glioma and N18TG2 neuroblastoma cells. It could be shown that a concentration of 10 µM CBD exerts cytotoxic effects in glioma and neuroblastoma cells, therefore in this study I also used a concentration of 10 µM CBD as a maximum. Additionally, the effects of a combination of CBD and 2-DG were tested to elucidate additive toxicity. As a result, no significant difference of the cytotoxic effects was observable (see supplementary material). Glioma cells responded more sensitively to the treatment than neuroblastoma cells.(Svatunek, 2020)

In the study presented here, cells prepared from embryonic mouse mesencephala were used. In the first part of the study, 2-DG induced inhibition of glycolysis in combination with the effects of CBD was investigated to evaluate whether this could serve as a potential new cancer treatment.

Additionally, it was tried to establish a primary cell culture for ketogenic diet. The outcome of the study was planned to trigger further studies for a cancer treatment based on CBD and substances inhibiting aerobic glycolysis.

Investigations were performed to answer the following questions:

1. What cytotoxic effects can be observed when adding CBD in various concentrations to murine mesencephalic primary cells?
   → Does cytotoxicity increase with higher concentrations of CBD?
2. How does a concomitant treatment of 2-DG and CBD affect cell degeneration?
   → Does CBD aggravate 2-DG induced cytotoxicity?
3. Can we develop a model that simulates a ketogenic diet *in vitro*?
   → How do murine mesencephalic primary cells react to a ketogenic diet?

2. Material and Methods

2.1. Material

2.1.1. Animals

Pregnant mice (OF1/SPF) at gestation day 14 were obtained from the Institute for Laboratory Zoology and Veterinary Genetics, Himberg, Austria. Animals were cared and handled in accordance with the guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals.

2.1.2. Chemicals

All chemicals required for buffers and solutions are listed below:

2-deoxy-D-glucose (2-DG), Sigma-Aldrich, Germany

2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride (Hoechst 33342), Sigma-Aldrich, Germany

3-hydroxybutyric acid (BHB), Sigma-Aldrich, Germany

3,3´-diaminobenzidine (DAB), Sigma-Aldrich, Germany

3,8-diamino-5-(3-(dieethylammonio) propyl)-6-phenyl-phenanthridinium-diiodide, Sigma-Aldrich, Germany

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), Sigma-Aldrich, Germany

Absolute ethanol, Merck KGaA, Germany

Accustain™, Sigma-Aldrich, Germany

Adenosine 5′-triphosphate disodium salt solution (ATP), Sigma-Aldrich, Germany

Anti-TH mouse antibody, Szabo, Austria

B27 supplement minus AO 50x, Thermo Fisher Scientific, USA

Cannabidiol, THC Pharm, Germany

CellTiter-Glo® ATP Assay Kit (Colorimetric/Fluorometric), Promega, USA

Colorless Dulbecco's modified eagle's medium (cDMEM) high glucose, Sigma-Aldrich, Germany

D-Glucose, Sigma-Aldrich, Germany

Dimethyl sulfoxide (DMSO), Merck KGaA, Germany

DNAse 1 %, Sigma-Aldrich, Germany

Dulbecco's modified eagle's medium (DMEM) high glucose, Sigma-Aldrich, Germany

Dulbecco's modified eagle's medium (DMEM) low glucose, Sigma-Aldrich, Germany

Dulbecco's modified phosphate buffered saline (DPBS) 1x, Thermo Fisher Scientific, USA

Foetal bovine serum, Sigma-Aldrich, Germany

Hank's balanced salt solution (HBSS) 1x, Thermo Fisher Scientific, USA

HEPES solution, Sigma-Aldrich, Germany

Horse serum, Sigma-Aldrich, Germany

Hydrogen peroxide (H_2O_2) 30 %, Merck KGaA, Germany

Nicotinamid adenine dinucleotide, Sigma-Aldrich, Germany

Peroxidase mouse IgG ABC-Kit, Vectastain, Vector Laboratories, USA

Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, USA

Poly-D-lysine hydrobromide (PDL), Sigma-Aldrich, Germany

Resazurin sodium salt, Sigma-Aldrich, Germany

Ripa Lysis Buffer 10x, Merck KGaA, Germany

Sodium-pyruvate (Na-pyruvate), Sigma-Aldrich, Germany

Triton X, Roche Diagnostics, Germany

Trypan blue, Sigma-Aldrich, USA

Trypsin-EDTA 0.5 %, Thermo Fisher Scientific, USA

Trypsin inhibitor soybean, Thermo Fisher Scientific, USA

2.1.3. Equipment

All single-use plastic material and further laboratory equipment is listed below:

48-well plates, Nunc, Denmark

96-well plates, Greiner bio-one, Germany

Centrifuge tubes, Cellstar Tube, Greiner bio-one, Germany

Centrifuge, Hettich Rotixa/AP, Germany

Counting chamber, Neubauer, Germany

Disposable syringe 5ml, B. Braun, Germany

Diessecting set, F.S.T., Germany

Easypet, Eppendorf, Germany

Examination gloves, Hartmann, Germany

Incubator, NAPCO Model 5410, Germany

Injection cannulas, B. Braun, Germany

Laminar Flow HERAsafe®, Kendro Laboratory Products, Germany

Microplate Reader Spark®, Tecan, Switzerland

Microscope, Nikon Diaphot 300, Japan

Multipette, Eppendorf, Germany

Multipurpose Container, Greiner bio-one, Germany

Petri dishes large, Nunc, Denmark

Petri dishes medium, Nunc, Denmark

Petri dishes small, Nunc, Denmark

Pipette tips, Greiner bio-one, Germany

Syringe filters, Whatman, UK

Vortex 1, IKA, Germany

2.1.4. Buffers and Solutions

Basic Medium (BM)	N4
50 ml DMEM	50 ml DMEM (w/o phenol red)
5 ml foetal bovine serum	1 ml B27 supplement (10x)
1 ml L-glutamine (200 mM)	1 ml L-glutamine (200 mM)
500 µl HEPES solution (1 M)	500 µl HEPES solution (1 M)
370 µl glucose solution (20 %)	370 µl glucose solution (20 %)
100 µl penicilline streptomycin	

Table 1. Composition of BM and N4

Trypsin Solution	Trypsin-inhibitor solution	HBSS + DNAse
1 ml trypsin-EDTA (0.5 %)	1 mg trypsin-inhibitor soybean	2.5 ml HBSS (1x)
9 ml DPBS (1x)	4 ml DPBS (1x)	50 µl DNAse (1 %)

Table 2. Composition of solutions for preparation of murine mesencephalic primary cells

2.2. Methods

2.2.1. Preparation of murine mesencephalic primary cells

For a minimum of one hour before the preparation, one 48-well-plate and two 96-well-plates were coated with 200 µl of a PDL:DPBS-dilution (50 µg/ml in DPBS). That step provides a reduced surface tension inside the wells due to the attachment factor PDL. The required dissecting set was autoclaved.

Before getting started with the preparation, BM (table 1) and the other required solutions were prepared as described in table 2 and stored in the incubator till required. Table 3 shows the setting needed for the preparation:

trypsin solution	2 ml/mouse
trypsin-inhibitor solution	2 ml/mouse
HBSS + DNAse	2 ml/mouse
basic medium	as much as required
3 large petri dishes	10 ml DPBS (1x) each
3 medium petri dishes	5 ml DPBS (1x) each
1 small petri dish	1 drop of DPBS (1x)

Table 3. Overview of the required equipment and solutions for the preparation of murine mesencephalic primary cells

The microscope and the dissecting set were put into the laminar flow. One large petri dish, a pair of scissors and one tweezer were taken out of the laminar flow to start the preparation.

The aim of the preparation was to isolate neurons of the mesencephalon of murine embryos. Thus, a pregnant mouse was dissected on gestation day 14. First, the mouse was put into a container and anaesthetized with CO_2. Next, the end of a spatula was placed just behind the skull and pressed down firmly. Then, the tail was pulled back- and upwards fast and relatively strongly until the cervical dislocation. The mouse was turned on the back and the abdomen disinfected with ethanol (70 %). A small part of the abdomen was opened, and the uterus horns were relocated to the large petri dish. The uterus horns were covered and quickly put into the laminar flow to perform the following steps under sterile conditions.

The uterus horns were opened with scissors and the embryos were extracted. To decrease the risk of contamination, the embryos were put into a new large petri dish. Next, the placenta and the extraembryonic membranes were removed, and the embryo was again transferred to

a new petri dish. Then, the embryos were decapitated, and the heads were transferred into the first medium sized petri dish.

The further steps were performed under the microscope. Therefore, the laminar flow had to be opened, and a mask was put on. The skulls were opened with small scissors and the brains were transferred into the second medium petri dish. Next, the mesencephala were isolated by removing the myelencephala and the prosencephala with a scalpel. The mesencephala were transferred to a new petri dish. For the cell culture only the cells from the mesencephalic tissue were required. Therefore, the meninges were removed carefully. The mesencephala were put into the small petri dish. Before cutting the mesencephalic tissue mechanically into small pieces a last look through the microscope guaranteed that the meninges had been successfully removed.

The following procedures did not require the microscope anymore. Next, 2 ml trypsin solution were used to transfer as many cells as possible to a centrifuge tube. 2 ml HBSS + DNAse were added. Immediately, the mix was put into the incubator where it was stored for 7 minutes at 37 °C.

The centrifuge tube was brought back into the laminar to add 2 ml trypsin-inhibitor solution. This step stops the trypsin-reaction. Then, the suspension was centrifuged at 100 g for 4 minutes before the supernatant was discarded.

After that, the trituration was done. 3 ml BM plus 60 µl DNAse were added to the remaining pellet. To separate the cells, the pellet was triturated with a fire-polished Pasteur pipette. After one trituration a pause of 10 minutes is required to allow the remaining cell cluster to sediment. These 10 minutes were used to discard the PDL:DPBS-dilution of the pre-coated wells and to replace it with DPBS. The trituration was performed three times, transferring 2 ml, 3 ml and 4 ml of the supernatant into a glass flask containing 6 ml BM, which in total makes 15 ml cell suspension.

The flask was put in the incubator while the cells were counted using trypan blue staining and a Neubauer counting chamber. BM was used to dilute the suspension to a final concentration of 750,000 cells/ml.

For seeding, the DPBS inside the wells was discarded and 340 µl of cell suspension for 48-well plates and 150 µl for 96 well-plates was pleated out. The cell cultures were put in the incubator at 37 °C.

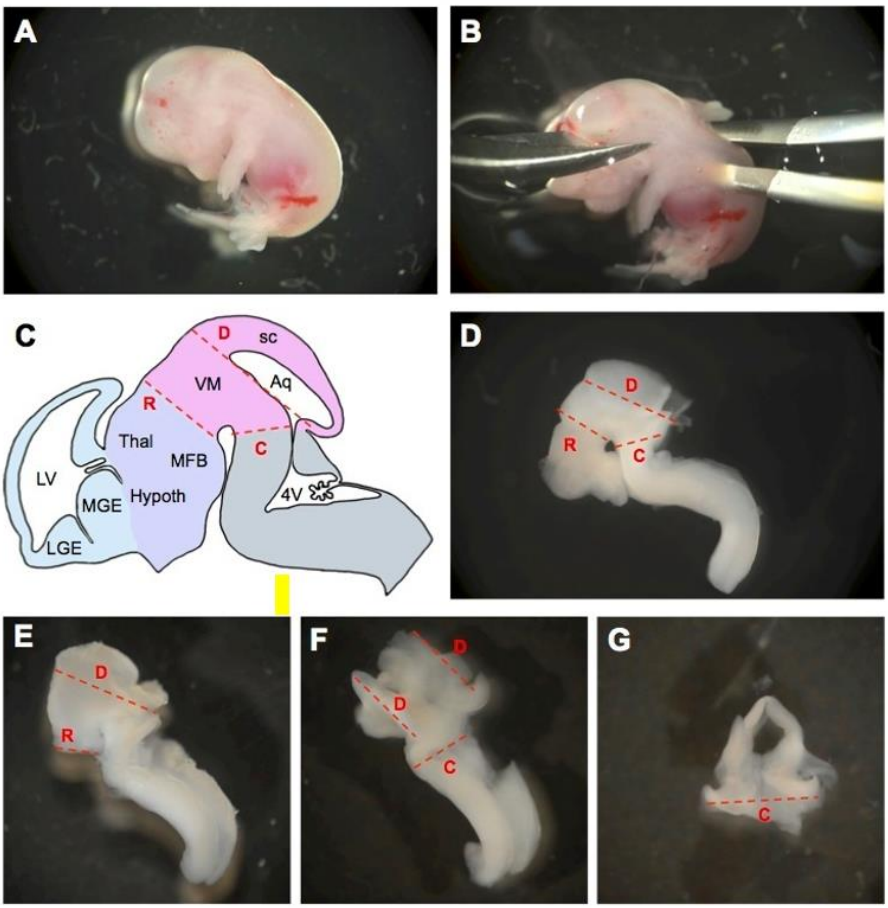

Figure 9. Execution of a murine mesencephalic dissection modified by (Gaven et al., 2014): **A** mouse embryo on gestation day 14; **B** Excision of the brain (in my studies embryo was beheaded before); **C** scheme of an embryonic mouse brain at this stage of development. Red lines symbol where the brain is cut to remove non-mesencephalic tissue; **D** isolated brain; **E-G** isolation of the mesencephalon

2.2.2. Murine mesencephalic primary cells

The generated mesencephalic primary cell culture was cultivated for 14 days. The cells were kept in the incubator at 37 °C and 5 % CO_2. On the 12th day after seeding, the cells were treated as described in 2.2.3. On the 14th and 15th day the immunocytochemistry staining of TH-positive cells and further biochemical analysis were performed.

The composition of the BM and the N4 medium for the cell culture is listed in table 1. Medium change was performed as recorded in table 4. The final volume per well before treatment was 300 µl/150 µl per well for 48 or 96 well plates, respectively.

Day	Timetable	Medium
0	preparation	BM
1	medium change	BM/BM day 0 (1:1)
2	medium change	BM
5	medium change	BM:N4 (1:1)
6	medium change	N4
9	medium change	N4
12	treatment	CBD/2-DG
14	staining, part 1/biochemical analysis	
15	staining, part 2	

Table 4. Protocol for cultivating murine mesencephalic primary cells

2.2.3. Ketogenic associated cell culture diet: medium containing reduced glucose supplemented with beta hydroxybutyrate (BHB)

To study the impact of a ketogenic and low glucose diet primary mesencephalic cells were cultivated. The cells were kept for either 14 or 21 days at 37 °C and 5 % CO_2. Biochemical analysis and immunocytochemistry staining of TH-positive cells were performed. Two types of mediums were used and prepared as described in table 5. N4 was used as a control medium. A final concentration of 33 mM glucose in N4 and the normal BM is more than the cells can ingest. A ketogenic medium with a concentration of 10 mM glucose is regarded as minimum for cell survival, and 7.3 mM in a low glucose medium will cause starvation. The concentrations have been tested in previous experiments and were acquired in this study.

low glucose medium LGM	ketogenic medium KM
50 ml DMEM low gluc (7,3 mM)	50 ml DMEM (no glucose)
1 ml B27 supplement (10x)	1 ml B27 supplement (10x)
500 µl L-glutamine (200 mM)	500 µl L-glutamine (200 mM)
500 µl HEPES solution (1 M)	500 µl HEPES solution (1 M)
	385 µl glucose solution (1.3 M)
500 µl BHB (2 M)	500 µl BHB (2 M)

Table 5. Composition of low glucose and ketogenic medium

Medium change was performed according to table 6. For 48-well plates, 300 µl fresh medium was added, for 96-well plates 150 µl. On day 14 one of the plates was remained and neither used for staining nor for biochemical analysis. This plate was further cultivated until day 21 when immunocytochemistry staining of TH-positive cells and further biochemical analysis were performed.

