University of Basel

Effects of *Rhodiola rosea* and Honeybush extracts on stress-induced defects in bioenergetics: from mitochondria to the synapse

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PREFACE

The following dissertation was written by the author.

Parts of the introduction are based on published reviews of the author.

The results section of this dissertation consists of one published manuscript, one submitted manuscript and one manuscript that will shortly be submitted for publication. Please refer to the author cotributions section of the manuscripts where the contribution of each co-author to this work is listed.

Στους γονείς μου

Στη γιαγιά μου Αναστασία και στον παππού μου Μανώλη

Σε όσους αγαπώ και με έχουν στηρίξει κατά τη διάρκεια αυτού του διδακτορικού

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Acknowledgments

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Summary

Mitochondria are essential organelles that are responsible mainly for the energy production in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) but also for various other cellular functions such as calcium and redox homeostasis, synaptic plasticity, neurotransmission and cell death. Cellular and organismal health is highly dependent on mitochondria because of the diverse role of the latter. The role of mitochondria is particularly prominent in the brain since neurons conduct a number of complex processes which require a great amount of energy. Mitochondria “fund” neuronal functions by providing ATP and thus neurons highly depend on these organelles. Mitochondria play pivotal roles not only in health but also in the pathogenesis of several diseases. Mitochondrial dysfunction, oxidative stress, impaired energy metabolism and synaptic dysregulation have been identified as common features in many neurodegenerative diseases but also in stress-related brain disorders. For example, tauopathies such as Alzheimer’s disease (AD) and chronic traumatic encephalopathy are characterized by decreased ATP levels, increased reactive oxygen species (ROS) levels and impaired synaptic transmission leading to neuronal death in the brain. Lately, there is an increasing interest on mitochondria-targeting pharmacological strategies with a particular focus on substances that increase bioenergetics (respiration and ATP levels) and decrease ROS.

Current pharmacological strategies against neurodegenerative and stress-related mental disorders are inadequate in stopping the progression of these diseases. They mainly treat symptoms but often present severe adverse effects. Nature has always been a source of therapeutic plants and phytochemicals which were used by local healers against a variety of diseases and discomforts. Of note, a few of the current AD medications are naturally-derived phytochemicals (e.g. galantamine and rivastigmine). Therefore, plant extracts and phytochemicals derived from plants could be used as medications or supplementations in order to alleviate symptoms as they do not present severe adverse effects while they exert multi-targeted functions. In order to develop novel plant-based strategies against complex diseases, such as neurodegenerative, we need to study and understand the molecular mode of action of phytochemicals.

Therefore, the aim of this PhD thesis was to identify phytochemicals that enhance mitochondrial functions and test their ability to counteract deficits caused by different types of stress. In this matter, we evaluated *in vitro* the effects of two extracts: Honeybush extract (*Cyclopia* species) and *Rhodiola rosea* extract as well as the Honeybush extract compound mangiferin against oxidative stress, glucocorticoid- or abnormal tau-induced stress respectively.

Honeybush (*Cyclopia* species) is a South African endemic plant and certain species have a long tradition of use as herbal tea, known as honeybush tea. Increased global demand for honeybush tea led to a scientific interest for the bioactivity of its extracts due to their phenolic composition. Indeed, honeybush extracts have been indicated to possess antioxidant activities. However, their neuroprotective properties have never been tested before. Due to their antioxidant activities we hypothesized that honeybush extracts would exert antioxidant properties as well as beneficial effects on mitochondria in neuronal cells. Therefore, we tested the effects of four honeybush extracts on human neuroblastoma SH-SY5Y cells. The effects of the aqueous extracts of *C. subternata*, *C. genistoides*, *C. longifolia* and a 70% ethanolic extract of *C. genistoides* in intrinsic health status were firstly evaluated. We showed that treatment with honeybush extracts had a beneficial effect on bioenergetics as it increased ATP production, respiration and mitochondrial membrane potential (MMP). Next, their ability to counteract oxidative stress-induced deficits was tested. The aqueous extracts of *C. subternata* and *C. genistoides*, in particular, showed a protective effect by rescuing the bioenergetic and mitochondrial deficits under H_2O_2-induced oxidative stress conditions. In detail, the extracts partially rescued the ATP levels and respiration and they completely rescued MMP levels under oxidative stress as well as reduced the H_2O_2-induced ROS. The most promising effects were exerted by the aqueous extract of *C. subternata* followed by the aqueous extract of *C. genistoides* which is relevant as these two extracts are largely used for the production of honeybush tea. Our data indicate the potential neuroprotective effect of Honeybush extracts against oxidative stress while we demonstrated for the first time their mitochondria-enhancing properties. Although more research is required, these findings set the basis for the development of a condition-specific nutraceutical.

Rhodiola rosea is a perennial plant growing in mountainous areas of Central Asia and Europe. Extracts of *Rhodiola rosea* have been characterized as adaptogens as they help organisms to adapt to new or stressful situations and have been used by Vikings and the Russian army to enhance performance during battles. Rhodiola has been shown to alleviate symptoms of stress-related mental disorders such as chronic stress and burnout in clinical studies. These conditions lead to the dysregulation of the stress system, namely the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the overproduction of glucocorticoids such as cortisol in humans as end-products. Overaccumulation of glucocorticoids causes detrimental cellular effects by compromising mitochondrial functions and overall cellular health. Due to its adaptogenic properties, we hypothesized that *Rhodiola rosea* extract (RRE) would be able to protect neuronal cells against glucocorticoid stress induced by the synthetic glucocorticoid dexamethasone. Therefore, we evaluated its effects against dexamethasone-induced deficits both in neuroblastoma SH-SY5Y cells and in murine hippocampal HT22 cells as well as the ability of RRE itself to enhance mitochondrial functions under non-stressful conditions. We showed that RRE protected both cell lines from dexamethasone-induced cell death and normalized the increase in ROS levels in an inverted U-shape and U-shape manner respectively. Also, we showed that RRE increased ATP production and metabolic activity in intrinsic health status in both cell lines and again in an inverted U-shape manner. These biphasic response curves reflect the activation of the «adaptive cellular stress response» and thus we confirmed that RRE has the typical features of an adaptogen with regard to the modulation of stress response and adaptive homeostasis. In addition, we showed that RRE induced neurite outgrowth in neuroblastoma SH-SY5Y as it increased the number and length of neurites as well as their endpoints and attachment points compared to the untreated control condition with a concomitant upregulation of the brain-derived neurotrophic factor (BDNF) mRNA and protein levels. Therefore, RRE promoted neurite outgrowth possibly via the BDNF pathway. Taken together, our results support the use of RRE as an adaptogen in stress-related mental disorders while we showed that the BDNF pathway might mediate RRE`s beneficial activities.

Mangiferin is a xanthonoid polyphenol present in the fruit, leaves and bark of *Mangifera indica,* but also in Honeubush extracts, and has been shown to exhibit antioxidant and neuroprotective properties including amyloid beta (Aβ)-related AD models. However, studies showing its benefits in tau-related AD models are missing. Tauopathies are a group of neurodegenerative diseases (e.g. AD) characterized by an abnormal accumulation of the protein tau within neurons, excessive tau hyperphosphorylation and formation into neurofibrillary tangles which are neurotoxic. As many other cases of neurodegenerative diseases, tauopathies are characterized by mitochondrial dysfunction, disturbed bioenergetics and excessive oxidative stress. We hypothesized that mangiferin could have beneficial effect on a cellular tauopathy model by boosting mitochondrial functions and by promoting neurite outgrowth in neuroblastoma cells stably transfected with the mutant tau P301L (P301L cells). Indeed, we demonstrated that mangiferin not only increased ATP levels and MMP in the P301L cells but also promoted neurite outgrowth and brought the P301L cells in a morphological state of resembling the healthy mock neuroblastoma cells (cells transfected with the empty vector). These findings suggest that mangiferin might be a promising candidate against tauopathies but of course more studies are required to establish such use.

Collectively, our data show that indeed plant extracts and phytochemicals can exert beneficial properties against various types of stress by enhancing mitochondrial functions, by scavenging ROS and by promoting neurite outgrowth. These benefits lead to the development of more complex neural networks, enhanced synaptic transmission and strengthened synaptic plasticity. Therefore, they exert benefits at the mitochondrial, cellular and at the synapse level. Overall, the studies performed in this PhD thesis helped to comprehend the molecular mode of action of phytochemicals both in healthy conditions and against different types of stress. We showed the promising effects of Honeybush extracts (*Cyclopia* species) against oxidative stress, of *Rhodiola rosea* extract against glucocorticoid stress and of the compound mangiferin against abnormal tau-induced cellular stress. All of those benefits were achieved through the improvement of mitochondrial functions. Our findings could contribute to the development of pharmacological strategies against specific conditions.

A. Introduction

1. Mitochondria

Mitochondria are essential yet independent organelles contained in the cytosol of eukaryotic cells and they are responsible for numerous functional activities within the cells. However, they were not always an intrinsic structure of eukaryotic cells. They occured through the endosymbiosis of an alpha-proteobacterium into a prokaryotic progenitor and this is why they contain their own DNA, namely, the mitochondrial DNA (mtDNA) [1]. Mitochondria have obtained the title of “powerhouse of the cell” due to their ability of producing energy in the form of adenosine triphosphate (ATP) mainly through oxidative phosphorylation (OXPHOS). This energy is required for the survival and functioning of the cell. Actually, they are more than just a “powerhouse” as they are the ultimate multitaskers which define the cell fate. Apart from producers of energy, mitochondria are the key modulators of brain cellular survival, differentiation and death by controlling calcium (Ca^{2+}) and reduction-oxidation (redox) equilibrium, by producing reactive oxygen species (ROS), by affecting neurotransmitter release, synaptic plasticity and by controlling cell apoptosis [2-4]. The nervous system (particularly the brain) consumes a considerable amount of oxygen and requires a remarkable amount of energy in order to operate, maintain, and enhance neuronal functions and plasticity. Neurons are postmitotic polarized cells with significant energy demands. OXPHOS, taking place in mitochondria, is the main energy provider in the form of ATP and neurons depend almost solely on this procedure in order to satisfy their energy needs. This is why mitochondria play a vital role in the nervous system [5].

1.1. Structure

Regarding the structural characteristics of mitochondria, they contain two structurally and functionally distinct membranes, the outer and the inner membranes. The space between the two membranes is called intermembrane space. The inner membrane smoothly folds inwards and forms cristae which encapsulate the matrix (Figure 1). The inner membrane carries the electron transport chain (ETC). The ETC is formed by 4 protein complexes (complexes I-IV) and 2 electron carriers (coenzyme Q or ubiquinone and cytochrome c). The transportation of electrons is accomplished via two pathways in the ETC: either through complex I/III/IV using NADH (reduced

nicotinamide adenine dinucleotide) as substrate or through complex II/III/IV using succinic acid as substrate. Complexes I-IV together with the complex V or else ATP synthase constitute the machinery that is responsible for the generation of ATP during OXPHOS [6, 7].

Complex I is the biggest complex in the ETC. The main role of complex I is to transport electrons from the electron carrier NADH to coenzyme Q (or else ubiquinone). Complex II is a unit of both Krebs cycle (tricarboxylic acid cycle-TCA) and ETC, acting as a nexus between metabolism and OXPHOS. Complex II facilitates the oxidation of succinate to fumarate as a part of the TCA cycle. Complex II is an additional point of entry for electrons in the ETC and passes them from succinate to coenzyme Q. Complex III is a symmetrical dimer and its principal role is to transfer electrons retained by ubiquinol (reduced form of coenzyme Q) to cytochrome c. Complex IV moves electrons from cytochrome c to O_2 (the final electron acceptor) in order to produce H_2O. Complex IV in mammals contains 13 subunits. Cytochrome c is an electron carrier that is loosely bound to the outer part of the inner mitochondrial membrane permitting it to interact with complex III and to receive electrons. The reduced form of cytochrome c moves across the membrane and interacts with complex IV [6].

mtDNA is a circular genome located in the matrix (Figure 1). Proteomic in conjunction with bioinformatic research revealed that there are around 1500 proteins in mammalian mitochondria. However, mtDNA encodes only 13 proteins which are used as structural components of the ETC complexes while the rest of the proteins are encoded in the nucleus of the cells [2]. Interestingly, mtDNA follows a uniparental inheritance transmission in which only the maternal mtDNA is inherited. In several species, including humans, the paternal mitochondria are destroyed after fertilization [2, 8]. Although biparental mitochondrial inheritance was lately described, it is still under skepticism [9, 10].

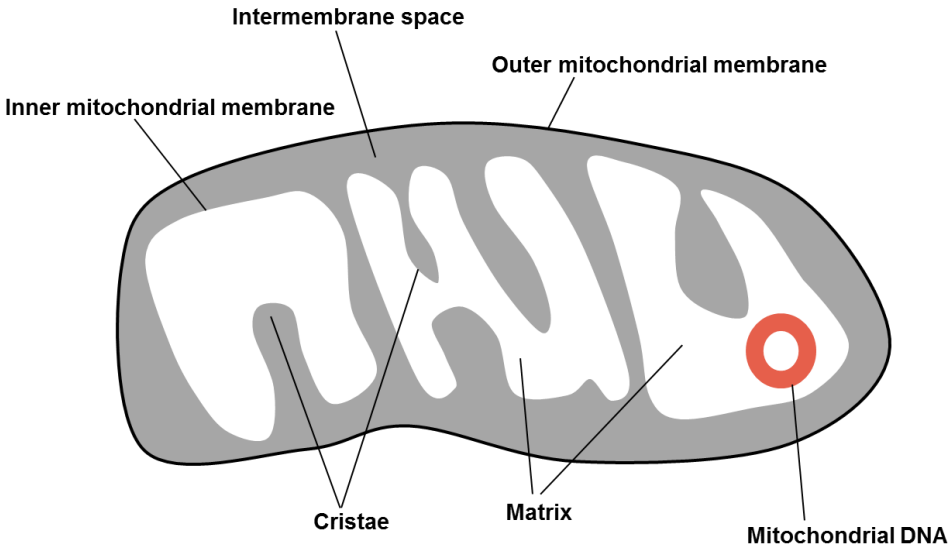

Figure 1. Structural characteristics of mitochondria. Mitochondria contain two structurally and functionally distinct membranes, the outer and the inner membranes. The space between the two membranes is called intermembrane space. The inner membrane smoothly folds inwards and forms cristae which encapsulate the matrix. The inner membrane carries the electron transport chain (ETC). Mitochondria have their own circular genome, namely the mtDNA that is responsible for the production of 13 proteins that constitute structural components of the ETC.

1.2. Function

1.2.1. Cellular energy generation

ATP is the universal molecular energy currency of the cell and absolutely necessary for the completion of all cellular processes such as biomolecule synthesis (e.g. DNA, RNA, proteins, lipids), cell signaling and macromolecule transportation to name a few. ATP has also been described to act as a signaling molecule among cells [11, 12]. ATP molecules consist of an adenosine, three phosphate groups (triphosphate) and a ribose sugar [11]. The energy release takes place upon hydrolysis of the high-energy bond between the second and the third phosphate groups, a process that results in adenosine diphosphate (ADP) formation. ADP can then re-obtain the phosphate group and form ATP again allowing the “storage of energy” [12]. There are two pathways

leading to the production of ATP molecules: a) glycolysis and b) oxidative phosphorylation.

1.2.2. Cellular glycolysis

Cellular glycolysis is the first of the two principal metabolic pathways leading to the production of ATP and it takes places in the cytosol of the cells. It is the first step of the cellular respiration and it starts with the oxidation of glucose. Within the 10 steps of glycolysis, 2 ATP molecules are initially “invested” and lead to the generation of 4 ATP molecules, 2 pyruvate molecules and 2 NADH (nicotine adenine dinucleotide) per single molecule of glucose [13]. Under aerobic conditions, pyruvate derived from glucose diffuses into mitochondria, converts into acetyl-CoA (an energy carrier) and enters the Krebs cycle in the mitochondria (TCA cycle). Under anaerobic conditions, pyruvate remains in the cytosol and is converted into lactate by the enzyme lactate dehydrogenase. Lactic acid fermentation is the major source of ATP in tissues with low metabolic needs and with few to no mitochondria such as erythrocytes. Cellular glycolysis results in only 2 ATP molecules versus 32 ATP molecules produced during oxidative phosphorylation making it an adequate backup pathway for emergency energy needs or a precursor of oxidative phosphorylation [13, 14].

1.2.3. Mitochondrial oxidative phosphorylation

The Krebs cycle is a connecting pathway taking place in the matrix of mitochondria and provides with molecules which facilitate oxidative phosphorylation. In detail, Krebs cycle is characterized by three conversions: a) three NAD+ (nicotine adenine dinucleotide) molecules are converted into three molecules of its respective reduced form (NADH), b) one molecule of FAD+ (flavin adenine dinucleotide) is converted into one molecule of its respective reduced form ($FADH_2$) and c) one molecule of GTP (guanosine triphosphate) is derived from one molecule of GDP (guanosine diphosphate) (Figure 2) [15].

Oxidative phosphorylation, or else mitochondrial respiration, is the second main pathway that leads to the generation of energy under the form of ATP. The co-factors NADH and $FADH_2$, produced during the Krebs cycle in the matrix, transfer electrons to O_2. However, electrons are not transferred directly to O_2 but are rather diffused through intermediate electron carriers in the ETC on the inner membrane of mitochondria. Upon oxidation of these two electron-carriers, conformational changes are taking place in the ETC complexes I, III and IV, triggering them to pump protons (H+) from the mitochondrial matrix into the intermembrane space [9, 16]. In this way, a proton-gradient is produced and an electrochemical potential is generated across the inner mitochondrial membrane, named mitochondrial membrane potential (MMP). This proton motif force that was created is finally used by complex V (ATP synthase) and drives the synthesis of ATP by phosphorylation of ADP (Figure 2) [17]. In more detail, the process is initiated when NADH transfers two electrons to complex I of the ETC (termed also NADH dehydrogenase) and is then converted to NAD+. The two electrons are subsequently transported to coenzyme Q, a lipophilic redox carrier, while in parallel four protons are exported from the matrix into the intermembrane space. Complex II (termed also succinate dehydrogenase) drives the reduction of FAD to $FADH_2$ and provides additional electrons to coenzyme Q. Interestingly, complex II is the only one of the complexes comprising proteins that are solely encoded by nuclear DNA while it does not pump protons in the intermembrane space. As a next step, the reduced form of coenzyme Q traverses the inner mitochondrial membrane and transfers its electrons to complex III (termed also ubiquinol-cytochrome c oxidoreductase). Complex III oxidizes the coenzyme Q and passes the freed electrons to cytochrome c (another electron carrier and soluble redox protein), a reaction that is accompanied by the pumping of four protons into the intermembrane space. Following that, electrons are transferred from cytochrome c to molecular oxygen (O_2) generating two molecules of water while four more protons are exported into the intermembrane space by complex IV. The motion of electrons through the complexes of the ETC maintains the MMP through the pumping of protons into the intermembrane space (Figure 2). The last step of OXPHOS is the synthesis of ATP at complex V (ATP synthase) which is also called F_0F_1 ATPase by using the electrochemical energy created by the proton gradient across the inner mitochondrial membrane. ATP production is the most ubiquitous chemical reaction in the biological world. As implied

by the name, ATP synthase F_0F_1 in humans is formed by two units, F_0 and F_1, and has a molecular weight of circa 550 kDa. The F_0 unit is the proton specific channel which allows protons to flow back into the matrix while the F_1 unit catalyzes the generation of ATP from ADP and Pi (inorganic phosphate) by using the energy generated from the earlier oxidation of electron carriers (NAD+ and coenzyme Q) [18-21]. ATP synthesis and MMP are two interdependent mitochondrial functions. ATP synthase acts like a motor that constantly produces ATP driven by the proton gradient and the MMP across the inner mitochondrial membrane. Polarized mitochondrial membranes allow the unbothered production of ATP with a net yield of 30-32 ATP molecules per molecule of glucose fueling all cellular activities [18-21]. Energy homeostasis is a dynamic equilibrium that depends on energy supply (ATP production) and energy requirement (ATP consumption). The energy homeostasis and bioenergetic activity is regulated in cells by molecular elements such as transcription factors and kinases in order to respond to cellular stress or to energy deprivation. In particular, AMP-activated protein kinase (AMPK) acts like an energy sensor which is activated by modifications in the AMP:ATP cellular ratio and is accountable for the activation of a phosphorylation cascade and the triggering of glycolysis and OXPHOS. AMPK triggers the phosphorylation of transcription factors and metabolic enzymes that activate ATP-generating catabolic pathways and deter ATP-consuming biosynthetic pathways. Of note, the AMP:ATP ratio is a more sensitive and efficient marker of energy status in cells than the ADP:ATP ratio [22].

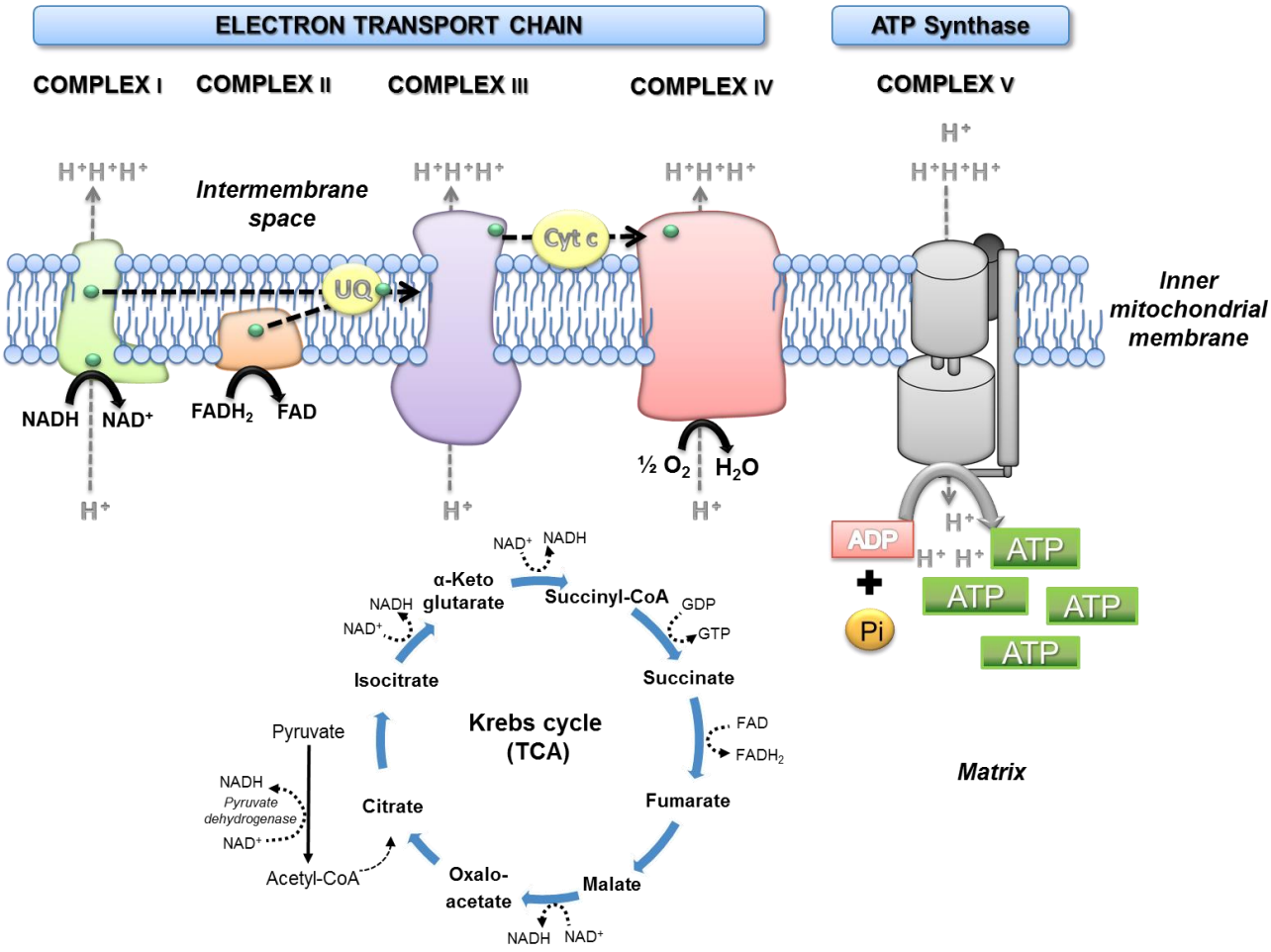

Figure 2. Krebs cycle and oxidative phosphorylation. Adapted from [19]. The Krebs cycle is a connecting pathway taking place in the matrix of mitochondria and provides with molecules such as NADH and $FADH_2$ which facilitate oxidative phosphorylation. Oxidative phosphorylation is the main pathway that leads to the generation of energy under the form of ATP. The co-factors NADH and $FADH_2$, produced during the Krebs cycle in the matrix, transfer electrons to O_2 through intermediate electron carriers in the ETC on the inner membrane of mitochondria. Upon oxidation of these two electron-carriers, conformational changes are taking place in the ETC complexes I, III and IV, triggering them to pump protons (H+) from the mitochondrial matrix into the intermembrane space. In this way, a proton-gradient is produced and an electrochemical potential is generated across the inner mitochondrial membrane, named mitochondrial membrane potential (MMP). This proton motif force that was created is finally used by complex V (ATP synthase) and drives the synthesis of ATP by phosphorylation of ADP.

1.3. Mitochondria: producing both the fuel and poison of life

As mentioned above, mitochondria are absolutely necessary organelles as they provide the energy for the accomplishment of all cellular functions, especially the extremely demanding ones of the central nervous system (CNS). However, mitochondria do not only contribute positive outcomes to the cell but also harmful ones as they are responsible for the production of reactive oxygen species (ROS). ROS, when produced in excess, are detrimental for mitochondria themselves and for the entire cell.

1.3.1. Production of reactive oxygen and nitrogen species

ROS are oxygen containing chemical entities of great reactivity that have been in the spotlight as a common feature in numerous diseases. They are implicated in neurodegenerative and cardiovascular diseases, cancer, atherosclerosis, diabetes and also in normal aging [5, 23-25]. ROS include mainly the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) of which superoxide anion and hydrogen peroxide are found in the most abundance in cells [26]. Other ROS are peroxyl and alkoxyl radicals as well as singlet oxygen. Mitochondria are the epicenter of ROS production and metabolism [5, 27, 28]. Despite an estimation of 31 existing ROS (mostly superoxide anion and H_2O_2) production sites in the entire cell versus 12 ROS emission sites in mitochondria, the majority of cellular endogenous ROS are produced by mitochondria as by-products of OXPHOS [26, 29]. Exposure to oxygen is not only unavoidable but also vital and necessary for organism and cell survival and for energy production. The synthesis of ATP in mitochondria demands around 90% of the O_2 used by the cell [27]. Most electrons are transferred by the electron carriers NADH and $FADH_2$ to O_2 through the ETC. As a result, O_2 is reduced to H_2O at complex IV. However, the transfer of electrons might not always be efficient. Due to electron leak, some electrons (amounted to 0.2-2%) in the ETC are not transferred to O_2 (the final electron acceptor) and the energy emitted by the electrons is not entirely coupled with ATP production [6]. Although there are many ROS-production sites in mitochondria, the majority of mitochondrial ROS are proved to be

produced by complexes I and III of the ETC (Figure 3). In detail, superoxide anion can be generated in the matrix during the transfer of electrons from NADH to coenzyme Q and complex I due to partial reduction of O_2. Complex III produces ROS via a non-enzymatic reaction while it releases superoxide anion both into the intermembrane space and in the matrix. The superoxide anion released by complexes I and III is quickly converted to H_2O_2, a relatively stable ROS form, by the antioxidant enzyme superoxide dismutase 1 (SOD1) in the intermembrane space and by superoxide dismutase 2 (SOD2) in the mitochondrial matrix. H_2O_2 can freely traverse the mitochondrial outer membrane or it can reach the cytosol via anion channels where it is quenched by cytosol antioxidant enzymes such as glutathione peroxidase, catalase and thioredoxin peroxidase (Figure 3) [24, 30]. Complexes II and IV are less susceptible to produce ROS. Complex II per se was not considered to produce ROS. However, it is described to boost their formation via succinate, its substrate. In multiple tissues, succinate enables the transportation of electrons to coenzyme Q through complex II and then back to complex I, a process called reverse electron transfer. Apart from that, it was suggested that complex II can be responsible for superoxide anion generation in aged or diseased tissue, especially in the case of skin aging and diabetes [6, 27].

Reactive nitrogen species (RNS) are a group of nitrogen entities linked to oxygen. They are produced when exogenous or endogenous nitric oxide (NO) interacts with ROS. NO is a free radical able to diffuse through membranes and is synthesized principally by the three types of nitric oxide synthase (NOS), namely the neuronal, endothelial and inducible NOS. NO is a greatly reactive free radical-carrying gas that can react with superoxide anion leading to the production of RNS and peroxynitrile (OONO-) which can lead to the synthesis of hydroxyl radical. The reaction of NO with reductants and oxidants can lead to the generation of more RNS including nitroxyl anion (NO^-) (when •NO is reduced), higher oxides of nitrogen such as NO_2 (nitrogen dioxide) and N_2O_4 (dinitrogen tetroxide), dinitrosyl Fe complexes and S-nitrosothiols. Nitrosyl halides are emitted when •NO reacts with halogens (fluorine, chlorine and bromine). Upon removal of a single electron from •NO, the nitrosonium cation (NO^+) is formed which can potentially interact with nucleophilic centers and produce nitroso compounds (Figure 3). The biological reactivity, solubility and half-life of RNS depend

on each of these entities. For example, peroxynitrite is extremely reactive and possess a half-life of approximately one second [31-34].

Although accountable for the production of the majority of ROS, mitochondria are not the only ROS-generating organelles. Microsomal enzymes such as peroxisomal enzymes, cytochrome P450 system [35], sarcosine oxidase, polyamine oxidase, xanthine oxidase, some types of acyl-CoA oxidases [36] and a few plasma membrane enzymes including NADPH (nicotinamide adenine dinucleotide phosphate) lipoxygenase and oxidase [37, 38], have been described as non-mitochondrial ROS producers. Other reported sources of ROS inside mitochondria are dihydroorotate dehydrogenase and monoamine oxidase [39, 40]. Moreover, glycerol phosphate dehydrogenase and acyl-CoA dehydrogenase (which are flavoproteins) can generate ROS during lipid-derived substrate oxidation [41, 42]. Finally, pyruvate as well as α-ketoglutarate dehydrogenase carry flavoenzyme dihydrolipoyl dehydrogenase subparts and are additional ROS producers [27, 43].

1.3.2. Physiological role of ROS

ROS such as H_2O_2 were once considered solely harmful and thus unwanted by-products of OXPHOS. However, they are now acknowledged to have an essential role as signaling molecules in the regulation of senescence, cell death and proliferation and in the defense against pathogenic microorganisms as long as they are maintained in physiological concentrations. In particular, H_2O_2 is often claimed to be a mitokine. Mitokines are secondary messengers and diffusible factors composed via normal mitochondrial metabolism and control many cell functions [44]. Additionally, it has been described that low levels of superoxide anion plays a similar role to that of H_2O_2, which is however not proven and in dispute [26, 45].

ROS act via the oxidative alteration of several protein types, particularly kinases, phosphatases, caspases, different receptors, transcription factors and ion channels [46]. ROS derived from complex III are essential to stabilize the hypoxia-inducible factor 1α (HIF-1α) and thus for the proliferation of cells [47]. Emerging evidence shows that ROS are also implicated in protein kinase signaling cascades, such as AMPK and

protein kinase B (AKT) and as a result largely affect cellular autophagy and apoptosis [48]. ROS activate AMPK under hypoxic states which can in turn upregulate and enhance cytoprotective autophagy by restraining rapamycin activity and its downstream targets [49]. ROS have also been described to modulate molecules involved in synaptic plasticity and especially channels and receptors such as cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) [50, 51], N-methyl-d-aspartate (NMDA) receptor [52], extracellular signal-regulated kinase (ERK) [53], Ca^{2+} channel [50, 54] and Ca2+/calmodulin-dependent protein kinase II (CaMKII) [55]. ROS also fulfil an important role during long-term potentiation, a process of synaptic plasticity responsible for learning and memory [51, 56]. ROS, in physiological levels, support the maintenance of neuronal polarity and can affect neuronal dynamics and organization by modulating intracellular Ca^{2+} release [6, 57-59].

On the basis of these findings, the term “oxidative stress” has been revised and two new terms are lately being used: oxidative eustress and oxidative distress. Oxidative eustress describes a slight oxidative challenge during which H_2O_2 is taking part in regulatory or adaptive signaling [60]. During this process, cellular ROS counter-oxidize parts of proteins that have been reduced and thus regulate metabolic, genomic and transcriptional processes in response to exposome modifications [61]. On the one hand, what oxidative eustress does is enabling cells to adapt to changes in the extra- and intra-cellular environment [62]. On the other hand, oxidative distress is related to excessively high ROS levels that leads to oxidative damage and ultimately to apoptosis [26, 29]. Oxidative stress is presented and explained in detail in section 3.