Day	Timetable	Medium
0	preparation	BM
1	medium change	BM/BM day 0 (1:1)
2	medium change	BM
5	medium change	BM:KM/LGM/N4 (1:1)
6	medium change	KM/LGM/N4
9	medium change	KM/LGM/N4
12	medium change	KM/LGM/N4
14	staining, part 1/biochemical analysis	
14	medium change remaining cells	KM/LGM/N4
15	staining, part 2	
16	medium change	KM/LGM/N4
19	medium change	KM/LGM/N4
21	staining, part 1/biochemical analysis	
22	staining, part 2	

Table 6. Protocol for cultivating murine mesencephalic primary cells

2.2.4. Treatment of murine mesencephalic primary cells

On day 12 the following treatment of murine mesencephalic primary cells was performed: The cells were treated with two test substances, i.e. the cannabinoid CBD and 2-DG, a substance known to inhibit glycolysis. Medium was discarded. Then, CBD in different concentrations, 0 (Cc), 0.01, 0.1, 1, 10 µM (final concentration) was added. The wells were filled up with N4 or 2-DG with a final concentration of 10 mM. The final volume after the treatment was 300 µl/150 µl per well for 48 or 96 well plates, respectively.

The pipetting scheme of the treatment is demonstrated in table 7. To ensure equal conditions, DMSO was added to the control medium (Cc), as the CBD stock was dissolved in DMSO. The treatment was performed in duplicates and the incubation time was 48 h at 37 °C.

Cc + N4	CBD [0.01] + N4	CBD [0.1] + N4	CBD [1] + N4	CBD [10] + N4
Cc + N4	CBD [0.01] + N4	CBD [0.1] + N4	CBD [1] + N4	CBD [10] + N4
Cc + 2-DG	CBD [0.01] + 2-DG	CBD [0.1] + 2-DG	CBD [1] + 2-DG	CBD [10] + 2-DG
Cc + 2-DG	CBD [0.01] + 2-DG	CBD [0.1] + 2-DG	CBD [1] + 2-DG	CBD [10] + 2-DG

Table 7. Pipetting scheme of treatment

2.2.5. Staining of dopaminergic neurons by Anti-tyrosine-hydroxylase immunocytochemistry

On day 14, 48 h after treatment, immunocytochemistry staining of tyrosine hydroxylase (TH) positive cells was performed to detect dopaminergic neurons. First, 50 µl treatment solution of each 48-well plate was transferred to a new 96-well plate for the LDH-determination, followed by discarding the treatment solution from the 48-well plate and adding Accustain™ to fix the cells. After incubation time of 15 minutes for fixation, Accustain™ was discarded and replaced by 0.2 ml Triton-X solution (0.4 %) per well for cell membrane perforation. The cells with Triton-X solution were incubated at RT for 30 minutes. Next, the wells were washed three times with PBS, for 2 minutes each. After this, the unspecific binding sites were blocked with 0.2 ml horse serum (1:50, mixed with PBS) for 90 minutes at RT. Finally, the horse serum was replaced by 0.2 ml mouse anti-tyrosine hydroxylase antibody (diluted 1:1,000 in horse serum solution) solution and incubated overnight at 4 °C.

On the next day, the first antibody solution was discarded, the wells were washed three times and incubated with 0.2 ml of the secondary antibody (biotinylated horse anti-mouse IgG, 1:200, and afterwards mixed with PBS) for 90 minutes at RT. In the meantime, the AB-solution was prepared by mixing one drop of avidin/biotin solution in a tube which had to react for a minimum of 30 minutes. After 90 minutes of incubation with the secondary antibody, wells were washed again three times with PBS for 5 minutes each. Just before using the AB-solution, 5 ml of PBS were added. Now, the AB-solution was administered to the wells and incubated for 90 minutes at RT. The wells were washed again three times while the diaminobenzidine solution (DAB) was being prepared as follows: 20 µl H_2O_2 and 500 µl PBS were added to an Eppendorf tube and mixed. 10 mg of DAB was added to 10 ml PBS. The hydrogen peroxide mix was added to the DAB solution, vortexed, and 0.2 ml of the final DAB solution was added to the wells.

After 10 minutes, the success of the staining process was controlled using a light microscope. Dopaminergic neurons are detectable due to a brown DAB oxidation product. When the

intensity was high enough, the last three washing steps were performed. Finally, the wells were sealed with Kaiser's glycerol gelatin, which had been melted inside a water bath at 60 °C.

To generate the data, dopaminergic neurons were counted with a Nikon Diaphot 300 microscope at 100x magnification.

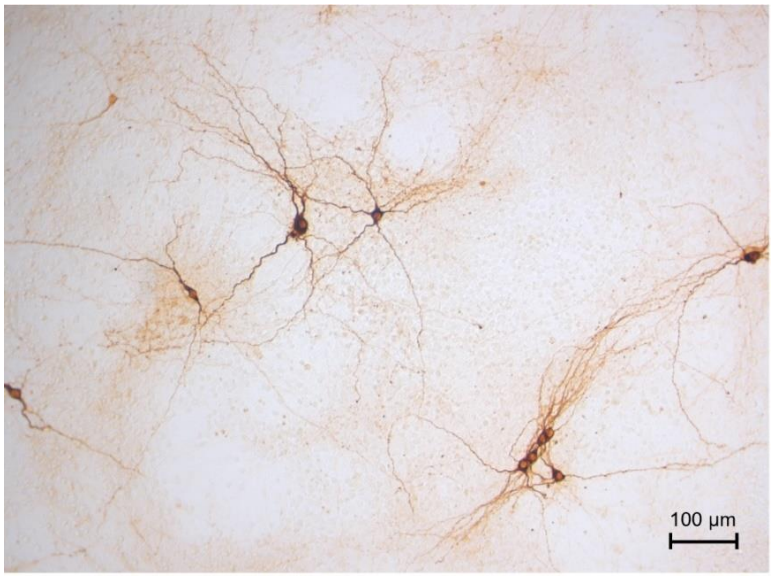

Figure 10. Dopaminergic neurons in murine mesencephalic culture stained by TH immunocytochemistry

2.2.6. Resazurin reduction cell viability assay

This colorimetric assay was performed in order to measure the overall metabolism. The assay is based on resazurin reduction. Resazurin is a redox-sensitive, non-toxic, cell-permeable blue dye. By adding resazurin to the cell culture, resazurin is reduced inside the mitochondria of viable cells to resorufin. Resorufin is a compound that is purple pink in color. With a microplate reader, the increased OD of resorufin can be detected by measuring the absorbance at 570 nm. As a reference wavelength 600 nm (resazurin) was used to increase the precision of the measurement.

48 h after treatment 15 µl of resazurin solution (500 µM in DPBS) was added to each well. Subsequently the first measurement was taken. A total of 4 measurements were performed after 0, 1, 2 and 3 hours. In between, the cell culture was kept in the incubator at 37 °C.

2.2.7. Bicinchoninic acid protein assay

The bicinchoninic acid (BCA) assay was performed to determine the protein concentration in the wells. The amount of protein correlates with the amount of cells. Proteins are detectable

by a color change of the sample solution from green to purple determined by the protein concentration, which can then be measured with a microplate reader.

The assay is based on two main reactions. First, on the reduction of Cu^{2+} to Cu^{+} because of the chelation of peptide bonds in an alkaline solution. Secondly, BCA reacts with the previous formed Cu^{+} and together they form a complex that has its absorption maximum at 562 nm. This is visible due to a color change of the solution from green to purple.

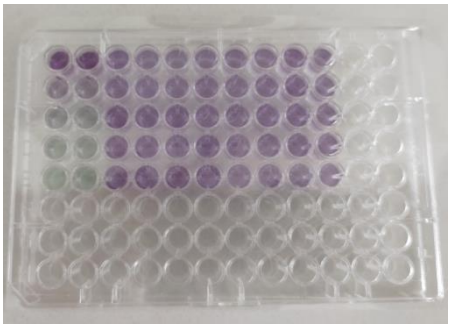

Figure 11. Protein detection BCA assay

The assay was started 48 h after the treatment. For cell lysis, the treatment solution was discarded from the wells and replaced with 200 µl of a solution consisting of RIPA lysis buffer (10x) that was mixed 1:10 with distillated water. While the cells were set aside for 15 minutes, the working reagent (WR) consisting of 15 ml reagent A and 0.3 ml reagent B was prepared. Then the standard probes were prepared as described in table 8.

BSA concentration	Vol. BSA	Vol. PBS (1x)
500 µg/ml	50 µl [of stock solution]	150 µl
125 µg/ml	50 µl [of 500 µg/ml solution]	150 µl
50 µg/ml	100 µl [of 125 µg/ml solution]	150 µl
25 µg/ml	100 µl [of 50 µg/ml solution]	100 µl
0 µg/ml	-	100 µl
BSA: stock solution 2000 µg/ml		

Table 8. Scheme of BSA standard probes

For the measurement, 25 µl cell lysate of BSA standards were taken from each well and transferred to a new 96-well plate. To all wells, 200 µl of WR was added. The plate was put on a plate shaker for 30 seconds before incubation at 37 °C for 30 minutes. Then, the plate was cooled down to RT and measured at the absorption maximum of 562 nm.

2.2.8. CellTiter-Glo® luminescent cell viability ATP assay

After quantifying ATP in the cell culture, measurements can be used as an indicator ability to form ATP without discriminating its origin. The ATP assay is based on reagents containing

detergent, stabilized luciferase and luciferin substrate. Adding detergent sets off cell lysis and as a further consequence ATP is released. ATP is needed as it provides energy for the luciferase, using luciferin to generate a luminescent signal. The assay was performed 48 h after treatment.

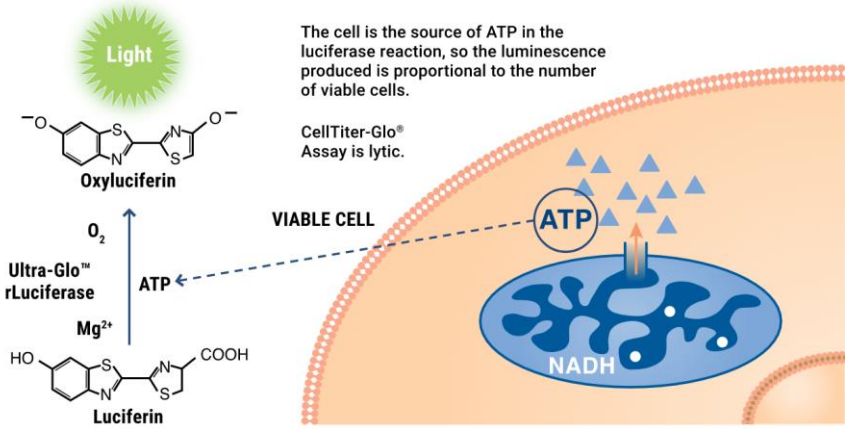

Figure 12. The CellTiter-Glo® Assay detects ATP as an indicator of viable cells. After cell lysis, ATP is released into the medium. Then, the luciferase uses luciferin to generate luminescence. The signal is dependent on the amount of ATP.(Lee & Mohns, 2019)

First, the light was turned off as the reaction is sensible to UV-radiation. Then, CellTiter Glo® reagent was taken out of the freezer and mixed 1:2 with PBS to get the WR. Standards were prepared as listed in table 9.

	ATP stock 100 µM	PBS	concentration
predilution	100 µl	900 µl	10 µM
standard A	10 µl	990 µl	1 µM
	predilution 10 µM	PBS	concentration
standard C	25 µl	975 µl	0.25 µM
standard D	10 µl	990 µl	0.1 µM
	standard A 1 µM	PBS	concentration
standard F	25 µl	975 µl	0.025 µM
standard G	10 µl	990 µl	0.01 µM
standard H	-	125 µl	0 µM

Table 9. Pipetting scheme of the ATP standards

Afterwards, 125 µl of each standard solution was transferred to a new Eppendorf tube and the remaining standard solutions were discarded. The treatment solution was removed from the plate and 125 µl WR was added to the emptied wells and to each standard tube. The standards and the plate were wrapped in aluminum foil and put on the plate shaker at 200 rpm for 30 minutes. Finally, 100 µl of the solution was taken out of every well and transferred to a new, black-walled 96-well plate. Standards were used in duplicates. Then, the luminescence was measured on a microplate reader.

2.2.9. Lactate dehydrogenase (LDH) activity determination

The LDH assay was performed 48 h after treatment to measure the activity of the oxidoreductase lactate dehydrogenase in the cell culture medium. The enzyme is expressed in nearly every living cell and released into the medium when cells are damaged. LDH can convert lactate into pyruvate and back to lactate. Therefore, it uses the cofactor NAD^+ as it converts it to NADH and back. Quantifying the amount of NADH in the media provides the possibility to evaluate the presence of damage or lysed cells.

First, the LDH-buffer consisting of 100 ml PBS (1x) plus 2 ml HEPES buffer (1 M) was made and 15 ml were stored at RT. Secondly, 4 mg of Na-pyruvate and 8 mg of NADH were weighed in and diluted in LDH-buffer. Thirdly, the prepared plate and the assay dilution were taken next to the plate reader. The next step had to be performed immediately. 85 µl of the LDH mix was quickly added to the wells filled with 50 µl of the treatment media with a multichannel pipette, and the plate was measured three times (after 30, 60, and 90 seconds) at 334 nm, the absorption maximum of NADH.

2.2.10. JC-1 mitochondrial membrane potential assay

The JC-1 mitochondrial membrane potential assay was performed only with cells cultivated in low glucose and ketogenic media. Tetraethyl benzimidazolylcarbocyanine iodide (JC-1) is a cationic dye that enters viable mitochondria and aggregates inside. In cells with a normal membrane potential the dye accumulates in dimers and fluoresces at a range from red to orange with an emission spectrum of 590 nm. Whereas, mitochondrial depolarization is indicated by green fluorescence with an emission maximum of about 530 nm, since JC-1 is mainly present as a monomer.

JC-1 was taken out of the freezer and defrosted to RT. Then, 100 µl was diluted in 900 µl prewarmed colorless DMEM and mixed well. Next, 15 µl of the dilution was added to each well.

Cell culture was then incubated at 37 °C for 30 minutes. Afterwards, the supernatants were discarded, and wells were washed with colorless DMEM. Then, DMEM was discarded and replaced by 100 µl PBS per well. Finally, the plate was measured in a plate reader. First, the green fluorescence was measured. Then, the red fluorescence polymers were identified by a second measurement at the excitation wavelength of 590 nm and an emission wavelength at 610 nm.

2.2.11. Hoechst 33342 and propidium iodide assay

The Hoechst 33342 and propidium iodide (PI) assay kit was chosen because of its ability to directly analyze cells by a live/dead ratio. It is a double stain apoptosis detection assay containing two dyes that bind to DNA. Hoechst 33342 emits a blue light when bound to DNA in apoptotic cells with detectable emission maxima of around 350 to 461 nm. PI binds to dead cells only. PI is a dye emitting red light at emission maxima of around 617 nm. Therefore, it is possible to distinguish between healthy, apoptotic and dead cells in the cell culture. This assay was performed in ketogenic and low glucose cultures compared to untreated cells only.

Hoechst 33342 was taken out of the freezer without being exposed to light. When Hoechst 33342 was at RT 6 µl of the stock solution and 8 µl PI were diluted in 12 ml prewarmed, colorless DMEM. The medium was discarded from the wells and 150 µl PI/Hoechst 33342 dilution was added to each well. The cell culture was then incubated at 37 °C for 10 minutes. Next, the supernatants were discarded and replaced by 100 µl PBS and measured on a microplate reader.

2.2.12. Statistical analysis

To study the effects of CBD and 2-DG, data were obtained from a minimum of nine independent experiments. Data for a ketogenic diet was obtained from at least two independent experiments. Each parameter was analyzed separately and then examined for correlations. First, analysis was performed with EXCEL, further statistical evaluations were realized with StatView. Standard Deviation (SD) were used to express the variability of the data indicating dispersion from mean.

For experiments evaluating the survival of dopaminergic neurons and for the metabolic activity in mesencephalic primary cells, the Kruskal-Wallis test was used. A subsequent Chi2 -test was performed in order to compare the distribution of data belonging to different categories. A non-parametric pairwise comparison was performed with a non-parametric Mann-Whitney U-test. Data were regarded as statistically significant with a $p < 0.05$.

3. Results

3.1. Treatment of primary mesencephalic cultures with CBD ± 2-DG

For evaluating the effects of 2-DG and CBD, five parameters were measured: To semiquantify overall metabolism and the formation of ATP, a resazurin reduction assay and an ATP assay was conducted. To measure the amount of living cells, a bicinchoninic acid protein assay was used. The amount of dead cells was determined by lactate dehydrogenase activity measurements. TH immunocytochemistry of dopaminergic neurons was used to provide information on the cell count and of this specific cell type, which is highly vulnerable to oxidative stress and mitochondrial impairment.

Statistical analysis has revealed the following results.

3.1.1. Resazurin reduction assay

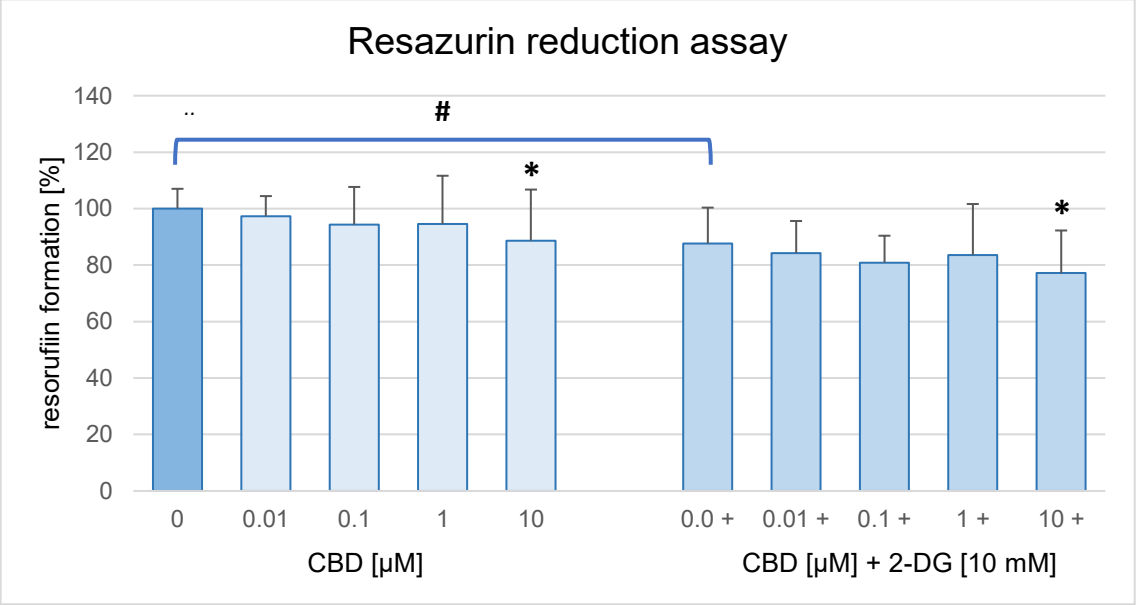

Figure 13. Resazurin reduction assay in mesencephalic primary cells 48 h after treatment with or without co-treatment with 10 mM 2-DG. 100 % corresponds to the untreated control after 14 DIV. Data are expressed as mean ± standard deviation (SD) of 8 independent experiments. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison between the control and the group that was treated with 2-DG and different levels of CBD. With the Kruskal-Wallis (H)-test followed by the Chi²-test, significant changes of CBD compared to the respective controls were examined $p < 0.05$ values were marked with (*).

A significant decrease of resorufin formation by 12.3 % between the cells treated with 10 mM 2-DG and the control group was observable after 48 h. This indicates that 2-DG has a cytotoxic

effect on the cells. Furthermore, a higher concentration of CBD induced lower resorufin levels. Treatment of neuronal cells with 10 µM CBD displays a decreased resorufin formation by 11.4 % compared to untreated control. However, the combination of 10 µM CBD and 10 mM 2-DG shows the highest diminishment of resorufin formation of all tested concentrations by 22.8 %.

3.1.2. Bicinchoninic acid protein assay

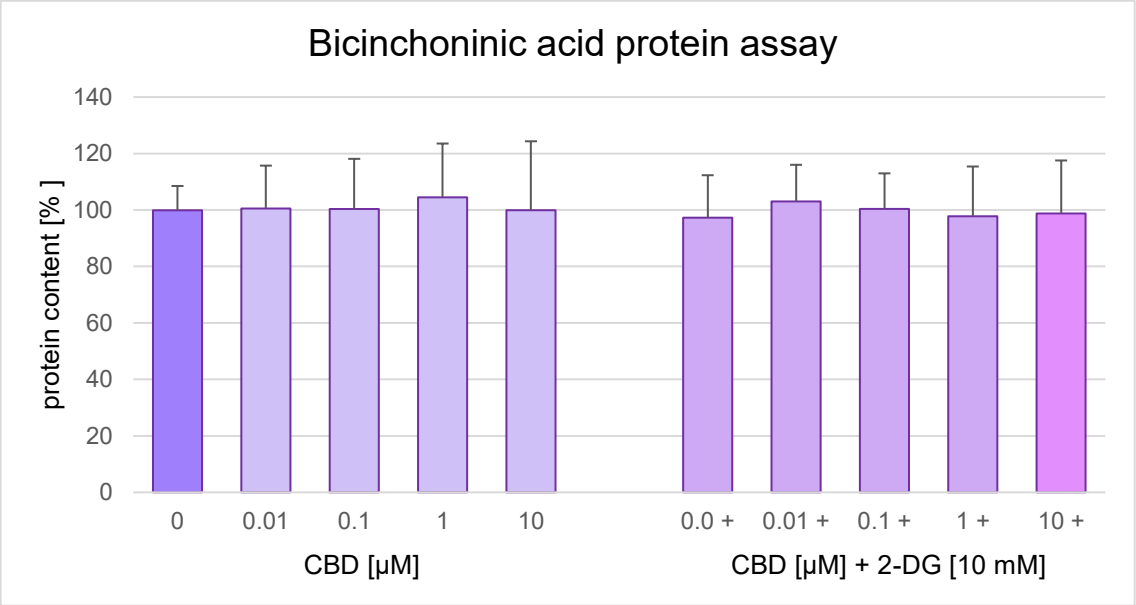

Figure 14. Protein measurement in CBD and CBD/2-DG treated cultures. Bicinchoninic acid protein assay for protein determination in mesencephalic primary cells 48 h after treatment with CBD alone of with concomitant treatment with 10 mM 2-DG. 100 % corresponds to the untreated control after 14 DIV. Data are expressed as mean ± SD of 10 independent experiments.

48 hours after treating mesencephalic primary cells with either CBD or with a combination of CBD and 2-DG, no significant changes of protein content can be observed.

3.1.3. ATP determination

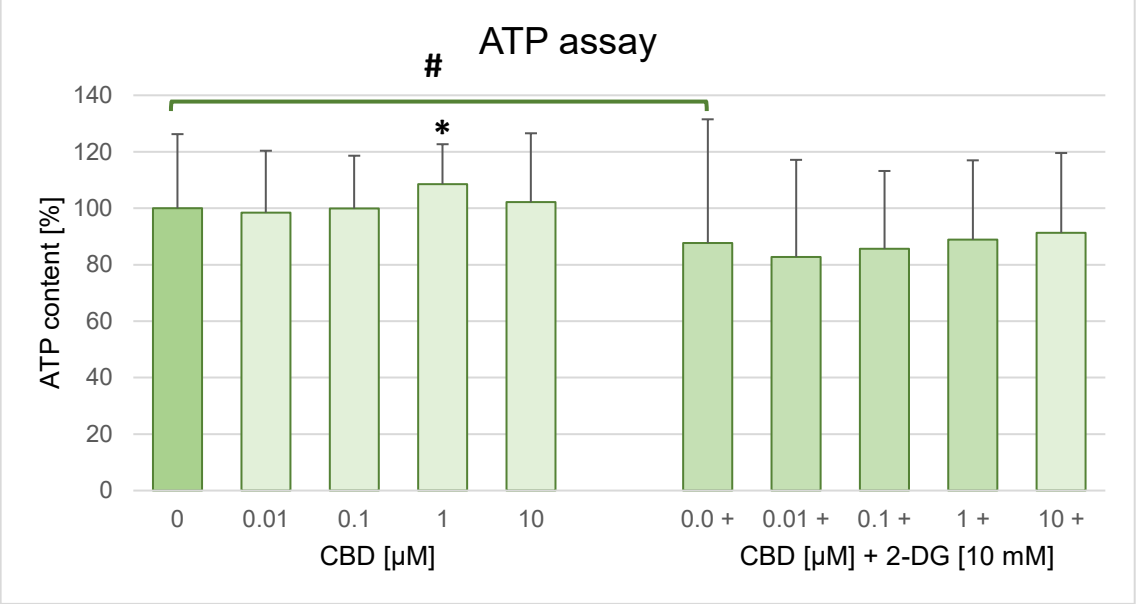

Figure 15. ATP determination in mesencephalic primary cells 48 h after treatment with CBD alone or with concomitant treatment with 10 mM 2-DG. 100 % corresponds to the untreated control after 14 DIV. Data are expressed as mean ± SD of 10 independent experiments. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison between the control and the group that was treated with 2-DG and different levels of CBD. With the Kruskal-Wallis (H)-test followed by the Chi²-test, significant changes of CBD compared to the respective controls were examined $p < 0.05$ values were marked with (*).

A significant difference between the control group and the cells treated with 10 mM 2-DG can be detected after 48 h. ATP content decreased in cells exposed to a concomitant treatment compared to the untreated control. Furthermore, data reveal a slight increase of ATP levels up to 108.6 % at treatment with 1 µM CBD compared to the untreated control.

3.1.4. LDH activity

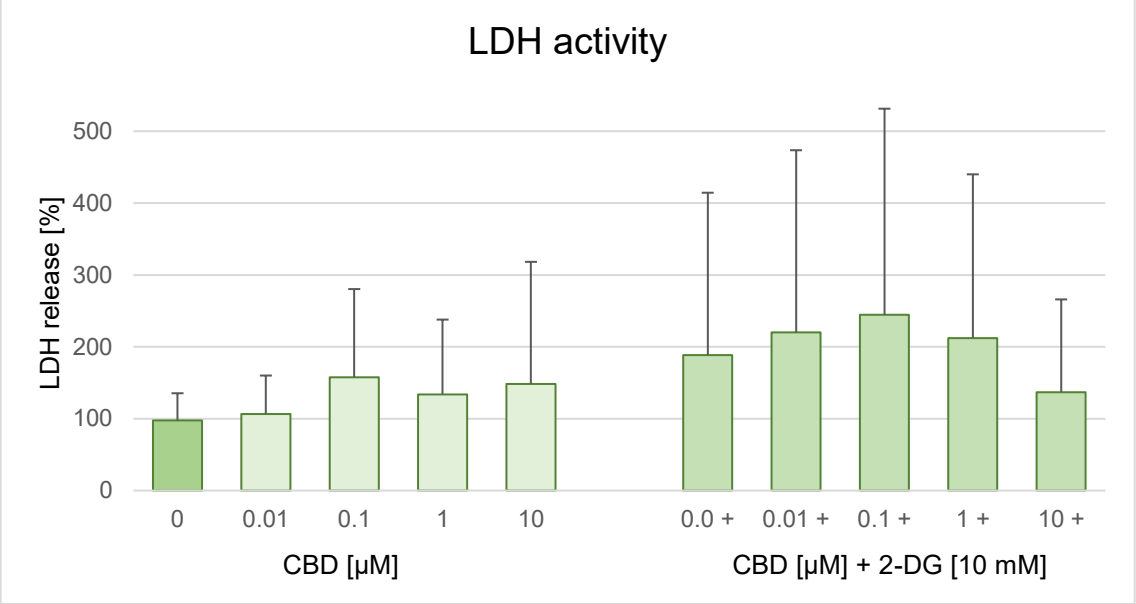

Figure 16. Effects on the release of LDH into the culture medium 48 h after a treatment with CBD with or without co-treatment with 10 mM 2-DG. 100 % corresponds to the untreated control after 14 DIV. Data are expressed as mean ± SD of 8 independent experiments.

The statistical values reveal no significances changes in LDH activities. Neither CBD nor a combination of CBD with 2-DG affected the cells after 48 h. However, a trend can be observed, indicating that a combinatory treatment has a higher cytotoxic effect on mesencephalic primary cells. Raw data display values near the lower detection limits, which is responsible for the high standard deviations (data not shown).

3.1.5. TH staining

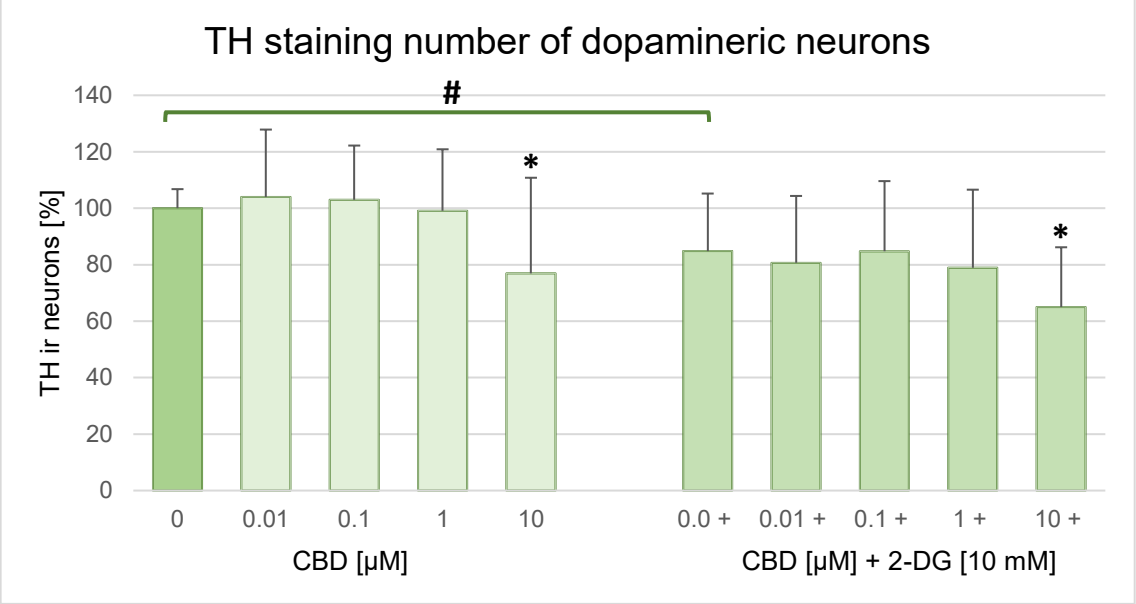

Figure 17. Number of TH positive cells in the mesencephalic primary cell culture 48 h after treatment with CBD. 100 % corresponds to the untreated control after 14 DIV. Data are expressed as mean ± SD of 8 independent experiments. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison between the control and the group that was treated with 2-DG and different levels of CBD. With the Kruskal-Wallis (H)-test followed by the Chi^2-test, significant changes of CBD compared to the respective controls were examined $p < 0.05$ values were marked with (*).

Treatment with 10 mM 2-DG for 48 h results in a significant difference between the control group and the group that was treated with 2-DG and different levels of CBD. This demonstrates that dopaminergic neurons are vulnerable to low glucose supply. Furthermore, the higher the concentration of CBD, the higher the cytotoxicity in the dopaminergic neurons. Especially, a concentration of 10 µM CBD reduces the number of dopaminergic neurons to 76.9 % in comparison to the control. Administration of 10 µM CBD and 10 mM 2-DG led to significantly decrease of dopaminergic neurons by 35 % indicating selective damage to dopaminergic neurons and absence of overall cytotoxicity in CBD and 2-DG treated primary mesencephalic cell culture.