1.4. Antioxidant defense strategies against ROS

Fortunately, cells developed antioxidant defense systems for their detoxification from the detrimental effects of excessive ROS production. The antioxidant defenses scavenge ROS in either an enzymatic or a non-enzymatic way. Enzymatic antioxidant systems include enzymes distributed in the cellular cytosol, extracellular space and within mitochondria that scavenge and neutralize ROS, mainly superoxide anion and H_2O_2. The first line of antioxidant enzymes consists of superoxide dismutase (SOD),

glutathione peroxidase (GPx) and catalase (CAT) while other enzymes able to defuse ROS are glutathione-S-transferase (GST) and glucose-6-phosphate dehydrogenase which provide a phase II detoxification (Figure 3) [27, 29, 63-65]. Neurons are high energy demanding cells. Therefore, mitochondria in the brain are “overworked” in order to fulfil the neuronal energy requirements meaning that they consume a lot of oxygen to conduct oxidative phosphorylation [24]. As a result, ROS are highly produced in the brain and therefore there is a great need for antioxidant defenses there. Surprisingly, the brain carries significantly lower antioxidant enzyme levels compared to other organs in the body [66]. Suboptimal antioxidant concentrations deters the counterbalance of the high quantities of cumulative ROS, iron and reactive polyunsaturated fatty acids in the brain [66], and especially in the hippocampus, cerebral cortex, hypothalamus and striatum [64, 67-70].

1.4.1. Enzymatic antioxidants-the gatekeepers of redox homeostasis

Superoxide dismutase (SOD)

Three types of SODs have been identified in the human brain each one containing a different metal co-factor and acting on a different cellular site. All three SODs convert the superoxide anion into H_2O_2. SOD1 (or else Cu-SOD) is a SOD containing copper (Cu) and is localized in the cytosol as well as in the mitochondrial intermembrane space. SOD2 (or else Mn-SOD) is found in the mitochondrial matrix and its metal co-factor is manganese (Mn). Finally, SOD3 (or else Zn-SOD) is containing zinc (Zn) and is localized in the extracellular matrix [29, 64]. Superoxide anion, produced at the ETC, is converted to H_2O_2 by SOD1 in the mitochondrial intermembrane space and cytosol and by SOD2 in the mitochondrial matrix [27]. SOD1, apart from being an enzyme, has also been described to act as a transcription factor upon increase in cellular ROS. When H_2O_2 is accumulated in cells, SOD1 has been shown to translocate to the nucleus, attach to promoters and upregulate the expression of oxidative repair genes [29]. SOD1 and SOD3 dysfunction has been involved in cardiovascular and neuromuscular diseases [71, 72]. However, SOD2 seems to be more important for brain integrity due to direct action in the mitochondrial matrix [73]. SOD2

overexpression protected against NO and NMDA neurotoxicity in cortical cell [74]. In line with this, insufficient SOD2 expression seems to induce mitochondrial impairment, neuronal atrophy, and increased CNS senescence as showed *in vivo* [64, 75].

Glutathione peroxidase (GPx)

GPx is a selenocysteine-carrying enzyme related specifically to organic peroxides. It transforms hydroxyl radicals and peroxides into nontoxic products in the cytosol and mitochondria. In addition, GPx is responsible for the oxidation of the reduced form of glutathione (GSH) into the oxidized one, namely glutathione disulfide (GSSG). GSSG is subsequently reduced back to GSH by the enzyme glutathione reductase [27, 65]. H_2O_2 can easily traverse the mitochondrial membranes and reach the cytosol, where cytosolic GPx or peroxisomal catalase turn it into water [27]. There are five GPx isoforms. GPx1 is the most common and present in abundance throughout the body while it is the most studied isoform [76]. Low levels of GPx1 are found in most neurons while higher cerebral concentrations of the enzyme are mainly found in microglia of the white matter. Studies have shown that neurons are able to guide GPx1 activity to pathogenic areas with increased cell toxicity [77]. Additional evidence showed that the knockout of GPx1 in mice caused an enhanced apoptosis and cell death [78] while GPx1 overexpression protected against post-ischemic infection and experimental stroke [64, 79].

Catalase (CAT)

Following conversion of superoxide anion to H_2O_2 by SODs, glutathione peroxidases and catalase accomplish a final conversion step and decompose H_2O_2 to water and oxygen thus preventing the formation of hydroxyl radicals. CAT is an essential antioxidant enzyme for maintaining H_2O_2 concentrations low in the intracellular space [80, 81]. CAT knockout mice produced ATP at a slower rate in brain mitochondria than that in mice comprising CAT [82]. Likewise, overexpression of mitochondrial CAT in

transgenic mice led to increased life span, reduced oxidative injury and neuroprotection against cerebral [64, 82, 83].

Glutathione-S-transferase (GST)

GSTs is an umbrella term referring to isoenzymes responsible for the neutralization of toxins, xenobiotics and reactive final products in the cytosol [84]. The GSTs consist of eight subclasses (Alpha, Kappa, Mu, Pi, Sigma, Theta, Zeta, and Omega) [85]. The Mu-GST gene (or else GSTM1) is highly expressed in human brain structures [64, 80]. In addition, GST acts as a catalyst during the coupling of glutathione (GSH) to electrophilic centers on different substrates using a sulfhydryl group. As a result, peroxide levels are decreased. Finally, glucose-6-phosphate dehydrogenase is responsible for sustaining a constant number of metabolic intermediates including nicotinamide adenine dinucleotide-phosphate (NADPH) and GSH which enable the proper function of the first line antioxidant enzymes [65].

1.4.2. Non-enzymatic antioxidants

As mentioned before, cells and particularly mitochondria are also equipped with non-enzymatic antioxidants. Those are mainly small molecules including ascorbate (Vitamin-C), tocopherol (Vitamin-E), glutathione (GSH), thioredoxin, retinoic acid, uric acid, melatonin and pyridine nucleotides which make the protection against oxidative stress more sufficient. The non-enzymatic antioxidants can directly scavenge free radicals (e.g. ascorbate and tocopherol) or can regulate the activity of enzymatic antioxidants (e.g. GSH, thioredoxin and pyridine nucleotides) [27, 86]. Ascorbate, found in the extracellular and intracellular aqueous phase, converts into a non-reactive and stable radical and scavenges ROS. It also converts tocopherol free radicals back to tocopherol [87]. In contrast to ascorbate, tocopherol is localized in the lipid phase of cells, specifically on the inner cell membrane surface and acts by neutralizing lipid peroxyl radicals produced during lipid peroxidation [86]. α-Tocopherol is the main

cellular membrane antioxidant as well as the most active type of vitamin E [87]. Uric acid is a strong peroxynitrite scavenger in the extracellular space. However, it cannot fulfil its scavenging properties unless ascorbic acid and thiols are present [88]. The reduced glutathione form (GSH), thioredoxin and pyridine nucleotides work synergistically with the antioxidant enzymes as they provide them with reducing equivalents to defuse ROS [86, 88]. GSH is ubiquitously found in all cellular compartments. The GSH/GSSG ratio is an important indicator of oxidative stress. GSH helps GPx to detoxify H_2O_2 and lipid peroxides while it can also directly donate its electron to H_2O_2 in order to decompose it to H_2O and O_2. GSH can also prevent oxidation of membrane lipids by providing them with protons [87]. Melatonin, generated in the pineal gland and referred to as the sleep hormone, was also shown to possess antioxidant protective properties in mitochondria, particularly by deterring loss of ETC activity and by obstructing cardiolipin oxidation [27, 89-91].

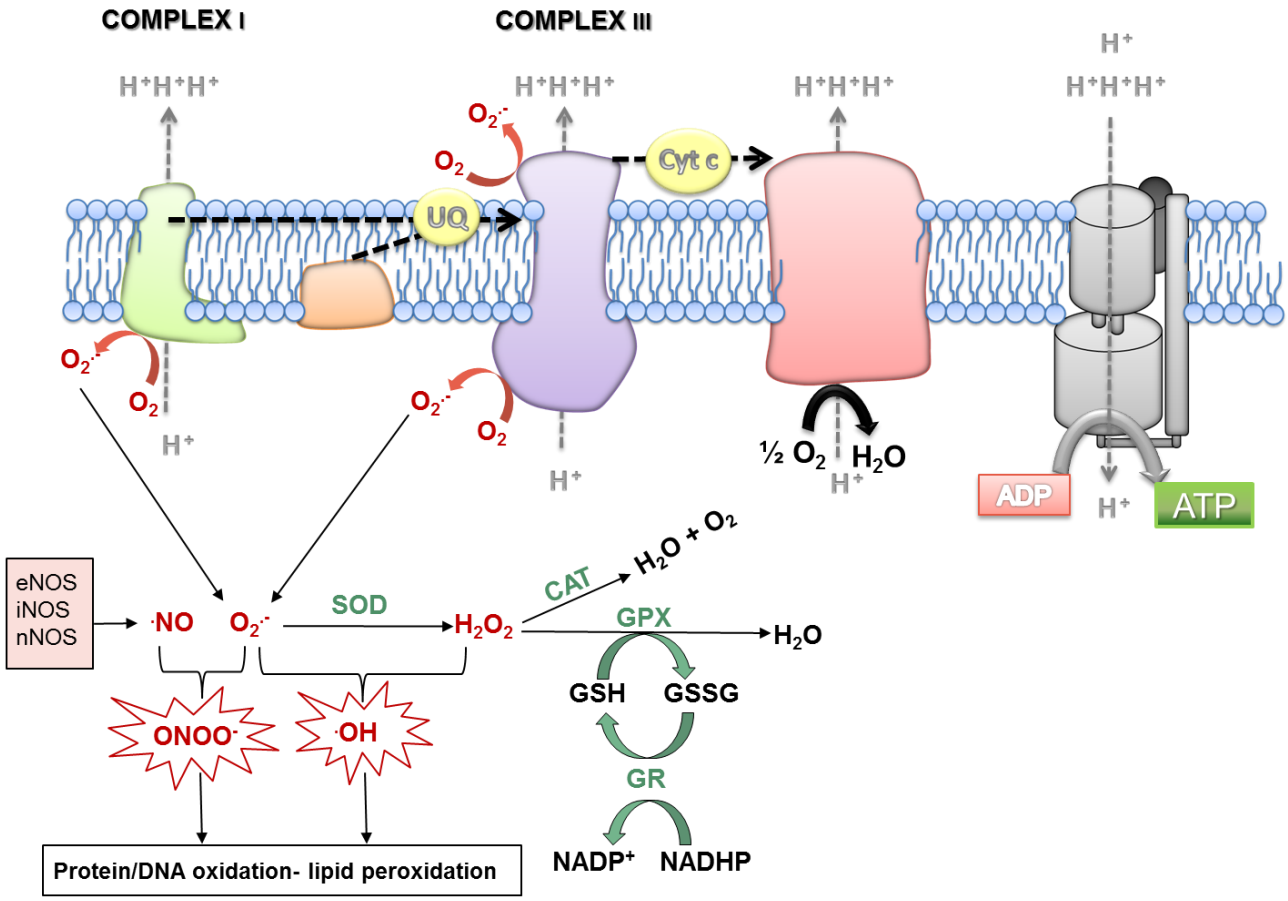

Figure 3. ROS and NOS production and antioxidant defenses. Mitochondria are the epicenter of ROS production and metabolism. Due to electron leak, some electrons in the ETC are not transferred to O_2 (the final electron acceptor). The majority of mitochondrial ROS are produced by complexes I and III of the ETC. Superoxide anion can be generated in the matrix during the transfer of electrons from NADH to coenzyme Q and complex I due to partial reduction of O_2. Complex III produces ROS via a non-enzymatic reaction while it releases superoxide anion both into the intermembrane space and in the matrix. The superoxide anion released by complexes I and III is quickly converted to H_2O_2 by the antioxidant enzyme superoxide dismutase SOD while H_2O_2 is turned into H20 bz catalase and glutathione peroxidase. RNS are produced when exogenous or endogenous nitric oxide (NO) interacts with ROS. NO is synthesized mainly by the three types of nitric oxide synthase (NOS). NO is a greatly reactive free radical-carrying gas that can react with superoxide anion leading to the production of RNS and peroxynitrile (OONO-) which can lead to the synthesis of hydroxyl radical. The reaction of NO with reductants and oxidants can lead to the generation of more RNS including nitroxyl anion (NO−) (when •NO is reduced), higher oxides of nitrogen such as NO2 (nitrogen dioxide) and N2O4 (dinitrogen tetroxide).

1.5. Mitochondria and cell death

Mitochondria enhance cell survival by generating ATP but they also monitor cell death, widely known as apoptosis or programmed cell death. Apoptosis happens via two main pathways: a) the intrinsic pathway (or mitochondrial) which is initiated by intracellular stimuli like over-accumulation of ROS and Ca^{2+} overload and b) the extrinsic pathway initiated by extracellular stimuli acting through plasma membrane receptors. Mitochondria are at the center of the apoptotic machinery and cascade by processing apoptosis signals via the Bcl (B-cell lymphoma) group of proteins and by monitoring caspase activation upon the release of cytochrome c [92]. Upon detection of the apoptotic signals, pores in the mitochondrial membrane, namely mitochondrial permeability transition pores (mPTP), are being opened causing the permeabilization of both mitochondrial membranes. The opening of the mPTP disturbs mitochondrial function and as a result the apoptotic signals are diffused from the mitochondrial intermembrane space into the cytosol. In detail, cytochrome c and apoptosis-inducing factor (AIF) which are modulated by anti- (bcl-2 and bcl-xl) and pro-apoptotic proteins

(bax, bad, bam, bid, bim) are released [93, 94]. Cytochrome c causes the formation of the apoptosome by binding to the apoptotic protease activating factor 1 (Apaf-1) which in turn triggers procaspace 9 protein upon the presence of dATP (deoxyadenosine triphosphate). This leads to the caspace 9 protein activation and sequentially to an activation cascade of other proteins such as caspase 3 which are responsible for cell death [92, 95].

Overall, mitochondria are absolutely essential for cell function particularly in neurons that are post-mitotic, polarized, excitable cells with high energy demands. Therefore, improper or disrupted mitochondrial function has detrimental consequences for neurons and is implicated in the pathophysiology of several brain-related disorders such as neurodegenerative, mental and stress-related diseases.

2. Synaptic plasticity and the role of BDNF

2.1. Synapses and their formation

Neurons form inter-connections and communicate with each other at junctions called synapses. There are chemical synapses in which exchange of information takes place via chemical messengers while there are electrical synapses in which communication takes place via ions. Synapse is the interface of communication between two neurons or between a neuron and a different cell type (e.g. muscle cell or astrocyte). Chemical transmission includes the release of messenger molecules termed neurotransmitters (such as neuropeptides, monoamines and endocannabinoids). Neurotransmitters forward information from the pre-synaptic (forwarding neuron) to the post-synaptic (receiving neuron) neuron but are also able to send signals from the brain to other cells in the body. Neurotransmitters are carried in spherical synaptic vesicles in the axon terminal and are being released via exocytosis upon precise alignment of the pre- and post-synaptic structures (Figure 4). The post-synaptic terminal senses the neurotransmitters through diverse receptors. At the synapse, the pre-synaptic neuron fires an action potential and triggers the transmission of a signal to the post-synaptic neuron. This might increase or decrease the potential of the post-synaptic neuron to fire its own action potential. Synapses are usually created between axon terminals of

the pre-synaptic neuron and the dendrites of the post-synaptic neuron (Figure 4). Although synapses can be also formed between two axons as well as between an axon and a soma [96-98].

In humans, the majority of synapses are formed during pre- and postnatal development. From then on, around half of the synapses are eliminated or “pruned” during the following two decades of life. For example, spine density in the human prefrontal cortex is 2-3 fold higher in prepubescent subjects compared to mature subjects [99]. Most synapses that were not pruned during adolescence, are stably conserved during adulthood although synapses are being formed and eliminated throughout life. Developmental synapse generation is predominantly activity-independent while synaptic elimination is mostly activity-dependent.

Experiments showed that an immense local γ-aminobutyric acid (GABA) or glutamate release can trigger the development of post-synaptic specializations indicating that pre-synaptic neurotransmitter release can induce synapse formation which is thus activity-dependent [98, 100, 101].

2.2. Structural and functional synaptic plasticity

Synaptic or neuronal plasticity is defined as structural, morphological and functional modifications in neural circuits including dendritic spine development and synaptogenesis as an adaptive response to external stimuli such as environmental influences, learning or brain injury [102, 103]. Those are dynamic processes in the neuronal system and underpin the capacity of the brain to behaviorally adapt to a constantly changing environment. Synaptic plasticity represents the cellular mechanism of learning and memory.

2.2.1. Structural synaptic plasticity

The human brain is a plastic area. Structural neuronal adaptive responses to different stimuli can affect dendrites, axons, spines and adult neurogenesis. Therefore, the main features of structural synaptic plasticity include a) neurogenesis b) neurite outgrowth and branching as well as c) spine density and morphology [104, 105].

Neurogenesis

Neurogenesis is the creation and growth of new neurons. Contrary to outdated beliefs claiming that neurogenesis is solely a developmental phenomenon, it is now undoubtedly proven that neurogenesis is taking place in the adult brain as well. Neurogenesis occurs in two main areas of the adult brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle which provides neurons to the olfactory bulb. The development of new neurons in these areas is an additional way by which the brain is able to alter and regulate its own circuitry and therefore neurogenesis constitutes a weapon in the arsenal of brain's plasticity. This cellular renewal system is not static but rather a dynamic procedure that depends on external or internal challenges [104]. New functional neurons are added to the existing neuronal network and are derived from endogenous neural stem cell pools during both development and adult life. In the hippocampal SGZ, astrocytes generate intermediate progenitors (e.g. aroung 9.000 new progenitors per day in young adult rats) [106] which mature into granule neurons of the dentate gyrus [107], eventually growing axonal projections into the hippocampal region CA3 and sending dendritic projections to the molecular layer [108, 109]. Similarly, astrocytes in the SVZ function as neural stem cells that divide slowly and are able to produce neuroblast precursors [110, 111]. These neuroblasts enter a migration path termed rostral migratory stream (RMS) and migrate from the RMS into the olfactory bulb. During adulthood, cells from the SVZ mature into two main subtypes of olfactory inhibitory interneurons: a) granule cells and b) periglomerular cells. Every step of the adult neurogenesis is tightly modulated indicating that the adult brain generates neurons in response to requirements of its environment [104].

Neurite outgrowth and branching

Neurite outgrowth is an essential procedure for the differentiation of neurons and fundamental for wiring the nervous system and forming neuronal networks during development and adult regeneration. Neurites sprout from the cell body and generate both dendrites and axons. The two main cytoskeletal proteins implicated in neurite

outgrowth are actin filaments and microtubules while the two principle mechanisms that characterize neurite outgrowth are a) the re-organization of the cytoskeleton and b) the outspread of the plasma membrane driven by the exo- and endocytosis of certain vesicles (different from neurotransmitter-carrying vesicles). A growth cone is a big actin-supported prolongation of a developing/regenerating neurite searching for its synaptic target. In axons, the exocytosis-driven surface-expansion is concentrated at the growth cones while in dendrites exocytosis might be mainly distributed across the shaft. Although axons are usually longer than dendrites, dendrites show larger morphological variability. Dendrites can be single or multiple and either smooth or full of spines. Branching of both dendrites and axons can vary in extension from low or higher extent of branching [112, 113]. The outgrowth process takes place through the dynamic interaction of the cytoskeleton with the expanding membrane [114-116]. These processes seem to be mediated by Rho GTPase (Ras homologue signaling G proteins) family members which additionally stabilize contact points of growth cones [113, 117]. The sprouting from the cell body is facilitated by structural modifications of microtubules and actin filaments and leads to the birth of a few homogenous neurites [118-120]. Shortly thereafter, these neurites are specified into dendrites and axons. Polarity is obtained in five sequential stages, each involving specific cellular events [121]. Firstly, neurons are smooth and covered with small lamellipodia all over their surface. Subsequently, four or five small and similar neurites emerge during sprouting. Eventually, one of the neurites grows quickly, gains significant length compared to the other neurites and ultimately evolves to become a typical axon. Then, the rest of the neurites start to differentiate, obtaining the characteristic properties of dendrites. Interestingly, upon severe damage of an axon, a new axon is generated from an already existing outgrown neurite. The last step of neurite outgrowth is the establishment of neuron-to-neuron interactions, the maturation of the dendrites and the axon, branching, development of dendritic spines and axonal terminals and emergence of synapses. The structural neuronal differentiation is maintained by a range of cellular and molecular processes triggered by neurotrophins and other growth factors [122, 123]. Several signaling molecules have been found to mediate neurite outgrowth such as membrane receptors and cytoskeleton components [1–3]. The neural growth cone contains a plethora of actin filaments as well as distinct adapter and filament remodeling proteins [124-126]. Intracellular kinases, such as

phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and ERK regulate the production of actin filaments while GTPases connect kinase signaling to the actin cytoskeleton machinery [127, 128]. It has been found that activation of the PI3K/Akt pathway promotes neurite outgrowth in hippocampal cultures [129-131].
A broad neurite branching phenotype is an essential feature of neuronal structure. Every dendrite and axon comprise several branches that enrich the complexity of neural circuits by enabling interaction with a plethora of target neurons as well as with non-neuronal cells. For instance, a neuron can form synapses with numerous target neurons due to the large-scale branching at the axonal shaft. Moreover, dendrites exhibit branching of high complexity and thus develop a broad dendritic area that is able to receive synaptic inputs. Neurite branches build networks and promote the development of largely complex neural circuits which process information and orchestrate certain functions of the nervous system. Evidence indicates that abnormal neurite branching might underlie several neurodevelopmental and neurological disorders such as schizophrenia and autism [132-134].

Spine morphology and density

Dendritic spines are multifunctional and specialized structures of the nervous system and key units of neuronal signaling and connectivity. These structures are largely dynamic and are able to alter their number, morphology, density and motility relatively fast. Synaptic plasticity, which mediates learning and memory processes [135-137], implicates *de novo* spine development on dendrites (a procedure called spinogenesis) as well as remodeling of existing dendritic spines in order to maintain neuronal interconnections. Dendritic spines aim to broaden the dendritic surface area and thus increase the capacity of dendrites to acquire synaptic inputs [138]. Spine functions include a) regulation of single synapse efficacy both pre- and post-synaptically, b) increase in neuronal computational capacity and c) designation of postsynaptic gene expression and signaling [139-142]. Although there are different morphological types of spines, a spine consists of a thin neck and a wider head resembling the shape of a mushroom. Synaptic strengthening is linked to increased spine head diameter and spine density while synaptic weakening is correlated to a decrease in spine number

and shrinkage [143-147]. Spine-carrying neurons are present in several brain regions. The most studied spine-carrying neurons include Purkinje cells in the cerebellum, pyramidal cells in the cerebral cortex, and medium neurons at the striatum. Early in life during neurodevelopmental stages, dendritic spines usually have a long (around 2 μm), “filopodia-resembling” shape carrying a small number of organelles and no detectable head structure. Such dendritic spines are largely dynamic in their capacity to extend or retract very shortly after chemical stimulation [148, 149]. However, their function during synaptic transmission in adulthood seems limited since they largely lack synaptic inputs. Therefore, this type of spine is regarded as an immature and unstable phenotype. Upon receipt of enhanced synaptic input, immature spines transform into more stable and mature phenotypes. Ultrastructural studies have indicated that a number of the newly developed spines have the ability to become mature and to form synapses from hours to days after *de novo* spine growth [138].

2.2.2. Functional synaptic plasticity

The two principal and most important functional features of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) and both can take place in neuronal synapses in response to stimuli. The molecular and cellular mechanisms responsible for those changes in the neuronal network involve pre- and post-synaptic membrane trafficking, neurotransmission modifications, cytoskeletal reorganization as well as gene transcription and protein synthesis [150-153]. Mitochondria are localized in dendrites and axonal terminals of neurons and have a notable role in synaptic plasticity [154].

LTP was initially observed in the dentate gyrus of the hippocampus however, it emerges in many regions and pathways of the brain. Most types of LTP are glutamatergic and predominantly induced after activation of the N-methyl-D-aspartate (NMDA) receptors [155]. LTP in the hippocampus typically consists of three separate and sequential phases: short-term potentiation, early LTP and late LTP. Short-term potentiation and early LTP are temporary and include the alteration of pre-existing proteins. However, late LTP requires modifications in gene expression and therefore protein synthesis and lasts from a few hours to days [156]. LTP events are initiated

during synaptic transmission of low-frequency. Glutamate is secreted from the presynaptic neuronal terminal and binds both the NMDA and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Due to Mg^{+2} blocking the NMDA receptor channel, Na^{+} and K^{+} diffuse solely through the AMPA receptor channel. The Mg^{+2} channel blockage is relieved upon depolarization of the postsynaptic membrane which leads to the flow of Na^{+}, K^{+} but most importantly Ca^{2+} through the NMDA receptor and an increase in spine calcium. This Ca^{+2} increase in the dendritic spines is shown to trigger subsequent processes resulting in LTP. Those events are implicated in the induction of LTP but not in its constant maintenance [157]. In total, induction of LTP requires NMDA receptor activation, an increase in postsynaptic calcium, postsynaptic depolarization and a voltage-dependent block of NMDA receptor by Mg^{+2} and its subsequent permeability to Ca^{+2}. Repetitive NMDA receptor activation on a single spine leads to a long-lasting rise in the AMPA receptor response in the same spine. As a result, the spine volume is enhanced at the same time with the increase in the AMPA receptor response. Interestingly, processes that block structural plasticity also suppress LTP. Therefore, structural plasticity can be an indication for LTP [158]. NMDA and AMPA receptors are highly localized at excitatory synapses and interact with synaptic structures including the postsynaptic density (PSD) in order to preserve this local high density. Protein kinase activity plays a key role in LTP and LTD. Evidence showed that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is the main downstream target after entry of Ca^{+2} via the NMDA receptor and is required for LTP. CaMKII is activity-dependently translocated to the synapse and in particular to the PSD upon increased calcium concentration in the spine. This implicates activity-dependent binding of CaMKII to the NMDA receptor (on the GluN2B subunit) and facilitates phosphorylation of PSD proteins. Obstruction of this binding diminishes LTP. The maintenance of LTP depends on signaling cascades downstream of CaMKII. Protein phosphorylation and dephosphorylation might be crucial for LTP and LTD and other types of synaptic plasticity. Phosphorylation of certain AMPA receptor subunits might control receptor function and enhance synaptic transmission. Phosphorylation is orchestrated by protein kinases such as protein kinase A (PKA), protein kinase C (PKC), CaMKII), c-Jun N-terminal Kinase (JNK) and tyrosine-protein kinase (FYN). LTP increases cGMP-dependent protein kinase (PKG) phosphorylation while LTD decreases phosphorylation [158].

Long-term depression (LTD) was initially observed in the CA1 hippocampal region but has been apparent also in the amygdala and cortex [159] and is an activity-dependent decline in synaptic efficacy. LTD can be NMDA-receptor dependent or independent [155]. The difference between LTP and LTD is claimed to be on the duration and the extent of calcium signaling [160]. High calcium levels activate CaMKII (low-affinity kinase) which facilitates phosphorylation of PSD proteins leading to increased transmission. Low to modest calcium levels selectively recruit and activate calcineurin (high-affinity phosphatase) leading to dephosphorylation of PSD proteins and to decreased transmission. Additionally, studies have indicated that PKA and PKC substrate dephosphorylation is implicated in LTD [161]. It has also been proposed that the decrease in synaptic transmission observed during LTD is due to an elimination of synaptic AMPA receptors [158].

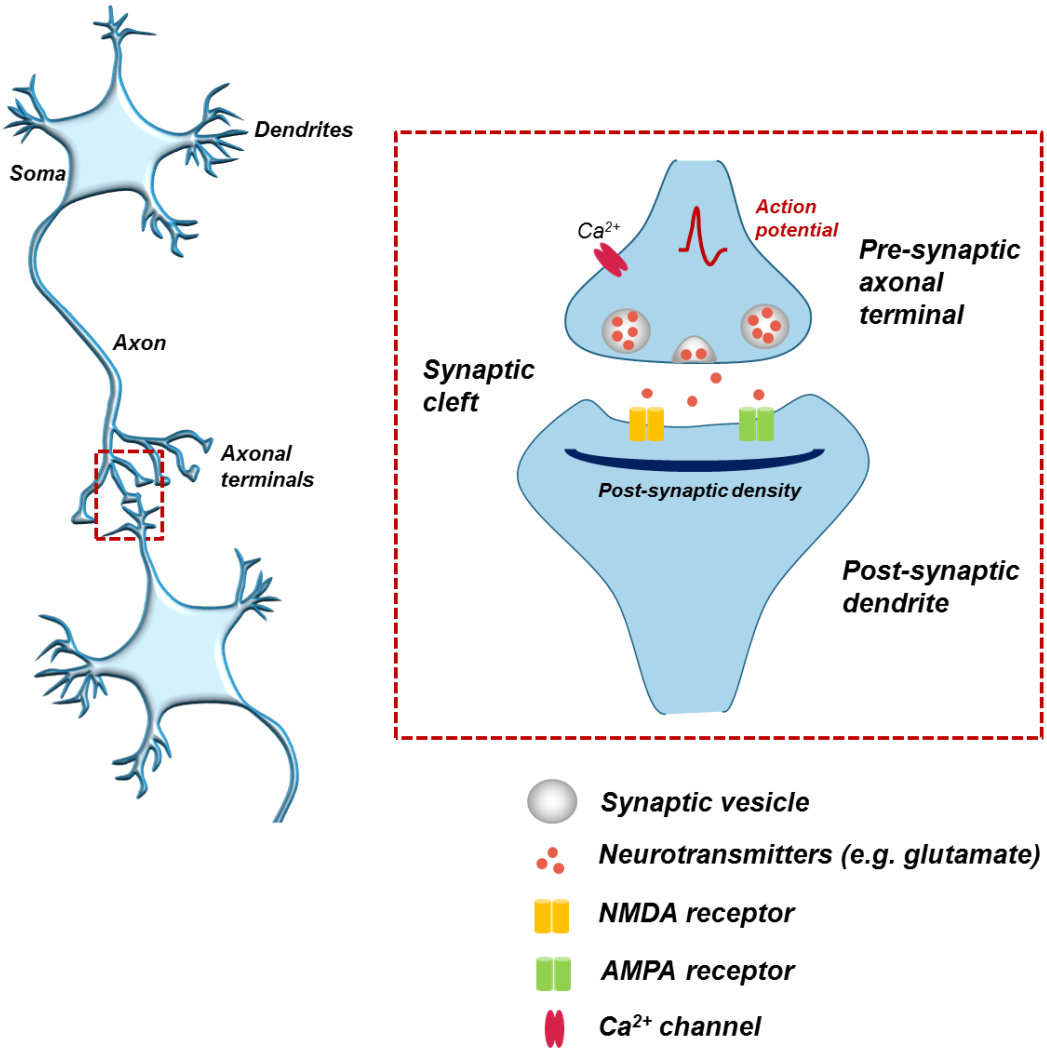

Figure 4. Synapse formation. Synapse is the interface of communication between two neurons or between a neuron and a different cell type (e.g. muscle cell or astrocyte). Chemical transmission includes the release of messenger molecules termed neurotransmitters (such as glutamate). Neurotransmitters forward information from the pre-synaptic (forwarding neuron) to the post-synaptic (receiving neuron) neuron but are also able to send signals from the brain to other cells in the body. Neurotransmitters are carried in spherical synaptic vesicles in the axon terminal and are being released via exocytosis upon precise alignment of the pre- and post-synaptic structures. Synapses are usually created between axon terminals of the pre-synaptic neuron and the dendrites of the post-synaptic neuron.

2.3. Neurotrophins and their role in synaptic plasticity

Neurotrophins is a family of proteins/growth factors that mediate survival, proliferation, differentiation and death of neuronal and non-neuronal cells. Neurotrophins include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) as well as the newly established neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7). These proteins possess a long tridimensional arrangement containing three disulfide bonds one of which is shaped into a distinguished connecting cystine knot. Neurotrophins are synthesized in their precursor forms termed proneurotrophins. They are then processed by several types of enzymes which perform proteolytic cleavage and transform the proneurotrophins into mature neurotrophins upon their release in the extracellular space. The respective mature form of each neurotrophin creates a complex with an identical molecule and they are shaped into a dimeric structure that is able to activate specific receptors [162]. Neurotrophins exert their actions via two types of receptors: tyrosine kinase receptors which show high affinity for mature neurotrophins and p75 receptors which show high affinity for proneurotrophins and a very low affinity for mature neurotrophins [163]. While mature neurotrophins bind to tyrosine kinase receptors (Trk) and exert several beneficial effects in the nervous system, binding of proneurotrophins to p75 receptors exhibits the opposite biological effects to those of mature proteins. There are distinct types of Trk receptors which are activated by certain neurotrophins. In detail, NGF binds to and activates TrkA, BDNF and NT-4/5 bind to and activate TrkB and NT-3

activates TrkC. Neurotrophins are able to autoregulate their generation as well as to regulate the generation of other members of this protein family [164, 165].