3.2. Simulating a ketogenic diet by administration of BHB and low glucose/glutamine treatment

In the second part of the study, seven parameters were measured: To semiquantify overall metabolism, resazurin reduction and ATP assays were conducted. Quantification of mitochondrial membrane potential was performed by using a JC-1 assay. To measure the amount of living cells, a bicinchoninic acid protein assay was implemented. The amount of dead cells was determined by lactate dehydrogenase activity measurements. Hoechst 33342 and propidium iodide fluorimetry was used to measure the living and dead cells. TH staining of dopaminergic neurons provides information on the amount of this specific cell type under ketogenic conditions.

Statistical analysis has revealed the following results.

3.2.1. Resazurin reduction assay

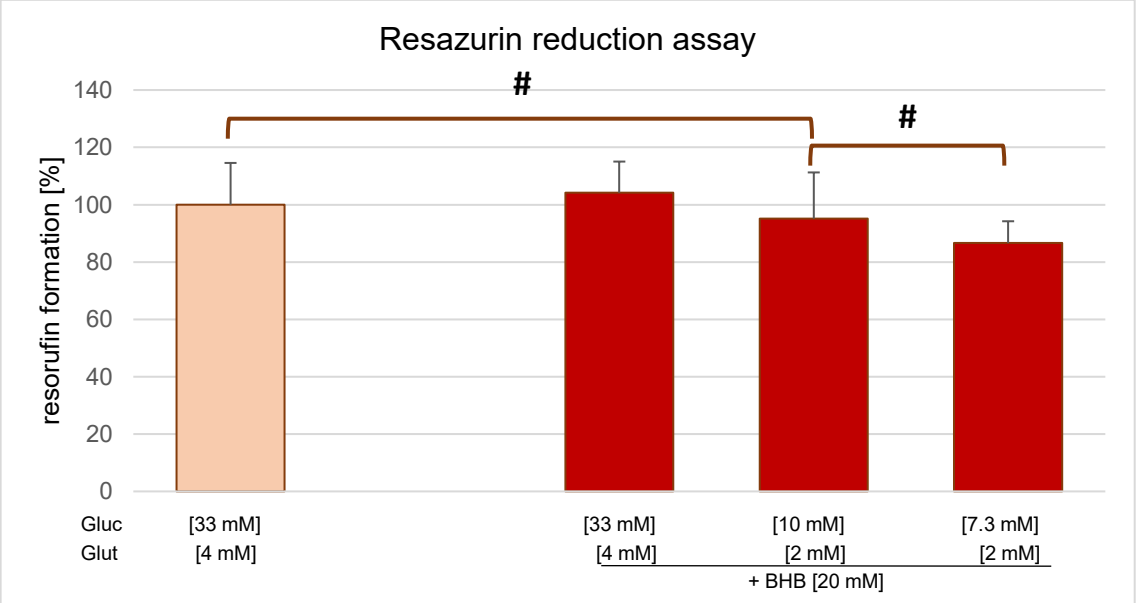

Figure 18. Resazurin reduction assay in mesencephalic primary cells incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of five independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

A significant difference between the control group and the cells incubated with glucose and glutamine deprived medium was observable after seven days. Deprivation of glucose and glutamine simulates a ketogenic and a low glucose diet and causes a mildly decreased

metabolism. Incubation with a fc. of 10 mM glucose/2 mM glutamine lead to a reduction of resorufin formation to 95.2 %. Whereas, least metabolic activity is observable in cultures exposed to a 7.3 mM medium, resorufin formation decreases to 86.6 %. Nevertheless, BHB does not seem to damage cellular metabolism as the resorufin levels remained stable when incubated with N4 and BHB.

3.2.2. Bicinchoninic acid protein assay

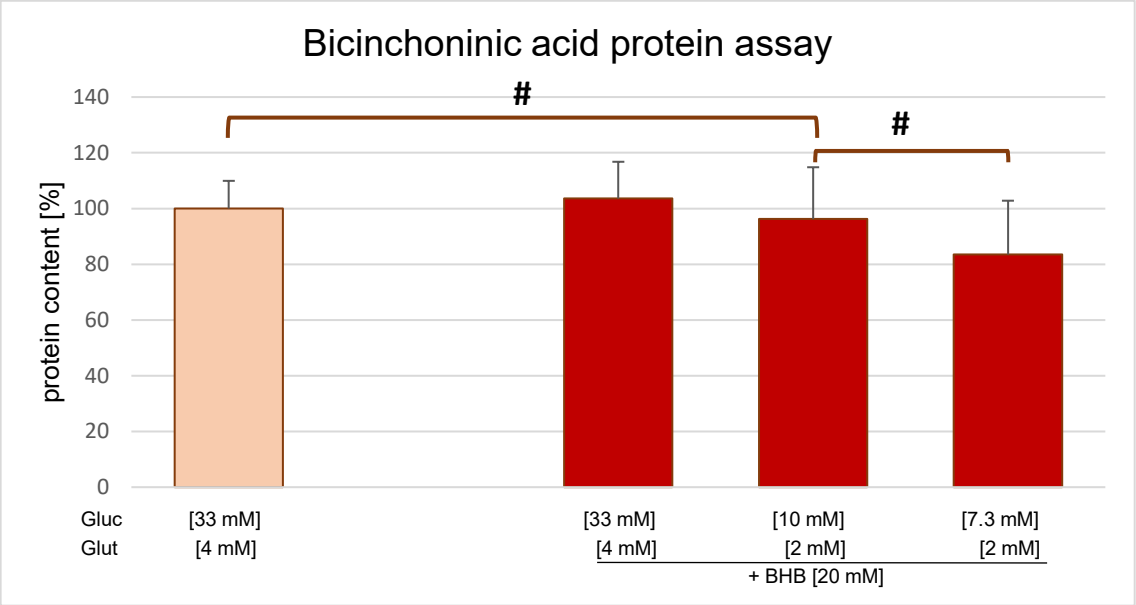

Figure 19. Bicinchoninic acid protein assay for protein determination in mesencephalic primary cells incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of six independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

The protein content corresponds to the resazurin reduction. Significant differences between the control group and the cells exposed to glucose and glutamine deprivation are observable. Incubation in a medium containing 10 mM glucose resulted in a diminished protein content to 96.3 %, whereas, a concentration of 7.3 mM leads to a reduction to 83.5 % compared to controls.

3.2.3. Hoechst 33342 / propidium iodide

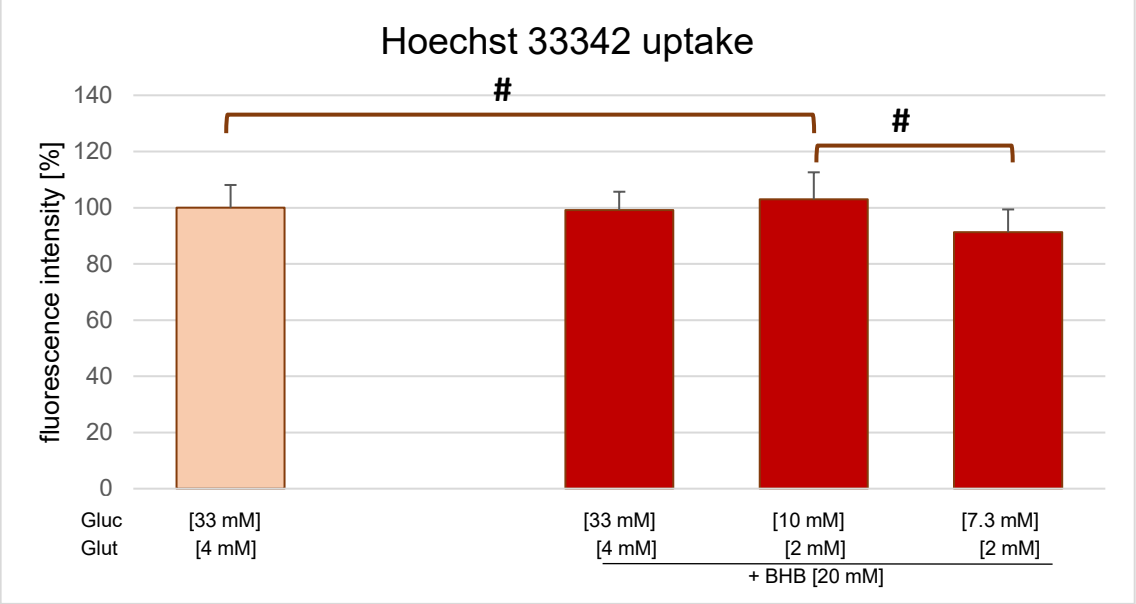

Figure 20. Hoechst 33342 fluorescence in mesencephalic primary cells incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of four independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

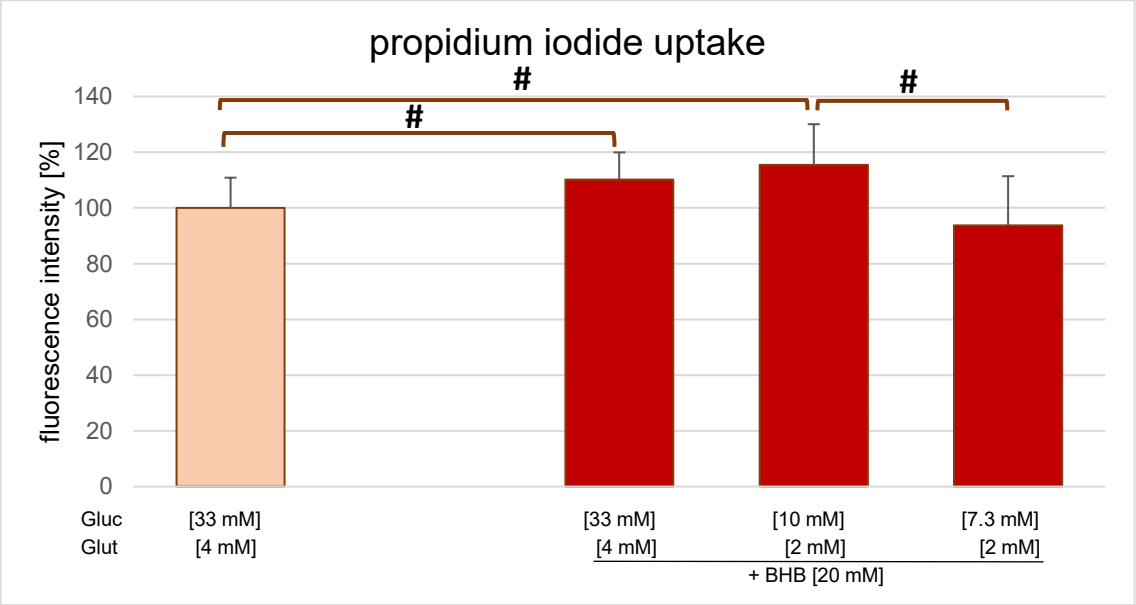

Figure 21. Propidium iodide uptake in mesencephalic primary cells incubated for one week with either N4 or glucose and glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of four independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

Detection of apoptotic cell death by Hoechst 33342 staining revealed that there is a significant loss of cells in the culture exposed to glucose/glutamine deprivation. PI staining showed increased fluorescence intensity up to 115.5 % in cells incubated with a concentration of 10 mM glucose and BHB (Fig.: 21) Cells incubated with a concentration of 7.3 mM glucose can be assumed to have higher cell loss than expressed by 93.7 % fluorescence intensity for PI staining. An explanation for this result might be that cell damage that occurred at an earlier stage and leads to detachment cannot be detected by PI fluorescence measurements (Fig.: 21)

3.2.4. ATP

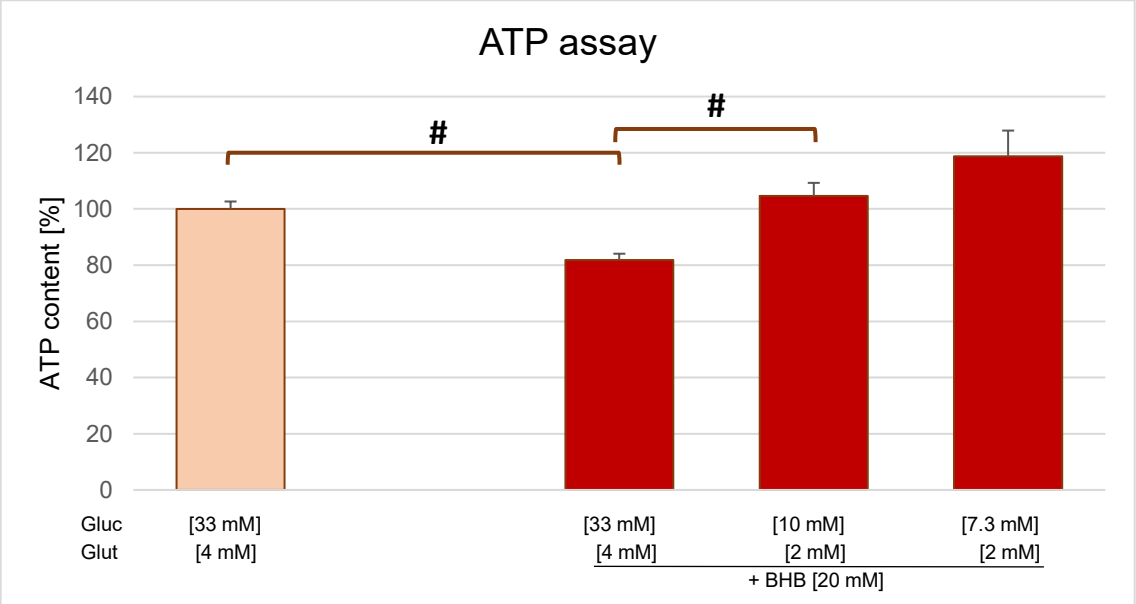

Figure 22. ATP determination in mesencephalic primary cells incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of six independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

The data reveal that a significant difference between the control group and the cells incubated with additionally BHB. 2-DG. It seems as if a low glucose medium increases the ATP formation by 18 %. These findings do not correspond to the other parameters.

3.2.5. TH staining

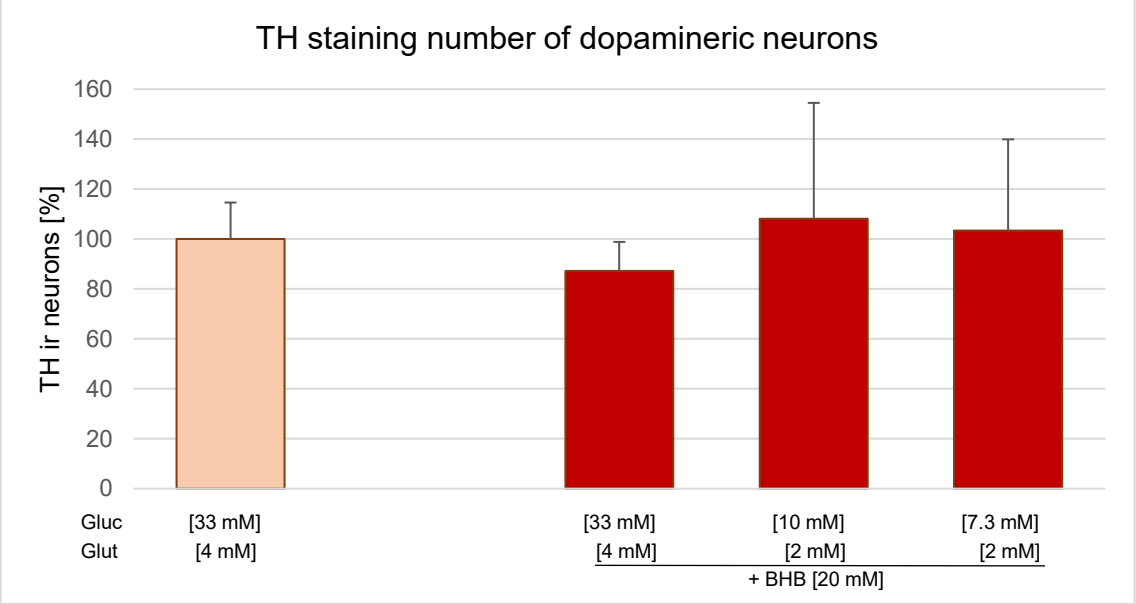

Figure 23. Number of TH positive cells in the mesencephalic primary cell culture incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of three independent experiments for control and glucose/glutamine deprived medium and one independent experiment for N4 with BHB.

Exposure of cultures to glucose and glutamine deprivation supplemented with BHB for a week shows little effect on the number of TH immunoreactive neurons. Incubation of cultures with N4 medium and BHB for seven days resulted in an insignificant loss of dopaminergic neurons to 87.2 % compared to the control.

3.2.6. LDH activity

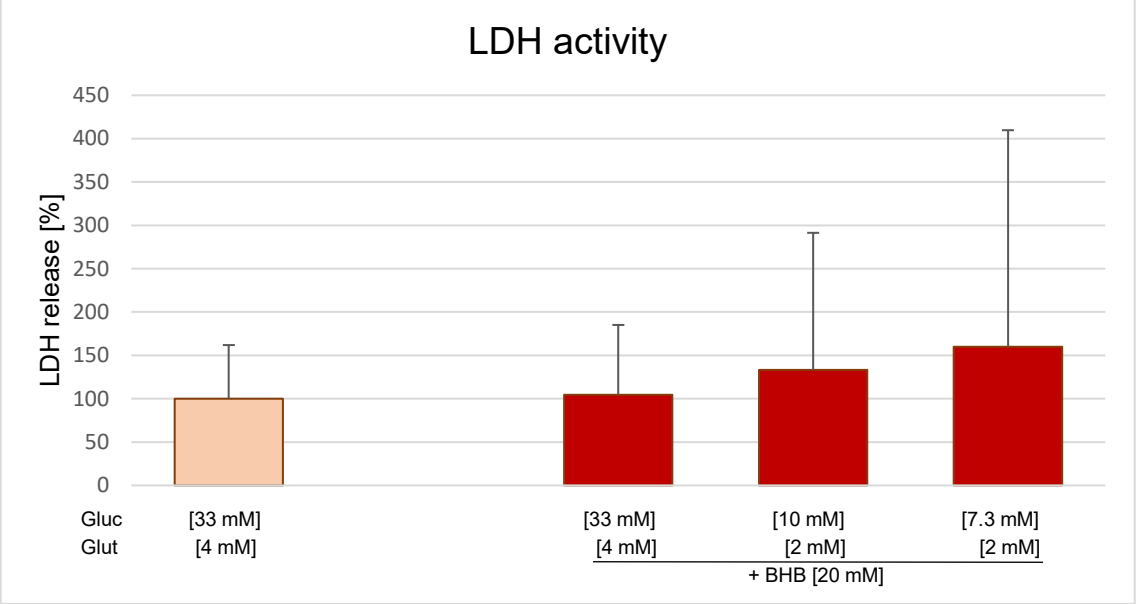

Figure 24. LDH activity in mesencephalic primary cells incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of four independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB.

Incubation of mesencephalic primary cells with N4 medium with or without BHB and medium deprived of glucose and glutamine supplemented with BHB has no significant effects on LDH release into culture media.

3.2.7. JC-1

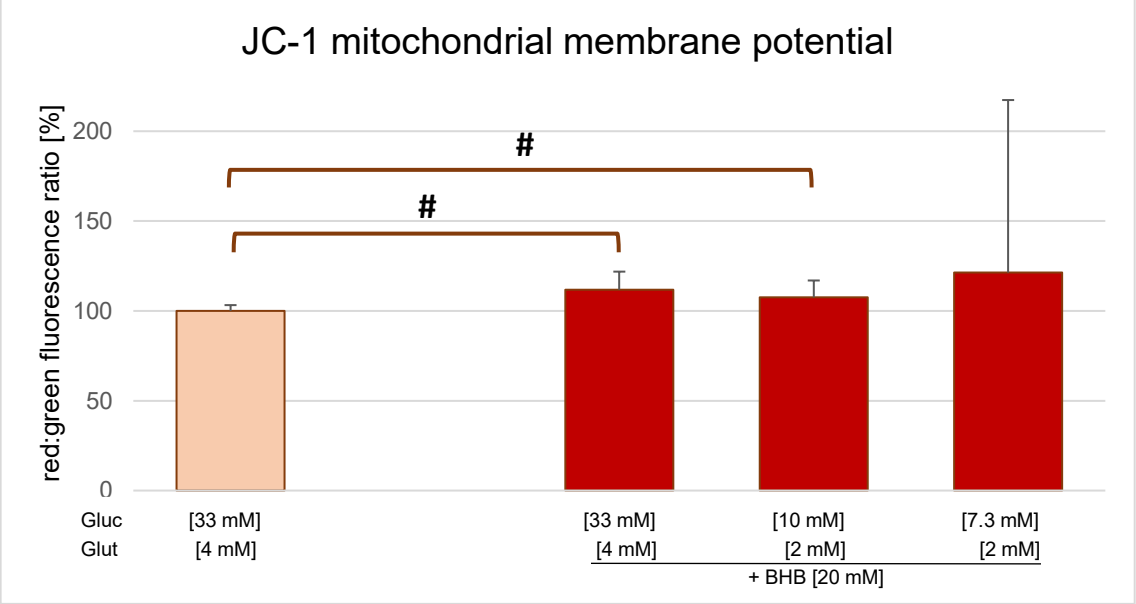

Figure 25. Measurement of Δψm in primary mesencephalic cell culture incubated for one week with either N4 or glucose/glutamine depleted medium and supplementary BHB. 100 % corresponds to the control incubated with N4 after seven DIV. Data are expressed as mean ± SD of six independent experiments for control and glucose/glutamine deprived medium and two independent experiments for N4 with BHB. Significance was defined at a $p < 0.05$ (#) using a Mann-Whitney-U-test for a pairwise comparison.

Fluorescence staining of cultured cells with JC-1 respectively revealed that glucose and glutamine deprivation supplemented with BHB does not influence mitochondrial integrity. Mitochondrial membrane potential even significantly increases in cells incubated with N4 and BHB up to 111.8 % compared to the control.

4. Discussion

4.1. The effects of 2-DG and CBD in mesencephalic primary cells

Neurons have a high demand for glucose, which is reflected by the fact that in humans, around 20 % of the glucose uptake is consumed by the brain.(Koepsell, 2020) This high demand of glucose might explain why the mesencephalic primary cells in our study reacted sensibly to a treatment of 10 mM 2-DG than. However, statistical evaluation does not reveal great changes in the survival of the cultures after a simultaneous treatment with CBD and 2-DG. Neither overall cell metabolism nor the cell viability decreased significantly in our *in vitro* model. Nevertheless, results have shown that the effect of CBD at least at a concentration of 10 µM is cytotoxic to some extent. Our study reveals significant cell loss of dopaminergic neurons, down to 65 % in primary mesencephalic cell culture exposed to a treatment with 10 µM CBD and 10 mM 2-DG (Fig.: 17). A significant decrease in resazurin reduction by 22.8 % corresponds to a mild to moderate inhibition of cellular metabolism (Fig.: 13). A concomitant treatment of CBD and 2-DG has shown a tendency to reduce resorufin formation, but the cytotoxic potential is not enough for overall cell degeneration in mesencephalic primary cell culture. It should be further studied if extended incubation duration could also lead to a loss of other cells than dopaminergic neurons in the mesencephalic primary cell culture.

4.2. Comparison of the effects of 2-DG and CBD on mesencephalic primary cells and glioma and neuroblastoma cells

A main characteristic of cancer cells is an altered metabolism leading to a dependency on glucose.(Voss et al., 2018) Several studies have shown that 2-DG has the potential to inhibit glycolysis. This leads to ATP-depletion in cancer cells as their ATP production is already reduced due to downregulated mitochondrial activity.(Voss et al., 2018) Monotherapy of 2-DG did not succeed *in vivo* experiments in mice and humans as several studies have shown.(Voss et al., 2018) Therefore, new models with additional substances need to be established. Previous studies have revealed that a concomitant treatment of CBD and 2-DG has cytotoxic effects on glioma cells and neuroblastoma cells. Glioma cells reacted more sensitively.(Svatunek, 2020) In this study we found similar cytotoxic effects on primary cells. CBD and 2-DG mediated cytotoxicity leads to a reduced metabolism by 22.8 %. (Fig.: 13). Protein content remained stable in all three cell cultures (Fig.: 14, 34, 37), same as insignificant changes of LDH activity (Fig.: 16, 32, 25).

This is an unexpected and interesting aspect as it was hypothesized that cancer cells would react more sensitively to an induced inhibition of glycolysis. It seems that cancer cells found an alternative way to cover their energy needs. CBD is considered to affect and inhibit mitochondrial complex I, also effects on complex II and III are being discussed.(Singh et al., 2015) Since CBD inhibits mitochondria, its application at a concentration of 10 µM resulted in a reduced metabolism, down to 87.7 % in primary cells (Fig.: 13). We found that resorufin levels were lowered by 22.8 % when primary cells were treated simultaneously with 10 µM CBD and 10 mM 2-DG, suggesting increased cell stress (Fig.: 13). Similar effects could be observed in previous studies on glioma cells as overall cellular metabolism decreased by 20 % (Fig.: 36).(Svatunek, 2020) However, a concomitant treatment could not alter the overall cellular metabolism in neuroblastoma cells compared to a treatment without CBD (Fig.: 33).(Svatunek, 2020)

In conclusion, our study presents that a simultaneous therapy of CBD and 2-DG, showed only little potential to significantly decrease the number of neuroblastoma and glioma cells *in vitro* but the treatment affected dopaminergic neurons in primary mesencephalic cell culture negatively.

4.3. The effects of BHB and low glucose in mesencephalic primary cells

A ketogenic diet is a diet very low in carbohydrates with a moderate-protein and a high fat intake. Animals and humans can adapt their metabolism and produce ketone bodies as an alternative source of energy. Ketone bodies are also able to pass the blood-brain barrier and can therefore also be utilized for energy metabolism in the brain.(Masood et al., 2020) A ketogenic diet increasingly is discussed to potentially help fighting against several diseases, ranging from neurological disorders to diabetes and cancer.(Li et al., 2020) Therefore new models to study the biochemical mechanisms are required.

Treatment with BHB instead of glucose for one week was not successful to simulate such a ketogenic diet in our culture model. BHB, used as a supplementary ketone body, could be ingested by mesencephalic primary cells and metabolized as a source of energy. BHB cannot compensate the lack of glucose in mesencephalic primary cells as shown by measurements on cell count and overall metabolism and protein assay (Fig.: 23, 18 and 19). Nonetheless, red:green fluorescence ratio of JC-1 did not reveal mitochondrial damage when BHB was added to the medium (Fig.: 25). Therefore, BHB can be considered as an unharmful substrate.

However, results do not correlate with the data of ATP production measured (Fig.: 23). This effect cannot be explained yet, so further experiments are needed, since based on the data presented here, a discussion of this effect would be too speculative. However, TH-staining showed that dopaminergic neurons do not seem to be affected by a restriction of glucose (Fig.: 23). A possible explanation might be that glial cells (astrocytes) are able to provide still enough lactate to the neuron.(*Brain Basics: The Life and Death of a Neuron | National Institute of Neurological Disorders and Stroke*, 2019)

It is known that the changes in energy metabolism by ketogenic diet in humans take several weeks. Genes that encode for the regulation of metabolic pathways are upregulated, which leads to an increased mitochondrial density in oxidative tissues.(Gershuni et al., 2018) Likely, the incubation time of only one week with low glucose and BHB is insufficient to allow the cells necessary metabolic adaptations. The results presented here put in question the usefulness of primary cell cultures to provide a tool for investigating the effects of ketogenic diet. Nonetheless, further research on cultures with longer incubation times, other media compositions, and measurements on more specific parameters related to energy metabolism, might be useful if research on ketogenic diet in cancer treatment lead to auspicious results.

4.4. Limitations

Cellular responses to certain treatments have been measured in the present study. However, we did not perform animal experiments, but provided a cellular animal replacement model. The *in vitro* environment cannot represent the complex interactions and mechanisms a multicellular system undergoes *in vivo*.

Another aspect of our study needs to be mentioned. Considering the results, the LDH activity assay does not seem to suit as a useful parameter. If cell damage occurs resulting in cell lysis LDH is released into the medium. Therefore, the amount of LDH measured correlates to the amount of dead cells. Raw data of LDH measurements indicate that the LDH activity values are nearly the detection limit (data not shown). Thus, cells neither get extremely damaged nor do they undergo apoptosis due to the treatment. Figures 16 and 24 are expressed in percent of controls, they probably reflect measured noise rather than reliable LDH levels.

5. Conclusion and outlook

CBD is being discussed as a potential anti-cancer drug. Due to that, the aim of this study was to generate an *in vitro* model to study the neurotoxic effects of CBD on murine mesencephalic primary cells in combination with glycolytic inhibitor 2-DG. For this purpose, data from mesencephalic culture studies were compared to data from previous studies in this lab, investigating the effect of the same treamebnt on glioma and neuroblastoma cells. Additionally, it was aimed to establish an *in vitro* model for ketogenic diet in murine neural primary cells.

The study provides answers to the following questions:

What cytotoxic effects can be observed when adding CBD in various concentrations to murine mesencephalic primary cells? Does cytotoxicity increase with higher concentrations of CBD?

→ Treating murine mesencephalic primary cells with CBD showed minor effects. The results revealed that the effect of CBD at a concentration of 10 µM is cytotoxic. Similar cytotoxic effects were observed on glioma and neuroblastoma cells.(Svatunek, 2020)

How do the effects of 2-DG and CBD correlate? Is a multiplicative effect observable?

→ The simultaneous treatment of CBD and 2-DG showed minimal additive cytotoxic effects on all three cell cultures (glioma and neuroblastoma cells, and primary mesencephalic cultures). Significant damage could only be observed with a combinatory treatment, of which inhibiting glycolysis with 2-DG seems to cause greater harm. A specific toxicity in cell line could not be detected.

How do murine mesencephalic primary cells react to a ketogenic diet? Can BHB safe the cells from glucose deprivation?

→ Adding beta hydroxybutyrate and limiting glucose/glutamine in the cell culture medium revealed significant negative effects on cell survival. Mesencephalic primary cells seem to be unable to compensate the lack of glucose with BHB. Therefore, this approach should undergo further studies.

The cytotoxic effects of a CBD and 2-DG treatment on mesencephalic primary cells which were observable especially on dopaminergic neurons leads to the conclusion that the combination of CBD and 2-DG shows little potential to serve as an anti-cancer treatment. Further research in animal models could show the effects of the treatment *in vivo,* since the complexity of a multicellular organism cannot be simulated *in vitro.*

As the results show little anti-cancer effects in glioma and neuroblastoma cells, and relatively high cytotoxic effects on primary cells, further experiments with animals investigating the effects of CBD and 2-DG cannot be recommended.

The ketogenic model on murine mesencephalic primary cells revealed that the cells are unable to compensate the lack of glucose with the provided BHB, however, few experiments have been conducted and further studies including more parameters to confirm this finding must be done.

6. Summary

6.1. English

In the last decade, research on the phytocannabinoid CBD has intensified. CBD originates from the plant *Cannabis sativa* and seems to have promising therapeutic efficacy on neurological disorders, inflammation and epilepsy. It is even considered to provide a new way of targeting cancer cells. Therefore, CBD is being discussed as a potential anti-cancer drug, while the effects of CBD have not yet been fully understood.