BDNF stands out among neurotrophins due to its wide expression in many brain regions and due to its potent effects on synapses. Biological properties of BDNF, including its release, are regulated by neuronal activity. BDNF has been proved to be a crucial factor involved in the regulation of neuronal survival and differentiation, synapse formation as well as in the regulation of activity-dependent modifications in synapse structure and function [166]. Additionally, BDNF is a principal modulator of LTP in the hippocampus and other brain regions [167-169]. BDNF protein is synthetized as a pre-form that includes a pro-segment and the mature BDNF part. The pro-segment undergoes activity-dependent removal by proteases and the secreted mature BDNF binds to its receptor TrkB causing signal transduction and inducing LTP while the pro-BDNF binds to the receptor p75 causing LTD (Figure 5) [156, 170]. Although the proteolytic cleavage of the pro-segment is believed to take place after the secretion of BDNF, a study indicated that BDNF and its removed pro-peptide were present in large vesicles at presynaptic terminals of excitatory neurons in the mammalian adult hippocampus. This proposes that the cleavage of the pro-segment might take place already inside the secretory granule [156, 171]. The expression, synthesis and release of BDNF are triggered by certain neuropeptides and hormones after excitatory synaptic activity [172]. As mentioned above, glutamate secreted from excitatory synapses causes the influx of Na^+ and Ca^{2+} via AMPA and NMDA receptors as well as through voltage-dependent Ca^{2+} channels. Elevated Ca^{2+} levels activate CaMKII, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) which sequentially trigger the transcription factors nuclear factor κB (NF-κB) and cAMP response element-binding protein (CREB) which finally induce BDNF gene transcription [173]. BDNF is located in vesicles that are recruited to neuronal axons and dendrites from which it is secreted upon activation of glutamate receptors (Figure 5) [174]. BDNF mRNA is also concentrated in dendrites where synaptic activity can stimulate protein translation. Local BDNF secretion activates the TrkB or the p75 receptor on synaptic neurons or other cells in close proximity. TrkB is a tyrosine kinase receptor that stimulates phospholipase Cγ (PLC-γ), PI3K and MAPK signaling pathways in response to its activation and ultimately engages transcription factors that modulate the expression of proteins important for synaptic plasticity, neuronal survival,

cellular energy homeostasis and mitochondrial biogenesis [175, 176]. BDNF obstructs neuronal apoptosis by promoting anti-apoptotic Bcl-2 family protein expression and caspase inhibitors as well as by restraining pro-apoptotic proteins including Bax and Bad (Bcl-2-antagonist of cell death). It can also upregulate antioxidant enzymes and boost repair of damaged neuronal DNA [175, 177]. BDNF induces synaptogenesis and neurite outgrowth in the brain as well as in the periphery by activating RAS p21 protein activator 1, by strengthening cytoskeletal dynamics, by promoting mitochondrial biogenesis and by regulating cell adhesion. Additionally, BDNF signaling exhibits rapid effects on synaptic transmission and membrane excitability through modifying the kinetics leading to activation of NMDA receptors and by causing accumulation of synaptic vesicles in the presynaptic terminal [178].

Memories are thought to be stored in synapses in the brain while synaptic plasticity is believed to represent the cellular mechanism for memory and learning. Hippocampal LTP is the most studied type of synaptic plasticity. Late LTP, in contrast to early LTP, requires *de novo* protein synthesis which is associated with structural changes of synapses [179-184]. Stimuli leading to early and late LTP trigger distinct but partly overlapping pathways that lead to different changes at synapses. In particular, advent of late LTP leads to activation of PKA and MAPK/ERK (mitogen-associated protein kinase/extracellular signal-related protein kinase) pathway [181, 185]. Subsequently, a number of sequentially expressed transcription factors such as CREB and ELK-1 (ETS transcription factor ELK1) are phosphorylated and they promote the transcription of downstream genes that might regulate the structural and functional changes of synapses [181, 186]. BDNF is one of the downstream genes stimulated by the late LTP-inducing tetanus. BDNF is concentrated in vesicles in both presynaptic axon terminals and postsynaptic dendrites of glutamatergic neurons (mainly granule cells and pyramidal cells).

The first evidence associating BDNF with LTP in the hippocampus was supplied by studies indicating that mice carrying a target disruption on BDNF show a significant LTP impairment at the Schaffer collateral-CA1 synapses [187, 188]. Interestingly, the damage in LTP in BDNF knockout mice was improved upon acute administration of recombinant BDNF [188] or upon re-expression of the BDNF gene mediated by virus [189]. Plenty of evidence shows an important role of TrkB receptors as the main candidates mediating BDNF effects during LTP. BDNF is secreted during or directly

after LTP induction and possesses a principal role during early LTP and short-term memory but most importantly during late LTP and long-term memory [156, 169, 190-192]. BDNF regulates vesicle mobilization at the synapses possibly by modulating the phosphorylation and dispersal of synaptic proteins. As a result, BDNF enables the induction of early LTP by intensifying synaptic responses to tetanus stimulation. Apart from its role in the induction of early LTP, BDNF additionally affects its maintenance either by activating “silent synapses” and/or by modulating the actin motor complex [169, 192].

Initial evidence showing the BDNF-induced effects on the maintenance of late LTP was provided from studies conducted on BDNF knockout mice [193] using TrkB antibodies, TrkB-IgG fusion proteins [193, 194] as well as TrkB knockout mice [195]. The effects shown in these studies were extensively attributed to the role of BDNF in the upregulation of protein synthesis which subsequently causes structural and functional changes at synapses [156, 192]. While these studies indicate that endogenous BDNF is essential for the maintenance of late LTP, a crucial question would be whether an activity-dependent rise in endogenous BDNF is accountable for the maintenance of late LTP. To begin with, transcription of BDNF was increased by late LTP-causing stimuli. Using *in situ* hybridization, BDNF mRNA level was shown to increase in the CA1 region and dentate gyrus of the hippocampus within 2-4 h after the late LTP-inducing tetanus stimulation [151, 196, 197]. Therefore, BDNF level is enhanced either as a cause or as a result of late LTP induction. Additionally, the damage in late LTP witnessed in heterozygous BDNF mutant mice (BDNF+/−) could be rescued using perfusion of exogenous BDNF. This demonstrates that a rise in BDNF level might indeed contribute to the maintenance of late LTP [189, 198]. Further studies showed that late LTP was selectively disturbed unlike early LTP which remained intact in mutant mice with a blocked activity-dependent increase in BDNF mRNA [169]. These findings propose that without a rise in BDNF levels, an intense tetanus is not adequate to induce late LTP. Finally, upon anisomycin/emitin-induced complete inhibition of protein synthesis, late LTP was obstructed in the hippocampus [199, 200]. Notably, exogenous BDNF administration restored late LTP even when new protein synthesis was inhibited [185, 198]. Therefore, BDNF might be the key product derived from protein synthesis that is responsible for the maintenance of late LTP. Collectively, these findings suggest that late LTP-inducing stimuli enhance

endogenous BDNF levels and this enhancement might support the expression of late LTP.

Despite its essential role in late LTP, BDNF *per se* is not sufficient to induce synaptic potentiation. Late LTP demands a constant increase in endogenous BDNF in order to be maintained. Another interesting question is whether endogenous BDNF is produced pre- or postsynaptically. Initial studies supported that early LTP taking place at the Schaffer collateral-CA1 synapses required BDNF obtained solely from presynaptic CA3 neurons and not from postsynaptic CA1 neurons [201]. The initial increase in BDNF could occur due to the release of already existing BDNF-carrying vesicles from presynaptic CA3 neurons caused by high frequency stimulation. This could be significant for the induction of early LTP. However, the preexisting BDNF would be ultimately exhausted due to the low neuronal BDNF expression level. Therefore, for the maintenance of late LTP, a constant BDNF supply may principally occur from new protein synthesis induced by recurrent strong synaptic stimulation. This model is supported by studies showing that hippocampal BDNF mRNA levels are significantly enhanced 1-3h following the late LTP-inducing tetanus stimulation [151, 169, 196, 197, 202].

The effects of BDNF include its ability to modulate structural organization by contributing to the stabilization and maturation of preexisting synapses as well as to the generation of new synapses. BDNF has appeared to be a crucial factor required for spine remodeling in LTP and studies support that it might be implicated in the regulation of activity-dependent structural modifications both during early and late LTP. In detail, BDNF administration enhanced θ threshold level-induced rise in actin polymerization in dendritic spines via modulation of the p21-activated kinase (PAK) and actin-depolymerising factor (ADF) [203]. The endogenous BDNF secreted upon LTP-inducing stimuli is also necessary for the protein synthesis-dependent rise in spine volume at CA3-CA1 synapses [204]. In agreement with that, BDNF facilitated activity-dependent spine growth [205]. Moreover, studies conducted using exogenous BDNF administration to hippocampal slices and cultured hippocampal neurons strengthen the evidence supporting the role of BDNF in the control of dendritic spine plasticity [206-209]

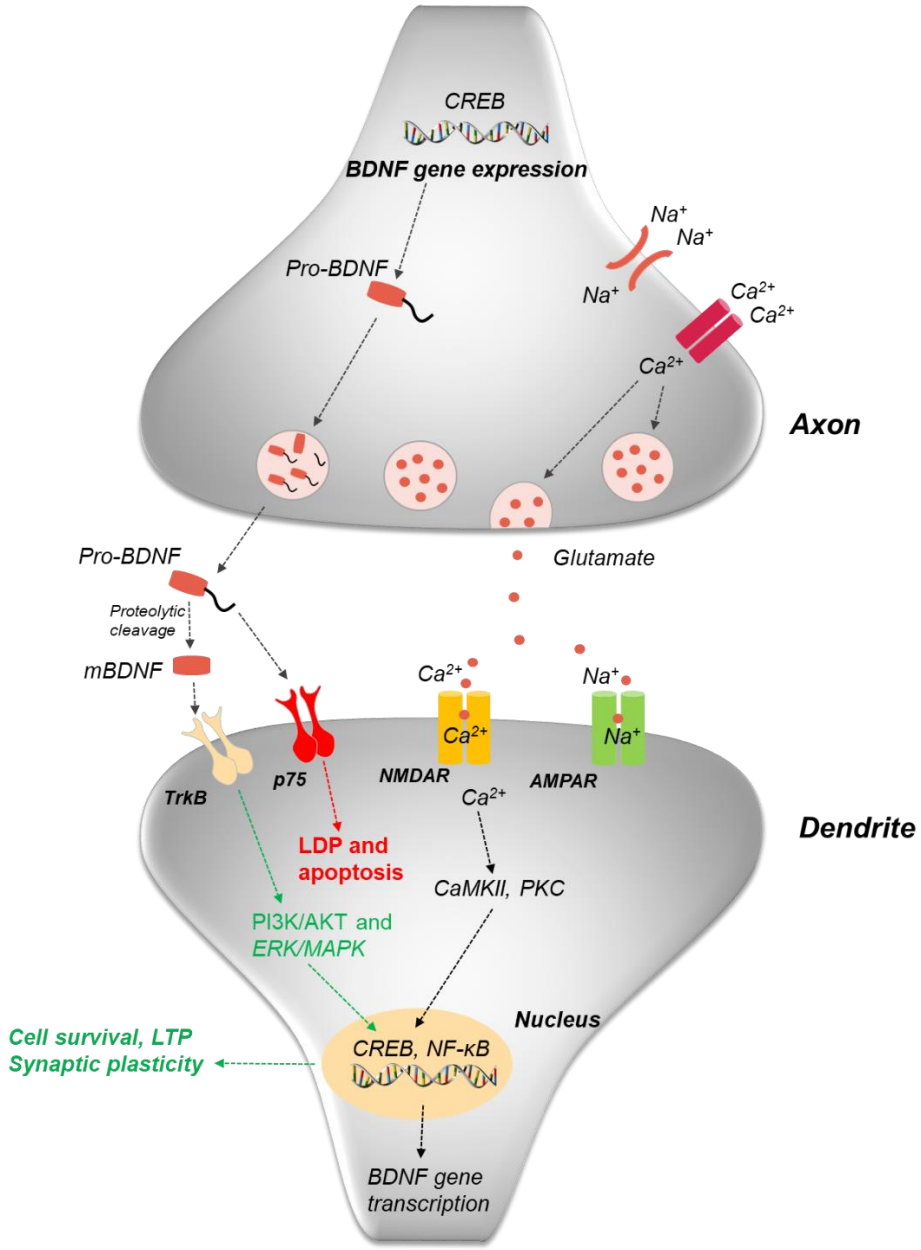

Figure 5. BDNF secretion at the synapse. The expression, synthesis and release of BDNF are triggered by certain neuropeptides and hormones after excitatory synaptic activity. Glutamate secreted from excitatory synapses causes the influx of Na^+ and Ca^{2+} via AMPA and NMDA receptors as well as through voltage-dependent Ca^{2+} channels. Elevated Ca^{2+} levels activate CaMKII, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) which sequentially trigger the transcription factors nuclear factor κB (NF-κB) and cAMP response element-binding protein (CREB) which finally induce BDNF gene transcription. BDNF is located in vesicles that are recruited to neuronal axons and dendrites from which it is secreted upon activation of glutamate receptors. BDNF protein is synthetized as a pre-form that includes a pro-segment and the mature BDNF part. The pro-segment undergoes activity-

dependent removal by proteases and the secreted mature BDNF binds to its receptor TrkB causing signal transduction and inducing LTP while the pro-BDNF binds to the receptor p75 causing LTD. NMDAR: NMDA receptor, AMPAR: AMPA receptor

2.4. Synaptic plasticity and mitochondria

Mitochondria are the main energy suppliers and calcium concentration regulators among other functions [154]. Given these functions of mitochondria, they are key candidates in the regulation and modification of synaptic transmission and consequently in processes of structural and functional plasticity. Plenty of evidence has shown that synaptic function as well as plasticity largely depend on mitochondria [103, 210-214]. Accordingly, studies have demonstrated that the inhibition of mitochondrial activity leads to damaged neurotransmission and synaptic potentiation [215, 216]. Acute inhibition of mitochondrial function during strong stimulation also results in reduced synaptic transmission [217, 218]. Conversely, increase of mitochondrial respiration pharmacologically caused an enhancement of synaptic density *in vitro* [219]. These findings support that mitochondria regulate synaptic plasticity apart from being affected by synaptic activity [220].

Mitochondria are found both in axonal and dendritic terminals of neurons and are largely dynamic organelles that fuse, divide and move through the cell compartments of the neuron in order to support cellular functions during synaptic transmission [220]. The mitochondrial transportation and recruitment to neuronal areas with high metabolic requirements are crucial for the adequate functioning of the neuronal network [221]. In dendrites, mitochondria are mostly present in the dendritic shafts and are additionally related to spines [222-224]. Upon synaptic stimulation, mitochondria are transported towards dendritic projections and boost their activity. A study demonstrated that the number of mitochondria in dendrites increased at the same time with synapse and spine formation, either in response to electrical stimulation or after recurring depolarization. In the same study, reduction of the number of dendritic mitochondria caused a loss of spines and synapses. In contrast, spines and synapses were significantly more apparent upon gathering of mitochondria in the dendrites

[219]. Moreover, *in vitro* studies indicated that the decrease in dendritic mitochondria caused by enhanced mitophagy resulted in obstruction of dendritic outgrowth during neuronal polarization [211] and in dendrite shrinkage in mature neuronal cultures [225]. Thus, adequate number of dendritic mitochondria is necessary for proper formation, development and conservation of dendrites as well as for spine and synapse genesis. Electric stimulation in hippocampal slices caused dendritic spine growth and accumulation of mitochondria to the respective active site. Additionally, novel spine and synapse formation is increased by concentration of mitochondria in dendrites [219]. Therefore, there are mechanisms for reciprocal modulation of synaptic plasticity and distribution and activity of mitochondria [154].

2.4.1. ATP production and synaptic plasticity

Neuronal functions including the arrangement of the actin cytoskeleton necessary for the formation of pre- and postsynaptic sections, membrane potential production, synaptic vesicle accumulation and secretion as well as protein phosphorylation processes are energetically funded by mitochondrial ATP production [224, 226-228]. These actions are crucial for neuroplasticity and can be affected by modifications in the ATP generation [229]. Mitochondria have major roles in modulating neuroplasticity procedures including remodeling of dendrites, neurotransmitter release and especially by producing energy [103, 224]. Inhibition of mitochondrial OXPHOS caused significant impairment of LTP [230]. Uncoupling of ETC from OXPHOS using FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone) as well as inhibition of mitochondrial complex I using rotenone in synaptosomes caused a decrease in synaptic vesicle secretion as a result of impaired mitochondria and obstructed ATP production [213]. A study showed that the accumulation of backup synaptic-pool vesicles is adequate for the maintenance of proper neurotransmission during strong stimulation and highly depends on ATP production in *Drosophila* neuromuscular junctions. A deficiency in synaptic mitochondria but also treatment with oligomycin (inhibitor of mitochondrial ATP synthesis) led to an incapability to recruit backup/reserve pool vesicles. This was partly rescued by treatment with ATP [227]. These results support that mitochondrial ATP generation is essential for a normal

neurotransmitter secretion through vesicle exocytosis as well as for transportation and distribution of backup synaptic-pool vesicles and therefore for the regulation of synaptic strength [154].

2.4.2. Calcium homeostasis regulation and synaptic plasticity

Synaptic plasticity mechanisms are apart from ATP also calcium-dependent procedures. A decrease in extracellular calcium and intracellular administration of EGTA (calcium binding ethylene glycol tetra-acetic acid) inhibited LTP in hippocampal neurons [231, 232]. In addition, a constant increase in calcium uptake and retention characterizes hippocampal LTP [233]. In that matter, cellular but especially mitochondrial calcium regulation is key during LTP induction and maintenance. Neuronal calcium levels are regulated by cytoplasmic buffers, by pores and pumps on plasma membranes as well as by two organelles: endoplasmic reticulum and mitochondria [234]. For example, enhanced mitochondrial pump activity was linked to hippocampal LTP as calcium uptake in mitochondria was continuously increased following LTP induction in dentate gyrus synapses [235]. The slow efflux of calcium out of mitochondria causes a plateau of calcium concentration which lasts a few minutes and facilitates the synaptic response [236]. As a result, mitochondria largely affect neurotransmitter release from presynaptic axon terminals which can be hindered by inhibition of the mitochondrial calcium release [220, 237]. The capacity of mitochondria to control regional calcium levels depends on the permeability of their outer membrane [212] and therefore on the mitochondrial permeability transition pore (mPTP) [238]. In hippocampal slices, cyclosporine A-induced blockage of the mPTP caused a rise in basal synaptic transmission and a decline in synaptic plasticity. This occurred due to a rise in the resting calcium levels in presynaptic terminals [239]. In agreement, additional studies demonstrated that mitochondria were able to modulate synaptic plasticity and function due to their capacity to uptake and release calcium [216, 217]. Porin is the structural component of the mPTP and the importance of mitochondria in buffering calcium and therefore in controlling synaptic plasticity was demonstrated in porin knock out mice lines [212, 238]. Mitochondrial membrane permeability also affects MMP and therefore major mitochondrial functions including

OXPHOS and release of apoptotic agents. Therefore, improving the outer membrane permeability of mitochondria might not only benefit cellular functions and survival but also synaptic transmission due to control of calcium levels. Overall, mitochondrial calcium efflux and influx has a predominant role in regulating synaptic plasticity [154].

3. Oxidative stress

As mentioned previously in section 1.3, ROS at physiological, non-elevated concentrations have essential roles in cellular signaling, in the regulation of senescence, cell death and proliferation and lead to an oxidative eustress forcing cells to adapt to challenges [240, 241]. However, ROS become harmful and disease-causing agents upon their overproduction leading to the so-called oxidative (di)stress. For their protection, cells are equipped with antioxidant defense systems in order to fend off ROS [242, 243]. The redox state of cells is dynamic and depends on the one hand on the production of ROS and on the other hand on the existence and functionality of antioxidant defense systems. As mitochondria are the main superoxide anion and hydrogen peroxide producers, they largely affect reduction-oxidation (redox) homeostasis. When there is an overproduction of ROS, the antioxidant defense systems are overwhelmed and they are not able to neutralize them. Therefore, oxidative stress is the over-accumulation of ROS (mainly superoxide anion and H_2O_2) due to their overproduction or due to overburdened antioxidant defense systems (or both) leading to a serious imbalance in redox homeostasis favoring oxidation [23, 26]. Apart from mitochondrial metabolic activity, there are other causes of ROS overproduction and oxidative stress: environmental and lifestyle stimuli such as growth factors or smoking/drinking, ultraviolet radiation (UV), inflammatory cytokines, toxins, exposure to metals or chemical oxidants and chemotherapy to name a few [30]. Oxidative stress is a common feature in many chronic diseases such as neurodegenerative, mental and stress-related disorders as well as in normal aging having devastating consequences for the functionality and well-being of biomolecules (e.g. DNA and lipids) and, therefore, of neuronal cells [3, 24, 240, 244-246].

ROS react with and damage many cellular and mitochondrial macromolecules. Of note, superoxide anion and H_2O_2 can easily diffuse through membranes, spread

throughout the cell and cause lipid peroxidation and membrane damage, protein mis-folding as well as DNA damage compromising cellular health [24]. ROS target DNA and cause a range of DNA lesions including breaks in DNA strand, oxidation of DNA bases, removal of nucleotides, crosslinking DNA-protein and abasic sites inevitably resulting in genomic instability. One of the most common and well-studied ROS-induced DNA lesions is 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG) which is also used as a biomarker of DNA oxidative damage. It is a largely mutagenic aberration that leads to G : C to T : A transversions [30, 244, 246]. In addition, DNA damage can potentially interfere with gene transcription and negatively impact promoter activity leading to disrupted transcription of important genes and finally to mutations. ROS can also cause RNA damage which in turn damages protein translation and obstructs the synthesis of crucial proteins [244]. ROS-induced protein damage includes changes in site-specific amino acids as well as in electric charge, enzymatic inactivation, aggregation of cross-linked reaction products, fragmented peptide chains and proteolysis [246]. Oxidative stress causes peroxidation of lipids on cellular and mitochondrial membranes thus compromising their biological properties (e.g. the level of fluidity) and can result in inactivation of enzymes and receptors localized on these membranes. As a consequence, cells and tissues become more permeable and susceptible to cell death. Apart from the damage in membranes *per se*, lipid peroxidation can also increase cellular impairment due to formation of chemically reactive oxidized products such as malondialdehyde (MDA), isoprostanes (F2-IsoPs), 2-propenal (acrolein) and 4-hydroxy-2-nonenal (HNE). These lipid peroxidation end-products have been widely used as oxidative stress biomarkers. Lately, the use of microRNAs as biomarkers of oxidative stress is being tested and might offer novel strategies for early detection and therefore prevention of oxidative stress-related diseases [247].

mtDNA is found in the mitochondrial matrix and encodes for 13 proteins which are structural parts of the ETC. mtDNA is in very close proximity to the ROS production sites (mainly complexes I and III) and is therefore directly affected and mutated, leading to faulty ETC components which leads back to impaired OXPHOS and more production of ROS [3, 243]. Several studies have proved that 8-oxo-dG, a major oxidative biomarker, is found at higher degree in mtDNA than in nuclear DNA. This

suggests that mtDNA is more vulnerable to oxidative damage that nuclear DNA possibly due to its localization near the ROS sources [248-253].

3.1. Oxidative stress in aging and neurodegeneration

Aging features an increase in ROS and a reduction in antioxidant defenses resulting in mitochondrial impairment and ultimately to senescence, cellular dysfunction and apoptosis. Normal aging and neurodegenerative diseases share these characteristics although to a different extent. When the ROS levels surpass a certain threshold then they become mitochondria-damaging and disease-causing agents [27]. In neurodegeneration the damaging effects are even more profound [4, 24, 26, 254]. During aging there is an accumulation of mtDNA mutations, decrease in ETC function and dysregulation of calcium homeostasis. Therefore, aging is one of the main risk factors for developing neurodegeneration [255]. The highly oxidized DNA, proteins and lipids discovered in the post mortem brain of patients with neurodegenerative diseases underpin the major role of oxidative stress in these diseases. Mutations in the antioxidant enzyme SOD1 have been linked to familial amyotrophic lateral sclerosis, another neurodegenerative disorder [246, 256]. Increased lipid peroxidation, by means of increased HNE and isoprostanes as well as increased 8-oxo-dG levels have been found in patients with AD and mild cognitive impairment [244]. Mechanisms in the pathogenesis of neurodegeneration involve misfolding and aggregation of proteins, damaged synaptic transmission, disturbed kinase-signaling pathways and dysregulation of neuronal calcium [257]. Neurodegenerative diseases are often characterized by pathogenic aggregation of different proteins in neurons [258]. ROS can highly affect these proteins by converting them in oxidative forms which intensifies the formation of aggregates [259]. Protein aggregates subsequently restrain proteasomes, the organelles responsible for the quality control and removal of cellular abnormal proteins. Over-accumulation of abnormal proteins that are not degraded by the proteasome triggers more ROS formation creating a vicious cycle, a common phenomenon in oxidative stress-associated neurodegenerative diseases [246, 258,

260]. Depression has also been characterized by increased ROS and decreased antioxidant levels [240].

3.2. Supporting theories

Several decades ago, Denham Harman proposed the free radical theory of aging for the first time. The theory supports that aging is caused due to accumulation of free radicals which exert all the detrimental effects mentioned in the previous sections. Accordingly, lifespan is determined by the capacity of an organism to survive ROS-induced cellular damage [261]. In line with this theory, increased mitochondrial ROS generation with concomitant increased 8-oxo-dG levels in the mtDNA are often evidenced in aged tissues [262-266] suggesting that gradual accumulation of oxidative stress-induced DNA damage throughout lifespan contributes to the aging process. Similarly, studies using cellular models have shown that increased oxidative injury is closely related to aging [248, 267, 268]. In addition, genetic studies in mice, worms and flies have correlated increased lifespan to lower free radical levels [269]. *C. elegans* strains carrying mutations that make them resistant to oxidative stress have exhibited increased lifespan while strains more vulnerable to free radical toxicity have demonstrated shorter lifespan [270, 271].

Mitochondria are both the generators and targets of ROS and thus they are key role-players in the pathomechanisms of aging and neurodegeneration. The mitochondrial theory of aging is an extension of the free radical theory. It supports that oxidative impairment of mitochondrial biomolecules including mtDNA, proteins and lipids is the cause of aging [30].

4. Glucocorticoid/hormonal stress and the stress system

4.1. HPA axis and the stress response system

Stress is defined as a state of threatened or recognized as threatened homeostasis. In response to this threatened situation, organisms are armed with a highly sophisticated system, namely the stress system, which supplies the proper central and peripheral neuroendocrine adaptive responses. If these responses are excessive, insufficient or prolonged, they might be harmful for essential physiological functions, including metabolism, growth, circulation, immune responses as well as reproduction to name a few [272]. The main elements of the stress system are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. The HPA axis consists of the hypothalamus, the pituitary gland and the adrenal cortex. Stressful experiences trigger a hormonal cascade response that enables adaptation (Figure 6). Shortly after stress, brainstem circuits activate the sympathetic nervous system leading to a burst of adrenaline release from the adrenal medulla in the adrenal gland which subsequently leads to noradrenaline release in the brain [273, 274]. After that, the process at the HPA axis is initiated in the paraventricular nucleus of the hypothalamus with the release of corticotropic-releasing-hormone (CRH) as well as the secretion of arginine vasopressin. CRH reaches the pituitary gland via the hypophysial portal system and binds to its G-protein-coupled receptor [272]. CRH and arginine vasopressin enhance each other`s expression while they both stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the systemic circulation. At the same time, CRH and arginine vasopressin activate noradrenergic neurons in the locus caeruleas/norepinepherine (LC/NE) system of the brain. The LC/NE system primarily activates the immediate “fight or flight” reaction in the brain which is triggered by the secreted epinephrine and norepinephrine. The ACTH subsequently stimulates the synthesis and release of corticosteroids, cortisol in humans and corticosterone in rodents, from the adrenal cortex after binding to its G-protein-coupled transmembrane receptor in the adrenocortical cells. Corticosteroids are the end-products of the stress response mediated by the HPA axis (Figure 6) [272]. The HPA axis contains a control mechanism which regulates the cortisol levels. Cortisol (and corticosterone in rodents) terminates the stress response by exhibiting a

negative feedback loop on the release of CRH and ACTH at the hypothalamus and the pituitary gland respectively. These negative feedback systems, particularly for ACTH, seem to be controlled additionally by non-genomic fast glucocorticoid actions. The negative feedback of cortisol on its own release is developed in order to inhibit long-term exposure of tissues to glucocorticoids and allows re-establishment of homeostasis. Chronic exposure to stressors can result in HPA axis dysregulation impacting the end-organ health and function [272, 274]. It has been proved that the stress response is mediated by fast non-genomic as well as by slower genomic pathways (for detailed analysis of these pathways through activation of glucocorticoid receptors and their impact on mitochondria see section 4.5). In detail, corticosteroids appear to exhibit rapid non-genomic actions in the hippocampus which demand increased levels of the hormones. These fast effects in CA1 neurons induce excitability and enhance the effects of several other stress hormones leading to alertness. Concomitantly to the non-genomic responses, gene-mediated pathways are initiated with a delayed response when hormone levels are already decreasing and lead to weakened neurotransmission. This late response is essential for developing resilience and adaptation [274].

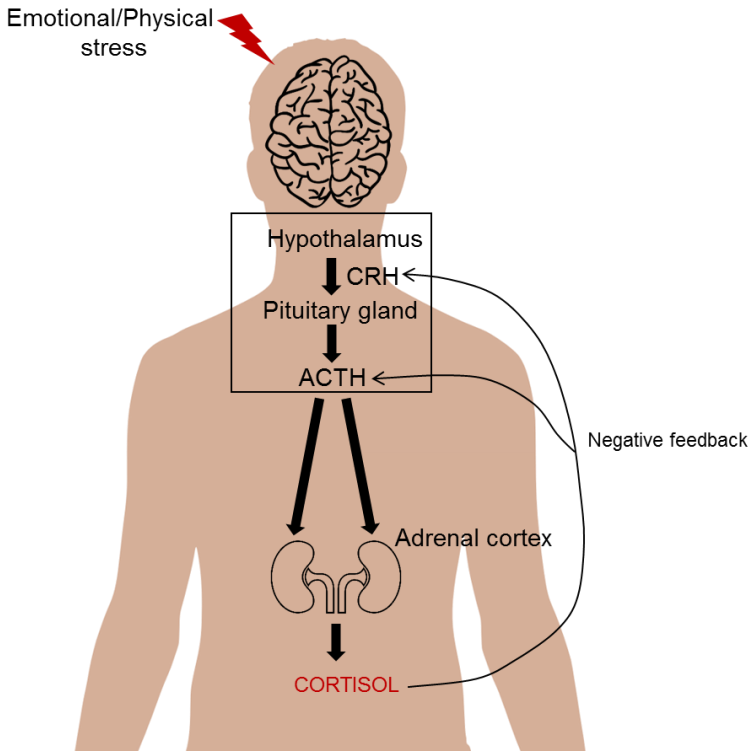

Figure 6. The HPA axis. The HPA axis consists of the hypothalamus, the pituitary gland and the adrenal cortex. Stressful experiences trigger a hormonal cascade response that is initiated in the paraventricular nucleus of the hypothalamus with the release of corticotropic-releasing-hormone (CRH). CRH reaches the pituitary gland via the hypophysial portal system and stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the systemic circulation. ACTH then triggers the synthesis and release of corticosteroids (cortisol in humans) which are the end-products of the stress response mediated by the HPA axis. The HPA axis contains a control mechanism which regulates the cortisol levels. Cortisol terminates the stress response by exhibiting a negative feedback loop on the release of CRH and ACTH at the hypothalamus and the pituitary gland respectively.

4.2. Cortisol and its receptors

Corticosteroids are a class of steroid hormones and they contain two subclasses: the glucocorticoids and the mineralocorticoids. Glucocorticoids are hormones derived from cholesterol and have an elemental role in the maintenance of stress-associated homeostasis. These hormones are involved in the maintenance of several physiological functions by influencing inflammatory and immune responses, by monitoring the intermediary metabolism and by maintaining the cardiovascular tone. In addition, non-genomic and genomic glucocorticoid actions can affect cell proliferation, reproduction, cognition, behavior, growth, survival as well as electrolyte and water equilibrium [272]. Glucocorticoids are produced and released by the adrenal gland in on oscillating manner meaning that their secretion takes place in hourly pulses throughout the day and is controlled by circadian, ultradian and stress responses. Cortisol levels peak prior to awakening which enables the coordination of processes during the active phase of the day [275]. Following that, cortisol levels progressively decrease and reach their lowest point prior to the inactive phase of the day [273]. Corticosteroids are able to passively traverse cellular membranes and are therefore present in all types of cells while they sufficiently cross the blood-brain-barrier (BBB) and reach all brain cells. Their neuronal effects depend on the distribution of their receptors as well as on their enzymatic metabolism [273, 276].

Corticosteroids bind to two types of receptors in the brain: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors act as hormone-

dependent transcription factors and they belong to the nuclear hormone receptor gene family. GR and MR bind endogenous natural as well as synthetic glucocorticoids each one with a different affinity. On the one hand, GR binds cortisol and corticosterone with a relatively low affinity amounting to Kd ~3–5 nM and synthetic glucocorticoids (e.g. dexamethasone) with a high affinity amounting to Kd ~ 0.1 nM and [276]. On the other hand, MR binds cortisol and corticosterone with high affinity (Kd ~ 0.5 nM) and most of the synthetic glucocorticoids with very poor affinity. Both basal and stress-provoked oscillating cortisol levels are highly affected by the hormone's different affinity for GR and MR. As MR possesses a 10 times higher affinity for cortisol than for GR, it is occupied much faster and more extensively by cortisol than GR. Therefore, MR is bound even at low systemic cortisol levels whereas GR in only occupied upon a significant increase in cortisol levels usually at the circadian cycle peak or during acute stress [276]. Both GR and MR are ubiquitously expressed in the limbic areas of the brain. Although most emphasis was granted in the past on the genomic pathways, later there is a focus on the glucocorticoid-induced non-genomic pathways [274]. Both receptors bind to specific genomic sites or they interfere with transcription factors. Activated corticosteroid receptors are able to modify gene expression slowly but persistently and thus impact brain activity in the long term from hours to days [277]. The cortisol-induced fast negative feedback on the HPA as well as the cortisol-dependent fast amplification glutamate release in the hippocampus are two examples of the non-genomic effects [278, 279]. The rapid non-genomic responses can be regulated by protein-protein interactions of GR and MR receptors with signaling agents [279, 280]. Although, evidence shows that there is a distinct G-protein-coupled glucocorticoid integral membrane receptor [276]. Both GR and MR are very important for glutamatergic neurotransmission. Upon stress, elevated glucocorticoid levels activate post-synaptic GR located on membranes which in turn activate the c-AMP-protein kinase A pathway and promote the secretion of the retrograde agents 2-arachidonoylglycerol and anandamide. 2-arachidonoylglycerol causes the activation of type 1 cannabinoid receptors present on the pre-synaptic neuronal membrane which stops the release of glutamate-containing vesicles [272]. Additionally, pre- and post-synaptic MR located on membranes enable glutamatergic transmission. Glucocorticoids bind to membrane MRs pre-synaptically which subsequently activate the extracellular signal-mediated kinase cascade, resulting in glutamate secretion in

the synaptic space. At the same time, glucocorticoids activate membrane MR post-synaptically causing obstruction of potassium IA-currents. This enables the membrane diffusion of AMPA receptors [281, 282]. These processes prove that glucocorticoids act via transcription factors but they also participate in the early and rapid non-genomic events of the adaptive response [272]. The corticosteroid that is widely used for *in vitro* and *in vivo* experiments is dexamethasone (Dexa), which is a synthetic corticosteroid that mimics the action of physiological and endogenous stress hormones and binds to the same receptors exerting similar effects to cortisol [283].