It is known that cancer cells shift their metabolism from oxidative phosphorylation towards glycolysis, referred to as the Warburg effect. 2-DG, a structural analog of glucose, is proposed to act anti-cancerogenic in a combinatory therapy but to prove this, further studies are required.

Based on previous studies from the Neurochemistry Group on neuroblastoma cells, we performed experiments to assess the cytotoxicity of a concomitant treatment of CBD and 2-DG in mesencephalic primary cells. Furthermore, possible benefits of a ketogenic diet *in vitro* were studied in this culture model.

Furthermore, in a second part of the thesis, it was tried to establish an in vitro model for ketogenic diet, which likewise is discussed to be beneficial in some kinds of cancer. A ketogenic diet is a diet very low in carbohydrates with a moderate-protein and a high fat intake. Thus, it directly targets glycolysis by partially blocking it. Moreover, some cancer types are not able to adapt their metabolism and cannot use ketone bodies as an alternative source of energy. The consequence for these cancers might be starvation of energy while normal cells adapt and compensate the missing glucose with ketone bodies such as BHB. Therefore, mesencephalic primary cells were incubated for a week with a glucose and glutamine deprived medium substituted with BHB.

Various biochemical analyses were performed to measure the effects of the experiments on the cells: To assess overall metabolism, resazurin reduction and ATP assays were conducted. To measure the amount of living cells, a BCA protein assay was used. The amount of dead cells was determined by LDH activity measurements. For analyzing the effects of a ketogenic diet, additionally JC-1 and Hoechst 33342/propidium iodide fluorimetry was used. TH immunocytochemistry staining was performed to detect the dopaminergic neurons represented in the culture.

The study has revealed that partial inhibition of glycolysis using 2-DG has a similar effect on both, neuroblastoma cells and mesencephalic primary cells. Results have shown that the effect of CBD at a concentration of 10 µM is cytotoxic. CBD and 2-DG in combination had minimal additive cytotoxic effect on both cell cultures. Therefore, our data do not support the hypothesis that CBD and 2-DG might serve as an anti-cancer treatment. Since the anti-cancer effects of CBD and 2-DG are low and the negative effects on primary cells are comparatively high, further animal experiments with the same treatment cannot be recommended, but it must be noted that *in vitro* experiments cannot represent the *in vivo* situation completely.

With our second model we developed a model that simulates a ketogenic diet *in vitro*. Our data have revealed that mesencephalic primary cells can not compensate the lack of glucose with the provided BHB. It will take further studies to confirm this finding.

6.2. German

Im letzten Jahrzehnt beschäftigte sich die Forschung intensiv mit dem Phytocannabinoid CBD. CBD, ein aus der Pflanze *Cannabis sativa* isolierbarer Wirkstoff, scheint eine vielversprechende Wirkung bei der Therapie von neurologischen Störungen, Entzündungen, Suchtverhalten und Epilepsie zu haben. Weiters wird untersucht, ob mit CBD auch Krebszellen behandelt werden können, in der Hoffnung, mit CBD neue Krebsmedikamente entwickeln zu können.

Der Warburg-Effekt beschreibt, dass Krebszellen ihren Metabolismus von oxidativer Phosphorylierung primär auf Glykolyse umstellen. 2-DG, ein strukturelles Analogon der Glucose, hemmt die Glykolyse, was manche Krebszellen schädigen kann. Es wird daher geprüft, ob 2-DG als Mittel gegen Krebs eingesetzt werden kann.

Die Gruppe der Neurobiochemie prüfte in vorangegangenen Untersuchungen die toxischen Effekte einer gleichzeitigen Behandlung mit CBD und 2-DG auf Neuroblastomzellen. In unserer Studie wurden die Effekte dieser Behandlung auf murine mesencephale Primärzellen im Hinblick darauf untersucht, ob die Primärzellen durch die Behandlung mit CBD und 2-DG weniger geschädigt werden als Krebszellen, was Voraussetzung für eine Eignung als Krebsmedikament ist.

Weiters wurde im zweiten Teil dieser Arbeit versucht ein *in vitro* Modell für eine ketogene Diät zu entwickeln. Eine solche Diät wird ebenfalls als Behandlung von Krebs diskutiert. Bei einer

ketogenen Diät wird die Kohlenhydratzufuhr stark reduziert. Die Energie wird vorrangig aus Fett und zu einem geringen Anteil aus Protein gewonnen, wodurch Glykolyse stark gehemmt wird und Ketonkörper, wie z. B. BHB als Ersatz verstoffwechselt werden. Manche Krebszellen können ihren Stoffwechsel nicht derart anpassen und werden dadurch geschädigt.

In den vorrangegangenen Studien der Gruppe der Neurobiochemie konnten moderate zytotoxische Effekte durch eine Behandlung mit CBD und 2-DG auf Neuroblastomzellen gezeigt werden. Darauf basierend untersuchten wir vorrangig die Wirkung auf Primärzellen. Zudem wurde überprüft, wie sich die ketogene Diät auf Primärzellen auswirkt.

Anhand verschiedener Analysemethoden wurden die Auswirkungen auf die Primärzellen gemessen. Um Veränderungen des Zellmetabolismus quantitativ zu bestimmen, wurden Resazurin-Reduktion und ATP-Bestimmungen durchgeführt. Um die Menge der lebenden Zellen zu bestimmen wurden ein BCA-Protein-Assays eingesetzt. Die Zahl der abgestorbenen Zellen wurde anhand der LDH-Aktivität gemessen werden. Um die Auswirkungen der ketogenen Diät zu untersuchen, wurde JC-1 und Hoechst 33342/Propidiumiodid-Fluorimetrie als weitere Parameter herangezogen. Immunozytochemische TH-Fixierung wurde zur Ermittlung der dopaminergen Neuronen angewandt.

Die Studie zeigt, dass durch eine partielle Hemmung der Glykolyse mittels 2-DG Neuroblastomzellen und Primärzellen gleichermaßen geschädigt werden. Eine Konzentration von 10 µM CBD ist nachweislich zytotoxisch. Die Kombination von CBD und 2-DG hat minimale zellschädigende Effekte in allen untersuchten Zellkultursystemen erzielt. Da die Behandlung sich jedoch auch negativ auf mesencephale Primärzellen ausgewirkt hat, lässt sich aus unseren Daten schließen, dass sich aus einer Kombination von CBD und 2-DG mit dieser Datenlage keine vielversprechende Behandlung ableiten lässt.

Das *in vitro* Modell für eine ketogene Diät zeigt, dass mesencephale Primärzellen nicht in der Lage sind, die entzogene Glukose mit BHB zu substituieren. Um detaillierte Aussagen zu tätigen, müssen weitere Experimente durchgeführt werden.

7. References

Adjiri, A. (2016). Identifying and Targeting the Cause of Cancer is Needed to Cure Cancer. *Oncology and Therapy*, *4*(1), 17–33. https://doi.org/10.1007/s40487-015-0015-6

Aerobic Glycolysis and the Warburg Effect. (2012). Sigma-Aldrich. https://www.sigmaaldrich.com/technical-documents/articles/biofiles/aerobic-glycolysis-and-warburg-effect.html

Aft, R. L., Zhang, F. W., & Gius, D. (2002). Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: Mechanism of cell death. *British Journal of Cancer*, *87*(7), 805–812. https://doi.org/10.1038/sj.bjc.6600547

Ahmad, M., Wolberg, A., & Kahwaji, C. I. (2020). Biochemistry, Electron Transport Chain. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK526105/

Alberts, B., & Johnson, A. (2015). *Molecular biology of the cell* (Sixth edition.). Garland Science.

Alger, B. E. (2004). Endocannabinoids: Getting the message across. *Proceedings of the National Academy of Sciences*, *101*(23), 8512–8513. https://doi.org/10.1073/pnas.0402935101

Alharris, E., Singh, N. P., Nagarkatti, P. S., & Nagarkatti, M. (2019). Role of miRNA in the regulation of cannabidiol-mediated apoptosis in neuroblastoma cells. *Oncotarget*, *10*(1), 45–59. https://doi.org/10.18632/oncotarget.26534

AMPK Signaling. (2020). Cell Signaling Technology. https://www.cellsignal.com/contents/science-cst-pathways-cellular-metabolism/ampk-signaling/pathways-ampk

Andre, C. M., Hausman, J.-F., & Guerriero, G. (2016). Cannabis sativa: The Plant of the Thousand and One Molecules. *Frontiers in Plant Science*, *7*. https://doi.org/10.3389/fpls.2016.00019

Brain Basics: The Life and Death of a Neuron | National Institute of Neurological Disorders and Stroke. (2019). https://www.ninds.nih.gov/Disorders/patient-caregiver-education/life-and-death-neuron

Burggren, A. C., Shirazi, A., Ginder, N., & London, E. D. (2019). Cannabis effects on brain structure, function, and cognition: Considerations for medical uses of cannabis and its derivatives. *The American Journal of Drug and Alcohol Abuse*, *45*(6), 563–579. https://doi.org/10.1080/00952990.2019.1634086

Chilakapati, J., & Farris, F. F. (2014). Cannabinoids. In P. Wexler (Ed.), *Encyclopedia of Toxicology (Third Edition)* (pp. 649–654). Academic Press. https://doi.org/10.1016/B978-0-12-386454-3.00267-0

Chinta, S. J., & Andersen, J. K. (2005). Dopaminergic neurons. *The International Journal of Biochemistry & Cell Biology*, *37*(5), 942–946. https://doi.org/10.1016/j.biocel.2004.09.009

Correia-Sá, I. B. (2020). A new role for anandamide: Defective link between the systemic and skin endocannabinoid systems in hypertrophic human wound healing. *Scientific Reports*, 11.

Dariš, B., Verboten, M. T., Knez, Ž., & Ferk, P. (2019). Cannabinoids in cancer treatment: Therapeutic potential and legislation. *Bosnian Journal of Basic Medical Sciences*, *19*(1), 14–23. https://doi.org/10.17305/bjbms.2018.3532

DeBerardinis, R. J., & Chandel, N. S. (2020). We need to talk about the Warburg effect. *Nature Metabolism*, *2*(2), 127–129. https://doi.org/10.1038/s42255-020-0172-2

Deutch, A. Y. (2013). Chapter 6—Neurotransmitters. In L. R. Squire, D. Berg, F. E. Bloom, S. du Lac, A. Ghosh, & N. C. Spitzer (Eds.), *Fundamental Neuroscience (Fourth Edition)* (pp. 117–138). Academic Press. https://doi.org/10.1016/B978-0-12-385870-2.00006-8

Elsaid, S., & Le Foll, B. (2020). The complexity of pharmacology of cannabidiol (CBD) and its implications in the treatment of brain disorders. *Neuropsychopharmacology*, *45*(1), 229–230. https://doi.org/10.1038/s41386-019-0518-1

Fadaka, A., Ajiboye, B., Ojo, O., Adewale, O., Olayide, I., & Emuowhochere, R. (2017). Biology of glucose metabolization in cancer cells. *Journal of Oncological Sciences*, *3*(2), 45–51. https://doi.org/10.1016/j.jons.2017.06.002

Fisar, Z. (2008, December 31). *Phytocannabinoids and Endocannabinoids*. Current Drug Abuse Reviews. https://www.eurekaselect.com/93058/article

Frezza, C. (2020). Metabolism and cancer: The future is now. *British Journal of Cancer*, *122*(2), 133–135. https://doi.org/10.1038/s41416-019-0667-3

Gaven, F., Marin, P., & Claeysen, S. (2014). Primary Culture of Mouse Dopaminergic Neurons. *Journal of Visualized Experiments : JoVE*, *91*. https://doi.org/10.3791/51751

George T Griffing, & Thai, A. (2019). *Endocannabinoids: Overview, History, Chemical Structure*. https://emedicine.medscape.com/article/1361971-overview

Gershuni, V. M., Yan, S. L., & Medici, V. (2018). Nutritional Ketosis for Weight Management and Reversal of Metabolic Syndrome. *Current Nutrition Reports*, *7*(3), 97–106. https://doi.org/10.1007/s13668-018-0235-0

Ghimire, P., & Dhamoon, A. S. (2020). Ketoacidosis. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK534848/

Gordon, J., Amini, S., & White, M. K. (2013). General overview of neuronal cell culture. *Methods in Molecular Biology (Clifton, N.J.)*, *1078*, 1–8. https://doi.org/10.1007/978-1-62703-640-5_1

Goshi, N., Morgan, R. K., Lein, P. J., & Seker, E. (2020). A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation. *Journal of Neuroinflammation*, *17*(1), 155. https://doi.org/10.1186/s12974-020-01819-z

Guo, J.-D., Zhao, X., Li, Y., Li, G.-R., & Liu, X.-L. (2018). Damage to dopaminergic neurons by oxidative stress in Parkinson's disease (Review). *International Journal of Molecular Medicine*, *41*(4), 1817–1825. https://doi.org/10.3892/ijmm.2018.3406

Harris, R. A., & Johnson, J. S. (2019). Glycolysis Overview☆. In *Reference Module in Biomedical Sciences*. Elsevier. https://doi.org/10.1016/B978-0-12-801238-3.11342-X

Huestis, M. A., Solimini, R., Pichini, S., Pacifici, R., Carlier, J., & Busardò, F. P. (2019). Cannabidiol Adverse Effects and Toxicity. *Current Neuropharmacology*, *17*(10), 974–989. https://doi.org/10.2174/1570159X17666190603171901

Koepsell, H. (2020). Glucose transporters in brain in health and disease. *Pflugers Archiv*, *472*(9), 1299–1343. https://doi.org/10.1007/s00424-020-02441-x

Lafaye, G., Karila, L., Blecha, L., & Benyamina, A. (2017). Cannabis, cannabinoids, and health. *Dialogues in Clinical Neuroscience*, *19*(3), 309–316.

Lambert, D. M. (2009). *Cannabinoids in Nature and Medicine*. Wiley-VCH.