4.3. Allostasis and allostatic load

Despite the fact that the term “stress” holds a negative meaning, exposure to stress can in fact increase an organism's performance and help in building resilience. The stress system helps in survival by activating the fight or flight mode. Acute activation of the HPA axis upon stressful events, the so-called eustress, increases the overall functions of the organism in a an inverted “U-shape” manner in which low to moderate levels of stress increase cellular, mitochondrial and organismal functions while intense or sustained stress exhibits harmful cellular, mitochondrial and organismal effects (Figure 7) [284-287]. In the brain, the term allostasis refers to the adaptive systemic and neural responses that are triggered by possibly threatening and stressful situations. These processes involve glucocorticoids such as cortisol, hormones of the sympathetic system such as adrenalin, hormones of the parasympathetic nervous system, metabolic hormones and inflammatory cytokines. Allostatic load is a paradoxical phenomenon in which the same hormones and other mediators that assist the body and brain to adapt to stressful situations are also able to trigger detrimental pathophysiologal mechanisms when overproduced [288, 289]. Allostasis can promote brain plasticity mechanisms and provide both the developing and the adult brain with considerable adaptive capacity through structural and functional plasticity. Therefore, a stressful situation can trigger alterations in neuronal architecture such as neuronal substitution and synapse turnover. Structural and functional plasticity due to allostasis is largely prominent in the hippocampus. The hippocampus is a brain structure important for learning and memory and one of the very few brain regions in which

neurogenesis takes place throughout life. Apart from the hypothalamus, hippocampus was also found to highly express both stress and sex hormone receptors, making it receive both the positive outcomes of allostasis but also quite susceptible to increased glucocorticoid levels and allostatic load [288, 289]. High levels of cortisol directly affects the hippocampus both structurally and functionally. Stressful life episodes have been linked to lower hippocampal volumes in humans, as well as structural changes in the hippocampus with stress provoking the shrinking of dendrites and loss of neurons [290-294]. As a result, there is an increased risk for the development of stress-associated disorders such as depression [283].

As mentioned above, sustained or intense stress leads to over-activation of the HPA axis and to allostatic overload during which over-accumulation of cortisol becomes deleterious for the brain but also for other systems in the body (Figure 7) [274]. Numerous pathological diseases are related to dysregulated HPA axis activity. Primarily, dysregulation of the HPA axis is strongly correlated to mental health disorders including depression, schizophrenia, bipolar disorder, anxiety disorders and posttraumatic stress disorder (PTSD) as well as to several other biomedical diseases including but not limited to hypertension, Type II diabetes, chronic fatigue syndrome, chronic pain and fibromyalgia. Persistent psychological stress is also linked to modified glucocorticoid hormone profiles which are manifested by changed patterns in basal glucocorticoid hormone release and/or changes in the response to acute stressors [288].

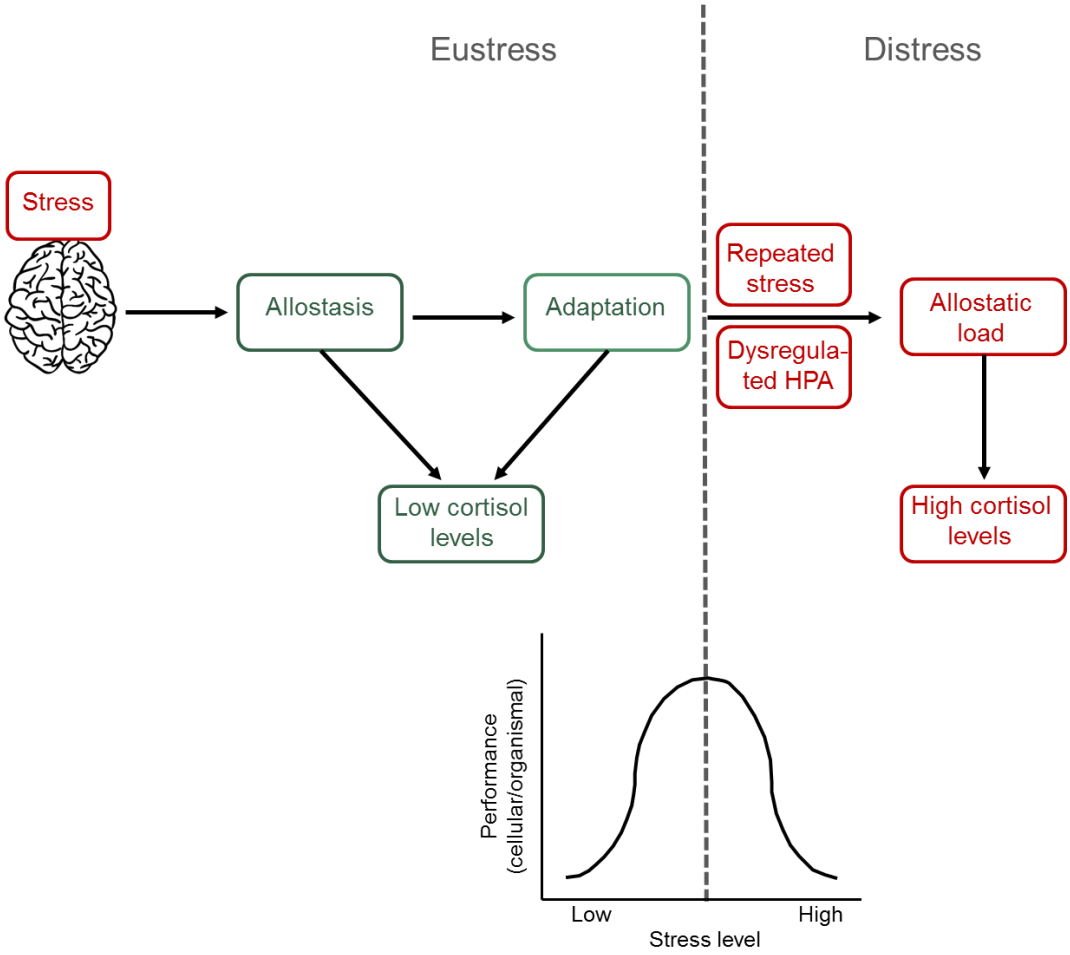

Figure 7. Eustress and distress. Stress can have both positive and negative effects on cellular and organismal health. Acute activation of the HPA axis upon stressful events, the so-called eustress, increases the overall functions of the organism in a an inverted “U-shape” manner in which low to moderate levels of stress increase cellular, mitochondrial and organismal functions while intense or sustained stress exhibits the opposite effects with harmful consequences. In the brain, allostasis refers to the adaptive systemic and neural responses that are triggered by possibly threatening and stressful situations. Sustained or intense stress leads to over-activation of the HPA axis and to allostatic overload during which over-accumulation of cortisol becomes deleterious for the brain but also for other systems in the body.

4.4. Corticosteroid stress and oxidative stress

Oxidative stress is also prominent during hormonal/corticosteroid stress while corticosteroid stress has been shown to increase oxidative stress. Interestingly, oxidative stress and mitochondrial dysfunction are common in mental, neurodegenerative and age-related diseases while additional corticosteroid stress intensifies these problems [284]. Corticosteroids such as cortisol have been shown to increase ROS production leading to an oxidative stress state [284, 295, 296] with increased oxidation markers such as 8-oxo-dG and 8-iso-prostaglandin F2α (IsoP) as demonstrated in the saliva of chronically stressed women [284]. Consistently, it has been shown that a non-genomic glucocorticoid receptor-mediated pathway can also lead to increased ROS production with concomitant activation of the ERK/CREB/PGC1-α signaling pathway as a stress compensation mechanism in the rainbow trout [296].

4.5. Corticosteroid stress and mitochondria

Mitochondria seem to fulfil essential roles during the stress response and adaptation due to 3 main reasons: a) they produce the energy required for the completion of all stress response processes on genetic, epigenetic, cellular and molecular level, b) they produce and metabolize glucocorticoids and steroid hormones, c) they respond to metabolic as well as to stress mediators. Every part of the stress response demands energy: hormone biosynthesis including gene expression, transcription and translation, neurotransmitter release, sympathetic and parasympathetic activation, brain structural and functional plasticity and behavioral adaptations are all dependent on cellular energy production which takes place primarily at mitochondria as previously discussed [289].

Mitochondria are also able to synthesize steroid hormones including estrogens, progestogens such as progesterone, androgens such as testosterone, glucocorticoids and mineralocorticoids such as aldosterone [297]. Glucocorticoid synthesis is conducted at the *zona fasciculata* of the adrenal cortex. It takes place through a series

of sequential molecular reactions within mitochondria via cholesterol transportation through the mitochondrial membranes and its subsequent metabolism. Nicotinamide nucleotide transhydrogenase (NNT) is a protein present on the inner mitochondrial membrane where it uses MMP to regenerate mitochondrial antioxidants as well as NADPH thus affecting energy metabolism [298]. Mutations on the NNT gene resulted in severe familial hypocortisolemia [299, 300]. Corticosterone levels were lower and release of the hormone was reduced by 50% during acute psychological stress in NNT-deficient mice [301] indicating that proper mitochondrial function is fundamental for steroidogenesis and corticosterone secretion during stress [289].

As in the case of ROS, mitochondria are not only the producers of glucocorticoids but are also largely impacted by them. Dexamethasone, a synthetic glucocorticoid mimicking the effects of cortisol, exhibited biphasic effects on mitochondrial functions in cultured neurons. Mitochondrial oxidation, MMP, and mitochondrial calcium-carrying capacity were modulated by long-term exposure to corticosterone in an inverted “U”-shape manner. Treatment with low concentrations of corticosterone exerted a neuroprotective effect while treatment with high corticosterone concentrations increased the kainic acid (KA)-caused toxicity in cortical neurons. The biphasic effects were explained by the formation of a complex between glucocorticoid receptors and Bcl-2 (anti-apoptotic protein) and its translocation into mitochondria upon acute corticosterone treatment (with both high and low concentrations). Long-term exposure (3 days) to high levels of corticosterone lead to lower GR and Bcl-2 complex levels in mitochondria [285]. In addition, the inverted U-shaped effect of glucocorticoids has been confirmed in other studies. Corticosterone regulated behavioral assays performance in an animal-model in an inverted U-shaped way [302]. Long-term potentiation also showed an inverted “U”-shaped relationship with glucocorticoid levels [303, 304]. Low glucocorticoid concentrations exerted trophic effects on neuronal dendrite formation and branching as well as on survival [304] while higher concentrations were harmful for neuronal survival [285]. Therefore, it can be said that the regulation of mitochondrial functions by chronic administration of high glucocorticoid concentrations mimics the allostatic load observed as a clinical result of chronic stress in organisms [285]. Moreover, in other cell lines (e.g. lymphoma cells) long-term glucocorticoid treatment was found to increase mitochondrial ROS levels and decrease the activity of mitochondrial ETC complexes [305, 306] possibly due to

binding of GRs on glucocorticoid response elements on the mtDNA and subsequent alterations on mtDNA gene expression [307, 308]. Glucocorticoids significantly decreased respiration state 3 and cytochrome c oxidase (complex IV) activity in isolated mitochondria from rat kidney. These results support that glucocorticoids target cytochrome c oxidase (complex IV) on the ETC [309].

Cortisol regulates genomic and non-genomic changes in MMP in hippocampal neurons. Regarding non-genomic actions, a stressful situation increases cortisol concentrations which binds to and activates GR. Activated GR binds to mitochondrial membranes and affects MMP. Regarding genomic actions, GR interacts with other cellular molecules (e.g. heat shock proteins 90/70/40) translocates to the nucleus, attaches to DNA and exhibits transcriptional activity. Downstream of GR stimulation, Bax (pro-apoptotic protein) can be upregulated and translocate to mitochondria where it binds to modulator of apoptosis-1 (MAP-1) and causes MMP alterations. These genomic and non-genomic cortisol-induced alterations in the MMP can lead to the release of cytochrome c from mitochondria to the cytoplasm and to further activation of caspases cascade and apoptosis (Figure 8) [310].

A direct action of glucocorticoids on mitochondrial transcription has been proposed according to studies on hormone distribution in liver cells which showed a very fast uptake of 3H-cortisol in mitochondria in a similar cortisol uptake in nuclei [311-313]. GR was found in liver mitochondria of adrenalectomized rats that translocated from the cytoplasm after hormonal induction [314]. Chromatin immunoprecipitation (ChIP) assay verified GR binding locations in the D-loop of the mitochondria on isolated mitochondria from hepatocarcinoma HepG2 cells, suggesting a direct action of GR on mitochondrial transcription [308].

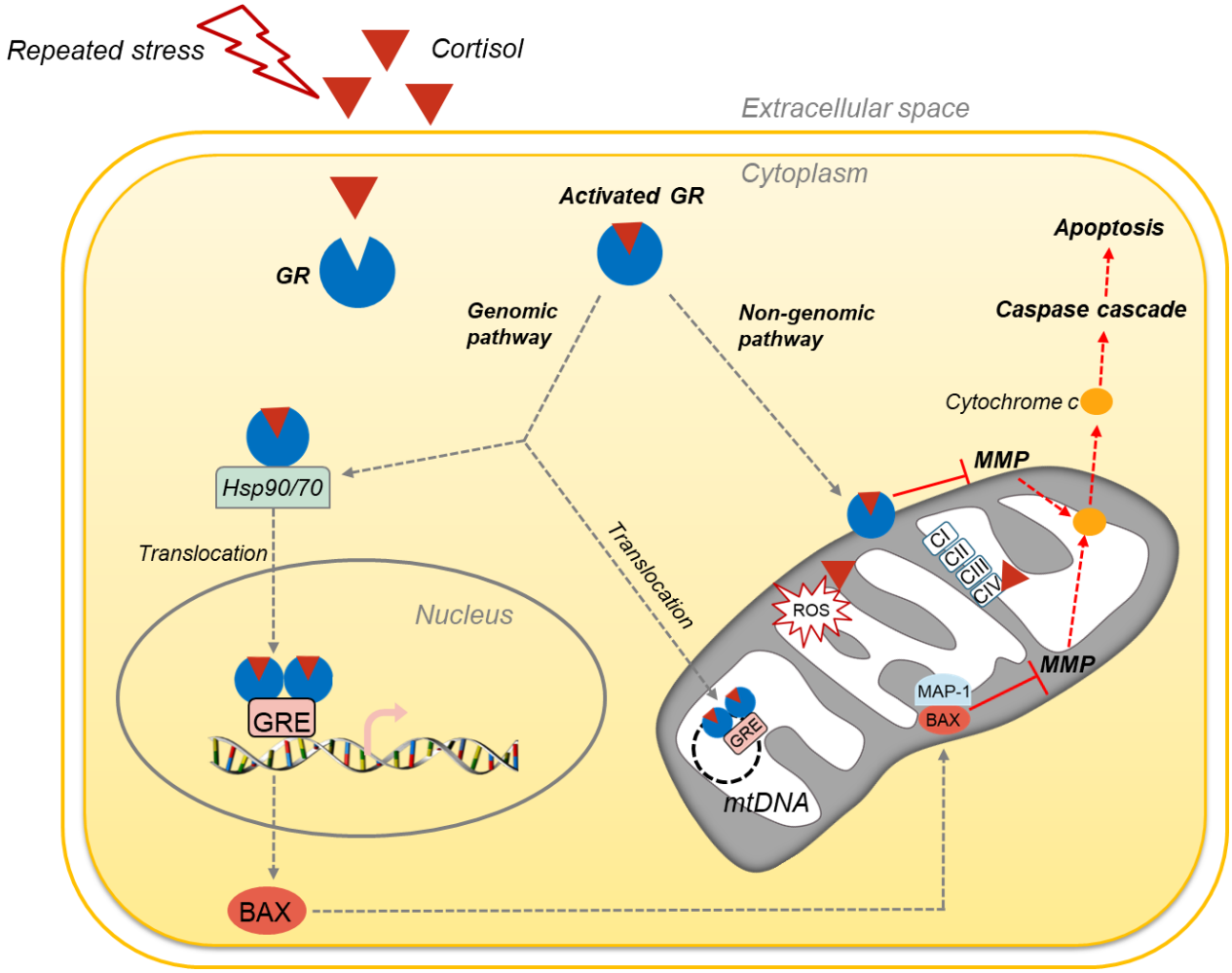

Figure 8. The genomic and non-genomic effects of corticosteroids. Cortisol regulates genomic and non-genomic changes in MMP in hippocampal neurons. Regarding non-genomic actions, a stressful situation increases cortisol concentrations. Cortisol binds to and activates GR. Activated GR binds to mitochondrial membranes and negatively impacts MMP. Cortisol also has been shown to increase the production of ROS and to impair mitochondrial complex IV. Regarding genomic actions, GR interacts with other cellular molecules (e.g. heat shock proteins 90/70/40) translocates to the nucleus, attaches to DNA and exhibits transcriptional activity. Downstream of GR stimulation, Bax (pro-apoptotic protein) can be upregulated and translocate to mitochondria where it binds to modulator of apoptosis-1 (MAP-1) and causes MMP alterations. Activated GR also translocate into the mtDNA and cause transcriptional changes which lead to impaired ETC structural parts. These genomic and non-genomic cortisol-induced alterations in the MMP can lead to the release of cytochrome c from mitochondria to the cytoplasm and to further activation of caspases cascade and apoptosis.

5. Tau protein and tauopathies

Neurodegenerative diseases are often characterized by over-accumulation of intracellular insoluble proteins in neurons (e.g. amyloid-beta aggregates in Alzheimer`s disease, alpha-synuclein clumps in Parkinson`s disease and huntingtin aggregates in Huntington's disease) [315, 316]. Tau is a microtubule-associated protein mainly expressed in neurons and in physiological conditions has an essential role in microtubule dynamics as it binds to and stabilizes the microtubules while free and bound tau are in a dynamic equilibrium that is modulated via phosphorylation and de-phosphorylation [317]. Phosphorylation of tau is facilitated by kinases which cause the detachment of the protein from microtubules and their subsequent depolymerization. De-phosphorylation of tau is mediated by phosphatases which leads back to the binding of tau to microtubules [9]. Tau is primarily found in the cytosol and is mainly localized in axons of neurons while it is implicated in the anterograde axonal transport (the transportation of proteins, lipids , nucleic acids, organelles and synaptic vesicles along microtubules) [317, 318]. Tau has been found in relatively low levels in cell types other than neurons such as oligodendrocytes and astrocytes [319]. In many neurodegenerative diseases, such as Alzheimer's disease, tau metabolism is modified [317, 320]. Tauopathies are a group of neurodegenerative diseases characterized by an abnormal accumulation of tau within neurons, excessive tau hyperphosphorylation at abnormal epitopes (and subsequent detachment from microtubules) and formation into neurofibrillary tangles [9]. There are two types of tauopathies. In primary tauopathies such as sporadic multiple system tauopathy, Pick's disease and frontotemporal lobar degeneration, tau is the principal contributing element of the neurodegenerative process and mutant variants of tau proteins have been identified [9, 321]. Secondary tauopathies such as Alzheimer`s disease, chronic traumatic encephalopathy and Creutzfeldt–Jakob disease are diseases with diverse causes in which tau has also a major role but other important factors are involved in the pathogenesis of the disease (e.g. amyloid-beta plaques in Alzheimer`s disease) [9, 322]. Although abnormal tau is linked to neurodegeneration, the mechanisms involved in tau-induced neuronal injury and death are not fully comprehended to date.

5.1. Tau structure

In humans, the different tau proteins are encoded by one gene: the microtubule-associated protein tau (MAPT) gene which contains 16 exons present on chromosome 17q21 [323-327]. Different splicing of exons 2, 3 and 10 leads to the expression of six distinct tau isoforms (0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R) which all exist in the adult human brain. These isoforms can be distinguished based on their N-terminal by the existence of none, one or two additions (0N, 1N or 2N respectively) as well as based on their C-terminal by the presence of either three (3R) or four (4R) microtubule-binding repeats [324, 328, 329]. Although the six tau variants seem to possess similar functions, in reality each one of them potentially has a defined and different physiological role. The scientific interest largely focuses on the 3R and 4R isoforms which are variants derived from the splicing of exon 10 and are being expressed physiologically to the same extent (1:1 ratio) in most mature brain areas [330, 331]. However, disruptions of this ratio are linked to specific tauopathies that can be categorized into three subgroups, namely 3R, 4R or 3R/4R, according to which isoform is identified in pathogenic aggregates thus enabling the onset of the disease [322]. 4R tau possesses a higher affinity for microtubules compared to the 3R isoform therefore inducing microtubule assembly more efficiently [328, 332]. Additionally, the two isoforms are able to differentially affect the mobilization of mitochondria within microtubules. Although the 4R isoform decreases the presence of mitochondria in axons more profoundly than the 3R isoform, 3R tau increases the number of mitochondria transportation towards the retrograde direction to a greater extend [323, 333].

Tau is structurally organized in an amino acid sequence that enables it to bind to and interact with different agents and to execute its functions. Tau contains mainly four subdomains: the N-terminal projection area, the proline-rich area, the microtubule-binding area and the C-terminal domain [334, 335]. As mentioned before, tau is able to bind and interact with microtubules which is facilitated by the microtubule-binding area in conjunction with the proline-rich surrounding domains. Tau is a largely soluble, naturally unfolded protein that possesses a highly flexible but limited secondary structure [336]. However, when it binds to and interacts with other proteins, tau is able to form local conformations [337]. Moreover, it has been demonstrated that soluble

tau changes its structure to a paperclip shape which might prevent tau aggregation [338]. Disruption of the paperclip shape leads to a higher proneness for tau aggregation [338, 339].

Tau often undergoes post-translational modifications. The most commonly occurred post-translational tau modification is phosphorylation. Tau is a phosphoprotein comprising 85 probable phosphorylation sites on the longest tau variant containing 35 threonine, 45 serine and 5 tyrosine residues [323, 340, 341]. Free and microtubule-bound tau are in a dynamic equilibrium that is modulated via phosphorylation and de-phosphorylation [317]. The phosphorylation state of tau depends on the activities of several protein kinases and phosphatases [334, 342]. Phosphorylation of tau is facilitated by kinases which cause the detachment of the protein from microtubules and their subsequent depolymerization. De-phosphorylation of tau is mediated by phosphatases which leads back to the binding of tau to microtubules [9]. Several different kinases have been shown to be implicated in the site-specific tau phosphorylation including PKA, PKC, CaMKII, MAPK, AMP-activated protein kinase (AMPK), the glycogen synthase kinase (GSK) 3α/β, tau-tubulin kinase 1/2 (TTBK1/2), cyclin-dependent kinase 5 (Cdk5) and tyrosine kinases of the Src family (e.g. Fyn and Src) [343]. In particular, Cdk5 and GSK3β are believed to have an important role in the pathogenesis of tauopathies by promoting increased phosphorylation [335, 344]. To balance the activity of kinases, a number of protein phosphatases such as protein phosphatase 1 (PP1), PP2A, PP2B, PP2C and PP5 are involved in tau de-phosphorylation [218, 342]. Of those, PP2 is thought to be the principal phosphatase in the brain contributing to around 70% of the total tau phosphatase activity there. Furthermore, it was indicated that PP2 activity was impaired by 50% in AD brains resulting in increased tau phosphorylation [345].

5.2. Tau physiological functions

Tau is a multi-purpose protein that has several important roles within neuronal cells. Tau is mainly located in neuronal axons in the adult brain of humans [324, 346]. Normally, around 90% of tau is bound to microtubules [347] thus one of tau's native and principal functions in axons is to promote microtubule assembly and stability [323,

348]. Tau is able to efficiently bind to microtubules through specific sites on its microtubule-binding and proline-rich areas called KXGS motifs [349]. Physiologically, the ability of tau to bind microtubules is a largely dynamic procedure and depends, among other factors, on the tau isoforms, post-translational modifications and mutations [335]. Regarding post-translational modifications, tau's binding capacity is primarily regulated by its phosphorylation state [340, 350]. Kinase-induced tau phosphorylation causes detachment of the protein from microtubules and additionally depolymerization. In contrary, phosphatases facilitate tau de-phosphorylation which enables the protein to attach to microtubules [351]. What reduces tau's binding capacity the most is the phosphorylation of the aforementioned KXGS motifs [341, 352]. Detachment and binding of tau to microtubules follows recurrent cycles that are essential for regulation of microtubule stability as well as for regulation of successful axonal transport [341]. Tau is implicated in the anterograde axonal transport which is the transportation of proteins, lipids, nucleic acids, mitochondria and synaptic vesicles along microtubules. Once tau binds microtubule, it regulates axonal transport by influencing the motility of two proteins: kinesin and dynein. Axonal transport of cargoes enables the distribution of organelles and biomolecules across neurons and is thus crucial for a healthy synaptic function. Consequently, pathological states of tau can result in the impairment of this transport compromising synaptic function and plasticity [353]. Furthermore, tau was also demonstrated to interact with and affect the actin cytoskeleton. Tau is able to bind to filamentous actin and promote the formation of aligned actin bundles which eventually causes changes in the organization of the cytoskeleton [323]. Under physiological conditions, dendrites contain a low quantity of tau which plays some roles there. It is suggested that tau post-synaptically targets Fyn kinase in dendrites where it binds it via PXXP motifs in its proline-rich domain ultimately promoting accumulation of Fyn to NMDA receptors [354, 355]. The emergence of tau in the post-synapse might possess a role in influencing synaptic plasticity and thus memory formation [356, 357]. Apart from axons and dendrites, tau has also been identified in the nucleus. Nuclear tau might be implicated in the protection of genomic DNA, nuclear RNA as well as cytoplasmic RNA, supporting their integrity, functionality and longevity [358-360].

5.3. Pathological aggregation of tau

In pathological situations, modifications in tau's properties can lead to the protein's aggregation which is a common feature in numerous neurodegenerative diseases. The transformation of normal soluble tau protein into pathological fibrillary tau aggregates is believed to be a process of multiple steps [324]. Since tau hyperphosphorylation and aggregation are both prominent in the AD brain, abnormal tau phosphorylation has been proposed as a triggering mechanism of tau aggregation [361]. Tau hyperphosphorylation may be a consequence of the imbalance between certain tau kinases and phosphatases and their activities, leading to either an increased phosphorylation rate and/or a decreased de-phosphorylation rate of tau [362]. As a result, hyperphosphorylated tau has a lower binding affinity to microtubules compromising tau's physiological microtubule-stabilizing function [363, 364] and eventually leading to microtubule depolymerization [351]. Tau's affinity for microtubules seems to be largely diminished by phosphorylation of KXGS motifs across the microtubule-binding area (especially S262) as well as across the flanking tau areas (especially S214) [365, 366]. The disconnection of tau from microtubules results in an aberrant rise of unbound free tau in the cytosol [367]. Increased cytosolic tau levels can make tau significantly more prone to misfolding and to the formation of non-fibrillar deposits of the protein, termed pre-tangles, which constitute an early pathological feature. Subsequently, tau undergoes structural changes resulting in the formation of paired helical filaments (PHF). The conversion from pre-tangles into PHF comprises the development of distinctive β-sheet-like structures [324, 368]. As a last step, PHF further accumulate to compose more organized aggregates and finally form insoluble neurofibrillary tangles (NFT) within neurons (Figure 9). The formation of NFT in conjunction with impaired cytoskeleton dynamics disturb the physiological axonal transport and thus promotes synaptic dysfunction and proneness to neurodegeneration [369, 370]. Also, modifications of tau *per se*, for example mutations of the MAPT gene, can also promote tau aggregation. Specifically, the tau mutations ΔK280, P301L and P301S are identified in the neurodegenerative disease frontotemporal dementia with parkinsonism-17 which also features the emergence of hexapeptide motif PHF6. However, the exact mechanism that underpins the pathogenesis of tau-induced neurodegeneration in tauopathies has not been

completely resolved yet. Although the increased levels of insoluble hyperphosphorylated aggregated tau, appearing as NFT, are regarded as a pathological characteristic in tauopathies [371] with their local accumulation being associated with the intensity of the cognitive impairment in AD [372], the neurotoxicity of NFT themselves is in dispute. Interestingly, evidence showed that small soluble tau oligomers are the most harmful tau forms inducing neurotoxicity and synaptic dysfunction [373]. Although, NFT seem to conduce to the progression of the disease [330]. Moreover, a growing body of evidence has been associating tau toxicity with neuroinflammation. As a matter of fact, aberrant tau species were linked to reactive microglia and to increased concentrations of pro-inflammatory cytokines including interleukin-1β [374]. Chronic neuroinflammation can promote synaptic loss and cognitive impairment (Figure 9). Prevalent therapeutic protocols targeting tau include therapeutic decrease of tau, anti-aggregation medicines (control of tau phosphorylation and obstruction of tau aggregation), regulators of tau gene expression in the form of antisense therapies, tau passive immunotherapy and tau vaccines [9].

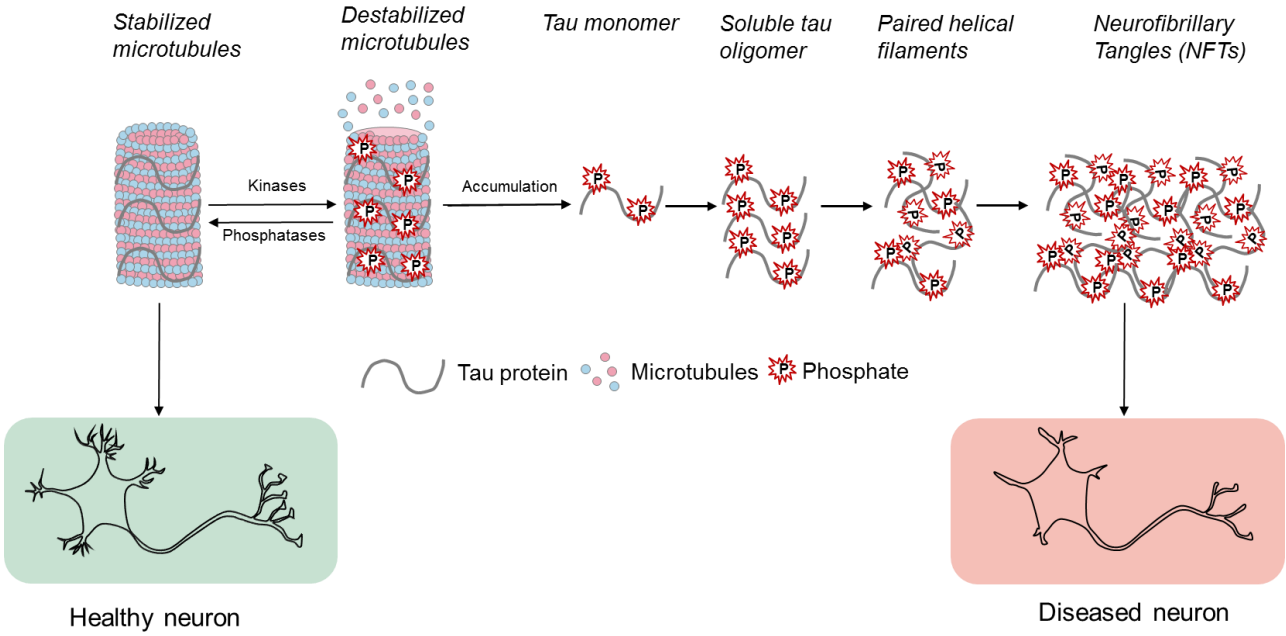

Figure 9. Stages of pathological tau hyperphosphorylation and aggregation. In physiological concentrations, tau facilitated microtubule assembly and contributes to the stabilization of the cytoskeleton. Tau is phorphorylated by kinases and in abnormal situations (e.g. mutations)

tau hyperphosphorylation is taking place leading to the destabilization of microtubules. Increased cytosolic tau levels can make tau significantly more prone to misfolding and to the formation of non-fibrillar deposits of the protein, termed pre-tangles, which constitute an early pathological feature. Subsequently, tau undergoes structural changes resulting in the formation of paired helical filaments (PHF). As a last step, PHF further accumulate to compose more organized aggregates and finally form insoluble neurofibrillary tangles (NFT) within neurons. Overaccumulation of hyperphosphorylated tau protein is toxic for the neurons and has been linked to many tauopathies including AD.

5.4. Tau and mitochondria

Emerging evidence underpins the impairment of mitochondria in tauopathies, including decreased bioenergetics, abnormal mitochondrial dynamics and morphology and hindered axonal transfer of mitochondria [375-380]. Abnormal tau species damage mitochondrial function promoting neuronal degeneration. The most relevant tau-affected mitochondrial functions will be discussed below.

5.4.1. Anterograde axonal transport

As mentioned before, tau facilitates and participates in the transport of cargoes across neuronal axons, including mitochondria. In K3 mice carrying the K369I tau mutation, mitochondrial anterograde axonal transport was negatively impacted leading to fewer mitochondria at the synapse [381]. Since mitochondria are accountable for the energy production and calcium buffering, a decrease in their number at the synapse might cause synaptic degeneration and even neuronal death [319]. One explanatory mechanism is that tau interacts with and isolates JIP1 (c-Jun N-terminal kinase-interacting protein 1) which is linked to the complex of kinesin motor protein [382]. By isolating JIP1 in the cell body, aberrant tau blocks its carriage to the axon which inhibits the kinesin motor complex formation and negatively influences the kinesin-mediated anterograde mitochondrial transport [381, 382]. Milton or Miro are adaptor proteins participating in the axonal mitochondrial transport. Their knockdown intensified tau

phosphorylation in transgenic human-tau-expressing *Drosophila* and caused neurodegeneration through the involvement of partitioning defective-1 protein (PAR-1) [383]. This finding suggested that a deprivation of axonal mitochondria induces tau phosphorylation and neurodegeneration. Neurons derived from mice carrying the P301L tau mutation demonstrated a reduction in mitochondrial number in the neurites paired with clustering of mitochondria around the nucleus [384]. In agreement, a 50% decrease in mitochondrial content was identified in neuronal axons of P301L tau knock-in mice (KI-P301L) [380]. Overall, these results suggest that abnormal tau highly influences the axonal transport of mitochondria causing a mitochondrial deprivation at the synapse which can result in synaptic degeneration and cell death.

5.4.2. Bioenergetics

Tau-mediated decline in bioenergetics was initially observed in mice expressing the P301L tau mutant (pR5 mice) in which components of the mitochondrial complexes I and V were downregulated, mitochondrial respiration was decreased in an age-dependent decrease manner, ATP concentrations and complex I activity were reduced and ROS levels were increased in comparison to wild-type littermates [375, 385]. Similar results were observed *in vitro* in P301L cells (SH-SY5Y cells carrying and overexpressing the P301L tau mutation) [317]. The same cells displayed a reduction in MMP as well as in the spare respiratory capacity and in the maximal respiration compared to wild-type tau cells [386] suggesting that overexpression of tau might inhibit the activity of complex I. Similarly, a lower mitochondrial complex I activity was additionally identified in the brain of mice carrying the P301L tau mutation (rTg4510 mice) but was accompanied with an increase in MMP [387]. In contrary, a recent study conducted in mitochondria isolated from SH-SY5Y cells indicated that tau reduces MMP through poration of mitochondrial membranes leading to impaired mitochondrial structural integrity such as swelling [388]. The MMP reduction was paired to the release of cytochrome c. Interestingly, those effects were not followed or related to the opening of the mPTP but were rather dependent on the emergence of non-selective ion-conducting tau nanopores induced by the attachment of oligomeric tau on cardiolipin-abundant membrane areas [388].

5.4.3. Mitochondrial permeability transition pore (mPTP)

mPTP opening causes permeability of mitochondrial membranes, disturbance of mitochondrial function and efflux of apoptotic agents into the cytosol. The precise composition of the mPTP is still unresolved but evidence claims that it contains proteins such as translocator protein (TSPO) and voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, adenine nucleotide translocator (ANT) in the inner mitochondrial membrane as well as cyclophilin D (CypD) inside the mitochondrial matrix. Genetic tau ablation restrained mPTP formation via reduction of the CypD protein in the hippocampus of homozygous tau-/- mice in comparison with wild-type littermates [389]. Additionally, interaction between phosphorylated tau and VDAC was apparent in the brain of AD subjects at different Braak stages (from I to V) and in 3xTgAD and 13-months-old APP/PS1 (human amyloid precursor protein/ mutant human presenilin 1) transgenic mice [390]. A recent study demonstrated that TSPO ligands enhanced MMP in both wild type tau- and P301L tau-overexpressing neuroblastoma cells [391].

In addition to those impairments, tau has been proved to inhibit mitophagy through its interaction with parkin [392-395] as well as to affect mitochondrial dynamics by promoting mitochondrial network elongation mediated by an increase in mitofusin 2 (MFN2) levels and a dynamin-related protein 1 (DRP1) mislocalization [378, 393, 396, 397]. In summary, tau interacts with mitochondria and highly affects their health and integrity by a) inhibiting anterograde axonal mitochondrial transport and therefore depriving the synapse of mitochondria and causing mitochondrial perinuclear clustering, b) disturbing mitochondrial bioenergetics by diminishing complex I activity, disrupting the MMP, reducing ATP levels and increasing ROS, c) affecting the mPTP opening via binding on mitochondrial proteins like voltage-dependent anion channel (VDAC) and complex V subunits, d) affecting mitophagy and mitochondrial dynamics [9].

6. Plants and natural compounds in the battle against diseases

Since ancient times, people turned to nature and plants in search for medicines that would alleviate their diseases. The traditional use of natural medicinal plants has led to the discovery of novel efficient agents used in the prevention or treatment of numerous diseases. Several thousands of natural compounds (~10.000) that are now used in modern medicine, had been initially used by traditional healers for centuries. An estimated annual $100 billion value is being spent on natural medicines globally while some countries highly depend on phytochemicals for the treatment of diseases because modern medicines are not easily and readily available there. Therefore, the use of medicinal plants as well as research conducted on them continues to prosper while the medicinal potential carried in nature is still vastly untapped. Currently, chronic diseases are at their peak possibly due to the modern human life style as well as eating habits. At the same time, the failure of synthetic drugs at our disposal to efficiently treat diseases in conjunction with their adverse and side effects make the use of natural drugs relevant and even needed. Thus, the use of medicinal plants and their extracts as well as plant-derived compounds could help in developing therapeutic or preventive strategies to combat chronic diseases [398]. The plant-derived compounds, called phytochemicals or natural compounds, are categorized according to their structural characteristics and include phenols and phenolic acids, tannins, flavonoids, terpenoids, alkaloids, steroids and saponins among others [399]. Flavonoids that are of particular interest can be further classified as flavonols, flavanols, flavones, flavanones, isoflavonoids and anthocyanidins. All these classes of compounds are secondary metabolites of plants and they present interesting and remarkable bioactivities [400]. Natural compounds have displayed potential preventive and therapeutic effects against many chronic conditions while their efficacy can be linked to a variety of molecular scaffolds found in plant extracts [401]. Plants are excellent sources or phenols and polyphenolics. Both plant extracts and plant-derived phenolics have exhibited bioactivities such as anti-inflammatory, anticancer, antibacterial, anti-diabetic, anti-aging and activities against cardiovascular disease and neurodegenerative diseases among other [402].

To understand the importance of phytochemicals and plant research, more than half of the anticancer drugs in use are derived from plants. Natural drugs have also been

shown to reduce toxicity of existing anti-cancer therapies such as the nephrotoxicity, hepatotoxicity and neurotoxicity which are adverse effects of some chemotherapeutics, namely doxorubicin and cisplatin. Moreover, two single compound used as AD drugs are derived from plants: (i) rivastigmine and (ii) the acetylcholine-esterase inhibitor, galanthamine, derived from the Galanthus species (*Galanthus caucasicus* and *Galanthus woronowii*). Some of the phytochemicals that have shown remarkable bioactivities are curcumin, resveratrol, quercetin, hesperetin, naringenin, silybin, genistein, berberine, cannabidiol, sinapic acid, lycopene, N-acetylcysteine, catechins and caffeic acid [24, 401].

The protective role of phytochemicals, especially of phenolics, can be linked to their antioxidant activity, since overproduction of ROS but also RNS is implicated in the pathogenesis of several chronic diseases while these compounds have been found to scavenge ROS, and enhance antioxidant defense mechanisms [400].

Moreover, the current mitochondrial cascade hypothesis postulates mitochondrial dysfunction as a central pathomechanism in many diseases including stress-related as well as neurodegenerative disorders [28, 403, 404]. Taking into account their primary role in those disorders as well as in aging and in the early stages of AD, mitochondria constitute promising targets for therapeutic strategies. For this reason, pharmacological studies are directed in enhancing mitochondrial functions such as ATP production and respiration or in reducing mitochondrial harmful by-products such as ROS [24].

Therefore, in the next parts we will describe specific plant extracts and natural compounds that were shown to possess a) anti-oxidant properties with emphasis on the case of Honeubush (*Cyclopia* species), b) anti-stress properties with emphasis on the case of *Rhodiola rosea* and c) effects against tauopathies with emphasis on the xanthone mangiferin.

6.1. Oxidative stress-directed phytochemicals

Overproduction of oxidants (ROS and RNS) is apparent in several diseases and is responsible for their pathogenesis as oxidants react with and damage biomolecules such as DNA, lipids and proteins. Scavenging of these oxidants is considered to be

an effective strategy to regulate oxidative stress levels in diseased organisms, thus, phytochemicals with antioxidant properties might possess a key role in the prevention and treatment of oxidative stress-related chronic diseases [405, 406]. Antioxidant phytochemicals are abundantly present in numerous foods (vegetables, fruits, cereal grains, microalgae and edible macrofungi) and in medicinal plants [407, 408]. For example, fruits rich in antioxidant compounds include berries, grapes, pomegranate, plum, dates and guava among many other [409-411]. The antioxidant and radical scavenging activities of natural polyphenols are linked to the presence of hydroxyl groups on their aromatic rings [412]. However, the presence and concentration of polyphenols in plants is not stable as it highly depends on the geographic region that the plant grows, on both the growing and harvesting seasons as well as on storage conditions [400]. An indicator of the antioxidant capacity of food, fruits, plants and their extracts is the total phenolic content (TPC) identified with the Folin-Ciocalteu method which measures the amount of polyphenols not only in foods but also in urine, plasma and even in different organs [413, 414]. There seems to be a direct relationship between TPC and the total antioxidant activity in extracts of different fruits with fruits demonstrating higher TPC resulting in stronger antioxidant activity [405, 415]. Phytochemicals including polyphenols have been shown to exhibit antioxidant effects not only *in vitro* but also in studies involving humans. For instance, a diet rich in fruits and vegetables containing high concentrations of antioxidant compounds has been demonstrated to enhance the antioxidant serum/plasma capacity [416, 417]. Additionally, polyphenols can bind to red blood cells and increase the total scavenging ability of human blood [418]. Both plants and their extracts as well as foods, are of complex phytochemical compositions and contain dozens to hundreds of compounds. Therefore, those compounds could have additive, synergistic or even antagonistic effects that would be responsible for their antioxidant properties [419, 420].

Many plant extracts and natural compounds have been proved to have both antioxidant as well as mitochondria-enhancing properties. Those agents are of particular interest in research studies because their properties make them effective in improving or preventing some of the symptoms in neurodegenerative diseases and stress-related diseases. As an example, resveratrol is a polyphenol from white hellebore (*Veratrum grandiflorum*) and a component of several dietary sources such as berries, peanuts and red grape skin or wine [24]. Resveratrol has been shown to

possess several benefits, including antioxidant, anti-aging, and neuroprotective properties. This polyphenol has emerged as a novel natural agent in the prevention and possible therapy of AD as *in vitro* and *in vivo* evidence demonstrated that it exerts a complex mode of actions through the protection of mitochondrial function (it increased activity of respiratory enzymes and oxygen consumption) and the activation of mitochondrial biogenesis (through overexpression of PGC-1α mRNA), through its effect on certain signalling pathways, through its antioxidant effects (by increasing the expression of the ROS-inactivating enzymes GPx1 and SOD1 and by reducing the expression of the ROS-producing enzyme NADPH oxidase 4) as well as through its anti-AD hallmark (Aβ clearance) properties [421-426].

Similarly, the very popular *Ginkgo biloba* extract (GBE) has shown some promising mitochondria-enhancing activities in conjunction with activity against oxidative stress and AD hallmarks. GBE consists of 24-25% flavone glycosides (mainly quercetin, kaempferol, and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B, and C and 2.6-3.2% bilobalide). Several findings through cellular and animal models demonstrate the mitochondria-modulating, antioxidant and free radical scavenging effects of GBE [28, 427-429]. In particular, in vitro studies have shown GBE (0.01 mg/ml) to ameliorate mitochondrial function by improving MMP and ATP levels in pheochromocytoma cells (PC12) cells [430]. GBE improved mitochondrial respiration, stimulated mitochondrial biogenesis and increased ATP production in an AD cellular model (amyloid precursor protein- (APP-) transfected human neuroblastoma cells) [431] as well as reduced Aβ-related increase in ROS/RNS and ameliorated complex I and IV activities [432-434]. It seems that those effects have been mediated via improvement of Hsp70 protein expression and subsequently by activation of the Akt (protein kinase B) pathways [435].

6.1.1. Honeybush (*Cyclopia* species)

Cyclopia is a genus containing 23 species native to the South African fynbos biome and belongs to the Fabaceae family [436]. Its distribution is extended from sandy coastal regions to mountainous areas, based on the species. Honeybush largely

grows between the Eastern and Western Cape areas of South Africa [437]. Several species including *C. subternata*, *C. genistoides* and *C. longifolia* have been traditionally used as herbal teas. Currently, these *Cyclopia* species constitute the bulk of cultivated plant material and supplement the plant material collected in the wild. This process is crucial to meet the growing demand of international markets. The principal product is “fermented” (oxidized) honeybush tea. However, the green (unoxidized) honeybush tea is preferred for nutraceutical extract production due to its increased phenolic content and antioxidant capacity. The stems, leaves and flowers of the plant are harvested and after being processed they are used for producing herbal tea infusion called Heuningtee in Afrikaans. Post harvesting processing includes cutting of the plant material and fermentation which is oxidation at elevated temperatures using baking ovens followed by drying. Fermentation causes a change in color of the plant material from green to dark brown as well as oxidation of the phenolic compounds [437]. The phenolic profile of honeybush varies qualitatively and quantitatively depending on the *Cyclopia* species. Major phenolic constituents belong to xanthone, benzophenone, flavanone, flavone, flavonol, isoflavone and dihydrochalcone subclasses and their respective glycosides. In addition, other types of phenolics have been identified within *Cyclopia* species such as a chalcone, a hydroxycinnamic acid, a flavan-3-ol, three benzaldehyde derivatives, three coumestans, four phenylethanols as well as two pterocarpans. More specifically, identified compounds from *C. subternata* [438], *C. genistoides* [439], *C. longifolia* [440], *C. maculata* [441], *C. pubescens* [442] and *C. intermedia* [443] belong to the xanthone (tetra- and penta-hydroxyxanthone glycosides), flavanone (eriodictyol glycosides), benzophenone (iriflophenone di-O,C-hexosides and maclurin di-O,C-hexosides) and dihydrochalcone (3-hydroxyphloretin-di-C-hexoside) subclasses. The lead compounds of honeybush are considered to be the xanthone mangiferin and the flavanone glycoside hesperidin since their biological activities (antioxidant, neuroprotective, anti-inflammatory among other) have been demonstrated and reviewed in several studies [444-451] but also because they are contained in increased concentrations within some *Cyclopia* species. Particularly, mangiferin has been found in high concentration in aqueous and 70% aqueous-ethanol extracts of *C. genistoides* (6.86 and 9.66 g/100 g extract respectively) and in the aqueous extract of *C. longifolia* (6.38 g/100 g extract) while the highest concentrations of hesperidin have

been found in the aqueous extract of *C. subtern*ata (1.43 g/ 100 g extract) and in the 70% aqueous-ethanol extract of *C. genistoides* (1.73 g/100 g extract) although in much lower concentrations than those of mangiferin [241, 436, 452, 453]. However, the phenolic composition of honeybush does not only depend on the species and the extraction solvent but also on whether the plant has been fermented or not and on the harvesting period and area. Unfermented honeybush species have been found to possess a higher phenolic content compared to their respective fermented form [454, 455]. In terms of time-related compositional changes, harvesting of *C. genistoides* in May, August or September during two successive years resulted in differences in its mangiferin and hesperidin concentration. May and September harvest of year one possessed higher mangiferin levels than the August harvest, however the plant material harvested in September of year two had a remarkably higher mangiferin content than the plant material harvested in May and August. The hesperidin concentration in the plant was mostly stable apart from the lower concentration of the plant material harvested in May of year two [456]. This was also observed in another study which showed that mangiferin content of the plant is reduced by about 12% during the 3-4 month harvesting period, whereas there are no significant modifications in the concentration of other main phenols [437]. Furthermore, different compounds are localized in different parts of the plant. A study on leaves and stems of *C. maculata* demonstrated that hesperidin was primarily found in the stems [457] while higher levels of mangiferin, isomangiferin and eriocitrin, were found in the leaves. In line, hesperidin concentration was higher in the stems of *C. subternata* than in the leaves while the rest of compounds were principally found in the leaves [438].

Increased worldwide consumption of honeybush caused a subsequent increased research interest in the plant in order to reveal new bioactivities and to test its potential use as a nutraceutical [437, 458]. Honeybush extracts have demonstrated anti-obesity [459, 460], anti-diabetic [461, 462], anticancer and antimutagenic [463-470], phytoestrogenic properties [471-474] as well as photoprotective properties [475, 476]. Quite predictably due to their phenolic composition, honeybush extracts have been shown to possess antioxidant activities which are of great importance and interest in the research of oxidative stress-related diseases [454, 477, 478].

In vitro studies

Pre-treatment with honeybush extract (40 and/or 60 µg/ml) protected human umbilical vein endothelial cells (HUVECs) against diesel exhaust particles-induced oxidative stress by reducing ROS, thiobarbituric acid reactive substances (TBARS), conjugated diene levels and inflammatory markers (IL-1α, IL-6, IL-8, VCAM-1 and ATF4) gene expression as well as by increasing the GSH levels and the GSH/GSSG ratio [477]. *C. maculata* and *C. genistoides* extracts attenuated lipid peroxidation in *in vitro* treated brain homogenates from male adult Sprague-Dawley albino rats which was assessed by the 2-thiobarbituric acid (TBA) assay by measuring the malondialdehyde (MDA) formation with *C. genistoides* at 2.500 mg/ml being the most effective. In contrast, the isolated compounds mangiferin and hesperidin did not exhibit any remarkable antioxidant activity as single entities in the TBA assay [479]. Unfermented *C. intermedia* extracts demonstrated the strongest antioxidant activity when compared to other fermented honeybush extracts assessed *in vitro* using the oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) assays with the methanol extract exhibiting the highest FRAP, ORAC and TEAC values. Generally, the unfermented honeybush extracts showed higher antioxidant capacity and higher total polyphenol content than that of the fermented honeybush ones [454]. Aqueous extracts of *C. subternata* and *C. genistoides* showed a total polyphenol content similar to that of green tea while they exhibited either similar or lower antioxidant activity compared to the rooibos, green, black and oolong teas when assessed with *in vitro* radical scavenging ($ABTS^{\bullet+}$), FRAP and inhibition of Fe^{2+}-induced microsomal lipid peroxidation assays. Again, unfermented honeybush demonstrated higher total phenolic content and therefore increased antioxidant activity in all the tested species, except *C. genistoides* whose ability to prevent lipid peroxidation was not impacted by fermentation while it also contained the highest quantities of mangiferin. In terms of single compounds, the most effective $ABTS^{\bullet+}$ scavenger was mangiferin, followed by equal activities of eriodictyol, eriocitrin and luteolin while hesperidin, naringenin and narirutin showed marginal activity in the assay [480]. An aqueous *C. genistoides* extract (240 µg/ml) exhibited a significant free radical scavenging activity *in vitro* measured with the DPPH ((2,2-diphenyl-1-picryl-hydrazyl-hydrate)) assay while both the aqueous and ethyl acetate

extracts significantly attenuated the $FeSO_4$-induced oxidative damage *ex vivo* in pancreatic rat tissue by increasing GSH levels and CAT and SOD activities as well as by decreasing MDA levels and lipid peroxidation. The most potent extract was the aqueous one but demonstrated a weaker activity that that of ascorbic acid [481].

In vivo animal studies

Unprocessed honeybush tea (*C. intermedia* aqueous extract) significantly increased the cytosolic glutathione S-transferase alpha activity and the GSH levels while significantly decreased the oxidized glutathione levels (GSSG) resulting in an increased GSH/GSSG ratio in the liver of rats treated orally for 10 days. Also, rats that received the unfermented honeybush tea showed the highest phenolic intake [455]. Polyphenol enriched extracts of *C. subternata* and *C. genistoides* fed (2.5 g/kg feed) to male Fischer rats significantly enhanced glutathione reductase activity after 28 days in the liver. Interestingly and in contrast to other studies, *C. subternata* decreased GSH content after 90 without significantly affecting the GSH/GSSG ratio despite showing the highest total polyphenol content. 28 day treatment with both *C. subternata* and *C. genistoides* modified the expression of antioxidant defence-related and oxidative stress genes, namely a) antioxidant-related genes: Kif9 (kinesin family member 9), Ptgs1 (prostaglandin-endoperoxide synthase 1) and Serpinb1b (serine peptidase inhibitor clade B member 1b), b) genes linked to ROS metabolism: Txnip (thioredoxin interacting protein) and Xpa (xeroderma pigmentosum complementation group A) and c) oxygen transporter-associated genes: vimentin and Fancc (Fanconi anemia complementation group C) [478].

6.2. Mangiferin- a promising natural compound

Mangiferin (2-C-b-D-glucopyranosyl 1,3,6,7-tetrahydroxyxanthone) is a xanthonoid polyphenol found mainly in the fruit, leaves and bark of *Mangifera indica* and has exhibited multifaceted therapeutic or preventing effects against various diseases. Notably, mangiferin has demonstrated antioxidant, neuroprotective, anti-inflammatory,

anti-diabetic, cardioprotective, anticancer, hepatoprotective, antiviral and immunomodulatory properties [481-489] as well as potent free radical scavenging properties through its ability to chelate iron in Fenton-type reaction [444, 490]. The bioactivities of mangiferin are mainly due to its ability to regulate a few pathways such as MAPK, AMPK, Nrf2 (Nuclear factor erythroid 2-like 2), NF-κB and mTOR (mammalian target of rapamycin) [491-494]. The therapeutic potential of mangiferin can be explained due to the many functional groups embedded in its structure. The compound consists of one glycosidic hydroxyl group, two aromatic rings, one lactonic carbonyl group and nonaromatic secondary hydroxyl groups (Figure 10). The catechol moiety of mangiferin forms a complex with iron and is responsible for the antioxidant action of the compound [446]. Mangiferin is also present in Honeybush plant extracts and especially in the aqueous-ethanol extracts of *C. genistoides* and *C. longifolia* [438-440] and is considered one of the lead compound in those extracts due to its proven bioactivities but also due to its high concentration therein. Mangiferin has been described to exert antioxidant properties [495, 496] in both cellular and animal models as well as neuroprotective and anti-dementia properties mainly in animal models which will be described below in detail [485, 497, 498].

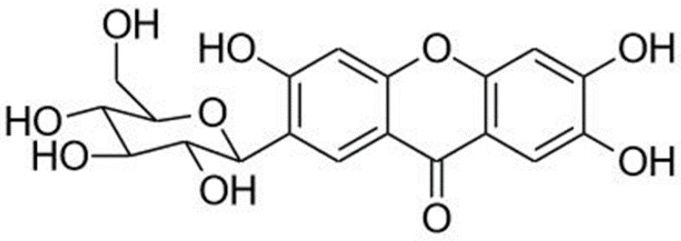

Figure 10. The structure of mangiferin

6.2.1. Mangiferin and neuroprotection

Mangiferin has demonstrated promising effects against neurodegenerative diseases, hypoxia ischemia, cerebral ischemia and other neuropathies via antioxidant, anti-inflammation pathways and especially via the Nrf2/HO-1(Nuclear factor erythroid 2-like 2/ heme oxygenase-1), PI3K/Akt (phosphatidyl inositol 3-kinase/protein kinase B) and ERK1/2 signaling pathways. Nrf2 is a transcription factor which regulates antioxidant pathways and the expression of antioxidant proteins downstream its activation including HO-1 which further fends off free radicals [499]. It has been shown that the Nrf2/HO-1 signaling pathway prevents ischemic injury in the brain by counterbalancing oxidative damage [500, 501]. Mangiferin enhanced the expression of Nrf2 and HO-1 proteins in a cerebral ischemia/reperfusion injury rat model [502]. Therefore, mangiferin is able to protect neurons and glia from oxidative injuries via the Nrf2/HO-1 pathway. Further antioxidant activities of mangiferin include the increase of GSH levels and antioxidant enzyme activities as well as decrease of lipid peroxidation (MDA levels) in Aβ-treated neurons in the prefrontal cortex and hippocampus [484, 491, 503, 504]. Mangiferin significantly enhanced numerous antioxidants enzyme levels such as catalase (CAT) and SOD as a response to oxidative stress [505, 506]. Mangiferin has also been proved to be more efficient in NO scavenging than curcumin [507].

The PI3K/Akt pathway modulates a range of cellular activities including cell survival, metabolism and protein synthesis [508] while it has also been linked to cerebral ischemia and hypoxia [509]. The ERK1/2 signaling pathway plays also important roles in cell death, cell proliferation and differentiation. The onset and development of several CNS diseases have been linked to the activation of ERK1/2 pathway cascades [510, 511] via modifications in neuronal cell death, neuroinflammatory responses, brain development and synaptic plasticity [445]. The PI3K/Akt pathway promotes anti-apoptotic and pro-survival processes via the phosphorylation of Bad, a pro-apoptotic protein. Mangiferin has been found to prevent cellular apoptosis by promoting the phosphorylation of Akt while it also blocks the over-excitability caused by the phosphorylated ERK1/2 kinases as well as decreases the calcium influx [512]. Mangiferin increased the Akt, GSK3β, mTOR complex 1 and mTOR complex 2 expression which subsequently enhanced cell cycle progression and cell survival in a

rat model of hypoxic–ischemic brain injury [504]. These results suggest that mangiferin promotes neuroprotection via the PI3K/Akt/mTOR signaling pathway. Additional studies have indicated that mangiferin prevents excitotoxicity-induced damage in cortical neurons and obstructs neuronal loss in the hippocampal CA1 pyramidal region as well as reduces ischemic impairment in the rat brain [513, 514].

Neuroinflammation is an immune response and a common feature present in a range of neurodegenerative diseases. The NF-κB signaling pathway is a major regulator of neuroinflammation. Mangiferin was shown to significantly inhibit the NF-κB activation, to decrease proinflammatory cytokine levels including IL-1β, IL-6, and TNF-α as well as to inhibit the activation of the NLRP3 (NLR family pyrin domain containing 3) inflammasome [515].

Mangiferin has shown some beneficial effects in neurodegenerative disease models (AD and PD) by three main mechanisms: scavenging free radicals, enhancing mitochondrial functions and rescuing metal toxicity by forming complexes [498, 516, 517]. A number of *in vitro* and *in vivo* studies have evaluated the neuroprotective activities of mangiferin in AD and mainly in Aβ-related models. Mangiferin exhibited neuroprotective effects in Aβ-treated cortical neurons through inhibition of ROS production and cellular apoptosis as well as through regulation of the mitochondrial calcium concentration [491, 518]. In addition, chronic mangiferin treatment (50 mg/kg/day) of APP/PS1 mice significantly decreased tau phosphorylation without impacting Aβ accumulation while mangiferin also prevented neuroinflammation [519]. Pretreatment with mangiferin for 14 days significantly reduced neuronal cell death, oxidative stress and cholinergic impairment in the hippocampus of scopolamine-exposed mice [497]. Furthermore, mangiferin rescued MMP and prevented cell death in rotenone-treated SK-N-SH neuroblastoma cells [520]. In addition, mangiferin inhibited the release of cytochrome C from mitochondria and therefore prevented the activation of the apoptotic cascade in a subarachnoid hemorrhage rat model [515]. Mangiferin has also shown protective effects against metal toxicity as it inhibited the mercury-provoked decline in GSH, SOD, CAT and GST antioxidant defense levels in human liver cancer cells (HepG2). Similarly, mangiferin obstructed DNA damage in methylmercury-treated human neuroblastoma IMR-32 cells by counterbalancing oxidative stress, by inhibiting changes in MMP as well as intra-cellular calcium ions influx and thus by diminishing apoptotic cell death [482]. Mangiferin administration (40

mg/kg) for 21 days stopped the $AlCl_3$-induced neurotoxicty and cognitive imairment in mice. The mechanism accountable for the indicated neuroprotective properties of mangiferin could be the increase of BDNF and the inhibition of AChE (acetylcholinesterase) in the hippocampus [485].

Overall, evidence suggests that mangiferin possesses remarkable neuroprotective properties and thus might have several potential clinical applications. Mangiferin is able to traverse the blood-brain barrier [521] implying that it could be used as therapy or prevention of neuronal damage. However, there is limited evidence on the pharmacokinetics of mangiferin and further research is needed and should concentrate on pharmacodynamic parameters such as half-life, bioavailability and adverse reactions. Also, clinical trials are needed to prove the efficacy of mangiferin in CNS disorders [445].

6.3. Strategies against glucocorticoid stress and anxiety-related disorders

Stress and especially early life stress has been linked to the development of stress-related and anxiety disorders which are characterized by dysregulation of the HPA axis and accumulation of cortisol. HPA axis hyperactivity and subsequent hypercortisolism have been noticed in patients with different chronic anxiety and mood diseases such as generalized anxiety disorder [522, 523] and panic disorder [524]. Therefore, pharmacological strategies often target the HPA stress system and its normalization. Current conventional anti-stress and anti-anxiety therapeutic approaches include benzodiazepines, selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs). Benzodiazepines (e.g. alprazolam) which are sedative-hypnotics have been shown to diminish CRH activity in neurons in the hypothalamus. SSRIs and TCAs are anti-depressants that are additionally effective anti-anxiety agents and could partly act via regulation of the HPA axis. In particular, escitalopram, a commonly used selective serotonin reuptake inhibitors (SSRIs), blocks CRH secretion in the amygdala while enhances glucocorticoid receptor density in the hypothalamus and hippocampus [525]. Although effective in reducing stress and anxiety, all the pharmacological strategies mentioned above present several adverse effects. Benzodiazepines could have been ideal anxiolytics if they inhibited the

hyperactive amygdala selectively. However, since GABA receptors are extensively found throughout the CNS, benzodiazepines act on the whole brain indiscriminately. Adverse effects of SSRIs, TCAs and benzodiazepines include sedation and fatigue, dizziness, nausea, agitation, restlessness, sexual dysfunction, poor concentration, headaches as well as dependence and abuse or misuse [526]. Therefore, due to the treatment gap of current medications, new therapeutic strategies could be of great interest and importance while plant based solutions could contribute to this need and will be discussed below.

Adaptogenic plants

Adaptogens are non-toxic agents that increase resistance against physical, chemical, biological and psychological stressors by normalizing the harmful effect of these stressors independently of the nature of the pathologic state. In this way, they help the organism to achieve a physiological equilibrium and homeostasis and to adapt during stressful situations as well as to improve performance [527]. Ideally, adaptogens should decrease stress-induced impairments, exert stimulating effects, be safe and have none or limited negative effects. Adaptogenic plants include *Eleutherococcus senticosus*, *Rhodiola rosea*, *Withania somnifera*, *Schisandra chinensis* and *Panax ginseng*. The need for agents that would boost physical and mental performance in healthy subjects emerged during World War II (WWII) when a range of stimulants were administered to pilots and other soldiers [527, 528]. For example, the Russian interest in the tonic effects of *S. chinensis* during WWII occurred from ethnopharmacological studies indicating that the seeds and berries of the plant were consumed by Nanai hunters (Goldes or Samagir) as a stimulant to reduce exhaustion, hunger and thirst as well as to enhance night vision [527]. Additionally, historically *Rhodiola rosea* had been used by Vikings to increase their endurance and physical strength in battles [529].

The stimulating and tonic effects of adaptogens (after an acute dose or after repeated administration respectively) are a result of their stress-protective properties. In contrary to commonly used stimulants, for example sympathomimetics (e.g.,

phentermine and ephedrine), adaptogens do not cause adverse effects such as abuse probability, addiction/withdrawal syndrome and psychotic symptoms when used in long term. Their stimulating effect is stronger under situations of stress and fatigue than under physiological conditions [527]. Adaptogens can be considered as anti-stress and anti-fatigue drugs that: a) enhance attention and stamina in fatigue situations that result in reduced performance and b) decrease stress-induced damage and proneness to stress-related disorders. Adaptogens exert their positive stress-protective effects to maintain homeostasis through various HPA axis related mechanisms of action by regulating key stress response factors including cortisol, nitric oxide (NO), stress-activated c-Jun N-terminal protein kinase (JNK1), molecular chaperons (Hsp70) and Forkhead box O (FoxO) transcription factor DAF-16 (Figure 11) [527].

Heat shock proteins such as Hsp70 and Hsp16 are molecular chaperons implicated in stress-provoked cytoprotection as well as in adaptation to reoccurring exposure of a stressor [530-534]. It is suggested that promotion of Hsp70 expression is a major mechanism of action of adaptogens [535]. The induction of heat shock protein expression is regarded as a defense mechanism against stress which promotes tolerance and resilience. Therefore, adaptogens act as inducers of eustress or as “stress vaccines” (stress-mimetics) which cause stress-preventive responses and protect against future stress [535, 536] preventing also malfunctions in the nervous, endocrine cardiovascular, gastrointestinal and immune systems [527, 537]. FoxO is a Forkhead protein that regulates protein synthesis associated with stress tolerance, cell survival and longevity. Adaptogens reduce cortisol and NO levels as well as JNK during stress and induce Hsp70 and p-FoxO1 expression (Figure 11). The activation of Hsp70 biosynthesis restores damaged proteins and obstructs the stress-provoked expression of NO genes. As a result, reduced NO levels are not able to inhibit energy supplying molecules and thus ATP levels are rescued and elevated in the adapted cells. Hsp70 also blocks JNK and thus apoptotic death as well as inhibits immune system suppression through activation of glucocorticoid receptors (GR). Proper GR function and elevated ATP levels are linked to the anti-fatigue effects of adaptogens. Finally, adaptogens induce the phosphorylation of FoxO and its subsequent translocation into the nucleus demonstrated in cells (human monocytes) or in simple

organisms (e.g. DAF-16 in *C. elegance*). This enhances resilience to stress and increases life span (Figure 11) [527, 535].