Lee, J., & Mohns, M. (2019). *Cell Viability Guide | How to Measure Cell Viability*. https://at.promega.com/resources/guides/cell-biology/cell-viability/

Li, R.-J., Liu, Y., Liu, H.-Q., & Li, J. (2020). Ketogenic diets and protective mechanisms in epilepsy, metabolic disorders, cancer, neuronal loss, and muscle and nerve degeneration. *Journal of Food Biochemistry*, *44*(3), e13140. https://doi.org/10.1111/jfbc.13140

Lin, K.-L., Lin, J.-J., & Wang, H.-S. (2020). Application of ketogenic diets for pediatric neurocritical care. *Biomedical Journal*, *43*(3), 218–225. https://doi.org/10.1016/j.bj.2020.02.002

Lu, H.-C., & Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biological Psychiatry*, *79*(7), 516–525. PubMed. https://doi.org/10.1016/j.biopsych.2015.07.028

Maccarrone, M. (2020). Missing Pieces to the Endocannabinoid Puzzle. *Trends in Molecular Medicine*, *26*(3), 263–272. https://doi.org/10.1016/j.molmed.2019.11.002

Maroon, J., & Bost, J. (2018). Review of the neurological benefits of phytocannabinoids. *Surgical Neurology International*, *9*. https://doi.org/10.4103/sni.sni_45_18

Masood, W., Annamaraju, P., & Uppaluri, K. R. (2020). Ketogenic Diet. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK499830/

Millán-Guerrero, R. O., & Isais-Millán, S. (2019). Cannabis and the exocannabinoid and endocannabinoid systems. Their use and controversies. *Gaceta Medica De Mexico*, *155*(5), 471–474. https://doi.org/10.24875/GMM.M20000334

Navarrete, F., García-Gutiérrez, M. S., Jurado-Barba, R., Rubio, G., Gasparyan, A., Austrich-Olivares, A., & Manzanares, J. (2020). Endocannabinoid System Components as Potential Biomarkers in Psychiatry. *Frontiers in Psychiatry*, *11*. https://doi.org/10.3389/fpsyt.2020.00315

Olivas-Aguirre, M., Torres-López, L., Valle-Reyes, J. S., Hernández-Cruz, A., Pottosin, I., & Dobrovinskaya, O. (2019). Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell Death & Disease*, *10*(10), 779. https://doi.org/10.1038/s41419-019-2024-0

Panhans, C. M., Gresham, G., Amaral, L. J., & Hu, J. (2020). Exploring the Feasibility and Effects of a Ketogenic Diet in Patients With CNS Malignancies: A Retrospective Case Series. *Frontiers in Neuroscience*, *14*. https://doi.org/10.3389/fnins.2020.00390

Park, J. H., Pyun, W. Y., & Park, H. W. (2020). Cancer Metabolism: Phenotype, Signaling and Therapeutic Targets. *Cells*, *9*(10). https://doi.org/10.3390/cells9102308

Piscitelli, F. (2015). Chapter 9 - Endocannabinoidomics: "Omics" Approaches Applied to Endocannabinoids and Endocannabinoid-Like Mediators. In V. Di Marzo & J. Wang (Eds.), *The Endocannabinoidome* (pp. 137–152). Academic Press. https://doi.org/10.1016/B978-0-12-420126-2.00009-2

Poyatos, L., Pérez-Acevedo, A. P., Papaseit, E., Pérez-Mañá, C., Martin, S., Hladun, O., Siles, A., Torrens, M., Busardo, F. P., & Farré, M. (2020). Oral Administration of Cannabis and Δ-9-

tetrahydrocannabinol (THC) Preparations: A Systematic Review. *Medicina*, *56*(6). https://doi.org/10.3390/medicina56060309

Pretzsch, C. M., Freyberg, J., Voinescu, B., Lythgoe, D., Horder, J., Mendez, M. A., Wichers, R., Ajram, L., Ivin, G., Heasman, M., Edden, R. A. E., Williams, S., Murphy, D. G. M., Daly, E., & McAlonan, G. M. (2019). Effects of cannabidiol on brain excitation and inhibition systems; a randomised placebo-controlled single dose trial during magnetic resonance spectroscopy in adults with and without autism spectrum disorder. *Neuropsychopharmacology*, *44*(8), 1398–1405. https://doi.org/10.1038/s41386-019-0333-8

Radad, K., Al-Shraim, M., Al-Emam, A., Moldzio, R., & Rausch, W.-D. (2018). Neurotoxic effects of domoic acid on dopaminergic neurons in primary mesencephalic cell culture. *Folia Neuropathologica*, *56*(1), 39–48. https://doi.org/10.5114/fn.2018.74658

Redalen, K. (2006). *In vivo Magnetic Resonance Spectroscopy and Diffusion Weighted Magnetic Resonance Imaging for Non-Invasive Monitoring of Treatment Response of Subcutaneous HT29 Xenografts in Mice*.

Rigoulet, M., Bouchez, C. L., Paumard, P., Ransac, S., Cuvellier, S., Duvezin-Caubet, S., Mazat, J. P., & Devin, A. (2020). Cell energy metabolism: An update. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1861*(11), 148276. https://doi.org/10.1016/j.bbabio.2020.148276

Rimmerman, N., Ben-Hail, D., Porat, Z., Juknat, A., Kozela, E., Daniels, M. P., Connelly, P. S., Leishman, E., Bradshaw, H. B., Shoshan-Barmatz, V., & Vogel, Z. (2013). Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: A novel mechanism for cannabinoid-induced cell death. *Cell Death & Disease*, *4*, e949. https://doi.org/10.1038/cddis.2013.471

Segeritz, C.-P., & Vallier, L. (2017). Cell Culture. *Basic Science Methods for Clinical Researchers*, 151–172. https://doi.org/10.1016/B978-0-12-803077-6.00009-6

Seltzer, E. S., Watters, A. K., MacKenzie, D., Granat, L. M., & Zhang, D. (2020). Cannabidiol (CBD) as a Promising Anti-Cancer Drug. *Cancers*, *12*(11). https://doi.org/10.3390/cancers12113203

Shilpa, J., & Mohan, V. (2018). Ketogenic diets: Boon or bane? *The Indian Journal of Medical Research*, *148*(3), 251–253. https://doi.org/10.4103/ijmr.IJMR_1666_18

Shoshan-Barmatz, V., Ben-Hail, D., Admoni, L., Krelin, Y., & Tripathi, S. S. (2015). The mitochondrial voltage-dependent anion channel 1 in tumor cells. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1848*(10, Part B), 2547–2575. https://doi.org/10.1016/j.bbamem.2014.10.040

Singh, N., Hroudová, J., & Fišar, Z. (2015). Cannabinoid-Induced Changes in the Activity of Electron Transport Chain Complexes of Brain Mitochondria. *Journal of Molecular Neuroscience: MN*, *56*(4), 926–931. https://doi.org/10.1007/s12031-015-0545-2

Svatunek, S. (2020). *Toxicity of cannabidiol on brain cancer cells and murine mesencephalic primary cultures under inhibition of glycolysis*. Vienna. http://media.obvsg.at/AC15758883-2001

Szabo, B., & Schlicker, E. (2005). Effects of Cannabinoids on Neurotransmission. In R. G. Pertwee (Ed.), *Cannabinoids* (pp. 327–365). Springer. https://doi.org/10.1007/3-540-26573-2_11

The Krebs cycle—Harnessing chemical energy for cellular respiration. (2007). Sigma-Aldrich. https://www.sigmaaldrich.com/technical-documents/articles/biofiles/citric-acid-cycle.html

Torii, M., Sasaki, M., Chang, Y.-W., Ishii, S., Waxman, S. G., Kocsis, J. D., Rakic, P., & Hashimoto-Torii, K. (2017). Detection of vulnerable neurons damaged by environmental insults in utero. *Proceedings of the National Academy of Sciences*, *114*(9), 2367–2372. https://doi.org/10.1073/pnas.1620641114

Voss, M., Lorenz, N. I., Luger, A.-L., Steinbach, J. P., Rieger, J., & Ronellenfitsch, M. W. (2018). Rescue of 2-Deoxyglucose Side Effects by Ketogenic Diet. *International Journal of Molecular Sciences*, *19*(8). https://doi.org/10.3390/ijms19082462

Weber, D. D., Aminazdeh-Gohari, S., & Kofler, B. (2018). Ketogenic diet in cancer therapy. *Aging (Albany NY)*, *10*(2), 164–165. https://doi.org/10.18632/aging.101382

Wu, J. (2019). Cannabis, cannabinoid receptors, and endocannabinoid system: Yesterday, today, and tomorrow. *Acta Pharmacologica Sinica*, *40*(3), 297–299. https://doi.org/10.1038/s41401-019-0210-3

Ye, L., Cao, Z., Wang, W., & Zhou, N. (2019). New Insights in Cannabinoid Receptor Structure and Signaling. *Current Molecular Pharmacology*, *12*(3), 239–248. https://doi.org/10.2174/1874467212666190215112036

Zhang, X. D., Deslandes, E., Villedieu, M., Poulain, L., Duval, M., Gauduchon, P., Schwartz, L., & Icard, P. (2006). Effect of 2-Deoxy-D-glucose on Various Malignant Cell Lines In Vitro. *ANTICANCER RESEARCH*, 7.

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., Babenko, V. A., Zorov, S. D., Balakireva, A. V., Juhaszova, M., Sollott, S. J., & Zorov, D. B. (2018). Mitochondrial membrane potential. *Analytical Biochemistry*, *552*, 50–59. https://doi.org/10.1016/j.ab.2017.07.009

Zou, S., & Ujendra Kumar. (2018). Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *International Journal of Molecular Sciences*, *19*(3), 833. https://doi.org/10.3390/ijms19030833

8. Appendix

8.1. List of abbreviations

AB-solution……..…avidin-biotin-horseradish peroxidase complex -solution

ATP……..….adenosine-5'-triphosphate

BHB……..……..……beta hydroxybutyrat

BM……..……...Basic Medium

CBD……...……..…….……cannabidiol

CNSs……..…….Central Nervous system

DMEM…….……....Dulbecco's modified Eagle's medium

DMSO……..……dimethylsulfoxide

DPBS…….…..…Dulbecco's modified phosphate buffered saline

GABA……..……γ-aminobutyric acid

Glut...……..……..glutamine

GPCR's……..……...G protein-coupled receptors

HBSS……..……...Hank's balanced salt solution

KM……..……...Ketogenic Medium

LDH……...…….....….lactate dehydrogenase

LGM……Low Glucose Medium

PBS……..……phosphate buffered saline

PPARs……peroxisome proliferator activated receptors

RT……..……..…...room temperature

TH……..……..……..tyrosine hydroxylase

TRP……...……Transient Receptor Potential channels

8.2. List of figures

8.3. List of tables

8.4. Effect of CBD ± 2-DG in N18TG2 neuroblastoma and in U-87 MG glioma cells

As mentioned in the introduction, the effect of CBD on 2-DG treated cultures was studied to evaluate whether CBD, which is discussed to possess anticancer characteristics, might be able to selectively damage cancer cells. In the study presented here, primary cells were chosen. It was expected that primary cells are not harmed by CBD/2-DG treatment in contrast to neuroblastoma N18TG2 cells and U-87 MG glioma cells. These were investigated in the laboratory of the neurochemistry group before, and the main outcome is presented here.

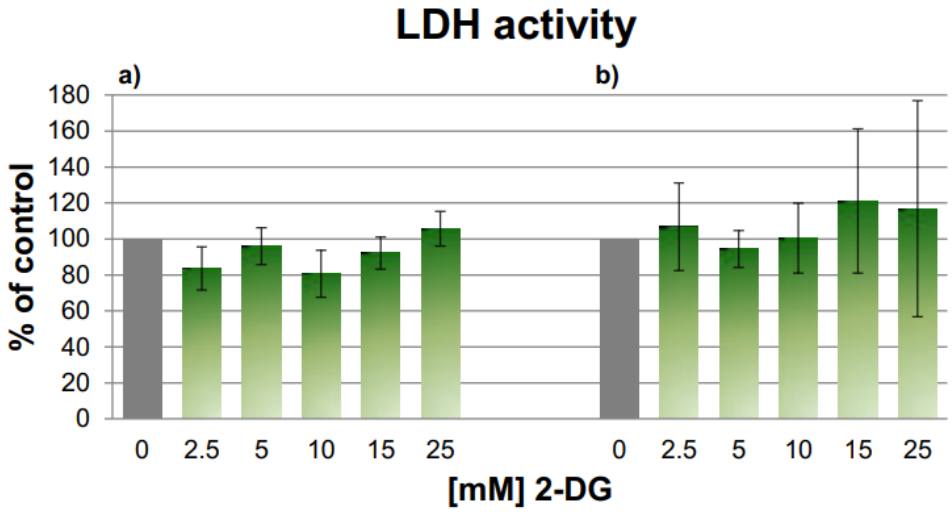

Figure 26. LDH activity in N18TG2 neuroblastoma after exposure to 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. Statistical evaluation does not reveal any significant changes.(Svatunek, 2020)

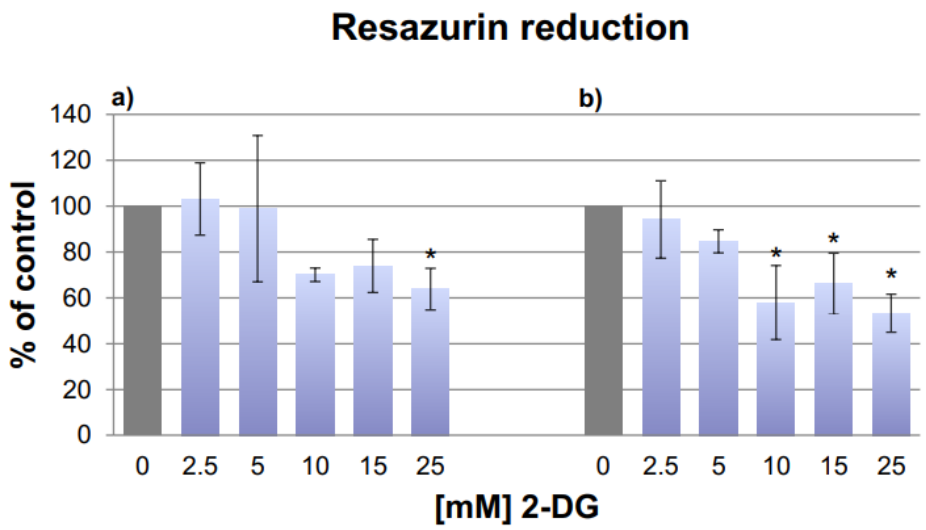

Figure 27. Resazurin reduction in N18TG2 neuroblastoma after exposure to 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. $P < 0.05$ (*) determined with Kruskal-Wallis (H)-test followed by Chi²-test.(Svatunek, 2020)

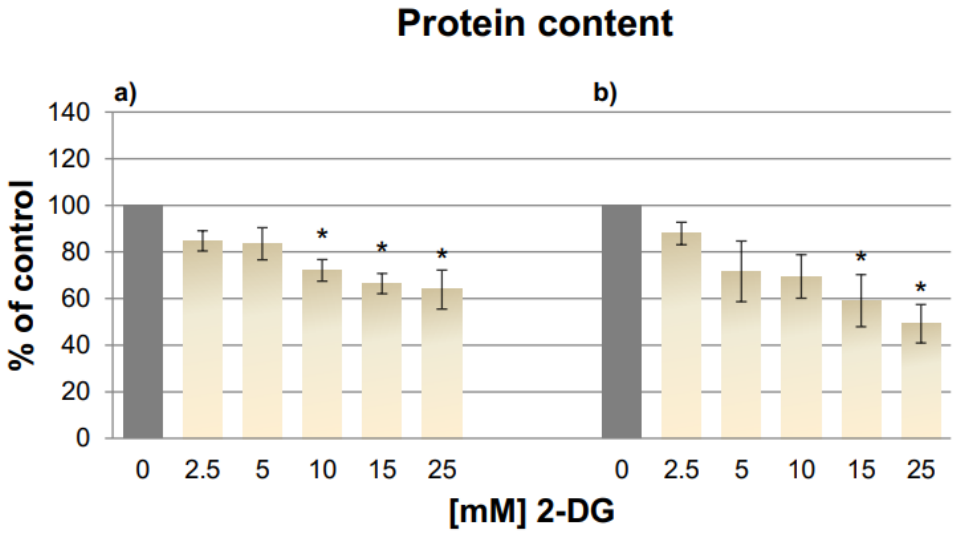

Figure 28. Protein content in N18TG2 neuroblastoma after exposure to 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. $P < 0.05$ (*) determined with Kruskal-Wallis (H)-test followed by Chi²-test.(Svatunek, 2020)

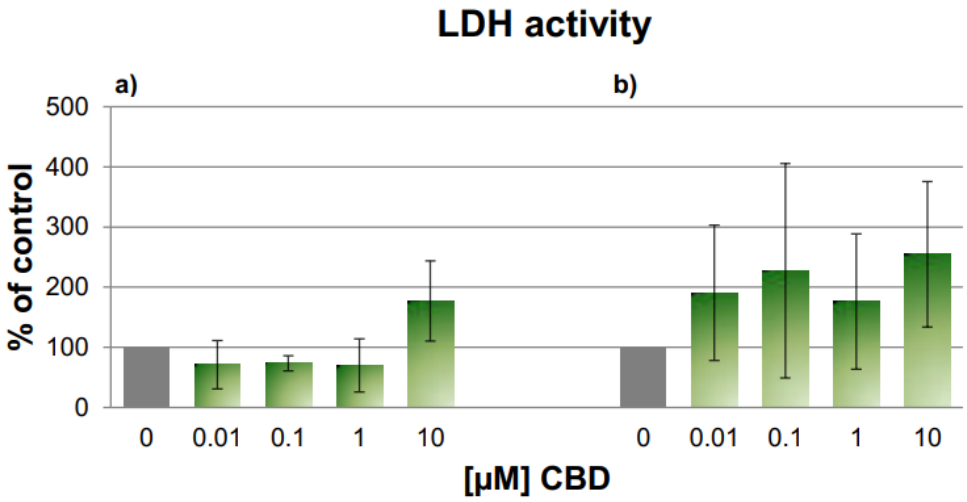

Figure 29. LDH activity in U-87 MG glioma after exposure to CBD for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. Statistical evaluation does not reveal any significant changes.(Svatunek, 2020)