Stress responses and adaptation to external challenges are complex processes that implicate extracellular and intracellular signaling pathways. Adaptogens regulate homeostasis both at the cellular and systems levels in a holistic manner that is correlated to several targets. Therefore, adaptogens display polyvalent and multitarget effects at the transcriptional, metabolomic and proteomic level [536].

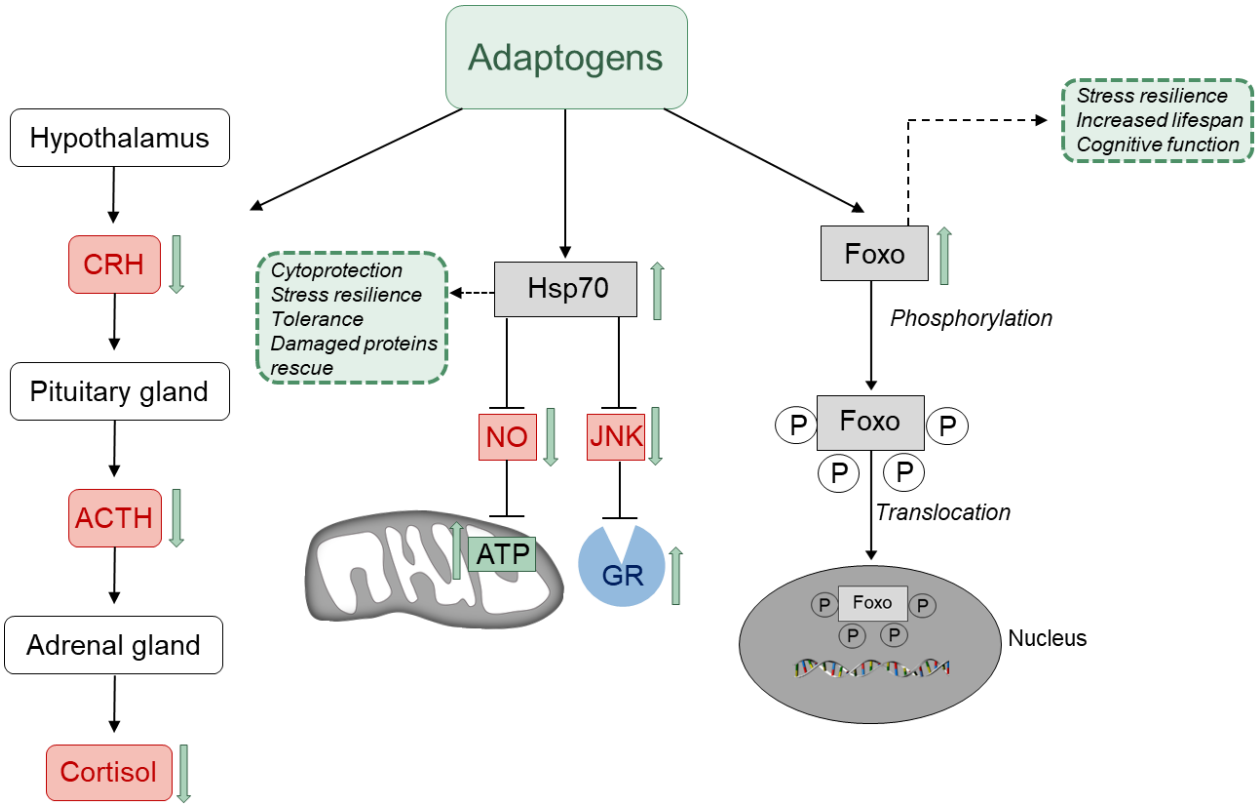

Figure 11. Mechanisms of action of Adaptogens. Adaptogens exert their positive stress-protective effects to maintain homeostasis through various HPA axis related mechanisms of action by regulating key stress response factors including cortisol, nitric oxide (NO), stress-activated c-Jun N-terminal protein kinase (JNK), molecular chaperons (Hsp70) and Forkhead box O (FoxO). The induction of heat shock protein expression is regarded as a defense mechanism against stress which promotes tolerance and resilience. Adaptogens reduce cortisol and NO levels as well as JNK during stress and induce Hsp70 and p-FoxO1 expression. The activation of Hsp70 biosynthesis restores damaged proteins and obstructs the stress-provoked expression of NO genes. Reduced NO levels are not able to inhibit energy supplying molecules and thus ATP levels are rescued and elevated in the adapted cells.

Hsp70 also blocks JNK and thus apoptotic death as well as inhibits immune system suppression through activation of glucocorticoid receptors (GR). Finally, adaptogens induce the phosphorylation of FoxO and its subsequent translocation into the nucleus which enhances resilience to stress and increases life span. The green arrows demonstrated the activity of adaptogens.

6.3.1. Rhodiola rosea

Rhodiola rosea, a member of the genus Rhodiola which contains approximately 24 different species, is a perennial flowering plant belonging to the Crassulaceae family and is known by the names Rhodiola, Golden Root, Rosenroot and Arctic Root. It grows naturally in the wild at high altitude and can be found in the Arctic and in mountainous areas of Central Asia and Europe [538]. Extracts from *Rhodiola* rhizomes and roots are considered adaptogens and have been characterized as ``traditional herbal medicinal products for temporary relief of symptoms of stress, such as fatigue and sensation of weakness`` by the European Medicines Agency (EMA) (EMEA/HMPC/102655/2007). Historically, *Rhodiola rosea* had been used by Vikings to increase their endurance and physical strength [529]. Currently, *Rhodiola rosea* is among the most popular adaptogenic plants used in Russia. It was initially suggested for usage as a stimulant to combat fatigue in patients who underwent asthenic situations and in healthy subjects who demonstrated weakness and exhaustion during periods of intense mental or physical effort. *Rhodiola* has also been indicated for use in cases of stress-related borderline mental disorders, neuroses and psychopathies [538]. Phytochemically, extracts of *Rhodiola rosea* roots and rhizomes contain mainly six classes of compounds: flavonoids, phenolic acids, phenylethanoloids, phenylpropanoids, monoterpenes and triterpenes as well as their glycosides. Salidroside, a phenylethanol glycoside, is considered to be the main pharmacologically active compound of *Rhodiola rosea*, although it is also found in many other *Rhodiola* species. The compound is metabolized to its aglycone p-tyrosol which has been identified in the rat plasma after treatment with salidroside [539-541]. Recently, the research interest has been focused on the neuroprotective effects of

salidroside with a plethora of *in vitro* and *in vivo* studies having been conducted on this matter. In summary, salidroside exerts its neuroprotective effects through: antioxidant and ROS scavenging properties, enhancement of antioxidant defense systems (GSH, SOD etc), decrease in lipid peroxidation (MDA levels), promotion of mitochondrial biogenesis and protection of mitochondrial functions, promotion of neurogenesis and reduction of Aβ, D-galactose and glutamate toxicity [542]. Those effects are due to the ability of salidroside to regulate certain signaling pathways including upregulation of the PI3K/Akt/mTOR and PI3K/Akt/GSK3β pathways, increase of anti-apoptotic Bcl-2 proteins and decrease of pro-apoptotic Bax proteins among other [542, 543].

In contrast to salidroside, some cinnamic alcohol glycosides such as rosavin, rosarin and rosin have been solely found in *Rhodiola rosea*. These compounds are referred to as 'rosavins' and are represented by the phenylpropanoid rosavin [538]. Both salidroside and rosavin represent the lead marker compounds of the RRE while RREs used for medicinal purposes are standardized to contain at least 3% of rosavins and 1% of salidroside. Pharmacological effects of RREs demonstrated in studies include: stress-protective, CNS stimulating effect including cognitive functions, anti-fatigue, antioxidant, anxiolytic, antidepressive, cardioprotective, endocrine-normalizing and life-span enhancing effect [538]. Various mechanisms of action potentially conducing to the clinical adaptogenic and stress-protective effect of RRE have been identified. These include regulation of the HPA axis and its end products (cortisol-decreasing), upregulation of defense proteins (e.g. heat shock proteins Hsp70 and FoxO/DAF-16) as well as decrease in NO and protein kinase JNK [538, 544-549].

[redacted]*In vitro* studies

A very recent study showed that RRE (50 mg/ml) exhibited free radical scavenging and antioxidant capacity when assessed in the ABTS and DPPH assays while it also normalized dihydrochloride-induced increase in ROS in PC12 cells [550]. RRE (0.001-0.1 μg/ml) could rescue the paraquat, UV and H_2O_2 induced cytotoxicity without affecting antioxidant defense levels (SOD, CAT, and glutathione reductase and peroxidase, GSH, GSH/GSSG ratio and HO-1) in human neuroblastoma (IMR-32),

human diploid fibroblast (IMR-90) and human osteosarcoma-derived (143B) cell lines [551]. In contrary, RRE increased the activity of the antioxidant enzymes SOD and CAT in a human keratinocyte cell line (NCTC 2544) [552]. RRE (20 μg/ml) counteracted the CRH-induced neuroinflammation by obstructing NF-κB nuclear translocation through modulation of the kinases MAPK-activated protein kinase 2, ERK 1/2 and JNK (RRE decreased their phosphorylation) and by increasing the Hsp70 expression in BV2 microglial cells [553]. Similarly, RRE (10 μg/mL) protected C_2C_{12} myotubes (murine skeletal muscle cells) from H_2O_2-induced oxidative stress via upregulating the mRNA expression of Hsp70 [554]. RRE pre-treatment protected human cortical cells (HCN 1-A) against H_2O_2- and glutamate-induced cell apoptosis by normalizing the stress-induced increase in intracellular Ca^{2+} concentration. Salidroside exerted the same effects but in a lower extent than the extract indicating that the neuroprotective effect of RRE is a result of synergistic effect of its compounds [555]. An ethyl acetate RRE fraction (50 μg/mL) demonstrated the highest cell viability values and AChE inhibitory activity during methylglyoxal-induced apoptosis in neuro-2A (N2A) cells while salidroside (50 μM 24 h) exhibited the strongest neuroprotective activity among the tested compounds of the ethyl acetate fraction [556].

[redacted] *In vivo* animal studies

Several *in vivo* studies have demonstrated the stress-decreasing effects of RRE. RRE seems to be able to normalize the secretion of stress hormones while concomitantly enhances energy metabolism and ATP synthesis in mitochondria [547, 548, 557, 558]. Stress-activated protein kinase (SAPK) pathway is implicated in the development of glucocorticoid resistance which is present in some chronic inflammatory diseases as well as in patients with depression [536, 548, 559]. RRE (1 mg/kg for seven days) protected from an increase in blood levels of stress factors such as cortisol, NO and p-SAPK/p-JNK versus placebo measured in rabbits stressed with immobilisation [548]. Similarly, RRE possibly inhibited NO formation and the subsequent reduction in ATP production via inhibition of the SAPK/JNK pathway [535, 560]. RRE (50 mg/kg) enhanced the performance of rats in the exhaustive swimming assay versus the untreated rats while it also promoted resynthesis of ATP in mitochondria of rat skeletal muscles as well as promoted post exercise recovery [557]. The multicopy suppressors of the snf1 (sucrose non-fermenting 1) defect proteins Msn2/Msn4 (multicopy

suppressor of SNF1 mutation proteins 2 and 4) are regarded parts of the general stress response and are stimulated during different stress conditions [561, 562] while Yap1p belongs to the c-jun protein family that, in combination with fos, form the higher eukaryote AP1 transcription factor. Yap1p is a key factor in resistance of yeast *Saccharomyces cerevisiae* to oxidative stress [563, 564]. RRE lower doses increased the yeast lifespan, elevated its resistance to oxidative stress and to various stressors in exponentially growing cultures as well as inhibited oxidation of proteins under H_2O_2-induced oxidative stress possibly via the implication of Msn2/Msn4 and Yap1 proteins [565]. RRE also prolonged the lifespan of *C. elegans* and increased its resistance to stress [546]. RRE pretreatment decreased lipid peroxidation (MDA levels) and increased antioxidant defenses (GSH, SOD and glutathione reductase) in the hippocampus of rats with streptozotocin-induced [566] and MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced [567] neurotoxicity and cognitive impairment. Similarly, RRE treatment for four weeks inhibited stress-induced elevation in blood, skeletal muscle and liver superoxide anion as well as in plasma MDA levels in rats. Additionally, the same treatment (125 mg/day/rat) increased the Mn-SOD, Cu/Zn-SOD and CAT in rat livers [568]. Interestingly, RRE also induced proliferation and differentiation of hippocampal neural stem cells in depressive rats (induced by mild chronic stress) indicating its potential role in rescuing injured hippocampal neurons [569]. C-Fos is a protein involved in the stress response. RRE significantly reduced the stress-induced (caused by water-floating and treadmill exercise) increase in CRH and peripheral corticosterone levels as well as reduced c-Fos mRNA levels in the hypothalamus of stressed rats [570]. RRE seems to be able to upregulate the anti-apoptotic protein Bcl-2 and downregulate the pro-apoptotic protein Bax as RRE administration for 2-4 weeks significantly increased the Bcl-2/Bax ratio in male Wistar rats [571].

Clinical studies

The traditional uses of Rhodiola have been justified through clinical studies confirming the beneficial effects of *Rhodiola rosea* extracts in stress and fatigue management, in building resilience and in depression regulation [572-578]. In clinical studies, mood, attention, mental work ability and performance were boosted during treatment with

RRE while stress and mild anxiety were decreased [573, 579-581]. Treatment with RRE (200 mg, twice-daily for four weeks) reduced life-stress symptoms, fatigue and stress-related dysfunctions at social, work and family life as well as improved concentration and quality of life in adults (n = 101) with life-stress symptoms. The improvements emerged after only three days of treatment and were sustained throughout the whole four-week study duration [573]. RRE has also exhibited beneficial effects against symptoms of mild anxiety associated with stress as it reduced self-reported anxiety, stress, anger and confusion and enhanced the total mood after 14 days of treatment of students (n = 81) versus untreated subjects [579]. RRE effects on neurophysiological and neuropsychological parameters of attention have been assessed. RRE treatment for 12 weeks improved attention and overall performance in multi-tasking mentally demanding situations in healthy subjects (n=50) of 30-50 years old at high stress risk [581]. A two week RRE administration reduced fatigue during stressful situations in healthy physicians (n = 56) on night duty by decreasing mental fatigue and improving complex cognitive cerebral functions including short-term memory, associative thinking, concentration and calculation ability as well as audio-visual perception speed [580].

RRE has also shown positive effects on mental performance in patients with stress-related fatigue as well as on chronic fatigue symptoms [547, 582]. RRE (576 mg/day, four weeks) improved attention, concentration, mental performance and fatigue as well as decreased cortisol levels in patients with burnout and fatigue syndrome (n = 60) versus placebo in a double-blind study [547]. RRE administration (200 mg, twice-daily, 8 weeks) caused significant symptom improvements in patients with chronic fatigue (n=101) as demonstrated in an open-label, single-arm study while it also improved ailments related to chronic fatigue (concentration, mood and overall well-being and quality of life ([582].

Treatment with RRE has also been beneficial against burnout symptoms [583] [577]. RRE treatment (8 weeks) significantly alleviated burnout symptoms such as fatigue, depression, exhaustion, insomnia and lack of performance in patients with two or more of these burnout symptoms (n=330) in an non-interventional study conducted in 128 health practices [583]. RRE (200 mg twice-daily, 12 weeks) improved symptoms of fatigue, concentration difficulties, cynicism and sexual dysfunction as well as somatic

symptoms in burnout patients (n = 118) during an exploratory multi-centre single-arm study. Some of these symptoms were already improved after only one week of treatment. In this study, RRE treatment aimed not only at the symptoms of the syndrome but also at the source in order to help patients build stress resilience and to further prevent the onset of subsequent burnout-related diseases [577].

Besides its stress-reducing and adaptogenic effects, RRE treatment has also been proved well tolerated and with minimal adverse effects. Therefore, lack of interaction with other medicines and adverse effects during clinical trials make RRE a promising agent for use as a potential safe medication or supplementation [538].

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B. Results

1. Honeybush extracts (*Cyclopia* spp.) rescue mitochondrial functions and bioenergetics against oxidative injury.

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Abstract

Mitochondrial dysfunction plays a major role in the pathogenesis of many oxidative stress or age-related diseases such as neurodegenerative as well as mental disorders, but also in normal aging. There is evidence that oxidative stress and mitochondrial dysfunction are the most upstream and common events in the pathomechanisms of neurodegeneration. *Cyclopia* species are endemic South African plants and some have a long tradition of use as herbal tea, known as honeybush tea. Extracts of the tea are gaining more scientific attention due to their phenolic composition. In the present study we tested the *in vitro* mitochondria-enhancing properties of honeybush extracts under physiological conditions, but also their ameliorative properties under oxidative stress situations. Hot water and ethanolic extracts of *C. subternata*, *C. genistoides* and *C. longifolia* were investigated. Pre-treatment of human neuroblastoma SH-SY5Y cells with honeybush extracts, at a concentration range of 0.1-1 ng/ml, had a beneficial effect on bioenergetics as it increased ATP production, respiration and mitochondrial membrane potential (MMP) after 24 hours under physiological conditions. The aqueous extracts of *C. subternata* and *C. genistoides*, in particular, showed a protective effect by rescuing the bioenergetic and mitochondrial deficits under oxidative stress conditions (400 µM H_2O_2 for 3 hours). These findings indicate that honeybush extracts could constitute candidates for the prevention of oxidative stress with an impact on aging processes and age-related neurodegenerative disorders potentially leading to the development of a condition-specific nutraceutical.

1. Introduction

Reactive Oxygen species (ROS) are oxygen containing chemical entities of great reactivity that have been in the spotlight as a common feature in many diseases. They are implicated in neurodegenerative and cardiovascular diseases, cancer, atherosclerosis, diabetes, and also in normal aging [1-4]. ROS include mainly superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) of which superoxide anion and hydrogen peroxide are found in the most abundance in cells [5]. Mitochondria are organelles which are responsible for the majority of adenosine triphosphate (ATP) production through oxidative phosphorylation (OXPHOS) taking place at their electron transport chain (ETC). Neurons are high energy demanding cells and thus are highly dependent on mitochondria in order to survive and function. However, mitochondria are also the epicenter of ROS production and metabolism [2, 6]. Despite an estimation of 31 existing ROS (mostly superoxide anion and H_2O_2) production sites in the entire cell versus 12 ROS emission sites in the mitochondria, the majority of cellular endogenous ROS are produced by mitochondria as by-products of OXPHOS [5, 7].

Exposure to oxygen is not only unavoidable but also vital and necessary for organism and cell survival and for energy production [5]. Mitochondrial ROS are mostly generated by complexes I and III of the ETC when leaking electrons that are provided by NADH or $FADH_2$ react with oxygen. Interestingly, the two high-production sites releasing $O_2^{\bullet-}$ and H_2O_2 directly into the intermembrane space are the enzyme sn-glycerol-3-phosphate dehydrogenase and complex III of the ETC [5]. As a result, the presence of ROS in the intermembrane space may cause depolarization of the

membranes and hinder the free motion of electrons through complexes I-IV, thereby directly affecting the proton gradient and the mitochondrial membrane potential (MMP) and ultimately preventing the production of ATP [8].

As mitochondria are the main superoxide anion and hydrogen peroxide producers, they largely affect redox homeostasis [9]. For their protection, cells are equipped with antioxidant defense systems (superoxide dismutases, glutathione peroxidases, thioredoxin, catalase and glutathione GSH) in order to fend off ROS [10-12]. The redox state of the cells is dynamic and depends on the production of ROS and the functionality of the antioxidant defense systems. At normal non-elevated concentrations ROS act as signaling molecules and they participate in the regulation of senescence, cell death and proliferation. When there is an overproduction of ROS, the antioxidant defense systems are overwhelmed and they are not able to diffuse them. Therefore, oxidative stress is the over-accumulation of ROS (mainly superoxide anion and H_2O_2) due to their overproduction or overburdened antioxidant defense systems [1, 5]. ROS react with and damage many cellular and mitochondrial biomolecules. Of note, they cause lipid peroxidation and membrane damage, protein mis-folding as well as DNA damage [3]. Mitochondrial DNA (mtDNA) is located in the matrix of mitochondria and encodes for 13 proteins which are structural components of the ETC. MtDNA is in very close proximity to the ROS production sites and is therefore directly affected and mutated, leading to faulty ETC components which leads back to impaired OXPHOS and more production of ROS [10, 13]. When the ROS levels surpass a certain threshold then they become mitochondria-damaging and disease-causing agents [14]. Aging is characterized by an increase in ROS and a decrease in antioxidant defenses leading to mitochondrial damage and ultimately to cellular dysfunction, senescence and apoptosis. Normal aging and neurodegenerative

disorders have these characteristics in common although to a different extent. In neurodegeneration the damaging effects are even more profound [3, 5, 9, 15].

Hydrogen peroxide, which is endogenously produced in mitochondria, is considered the ROS with the most impact on the fate of the cell. It can easily diffuse through membranes and has the greatest life span [9]. Therefore, hydrogen peroxide was used as an oxidative stressor in this study.

Cyclopia species, belonging to the Fabaceae family, are endemic to South Africa. Old records describe the traditional use of several species including *C. subternata*, *C. genistoides* and *C. longifolia,* as herbal teas [16]. At present, these *Cyclopia* species form the bulk of cultivated plant material supplementing plant material harvested in the wild and crucial to meet the growing demand of international markets. The main product is “fermented” (oxidised) honeybush tea while the green (unoxidised) herbal tea is preferred for nutraceutical extract production due to a higher phenolic content and antioxidant capacity. The phenolic profile of honeybush varies qualitatively and quantitatively depending on the *Cyclopia* species. Major phenolic constituents belong to xanthone, benzophenone, flavanone, flavone and dihydrochalcone sub-classes [17]. Increased consumption and popularity of honeybush came along with increasing research interest in order to reveal new bioactivities and to examine its potential use as a nutraceutical and functional food [16, 18]. Quite predictably due to their phenolic composition, honeybush extracts have been shown to possess antioxidant activities which are of great importance and interest in the research of oxidative stress-related diseases [19-22]. Considering on one hand the evidence of its antioxidant capacity and on the other hand the need for mitochondria-targeting antioxidant substances for use in the prevention of oxidative damage or the amelioration of increased oxidative

stress levels, we hypothesized that honeybush could possess some beneficial mitochondria-enhancing properties. For this reason, this study aimed at examining the protective effects of honeybush extracts against H_2O_2-induced oxidative stress in SH-SY5Y neuronal cells with a focus on mitochondria. To our knowledge, this is the first study that evaluates the effects of honeybush extracts on mitochondrial function in a neuronal cell model.

2. Materials and Methods

Chemicals and reagents

Dulbecco's-modified Eagle medium (DMEM), phosphate buffered saline (PBS), fetal calf serum (FCS), Hanks' Balanced Salt solution (HBSS), penicillin/streptomycin, pyruvate, dihydrorhodamine 123 (DHR), 2',7'-dichlorodihydrofluorescein diacetate (DCF), dihydroethidium (DHE), tetramethylrhodamine methyl ester (TMRM), gelatin and H_2O_2 were from Sigma-Aldrich (St. Louis, MO, USA). MitoSOX and glutamax were from Gibco Invitrogen (Waltham, MA, USA), ATPlite1step kit from PerkinElmer (Waltham, Massachusetts, USA) and XF Cell Mitostress kit from Seahorse Bioscience (North Billerica, MA, USA). Folin-Ciocalteau reagent was purchased from Merck (Darmstadt, Germany). Authentic reference standards (purity >95%) for identification and quantification of phenolic compounds were obtained from Sigma-Aldrich (hesperidin), Extrasynthese (Genay, France; mangiferin, eriocitrin), Chemos (Regenstauf, Germany; isomangiferin) and Phytolab (Vestenbergsreuth, Germany; vicenin-2, 3-β-D-glucopyranosyliriflophenone). Compounds from the Plant Bioactives

Group library included 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone, 3-β-D-glucopyranosylmaclurin and (2*S*)-5-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]naringenin isolated from *C. genistoides,* as well as scolymoside and 3',5'-di-β-D-glucopyranosylphloretin isolated from *C. subternata*. HPLC gradient grade 'far UV' acetonitrile was supplied by Merck.

Plant material and extract preparation

Harvesting of aerial parts (shoots and leaves) occurred in March 2017. *Cyclopia subternata* was harvested on Elsenburg research farm (-34.30267, 19.13809) while *C. longifolia* and *C. genistoides* were harvested on Nietvoorbij research farm (-33.90619, 18.87031), both located in the Western Cape Province of South Africa. The fresh plant material was mechanically cut into small pieces (<3 mm) and dried at 40°C in a cross-flow, temperature-controlled drying tunnel to a moisture content <7% as for green honeybush tea production. The dried plant material was coarsely milled using a rotary mill equipped with a 1 mm sieve (Retsch, GmbH, Haan, Germany).

Hot water extracts were prepared from each batch of milled plant material by extracting 70 g plant material with 700 ml deionised water at 93°C for 30 min followed by filtration and freeze-drying of the filtrate as previously described [23]. Similarly, 40% EtOH-water (v/v) extracts were prepared by extracting the milled plant material at 70°C for 30 min. Ethanol was removed under vacuum using rotary evaporation and the remaining aqueous layer was freeze-dried. Prior to extraction using 70% EtOH-water (v/v), the plant material was subjected to exhaustive Soxhlet extraction with dichloromethane to remove chlorophyll. The defatted plant material was air-dried and further treated as for the 40% EtOH-water (v/v) extracts. The

freeze-dried extracts (>15 g/extract) were coded, aliquoted into glass vials (for testing and retention samples), sealed and stored under desiccation in the dark.

Quantification and identification of phenolic compounds

The major phenolic compounds in the extracts were quantified using the respective species-specific validated HPLC-DAD method for *C. subternata* [23], *C. longifolia* [24] and *C. genistoides* [25]. Samples were dissolved in water or 10% DMSO and filtered using 0.45 µm pore size PVDF syringe filters (Merck) for *C. subternata* while 0.22 µm pore size filters were used for *C. genistoides* and *C. longifolia*. Ascorbic acid was added to prevent compound degradation during analysis (final concentration ca 9 mg/mL). Peak areas at the appropriate wavelength together with external calibration curves were used for quantification (benzophenones, flavanones and dihydrochalcones at 288 nm; xanthones and flavones at 320 nm). In cases where authentic reference standards were not available, quantification was in equivalents of a similar compound.

Total polyphenols content of extracts was determined using the Folin-Ciocalteau assay as adapted for microplate by Arthur et al [26]. Values were expressed as g gallic acid equivalents per 100 g extract.

Extracts selected for further study after initial testing were also analyzed by LC-MS using a Waters Acquity ultra-performance liquid-chromatography (UPLC) instrument coupled to a Synapt G2 quadrupole-time-of-flight (Q-TOF) MS detector equipped with an electrospray ionization (ESI) source (Waters, Milford, USA). Mass calibration was performed using a sodium formate solution and leucine enkephalin was used as the

lockspray solution. Analysis was first performed in the MS^E mode with negative ionization: scanning range, 150–1500 am; capillary voltage, -2.5 kV; sampling cone voltage, 15.0 V; source temperature, 120°C; desolvation temperature, 275°C; cone gas flow (N_2), 650 l/h; desolvation gas flow (N_2), 50 l/h. For the MS/MS experiments, a collision energy of 30.0 V was used. Peaks were identified by comparing UV-Vis spectra, relative retention time, MS characteristics (molecular formula predicted by accurate mass) and MS/MS fragmentation spectra with those of authentic standards or literature data.

Cell culture

The human neuroblastoma SH-SY5Y cell line was selected as our cellular model in this study as it is a well-established and widely used neuronal model in biochemical studies in general. The cell line behaves as human neuronal network in a dish and has been largely used in research as it expresses neuronal receptors. The SH-SY5Y cells were kept and grown at 37°C in a humidified incubator chamber under an atmosphere of 7.5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamax and 1% (v/v) penicillin/streptomycin. Cells were passaged 1-2 times per week and the cells used for the experiments did not exceed passage 20. The cells were plated when they reached 80–90% confluence.

Treatment of cells

Evaluation of ATP production was conducted on SH-SY5Y neuroblastoma cells to determine the potential toxic concentration range of the nine honeybush extracts. Two screenings were performed. Initially, aqueous, 70% ethanolic and 40% ethanolic

extracts of the species *C. subternata*, *C. genistoides* and *C. longifolia* were screened at a very broad concentration range of 0.1 ng/ml to 1mg/ml (data not shown). Of note, all dry extracts were dissolved in DMSO for our experiments (final concentration of DMSO<0.005%, no effect of the vehicle solution alone compared to the untreated condition). The first screening revealed that the extracts were not toxic for the neuroblastoma cells up to a concentration of 10µg/ml. According to the results of the first screening, the concentration range was reduced down to that of 0.1 ng/ml to 1µg/ml and the number of extracts was reduced from nine down to four (according to the capacity of the extracts in increasing the ATP levels of the cells) and a second screening cycle was performed. The screening was conducted by using an ATP detection assay (ATPlite 1step kit was from PerkinElmer). For the experiments, cells were plated and treated 1 day after plating for 24 h either with DMEM (untreated cells-control condition) or with a final concentration of 0.1 ng/ml to 1 µg/ml of the extracts.

Because vehicle treatment was without any effect in our assays, we evaluated the effects of the honeybush extract concentrations in comparison to the untreated control condition in the following experiments. Cellular sensitivity of SH-SY5Y cells was confirmed by using the positive control estradiol as previously described in Grimm et al 2014 [27].

Hydrogen peroxide (H_2O_2) which belongs to the reactive oxygen species produced by mitochondria, was used as a stressor at the concentration 400 µM which was able to decrease mitochondrial and cellular functions. The H_2O_2 concentration was selected based on screening experiments conducted on SH-SY5Y cells. For the stress experiments, cells were firstly pre-treated for 24 h with the honeybush extracts and

then treated for 3 h with 400 μM H_2O_2. Each assay was conducted and repeated at least in triplicate.

ATP levels

Total ATP content was determined using a bioluminescence assay (ATPlite 1step) according to the instructions of the manufacturer and as previously described [28-30]. Cells were plated in 6 replicates into white 96-well cell culture plates at a density of 1×10^4 cells/well. The ATP was extracted from the cells upon lysis and it was transformed into light. The method measures the formation of light from ATP and luciferin catalyzed by the enzyme luciferase. The emitted light was linearly correlated to the ATP concentration and was measured using the multi-mode plate reader Cytation 3 (BioTek instruments, Winooski, Vermont, United States).

Determination of mitochondrial membrane potential (MMP)

The MMP was measured using the fluorescent dye TMRM, since its transmembrane distribution depends on the MMP. As previously described [31, 32], the cells were plated in 6 replicates into black 96-well cell culture plates at a density of 1×10^4 cells/well and were incubated with the dye at a concentration of 0.4 μM for 20 min. After washing three times with HBSS, fluorescence was measured at 548 nm (excitation)/574 nm (emission), using a Cytation 3 multi-mode plate reader (BioTek instruments).

Mitochondrial respiration

Mitochondrial respiration and cellular glycolysis were measured using the Seahorse Bioscience XF24 analyser as described before [28, 29, 33]. Briefly, XF24 cell culture microplates were coated with 0.1% gelatin and cells were plated at a density of 2.5×10^4 cells/well in treatment medium (100 µl) containing 1 g/l glucose, 4 mM pyruvate and 10% FCS. After treatment with honeybush extracts for 24 hours, the cells were washed once with PBS and then 500 µl of assay medium (DMEM containing 1 g/l of glucose and 4 mM of pyruvate) was added to each well. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured concurrently under basal respiration. The data were extracted from the Seahorse XF24 software and bioenergetic parameters (basal respiration, ATP production, maximal respiration, spare respiratory capacity, and glycolytic reserve) were calculated according to the guidelines of the manufacturer.

Determination of ROS levels

Mitochondrial and cytosolic ROS levels, as well as the specific levels of mitochondrial O2•–superoxide anion radicals and the total levels of O2•– superoxide anion radicals levels were assessed using the fluorescent dyes dihydrorhodamine 123 (DHR), 2′,7′-dichlorodihydrofluorescein diacetate (DCF), the Red Mitochondrial Superoxide Indicator (MitoSOX) and dihydroethidium (DHE), respectively as described before [30, 34]. SH-SY5Y cells were plated in 6 replicates into black 96-well cell culture plates at a density of 1×10^4cells/well. After treatment with honeybush extracts alone or after pre-treatment with honeybush extracts, followed by treatment with H_2O_2, cells were treated with 10 µM of one of the dyes: DCF, DHR or DHE for 20 min or 5 µM of

MitoSOX for 90 min at room temperature in the dark on an orbital shaker. After washing the cells three times with HBSS, the formation of green fluorescent products triggered by DCF and DHR, respectively, were detected at 485 nm (excitation)/535 nm (emission). MitoSOX triggers the formation of red fluorescent products which were detected at 531 nm (excitation)/595 nm (emission). DHE, which is permeable to cells, is used as a total O2•– superoxide anion detector as it is oxidised to the impermeable red fluorescent product ethidium, detected at 531 nm (excitation)/595 nm (emission). The intensity of fluorescence was proportional to mitochondrial ROS, cytosolic ROS and $O_2^{\bullet-}$ levels (total and mitochondrial). The fluorescence was measured, using the Cytation 3 multi-mode plate reader.

Statistical analysis

Data are given as the mean ± S.E.M. Statistical analyses were performed using Graph Pad Prism software (version 5.02 for Windows, San Diego, California, USA). For statistical comparisons of more than two groups, one-way ANOVA was used, followed by a Dunnett's multiple comparison tests versus the control for physiological conditions and versus H_2O_2 for stress conditions. $P<0.05$ were considered statistically significant.

3. Results

Two cycles of screenings were conducted with regard to the ability of each extract in increasing the ATP production of SH-SY5Y cells. The nine *Cyclopia* extracts produced by extraction of *C. subternata*, *C. genistoides* and *C. longifolia* with hot water and two

ethanol-water mixtures were screened (data not shown) and the four most promising extracts in terms of increased ATP production were selected for all subsequent experiments: the water extracts of all three *Cyclopia* species and the 70% ethanolic extract of *C. genistoides*. Table 1 gives the content of the major phenolic compounds, present in the selected extracts. Qualitative and quantitative differences in the phenolic profile are evident, notably the absence or presence of only trace levels of dihydrochalcones in *C. longifolia* and *C. genistoides* but substantial xanthone levels, compared to *C. subternata*. Mangiferin followed by isomangiferin was the predominant compound in the *C. longifolia* and *C. genistoides* extracts. Scolymoside, a flavone rhamnoside, followed by 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone, a benzophenone, was the main phenolic compound in *C. subternata* water extract. Scolymoside was not detected in the two *C. genistoides* extracts, but these extracts had substantially higher levels of the flavone di-glucoside, vicenin-2, compared to the *C. subternata* and *C. longifolia* extracts. Overall, the total phenolic content, based on the sum of individual phenolic compound content was highest in the 70% EtOH-water extract of *C. genistoides* and lowest in the water extract of *C. subternata*. The total polyphenol content determined using the Folin-Ciocalteau assay was highest in the 70% EtOH-water extract of *C. genistoides* and lowest in the water extract of *C. longifolia* with similar values for the water extracts of *C. subternata* and *C. genistoides* (Table1).

Honeybush extracts increase ATP production under physiological conditions and under H_2O_2-induced stress

ATP is the end product of mainly oxidative phosphorylation but also of glycolysis and is thus an indicator of mitochondrial and cellular viability and proper functioning. Therefore, we assessed the effect of the honeybush extracts on the ATP production of neuroblastoma cells. The concentration range of 0.1-1000 ng/ml for each extract was first tested under physiological conditions. The results indicated that the lower concentrations (0.1-1 ng/ml), but not the higher ones (50 ng/ml-100 mg/ml, data not shown), of the water extracts of the three *Cyclopia species* and of the 70% ethanolic extract of *C. genistoides* significantly increased ATP production up to 4% after treatment for 24 h under physiological conditions (Figure 1 A-D).

Regarding ATP levels under oxidative stress, H_2O_2 at 400 µM caused a 39.1% decrease in ATP production. According to the experimental design under physiological conditions, we tested the same broad concentration range for each extract under oxidative stress (data not shown). Again, the concentrations 0.1 and 1 ng/ml significantly protected against oxidative stress. Therefore, these concentrations were used in the following oxidative stress experiments. The harmful effect of H_2O_2 was partially, but significantly ameliorated by all the extracts up to 13.5% (Figure 2).

Honeybush extracts increase mitochondrial respiration under physiological conditions and under H_2O_2-induced stress

Mitochondria consume oxygen to perform respiration and oxidative phosphorylation. Thus, for an assessment of mitochondrial respiration, the oxygen consumption rate of

the cells was measured live under basal conditions. The results indicated that the water extracts of *C. subternata* and *C. genistoides* and the 70% ethanolic extract of *C. genistoides* increased the respiration under physiological conditions at baseline. However, upon closer analysis of data, it was found that only the water extracts of *C. subternata* and *C. genistoides* at 1 ng/ml significantly increased the respiration by 33.2% and 40.7%, respectively (Figure 3A). The extracts that significantly increased the other pathway leading to the production of ATP, glycolysis, were *C. genistoides* (1 ng/ml) and *C. longifolia* (at 0.1 and 1 ng/ml). This increase was up to 51.7% (Figure 3B). Upon correlation of the respiration with the glycolysis, an “energy map” was obtained (Figure 3C) which allows a visual representation of where each individual extract acted. Thus, *C. subternata* and *C. genistoides* increased the oxygen consumption rate of the cells (respiration) while *C. longifolia* increased the glycolysis.

H_2O_2 caused a significant decrease of 41.7% in respiration (Figure 4A, red bar). All extracts increased the oxygen consumption rate, bringing it closer to the levels of the untreated cells. However, only the water extract of *C. subternata* was able to significantly enhance respiration at baseline (increase of 25.9%) (Figure 4A). Regarding glycolysis, H_2O_2 caused a significant decrease of 38.9% which was completely rescued by the water extract of *C. genistoides* (1 ng/ml) (Figure 4B). The “energy map” confirmed that the most effective extract in rescuing the respiration under H_2O_2 stress was the aqueous extract of *C. subternata* (Figure 4C).

Honeybush extracts increase mitochondrial membrane potential (MMP) under physiological conditions and under H_2O_2-induced stress

The aqueous extracts of *C. genistoides* (1 ng/ml) and *C. longifolia* (0.1 and 1 ng/ml) significantly increased MMP up to 24% under physiological conditions after a treatment of 24 h (Figure 5 A).

H_2O_2 at 400 µM caused a significant reduction of 55.1% in MMP which was increased by up to 67.9% by the extracts. In this case all extracts completely rescued the MMP (Figure 5 B).

Overall, all extracts acted on the mitochondrial membrane potential by increasing it both under physiological condition and under H_2O_2 induced oxidative stress.

Honeybush extracts decrease different types of ROS under H_2O_2-induced stress

H_2O_2 at 400 µM caused an increase of 29.5% in mitochondrial ROS which was detected using the dye DHR (dihydrorhodamine 123). This increase was significantly ameliorated up to 23.1% by *C. subternata* water extract. *C. genistoides* also brought the ROS levels down but not significantly (Figure 6A).

Cytosolic ROS were detected using the dye DCF (2',7'-dichlorodihydrofluorescein diacetate). H_2O_2 at 400 µM caused an elevation of 31.2%. All extracts lowered cytosolic ROS levels but the water extract of *C. subternata* at 1 ng/ml (28.9% reduction of cytosolic ROS) and the 70% ethanolic extract of *C. genistoides* at 0.5 ng/ml (26.2% reduction of cytosolic ROS) were the most effective (Figure 6B).

H_2O_2 at 400 µM increased the mitochondrial superoxide anion levels by 43%. All extracts, except the ethanolic extract of *C. genistoides,* significantly lowered the mitochondrial superoxide anion levels. However, the water extracts of *C. subternata*, *C. genistoides* and *C. longifolia* at a concentration of 1 ng/ml completely neutralized the mitochondrial superoxide anion levels (reduction of 42%, 42.6% and 42.6%, respectively) (Figure 6C).

The total superoxide anion levels were elevated by 67.9% in the H_2O_2-treated cells. All four extracts ameliorated this increase but only the water extracts of *C. subternata* and *C. longifolia* at 1ng/ml, and the ethanolic extract of *C.genistoides* at 0.5 ng/ml significantly reduced the superoxide anion levels by 48.8%, 50.9% and 50.3%, respectively (Figure 6D).

4. Discussion

In this study we hypothesized that honeybush extracts might exert a beneficial effect on mitochondria of neuronal cells under physiological conditions as well as under oxidative stress due to their phenolic compound content. Neurons have high energy demands and are thus particularly dependent on functional mitochondria. For this reason, we assessed the effects of four different honeybush extracts in a well-characterized neuronal model, the neuroblastoma SH-SY5Y cells. The four extracts were the hot water extracts of *C. subternata*, *C. genistoides* and *C. longifolia* as well as the 70% ethanolic extract of *C. genistoides*. These extracts were selected after screening the water, 40% ethanolic and 70% ethanolic extracts of these *Cyclopia*

species. Hydrogen peroxide (H_2O_2) was used as an oxidative stressor as it is one of the most abundant and reactive endogenous ROS.

The beneficial effect of honeybush extracts on mitochondrial functions under physiological conditions and a protective effect under oxidative stress could be demonstrated. The four extracts showed different beneficial properties in different mitochondrial and cellular sites. ATP is the energy that is required for the survival and functionality of cells and especially of neurons which have high energy demands. At the lowest concentrations (0.1-1 ng/ml) all extracts improved the production of ATP under physiological conditions. This increase amounted up to 4% (Figure 1). Also, all extracts were able to significantly increase the ATP levels under H_2O_2-induced oxidative stress. This improvement was not a complete rescue but a partial increase of up to 13.5% (Figure 2).

Mitochondrial respiration is an intrinsic function of mitochondria and is essential for the survival of the cells as it results in the production of the majority of ATP. Respiration is taking place at the ETC which is located on the inner mitochondrial membrane (IMM). Glycolysis is the secondary pathway leading to production of ATP. The aqueous extracts of *C. subternata* and *C. genistoides* (both at 1 ng/ml) significantly increased the basal respiration of the mitochondria by up to 40.7% while those of *C. genistoides* and *C. longifolia* significantly increased glycolysis up to 51.7% under physiological conditions (Figure 3). However, only *C. subternata* aqueous extract (1 ng/ml) could significantly rescue the impaired respiration and only *C. genistoides* aqueous extract (1 ng/ml) could rescue the impaired glycolysis caused by H_2O_2 (Figure 4).The aqueous extracts of *C. subternata* and *C. genistoides* specifically acted on respiration. In addition, the *C. subternata* aqueous extract enhanced respiration under

oxidative stress. This could be explained by the fact that this extract was the only one that neutralized all four types of tested ROS and particularly the mitochondrial ROS and the mitochondrial superoxide anion which directly affect OXPHOS and respiration (Figure 6). This could be the reason why it was also the only extract to act on respiration under stress.

The aqueous extracts of *C. genistoides* and *C. longifolia* increased the MMP under physiological conditions while all four extracts completely rescued the MMP under oxidative stress (Figure 5), in addition to partly ameliorating ATP production (Figure 2). During OXPHOS at the ETC of mitochondria, electrons provided by NADH and $FADH_2$ are transferred through complexes I-IV. This motion of electrons drives the complexes I, III and IV to pump protons into the intermembrane space where they are finally used by ATP synthase (complex V) to produce ATP via the phosphorylation of ADP. MMP is an indicator for polarized mitochondrial membranes and therefore an indicator that the pumping of protons in the intermembrane space is not hindered so that they can drive the ATP production by complex V [35, 36]. Amelioration of ATP production under oxidative stress by the extracts could be as a result of their capacity to completely rescue the MMP under oxidative stress and supports this interdependence of MMP and ATP production.

In terms of ROS (Figure 6), pre-treatment with the aqueous extract of *C. subternata* (mostly at 1 ng/ml) decreased the four types of tested ROS and it was the only extract of those tested to significantly reduce the mitochondrial ROS (detected with the dye DHR). The result that *C. subternata* extract acted both on mitochondrial superoxide anion levels (detected with the dye MitoSOX) and on all other mitochondrial ROS, such as H_2O_2 (detected with the dye DHR), could mean that it either additionally

scavenges them or it enhances the activity of the antioxidant defenses that neutralize them (e.g. glutathione, catalase) [21]. The aqueous extract of *C. longifolia* lowered cytosolic ROS, total superoxide anion levels and mitochondrial superoxide anion levels. The two *C. genistoides* extracts differed, i.e. its aqueous extract neutralized cytosolic ROS and mitochondrial superoxide anion while its 70% ethanolic extract decreased cytosolic ROS and total superoxide anion levels but had no significant effect on the specific mitochondrial ROS. All extracts had thus a minimizing effect on ROS levels, though at different degrees and on different ROS types (Figure 6). This might be explained by different bioactive components in the specific extract depending on *Cyclopia* species and extraction solvent. While all the water extracts (*C. subternata*, *C. genistoides*, *C. longifolia*) act on mitochondrial superoxide anion levels, the ethanolic extract of *C. genistoides* only affects the cytosolic ROS and total superoxide anion levels. It is assumed that the latter extract acted specifically on cytosolic superoxide anions

The most beneficial concentrations of the honeybush extracts in this study were found to be as low as 0.1 and 1 ng/ml. Plant extracts are complex mixtures of a multitude of compounds of diverse chemistries and pharmacological activities at different concentrations. The different constituents in the plant extracts could have antagonistic, synergistic or allosteric effects [37]. For example, an active substance at the higher concentration could have blunted the activity of another bioactive constituent. Possibly, there is one or several constituents that are effective at a low concentration and a gradual increase in concentrations may gradually reduce the efficacy and might explain the observed effect at very low concentrations.

Considering the phenolic profiles of the different extracts, it is clear that no pattern emerged that could explain differential activity. The total polyphenol content often highly correlates with the antioxidant activity *in vitro* but was similar in the aqueous extracts of *C. subternata*, *C. genistoides*, *C. longifolia* (~ 26, 25, 24 g gallic acid equivalents per 100 g extract respectively). In the ethanolic extract of C. genistoides the phenolic content was slightly increased (~ 28 g gallic acid equivalents per 100 g extract). However, the phenolic content does not differ substantially between the different extracts to provide an explanation to our findings. In fact, mangiferin, shown to have beneficial effects in *in vitro* and *in vivo* models of neurodegeneration, as well as of oxidative stress [38-43], was lowest in the *C. subternata* water extract and highest in the 70% ethanolic extract of *C. genistoides*. According to these studies we expected that the *C. genistoides* 70% ethanolic extract would exert the most potent neuroprotective properties, while the aqueous extract of *C. subternata* would exert the least. Interestingly, the results of our experiments proved our assumption wrong as the opposite effect was observed with the aqueous extract of *C. subternata* being the most beneficial extract. A closer observation at the composition of the extracts (Table 1) reveals that the aqueous extract of *C. subternata* contains higher concentrations of flavones and dihydrochalcones. Scolymoside, present in the highest concentration in the *C. subternata* water extract and absent in detectable quantities in the two *C. genistoides* extracts, is a glycoside of luteolin, a flavone aglycone demonstrated to inhibit the production of neuronal mitochondrial superoxide anion $O_2^{\bullet -}$ [44]. While glycosylation of position C-7 of the A-ring of the flavonoid structure as for scolymoside would decrease its radical scavenging potency compared to luteolin, it does not abolish the activity [45]. Dihydrochalcones related to those in *C. subternata* not only act as radical scavengers [46] but they demonstrated neuroprotective effects [47, 48].

The flavanone, hesperidin, present in the highest level in the 70% ethanolic extract of *C. genistoides* could alleviate oxidative stress [49] and act as neuroprotective agent, amongst others by enhancing endogenous antioxidant defense functions [50].

Regarding the bioavailability of the plant extract it depends on the bioavailability of the single compounds contained in each extract. Extracts from different honeybush species vary in chemical composition. However, the main active constituents of honeybush have been reported to be mangiferin and hesperidin and there are some data available with regards to their bioavailability and their ability to cross the blood-brain barrier (BBB). Of note, trace amounts of mangiferin were found in the rat brain after an acute oral treatment with a single dose of a plant extract containing mangiferin indicating that the compound can cross the BBB [51],whereas in another study, mangiferin was not detected in the brain of rats after a single dose via intraperitoneal administration [52]. However, one has to take into consideration that different assays of different sensitivity were used in the two studies. In the study from Li et al 2008 a validated highly sensitive HPLC method was developed and applied to detect mangiferin after a single oral dose of *Rhizoma Anemarrhenae* extract while in the study of Zajac et al 2012 a much less sensitive detection method, a simple TLC method, was used. Similarly, the bioavailability of the therapeutically active constituents of *Ginkgo biloba* extract (GBE) in the brain was formerly questioned until recent studies demonstrated the distribution of GBE in the brain of rats after single and repeated oral administration of GBE [53, 54]. The compounds in this case were also successfully detected with an HPLC method. Hesperidin or its aglycone hesperetin seem to be able to traverse the BBB and directly exert their neuroprotective effect in the brain [55-57].

Furthermore, bioavailability in the brain might be affected by the route of administration and by whether the pure compound is administered or contained in a plant extract but we can assume that mangiferin and hesperidin exert neuroprotective effects on brain and peripheral neurons.

To sum up, the results obtained from this study indicate that *C. subternata* aqueous extract is the most effective in enhancing mitochondrial functions especially under oxidative stress situations. It was the only one to act on respiration under oxidative stress and the only one to lower all four types of ROS measured in this study. These findings are particularly relevant for the establishment of honeybush tea as nutraceutical as the species that is mostly cultivated for the production of the tea is currently *C. subternata*. The other two aqueous extracts (*C. genistoides* and *C. longifolia*) also exert a beneficial effect. *C. genistoides* acted more on respiration under physiological conditions while *C. longifolia* was more effective in neutralizing ROS (active against three types of ROS). Interestingly, in the tea industry honeybush tea is often prepared after blending of different species to obtain an optimum flavor and phenolics level ratio. Therefore, evaluating the activity of a mixture of different species extracts will be very interesting.

5. Conclusion

In this study the effects of honeybush extracts on enhancing mitochondrial and neuronal functions and on preventing the detrimental effects of oxidative stress were examined. The aqueous extract of *C. subternata* was superior to the other extracts in increasing mitochondrial functions and bioenergetics, especially under H_2O_2-induced oxidative stress. The aqueous extracts of *C. genistoides* and *C. longifolia* came next

in terms of efficacy on mitochondrial functions. Lower extract concentrations (0.1-1 ng/ml) were also more effective. Overall, our data are in line with existing literature reporting an antioxidant effect of honeybush [19-22]. However, the effects of honeybush extracts on neuronal cells and specifically on mitochondrial function have been investigated here for the first time. Further research is ongoing by our team in order to study more in depth the effect of honeybush in combatting stress and in enhancing neuronal function. These findings make honeybush a potential candidate for prevention of oxidative stress, laying the foundation for further research aimed at the development of a condition-specific nutraceutical.

Conflict of interest

The authors declare no conflict of interest.

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Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Abbreviations

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

BBB: Blood-brain barrier

DCF: 2',7'-dichlorodihydrofluorescein diacetate

DHE: Dihydroethidium

DHR: Dihydrorhodamine 123

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

ECAR: Extracellular acidification rate

ESI: Electrospray ionization

ETC: Electron transport chain

$FADH_2$: Reduced form of flavin adenine dinucleotide (FAD)

FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FCS: Fetal calf serum

GBE: *Ginkgo biloba* extract

GSH: Glutathione(γ-glutamylcysteinylglycine)

GSH: GSSG : Reduced glutathione to oxidised glutathione ratio

HBSS: Hanks' Balanced Salt Solution

HPLC-DAD: High Performance Liquid Chromatography with Diode Array Detector

IMM: Inner mitochondrial membrane

LC-MS: Liquid chromatography–mass spectrometry

MMP: Mitochondrial membrane potential

MS/MS: Mass spectrometry/mass spectrometry

MtDNA: Mitochondrial DNA

NADH: Reduced form of nicotinamide adenine dinucleotide

OCR: Oxygen consumption rate

OXPHOS: Oxidative phosphorylation

PBS: Phosphate-buffered saline

Q-TOF: Synapt G2 quadrupole-time-of-flight

ROS: Reactive oxygen species

S.E.M.: Standard error of the mean

SH-SY5Y: human neuroblastoma cell line

TMRM: Tetramethylrhodaminemethyl ester

UPLC: Ultra Performance Liquid Chromatography

UV-Vis: Ultraviolet–visible

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Table 1. Phenolic composition (g/100 g extract) of aqueous extracts of *Cyclopia subternata*, *Cyclopia longifolia* and *Cyclopia genistoides* and a 70% ethanolic extract of *C. genistoides.*

Compounds	*C. subternata*	*C. longifolia*	*C. genistoides*	
	Water	Water	Water	70% EtOH
Benzophenones				
Maclurin-di-*O*,*C*-hexoside (MDH)[a]	nd	nd	0.079	0.061
3-β-D-Glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone (IDG)	1.67	0.700	1.78	1.41
3-β-D-Glucopyranosylmaclurin (MMG)	nd	nd	0.400	0.373
3-β-D-Glucopyranosyliriflophenone (IMG)	0.536	0.076	1.52	1.12
Total benzophenones	*2.21*	*0.776*	*3.77*	*2.97*
Xanthones				
Tetrahydroxyxanthone-di-*O*,*C*-hexoside A (THXA)[b]	nq	0.168	nq	nq
Tetrahydroxyxanthone-di-*O*,*C*-hexoside B (THXB)[b]	nq	0.133	nq	nq
Mangiferin	1.16	6.38	6.86	9.66
Isomangiferin	0.458	1.84	1.97	2.36
Total xanthones	*1.62*	*8.53*	*8.83*	*12.0*
Flavones				
Vicenin-2	0.182	0.192	0.498	0.524
Scolymoside	1.84	0.497	nq	nq
Total flavones	*2.02*	*0.690*	*0.498*	*0.524*
Dihydrochalcones				
3-Hydroxyphloretin-di-*C*-hexoside (HPDH)[c]	0.458	nq	nq	nq
3',5'-Di-β-D-glucopyranosylphloretin (PDG)	1.22	nq	nq	nq
Total dihydrochalcones	*1.67*	*0.000*	*0.000*	*0.000*
Flavanones				
Eriodictyol-*O*-hexoside-*O*-deoxyhexoside (EHD)[d]	nd	0.186	0.297	0.195
(2*R*)-5-*O*-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]naringenin (2RNAR)[e]	nd	0.028	0.146	0.051
(2*S*)-5-*O*-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]naringenin (2SNAR)	nd	0.087	0.397	0.444
Eriocitrin	0.536	0.310	nq	nq
Hesperidin	1.43	0.839	0.988	1.73
Total flavanones	*1.96*	*1.45*	*1.83*	*2.42*
Total quantified phenolics	***9.48***	***11.5***	***14.9***	***17.9***
Total polyphenols (Folin-Ciocalteau)[f]	***25.5***	***23.7***	***25.3***	***27.8***

[a] expressed as MMG equivalents; [b] expressed as mangiferin equivalents; [c] expressed as PDG equivalents; [d] expressed as eriocitrin equivalents; [e] expressed as 2SNAR equivalents; [f] expressed as g gallic acid equivalents/100 g extract.
Abbreviations: nd, not detected using LC-MS; nq, present in extract, but not quantified due to co-elution of very low content.

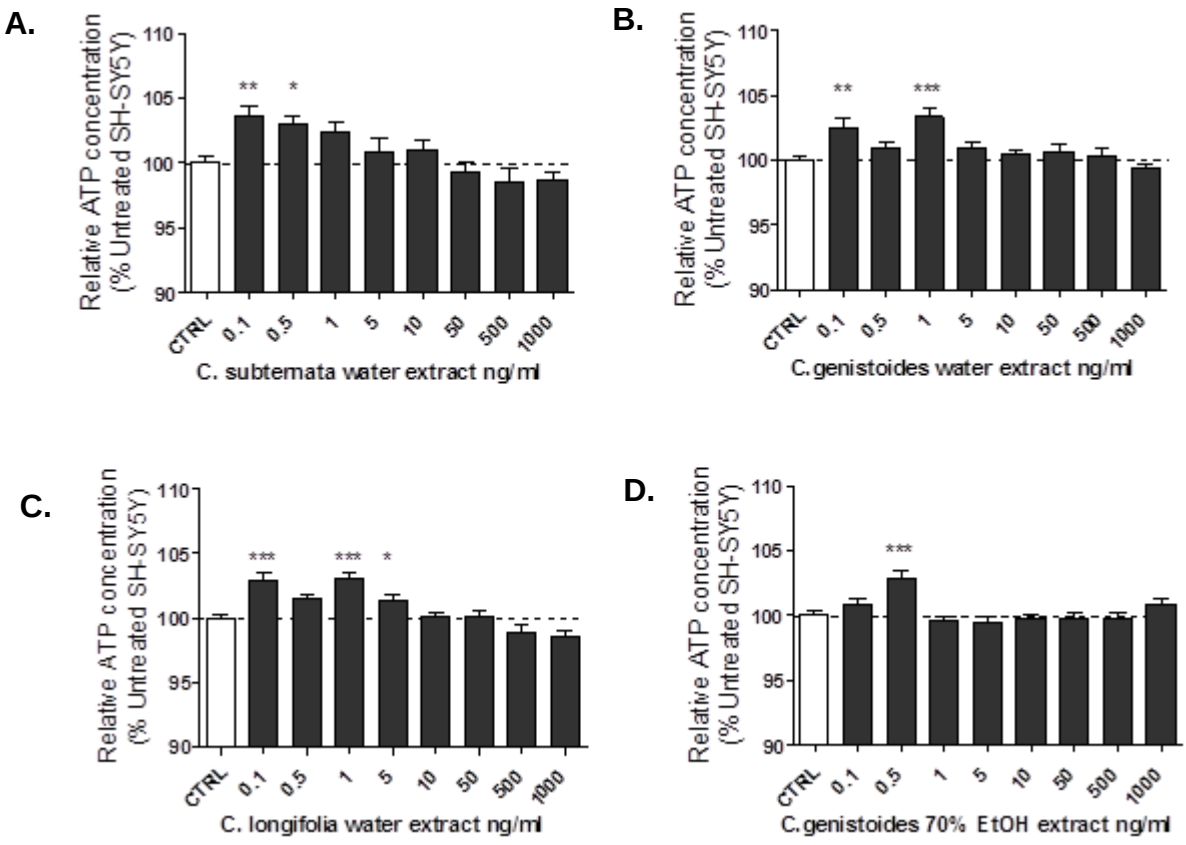

Figure 1.A-D) The water extracts of *Cyclopia subternata*, *C. genistoides* and *C. longifolia*, and the 70% ethanolic extract of *C. genistoides* significantly increased the ATP levels up to 4%. The cells were treated for 24 h with the extracts. Values represent the mean ± SEM of three independent experiments and were normalized on the untreated (CTRL) group (= 100%).One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells, *P<0.05, **P<0.01, ***P<0.001.

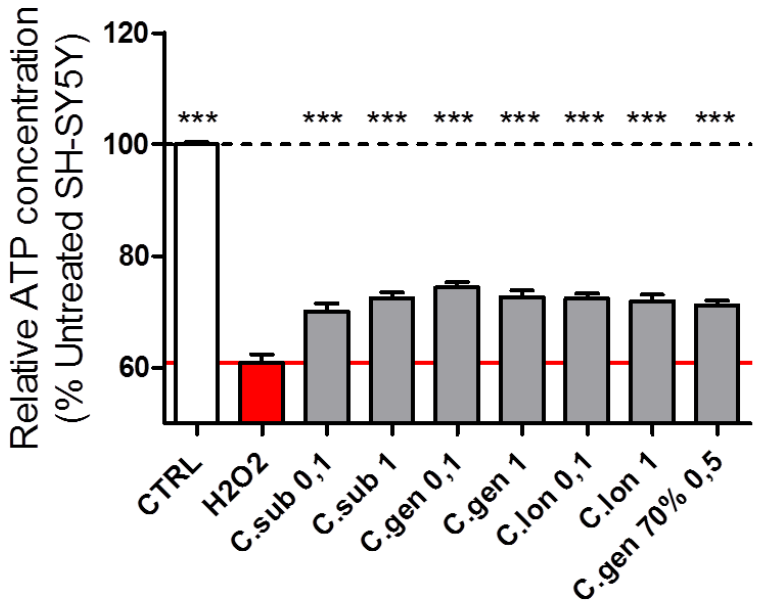

Figure 2. H_2O_2 treatment at 400 µM for 3h caused a 39.1% decrease in ATP production which was significantly increased up to 13.5% by a 24h pre-treatment with each of the extracts. The red bar represents the H_2O_2-treated cells and the grey bars represent cells that were pretreated for 24h with the indicated honeybush extract and then treated for 3h withH_2O_2. Values represent the mean ± SEM of three independent experiments and were normalized on the untreated group (= 100%). One way ANOVA and post hoc Dunnett's multiple comparison test versus H_2O_2 treated cells, *P<0.05, **P<0.01, ***P<0.001.

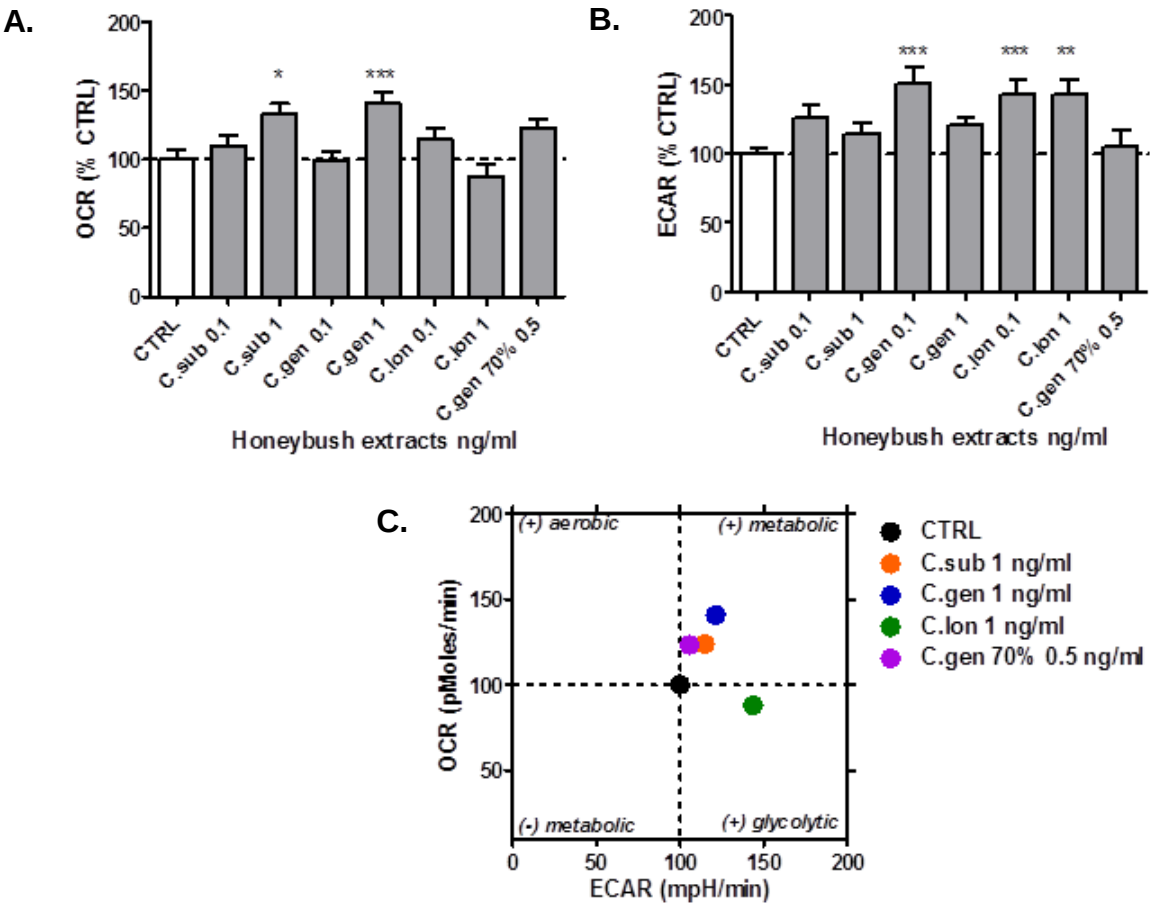

Figure 3. Respiration under physiological condition. A) 24h pre-treatment with the water extracts of *C. subternata* and *C. genistoides* (both at 1 ng/ml) significantly increased the oxygen consumption rate of the cells and therefore the respiration. B) The water extracts of *C. genistoides*(0.1 ng/ml) and *C. longifolia* (0.1 and 1 ng/ml) significantly increased the glycolysis in the SH-SY5Y cells. C) Energy map created after correlation of the OCR (respiration-y axis) with the ECAR (glycolysis- x axis). The aqueous extracts of *C. subternata* and *C. genistoides* acted on respiration (displayed as + metabolic on the figure) while the water extract of *C. longifolia* increased the glycolytic activity. Values represent the mean ± SEM of three independent

experiments and were normalized comparison test versus H_2O_2 treated cells, *P<0.05, **P<0.01, ***P<0.001.