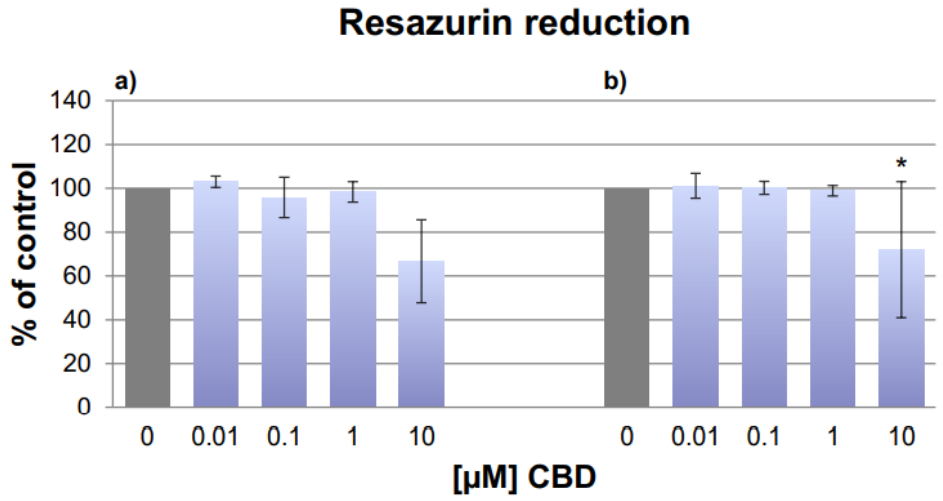

Figure 30. Resazurin reduction in N18TG2 neuroblastoma after exposure to CBD for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. $P < 0.05$ (*) determined with Kruskal-Wallis (H)-test followed by Chi^2-test.(Svatunek, 2020)

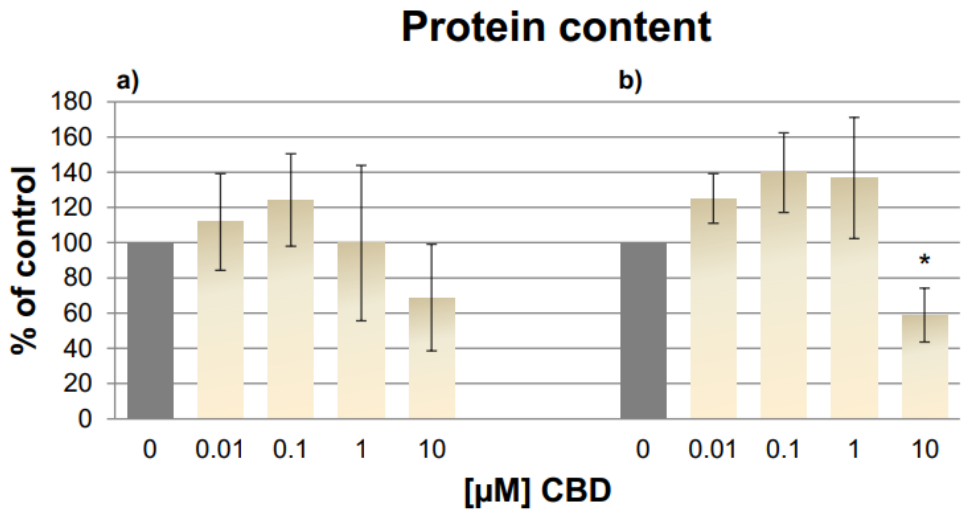

Figure 31. Protein content in N18TG2 neuroblastoma after exposure to CBD for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. P < 0.05 (*) determined with Kruskal-Wallis (H)-test followed by Chi²-test.(Svatunek, 2020)

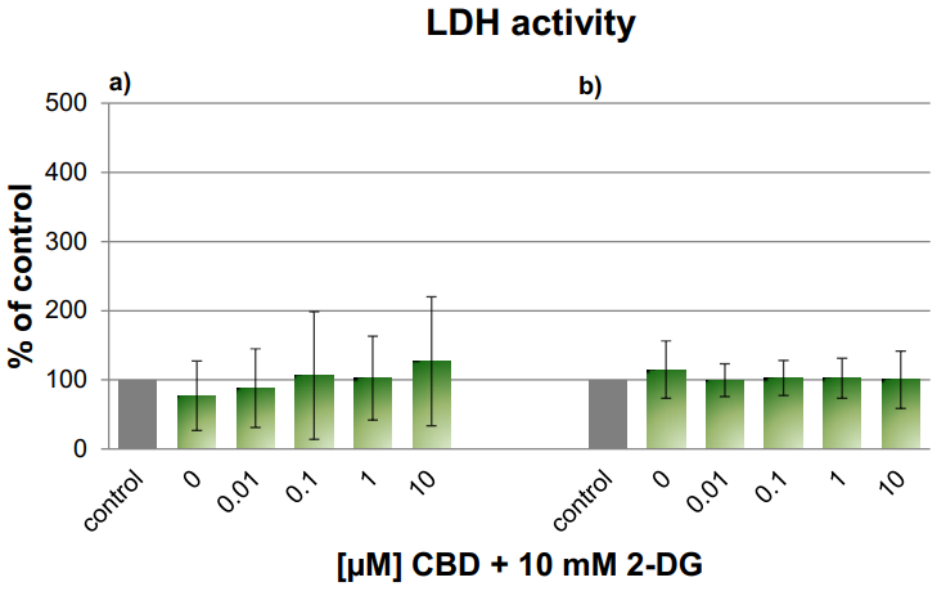

Figure 32. LDH activity in N18TG2 neuroblastoma after exposure to CBD and 10 mM 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 6 independent experiments. Statistical evaluation does not reveal any significant changes.(Svatunek, 2020)

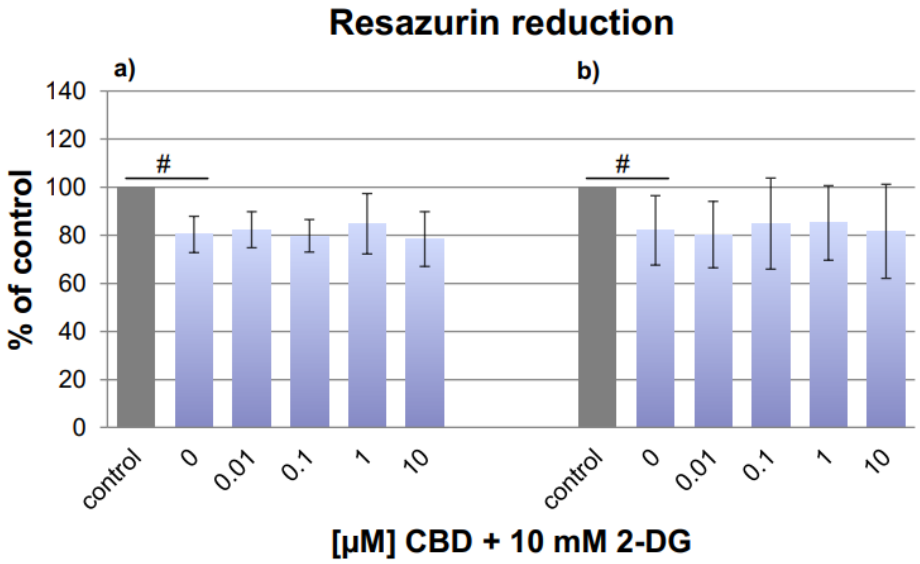

Figure 33. Resazurin reduction in N18TG2 neuroblastoma after exposure to CBD and 10 mM 2- DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 6 independent experiments. P < 0.05 (#) determined with Kruskal Mann-Whitney-U-test for comparison between the control group and the group that was treated with 2-DG.(Svatunek, 2020)

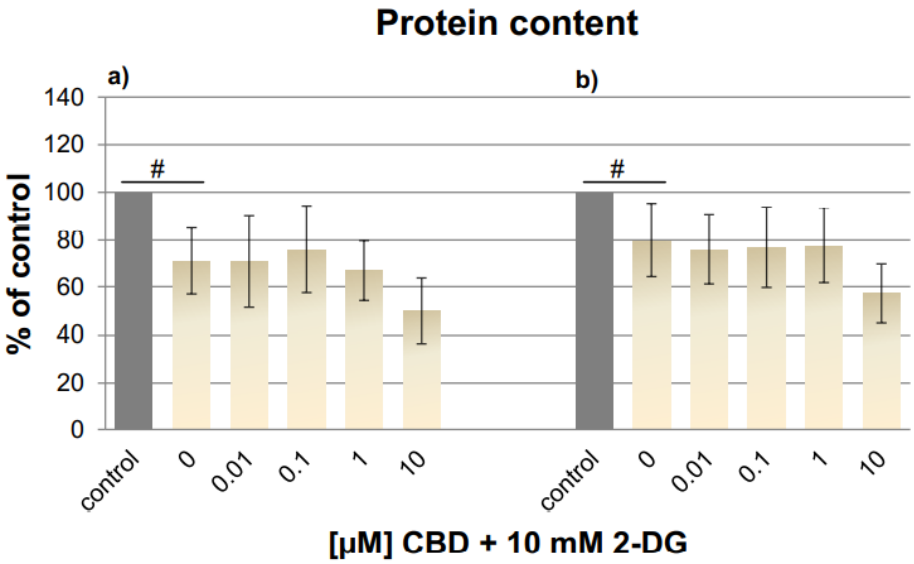

Figure 34. Protein content in N18TG2 neuroblastoma after exposure to CBD and 10 mM 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 6 independent experiments. P < 0.05 (#) determined with Mann-Whitney-U-test for comparison between the control group and the group that was treated with 2-DG.(Svatunek, 2020)

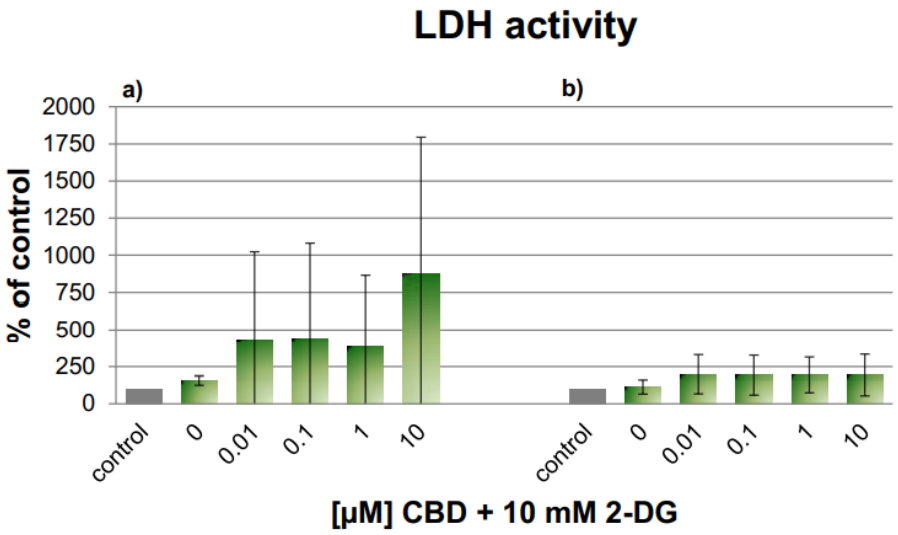

Figure 35. LDH activity in U-87 MG glioma after exposure to CBD and 10 mM 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. Statistical evaluation does not reveal any significant changes.(Svatunek, 2020)

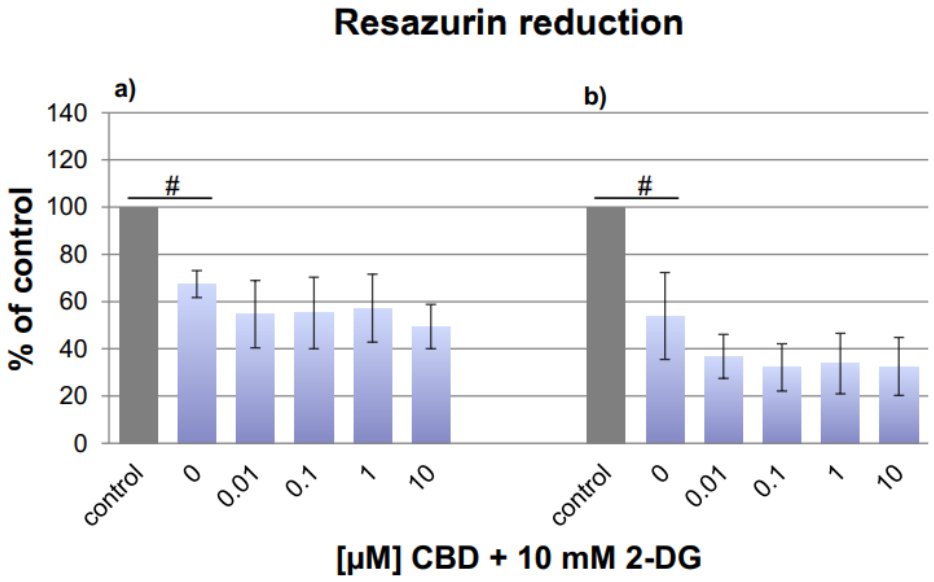

Figure 36. Resazurin reduction in U-87 MG glioma after exposure to CBD and 10 mM 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. $P < 0.05$ (#) determined with Mann-Whitney-U-test for comparison between the control group and the group that was treated with 2-DG.(Svatunek, 2020)

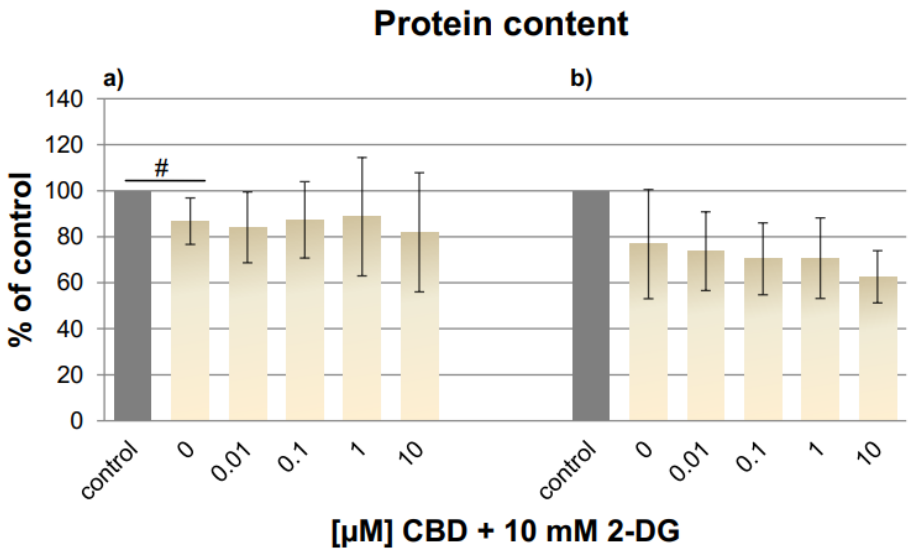

Figure 37. Protein content in U-87 MG glioma after exposure to CBD and 10 mM 2-DG for (a) 24 hours and (b) 48 hours. Data are expressed as means ± SD of 4 independent experiments. $P < 0.05$ (#) determined with Mann-Whitney-U-test for comparison between the control group and the group that was treated with 2-DG.(Svatunek, 2020)