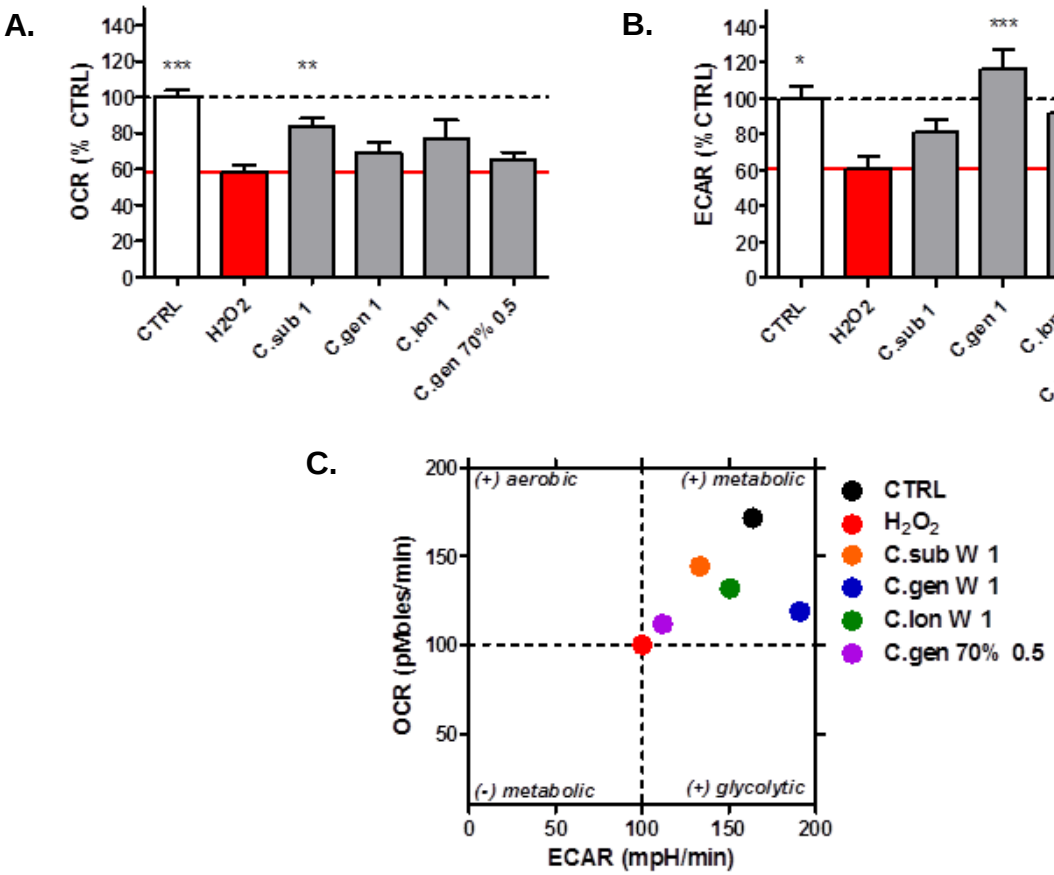

Figure 4. Respiration under oxidative stress condition. A) 3h treatment with H_2O_2 reduced the oxygen consumption rate by 41.7 % (red bars). 24h pre-treatment with the water extract of *C. subternata* (at 1 ng/ml) significantly ameliorated the oxygen consumption rate of the cells and therefore the respiration (grey bars). B) 3h treatment with H_2O_2 reduced the glycolysis by 38.9% (red bars). The aqueous extract of *C. genistoides* (1 ng/ml) significantly increased the glycolysis in SH-SY5Y cells. The red bar represents the H_2O_2-treated cells, and the grey bars represent cells that were pretreated for 24h with the indicated honeybush extract and then treated for 3h with

H_2O_2. C) Energy map created after correlation of the OCR (respiration-y axis) with the ECAR (glycolysis- x axis). This map helps in visually recognizing whether an extract predominantly increased the respiration (displayed as + metabolic on the figure) or the glycolytic activity compared to the H_2O_2 treated cells. Values represent the mean ± SEM of three independent experiments and were normalized on the untreated group (= 100%). One way ANOVA and post hoc Dunnett's multiple comparison test versus H_2O_2 treated cells, *P<0.05, **P<0.01, ***P<0.001.

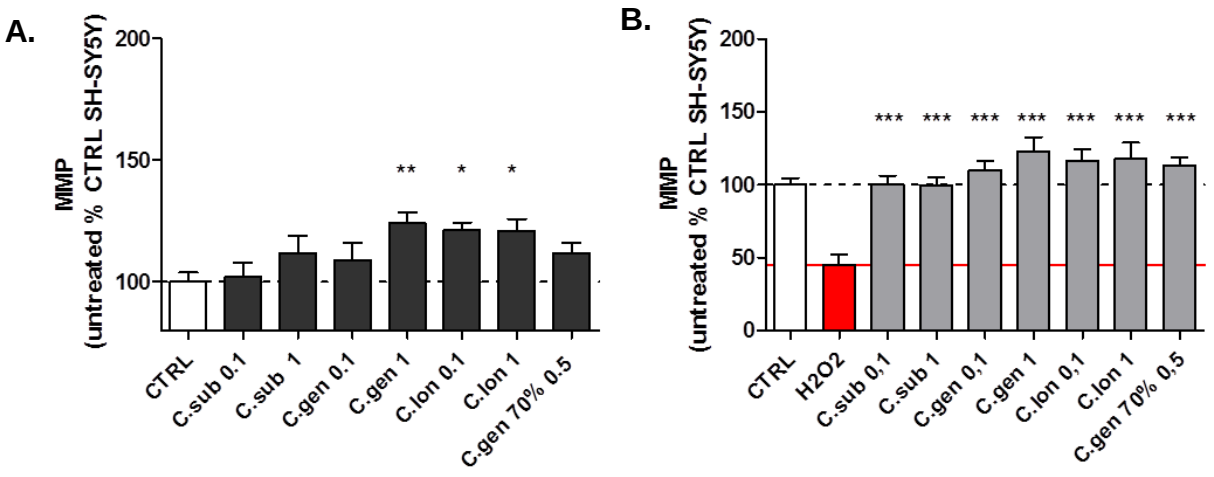

Figure 5.A) Honeybush extracts significantly increased the mitochondrial membrane potential (MMP) up to 24% under physiological conditions (black bars). B) 3h treatments with H_2O_2 at 400 µM caused a reduction of 55.1% in MMP which was rescued by the different honeybush extracts. The red bar represents the H_2O_2-treated cells, and the grey bars represent cells that were pretreated for 24h with the indicated honeybush extract and then treated for 3h with H_2O_2. Values represent the mean ± SEM of three independent experiments and were normalized on the untreated group

(= 100%). One way ANOVA and post hoc Dunnett's multiple comparison test versus A) untreated (CTRL) or B) versus H_2O_2 *P < 0.05, **P < 0.01, ***P < 0.001

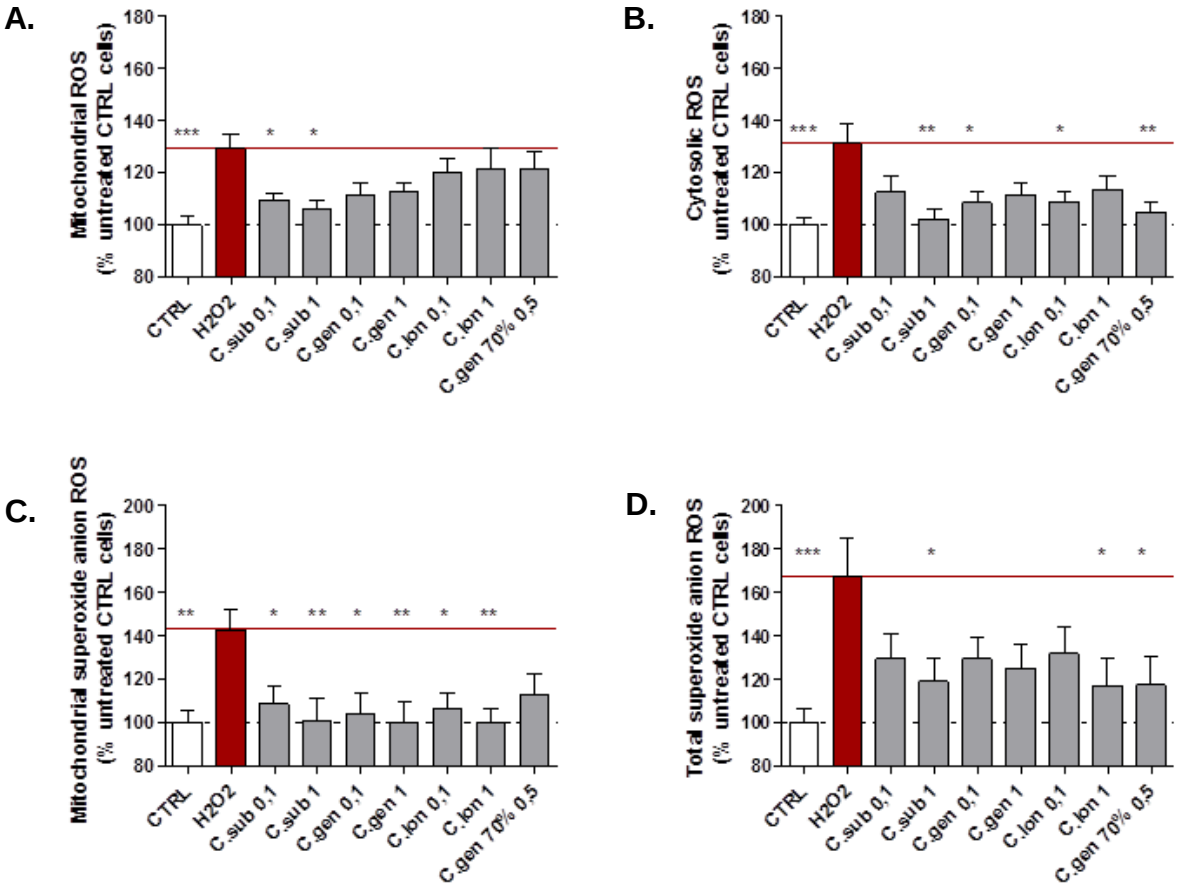

Figure 6.Effect of honeybush extracts on ROS levels under H_2O_2-induced oxidative stress. A) H_2O_2 treatment at 400 µM caused an increase of 29.5% in mitochondrial ROS which were detected using the dye DHR. This increase was significantly ameliorated up to 23.1% by *C. subternata* aqueous extract. B) H_2O_2 caused an

elevation of 31.2% in cytosolic ROS (detected with the dye DCF). All the extracts could bring the cytosolic ROS levels down, but the ones that reduced them significantly and most effectively were the aqueous extract of *C. subternata* at 1 ng/ml and the 70% ethanolic extract of *C. genistoides* at 0.5 ng/ml. C) H_2O_2 increased the mitochondrial superoxide anion levels by 43%. All the extracts, except the ethanolic extract of *C. genistoides,* significantly reduced the mitochondrial superoxide anion levels. The aqueous extracts of *C. subternata*, *C. genistoides* and *C. longifolia* at the concentration of 1 ng/ml each, completely neutralized the mitochondrial superoxide anion levels. D) The total superoxide anion levels were elevated by 67.9% in the H_2O_2-treated cells. All 4 extracts could ameliorate this increase but only the aqueous extracts of *C. subternata* at 1ng/ml, *C. longifolia* at 1ng/ml, and the ethanolic extract of *C. genistoides* at 0.5 ng/ml significantly reduced the superoxide anion levels. The red bar represents the H_2O_2-treated cells, and the grey bars represent cells that were pretreated for 24h with the indicated honeybush extract and then treated for 3h with H_2O_2.Values represent the mean ± SEM of three independent experiments and were normalized on the untreated group (= 100%). One way ANOVA and post hoc Dunnett's multiple comparison test versus A) untreated (CTRL) or B) versus H_2O_2 *P < 0.05, **P < 0.01, ***P < 0.001

2. *Rhodiola rosea* extract counteracts stress via improvement of bioenergetics, elimination of ROS and induction of neurite outgrowth

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Abstract

Background: Sustained stress with the overproduction of corticosteroids has been shown to increase reactive oxygen species (ROS) leading to an oxidative stress state. Mitochon-dria are the main generators of ROS and are directly and detrimentally affected by their over-production. Neurons depend almost solely on ATP produced by mitochondria in order to satisfy their energy needs and to form synapses while stress has been proven to deter synaptic plastici-ty. Emerging evidence underpins that *Rhodiola rosea*, an adaptogenic plant rich in polyphenols, exerts antioxidant, anti-stress and neuroprotective effects. Methods: In this study, the effect of *Rhodiola rosea* extract (RRE) WS®1375 on neuronal ROS regulation, bioenergetics and neurite out-growth, and its potential modulatory effect on the brain derived neurotrophic factor (BDNF) pathway was evaluated in the human neuroblastoma SH-SY5Y and the murine hippocampal HT22 cell line. Stress was induced using the corticosteroid dexamethasone. Results: Already in low concentrations (1-10 ng/ml) the RRE increased bioenergetics as well as cell viability and scavenged ROS with a similar efficacy in both cell lines and counteracted the respective cortico-steroid-induced dysregulation. The effect of RRE under dexamethasone-stress and the effect of RRE itself resulted in biphasic U-shape and inverted U-shape dose-response curves, respectively, a characteristic feature of adaptogenic plant extracts. Additionally, RRE treatment promoted neurite outgrowth and demonstrated an upregulating effect on the BDNF levels. Conclusion: These findings indicate that RRE could constitute a candidate for the prevention of stress-induced pathophysiological processes as well as oxidative stress with an impact on stress-associated mental disorders potentially leading to the development of a condition-specific supplementa-tion.

Keywords: *Rhodiola rosea* extract; mitochondria; oxidative stress; glucocorticoid stress; bioen-ergetics; neurite outgrowth; BDNF

1. Introduction

Reactive oxygen species (ROS), at physiological non-elevated concentrations, have essential roles in cellular signaling, in the regulation of senescence, cell death and prolif-eration, and in defense against pathogenic microorganisms [1, 2]. However, ROS become harmful and disease causing agents upon their overproduction leading to the so-called oxidative stress. For their protection, cells are equipped with antioxidant defense systems (glutathione GSH, superoxide dismutases, glutathione peroxidases, catalase and thiore-doxin) in order to fend off ROS [3-5]. The redox state of cells is dynamic and depends on the production of ROS and the existence and functionality of antioxidant defense systems. Oxidative stress is a state of serious imbalance between the production of ROS and the capacity of antioxidant defense systems to diffuse them. It is a common feature in many chronic diseases such as neurodegenerative, mental and stress-related disorders, as well as in normal aging having devastating consequences for the functionality and well-being of biomolecules (e.g. DNA and lipids) and, therefore, of neuronal cells [1-3, 6-10].

Oxidative stress is also prominent during hormonal/corticosteroid stress. The stress response is mediated by the stress system which consists of the hypothalamus, the pitui-tary gland and the adrenal cortex termed the HPA axis. The process in the HPA axis starts in the paraventricular nucleus of the hypothalamus with the release of cortico-tropic-releasing-hormone (CRH) as well as the secretion of the arginine vasopressin. These two factors enhance each other`s expression [11]. CRH stimulates the release of adreno-corticotropic hormone (ACTH) from the anterior pituitary gland. The ACTH subsequently stimulates the release of cortisol from the adrenal cortex which is the end-product of the stress response mediated by the HPA axis. The HPA axis contains a control mechanism which regulates the cortisol levels. In detail, the increased cortisol concentration inhibits the secretion of ACTH during a negative feedback to the brain which therefore stops the stress response and allows re-establishment of homeostasis [11]. The stress system helps in survival by activating the fight or flight mode. Acute activation of the HPA axis upon stressful events, the so-called eustress, increases the overall functions of the organism [12, 13]. However, sustained activation over a long period of time exerts detrimental effects as over-accumulation of cortisol becomes deleterious for the brain but also for other systems in the body. Cortisol negatively affects brain regions such as the hippocampus, which

is responsible for learning and memory and is one of the very few brain regions in which neurogenesis takes place throughout life. Cortisol directly affects the hippocampus both structurally and functionally. Stressful life episodes have been linked to lower hippocam-pal volumes in humans, as well as structural changes in the hippocampus with stress provoking the shrinking of dendrites and loss of neurons [14-18]. As a result, there is an increased risk for the development of stress-associated disorders such as depression [19]. The corticosteroid that is widely used for in vitro experiments is dexamethasone (Dexa), which is a synthetic corticosteroid that mimics the action of physiological and endogenous stress hormones [20].Additionally, corticosteroid stress has been shown to increase oxidative stress. Inter-estingly, oxidative and mitochondrial dysfunction are common in mental, neurodegener-ative and age-related diseases while additional corticosteroid stress intensifies these problems [21-23].*Rhodiola rosea* is a perennial flowering plant belonging to the Crassulaceae family. It grows naturally in the wild at high altitude and it can be found in the Arctic and in mountainous areas of Central Asia and Europe [24]. Extracts from Rhodiola are considered adaptogens (EMEA/HMPC/102655/2007). Adaptogens are non-toxic agents that increase resistance against physical, chemical, biological and psychological stressors by normal-izing the harmful effect of these stressors independently of the nature of the pathologic state. In this way, they help the organism to achieve a physiological equilibrium and ho-meostasis during stressful situations [25, 26]. Indeed, there have been clinical studies in-dicating beneficial effects of *Rhodiola rosea* extracts (including the WS®1375 extract) in stress and fatigue management, in building resilience and in depression regulation [27-35]. At the same time, RRE has shown antioxidant, anti-inflammatory and neuropro-tective effects in animal models [36-39] as well as in cellular models [40]. Phytochemical-ly, extracts of *Rhodiola rosea* roots and rhizomes contain mainly six classes of compounds: flavonoids, phenolic acids, phenylethanoloids, phenylpropanoids, monoterpenes and triterpenes. Salidroside, a phenylethanol glycoside, is considered to be the main pharma-cologically active compound of *Rhodiola rosea*, although it is also found in many other Rhodiola species. On the contrary, some cinnamic alcohol glycosides such as rosavin, ro-sarin and rosin have been solely found in *Rhodiola rosea*. These compounds are referred to as 'rosavins' and are represented by the phenylpropanoid rosavin [24]. Both salidroside and rosavin represent the lead marker compounds of the RRE. Extracts of

Rhodiola rosea rhizomes and roots have been characterized as `` traditional herbal medicinal product for temporary relief of symptoms of stress,such as fatigue and sensation of weakness`` by the European Medicines Agency (EMA) (EMEA/HMPC/102655/2007). Although there is a sig-nificant amount of research on the individual RRE compound markers [41-43] no study to date has examined cellular and molecular mechanisms underlying the action of the RRE extract using in depth in vitro methods. Therefore, in this in vitro study, we aimed to evaluate the effect of the RRE WS®1375 on neuronal ROS regulation, bioenergetics and neurite outgrowth, and its potential mod-ulatory effect on the BDNF pathway in the human neuroblastoma SH-SY5Y and the mu-rine hippocampal HT22 cell lines. Moreover, the potency of the RRE WS®1375 in neutral-izing corticosteroid stress-induced ROS generation and protecting cell viability was eval-uated.

2. Materials and Methods

2.1. Chemicals and reagents

Dulbecco's-modified Eagle medium (DMEM), phosphate buffered saline (PBS), fetal calf serum (FCS), Hanks' Balanced Salt solution (HBSS), penicillin/streptomycin, dihydrorhodamine 123 (DHR), 2',7'-dichlorodihydrofluorescein diacetate (DCF), thiazolyl blue tetrazolium bromide (MTT), neurobasal medium, retinoic acid, paraformaldehyde, bovine serum albumin (BSA), 4′,6-Diamidino-2-phenylindol (DAPI), Triton X-100 and dexame-thasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa fluor 488 and βIII-tubulin antibodies were from R&D Systems (Minnesota, USA). MitoSOX, glutaMax and B27 supplement were from Gibco Invitrogen (Waltham, MA, USA). NGF was from Lubio (Zürich, Switzerland), ATPlite1step kit from PerkinElmer (Waltham, Massachu-setts, USA) and horse serum (HS) from Amimed, Bioconcept (Allschwil, Switzerland). Recombinant human BDNF protein was from Novus Biologicals (Littleton, Colorado, United States). The mature BDNF rapid elisa kit was purchased from Biosensis (Adelaide, Australia), the RNA extraction kit from Qiagen (Hilden, Germany), the GoScript™ Reverse Transcription Mix, Oligo and the GoTaq® Master Mix for real time quantitative PCR (RT-qPCR) from Promega (Dübendorf, Switzerland), the BDNF primers from Microsynth (Balgach, Switzerland).

2.2. Characterization of RRE WS®1375

WS®1375, a proprietary dry extract from *Rhodiola rosea* roots and rhizomes harvested in Russia was supplied by Dr. Willmar Schwabe GmbH & Co KG (Karlsruhe, Germany). WS®13751 was obtained by extraction with 60% (w/w) aqueous ethanol (drug extract ratio 1.5-5:1). The quantification of rosavins (assessed as rosavin) and salidroside (assessed as p-tyrosol) in WS®1375 was conducted by reversed-phase high performance liquid chro-matography (HPLC) with UV-detection. For quantification of salidroside, p-tyrosol was used for calibration of the peak area. After integration of the salidroside peak, the value was multiplied with the factor of 2.173 to correct the amount according to the molecular mass difference between salidroside/p-tyrosol (300.31/138.16 = 2.173). The HPLC-UV chromatograms were recorded on Merck HPLC equipment using a Merck LiChrospher® 100 C18 (5 µM, 4 x 250 mm) column with pre-column conditioned at 25 °C. Eluent A con-sisted of demineralized water with 0.3% (v/v) o-phosphoric acid 85 %. Eluent B consisted of acetonitrile. At a flow rate of 1 ml/min, the gradient was as follows: from 0.0 – 30.0 min. linear from 5% to 20% eluent B, from 30.0 – 45.0 min. linear from 20% to 50% eluent B, from 45.0 to 45.5 min. linear from 50% to 5% eluent B, from 45.5 to 60.0 min. isocratic 5% eluent B as equilibration period, resulting in a total run time of 60.0 min. UV detection wavelength was 251 nm for rosavins and 275 nm for salidroside and a column tempera-ture of 25 °C was applied. The injection volume was 10 µL of a 4 mg/ml RRE WS®1375 dissolved in methanol. Calculations were conducted using the software EZChrom. Visu-alization of the chromatogram was done with ACD/Labs Spectrus Processor (v2017.2.1) software.

2.3. Cell culture

The human neuroblastoma SH-SY5Y cell line (ATCC® CRL-2266™ Manassas, VA, USA) was selected as our cellular model in this study as it is a well-established and wide-ly used neuronal model in biochemical studies in general as it expresses neuronal recep-tors. In parallel, the murine hippocampal cell line HT22 (SCC129, Sigma-Aldrich, St. Lou-is, MO, USA) was used for confirmation of results and because the hippocampus is di-rectly affected by corticosteroid stress. The SH-SY5Y cells were kept and grown at 37 °C in a humidified incubator chamber under an atmosphere of 5% CO2 in DMEM supplement-ed with 10% (v/v) heat-inactivated FCS, 5% HS, 2 mM glutaMax and 1% (v/v) penicil-lin/streptomycin. The HT22 cells were cultured in DMEM containing 10% FCS, 1% Peni-cillin-Streptomycin and 1% glutaMax. Cells were passaged 1-2 times per week and the cells used for the experiments did not exceed passage 20. The cells were plated when they reached 80–90% confluence.

For the 2D cell culture, cell plates were coated with collagen type I (Rat tail, Corning, Amsterdam, Netherlands) at 0.05 mg/ml.

2.4. Treatment of cells

Evaluation of ATP production and cell viability was conducted on SH-SY5Y and HT22 cells to determine the potential toxic concentration range of the WS®1375 RRE. Two screenings were performed. Initially, the RRE was screened at a very broad concentration range of 1 ng/ml to 1 mg/ml (data not shown). Stock solution of the RRE was prepared in DMSO (final assay concentration of DMSO <0.005%. There was no effect of the vehicle so-lution alone compared to the untreated condition. Therefore, only the untreated control condition is presented in the results section. The first screening revealed that RRE did not show toxic effects on the neuroblastoma and hippocampal cells up to a concentration of 400 ng/ml. According to the results of the first screening, a concentration range from 1 ng/ml to 100 ng/ml was tested in a second screening cycle. The screening was conducted by using an ATP detection assay (ATPlite 1step kit was from PerkinElmer) and a cell via-bility assay (MTT assay).

For the experiments with a scope to evaluate the effect of RRE per se, cells were plated and treated 1 day after plating for 24 h either with DMEM (untreated cells-control condi-tion) or with a final concentration of 1 ng/ml to 100 ng/ml of the extract (in particular 1, 5, 10, 100 ng/ml).

Dexa an agonist of the glucocorticoid receptor, was used to induce stress on the SH-SY5Y and HT22 cells. Dexamimics the effect of the stress hormone cortisol and they are both binding at the same receptors. Dexa was dissolved in DMSO and then subsequent working solutions were generated for the treatment of the cells. Dexa at 100 µM was able to induce stress in HT22 cells while the SH-SY5Y cells were stressed after treatment with Dexa at 400 µM. These Dexa concentrations were selected based on screening experiments conducted on SH-SY5Y and HT22 cells using the ATP production and MTT reduction as-says. For the ROS experiments, a much lower Dexa concentration could induce oxidative stress, namely, 5 µM for HT22 cells and 100 µM for SH-SY5Y cells.

For the stress experiments, cells were first pre-treated for 24h with RRE (1, 5, 10, 100 ng/ml) and then treated for another 24h with the respective Dexa concentration. Each as-say was conducted and repeated at least in triplicates.2.5. ATP levels

Total ATP content was determined using a bioluminescence assay (ATPlite 1step) according to the instructions of the manufacturer and as previously described [44-46]. SH-SY5Y and HT22 cells were plated in 6 replicates into white 96-well cell culture plates at a density of 1×104 cells/well and 5×103 cells/well respectively. The ATP was extracted from the cells upon lysis and it was transformed into light. The method measures the formation of light from ATP and luciferin catalyzed by the enzyme luciferase. The emitted light was linearly correlated to the ATP concentration and was measured using the mul-ti-mode plate reader Cytation 3 (BioTek instruments, Winooski, Vermont, United States).

2.5. Cell viability assay

Cell viability was investigated using a MTT cell proliferation assay. After treatment, the cells were incubated with MTT (3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl-tetrazolium bromide) in DMEM for 3 hours. MTT is reduced to a violet formazan derivative by mitochondrial enzymatic activity. At the end of the reaction, the cells were dissolved in DMSO 100%. MTT absorbance was measured at 595 nm using the multi-mode plate reader Cy-tation 3.

2.6. Determination of ROS levels

Mitochondrial and cytosolic ROS levels as well as the specific levels of mitochondrial superoxide anion radicals levels were assessed using the fluorescent dyes dihydrorhoda-mine 123 (DHR), 2′,7′-dichlorodihydrofluorescein diacetate (DCF) and the Red Mitochon-drial Superoxide Indicator (MitoSOX), respectively, as previously described [46, 47]. SH-SY5Y and HT22 cells were plated in 6 replicates into black 96-well cell culture plates at a density of 1×104 cells/well and 5×103 cells/well respectively. After treatment with RRE alone or after pre-treatment with RRE, followed by treatment with Dexa, cells were treated with 10 µM of one of the dyes: DHR or DCF for 20 min or 5 µM of MitoSOX for 90 min at room temperature in the dark on an orbital shaker. After washing the cells three times with HBSS, the formation of green fluorescent products triggered by DHR and DCF, re-spectively, were detected at 485 nm (excitation)/535 nm (emission). MitoSOX triggers the formation of red fluorescent products which were detected at 531 nm (excitation)/595 nm (emission). The intensity of fluorescence was proportional to mitochondrial ROS, cytosolic ROS and mitochondrial superoxide anion levels. The fluorescence was measured using the Cytation 3 multi-mode plate reader.

2.7. Neurite outgrowth determination

SH-SY5Y neuroblastoma cells were plated in black 96-well plates that were pre-coated with collagen 0.05 mg/ml. 24h post plating, cell differentiation was induced by adding neurobasal medium containing 2% B27 supplement, 1% penicillin-streptomycin, 1% glutaMax and 10 µM retinoic acid (RA) for 3 days as described previously [48]. Then, cells were treated either with the WS®1375 RRE at the concentrations 5 and 10ng/ml or with 50 ng/ml of NGF (positive control) or with 50 ng/ml of BDNF (positive control). After 3 days of treatment, cells were fixed with 2% paraformaldehyde.

2.8. Immunostaining

The usage of black 96-well plates with a clear bottom allows to directly image the samples. Immunolabeling of neurites was performed using an anti βIII-tubulin primary antibody and Alexa Fluor 488-conjugated secondary antibody (fluorescence emission in the green wavelength). Immunolabeling of the nucleus was conducted using DAPI (4′,6-diamidino-2-phenylindole) which emits blue fluorescence upon binding to adenine and thymine-rich regions of DNA.

2.9. Microscopy and analysis (software)

Images were taken using Cytation 3, a digital cell imaging multi-mode reader with a 20x objective. Analysis was performed using ImageJ (Neurophology plugin) software to evaluate parameters of neuroplasticity as described before [48]:

Soma count

Neurite count

Total neurite length

Number of branching points = attachment point

Number of contact points = endpoint

2.10. RNA Extraction and Quantitative Real-Time PCR

SH-SY5Y cells were plated at 6-well plates at a density of 4×105 cells/well. 24h post plating, the cells were treated either with DMEM (control condition) or with WS®1375 RRE at 5ng/ml. Cell were lysed after 1h, 2h, 3h, 4h, 12h and 24h of treatment. Total RNA was isolated with RNeasy Spin columns (Qiagen, Hilden, Germany). Quantitation was per-formed by spectrophotometry (Nanodrop, Thermoscientific). One microgram of RNA from each sample was subjected to reverse transcription using the GoScript™ Reverse Tran-scription Mix from Promega (Dübendorf, Switzerland). After reverse transcription the cDNA was diluted 1:5 with nuclease-free water and each cDNA sample was amplified using quantitative real-time PCR (StepOne™ System, Applied Biosystems). Each PCR contained 900 nM of each primer (forward and reverse), the PCR MasterMix (GoTaq® qPCR Master Mix, Promega) and CXR reference dye combined in 17 µl as well as 3 µl of the diluted cDNA. 40 cycles were performed of the following thermal profile: 2 min at 95 °C, 15 sec at 95 °C followed by 1 min at 60 y. Final quantitation was conducted using the comparative CT method (threshold cycle: number of cycle until sample signal is detected). GAPDH was used as control housekeeping gene to assess the validity of the cDNA mix-ture and the PCR reaction.

2.11. Determination of mature BDNF protein level

SH-SY5Y cells were plated in 10cm2 dishes. 24h post plating, the cells were treated either with DMEM (control condition) or with WS®1375 RRE at 5 ng/ml. Cell lysates were produced after 4h, 24h and 48h of treatment. The concentrations of mature BDNF were quantified in duplicate using the Biosensis Mature BDNF Rapid ELISA Kit (Thebarton, Australia) according to the instructions of the manufacturer. Prior to each immunoassay, cell lysates were diluted 1:4 using the provided sample buffer. After incubation, the ab-sorbance was measured at 450 nm using the Cytation 3 multi-mode plate reader and the levels of mature BDNF were calculated in pg/ml and normalized to 1 mg of total protein level.

2.12. Statistical analysis

Data are given as the mean ± SEM, normalized to the untreated control group (=100%). Statistical analyses were performed using the Graph Pad Prism software. For statistical comparisons, one-way ANOVA was used, followed by Dunnett's multiple comparison tests versus the control or versus Dexa (for the experiments under stress con-ditions). For the PCR and the Elisa experiments unpaired Student t test was used versus control. p values < 0.05 were considered statistically significant. The adequacy of fits was estimated by the R-squared value (>0.9) using Pearson correlation and linear regression analysis.

Results

3.1. Quantification of rosavins and salidroside in RRE WS®1375

The lead compounds in the RRE WS®1375 were determined by reversed-phase HPLC with UV-detection (Figure 1). The single WS®1375 batch used for the present study con-tained 1.04% rosarin, 2.63% rosavin, 1.04% rosin (all assessed as rosavins) and 1.16% salidroside (assessed as p-tyrosol) that is in agreement with the concentration range of those lead compound in RREs used for medicinal purposes in other studies [49, 50].

3.2. RRE eliminated ROS and rescued cell viability caused by dexamethasone in two cell lines

The capacity of RRE to counteract the detrimental effects of corticosteroid stress were examined in two cells lines, the human neuroblastoma SH-SY5Y and the murine hippo-campal HT22 cells. Dexa, a synthetic agonist of the glucocorticoid receptor which mimics the effect of cortisol, was used to induce stress. Dexa 100 µM caused a 16.7%, 38.1% and 22.2% elevation in mitochondrial ROS, cytosolic ROS and mitochondrial superoxide ani-on levels respectively in SH-SY5Y cells (Figure 2A-C). RRE (1, 5, 10, 100 ng/ml) was able to reduce the Dexa-induced mitochondrial, cytosolic and mitochondrial superoxide ROS levels by up to 16,9%, 22,0% and 22,1% respectively. The most effective concentrations of the extract that significantly decreased ROS levels to levels comparable to the control con-dition were the 5 ng/ml for mitochondrial ROS, 5 and 10 ng/ml for cytosolic ROS, and 10 ng/ml for mitochondrial superoxide anion. A similar effect was observed in the HT22 cells in which Dexa 5uM treatment caused an elevation of the same type of ROS by 18.7%, 13% and 36.2% respectively (Figure 2E-G). Treatment with RRE 5 and 10 ng/ml significantly reduced mitochondrial ROS by up to 14.4%, treatment with RRE 5 ng/ml significantly re-duced cytosolic ROS by 15.2%, and RRE 1, 5 and 10 ng/ml significantly reduced mito-chondrial superoxide anion by up to 25.6%. Treatment with the same concentrations was able to rescue the Dexa-induced cell death in the two cell lines. Dexa 400 µM caused a 14% reduction in cell viability of SH-SY5Y (Figure 2D). Treatment with 1, 5 and 10 ng/ml of the RRE rescued the cell viability by up to 12.2%, almost a complete rescue. Dexa 100 µM caused cell death of 19.2% compared to the control in HT22 cells. Treatment with 5 and 10 ng/ml of the RRE rescued cell viability up to 16.1% (Figure 2H). The two RRE concentra-tions of 5 and 10 ng/ml seemed to be the most efficacious in rescuing cell viability and in decreasing ROS under corticosteroid stress.

3.3. RRE increased bioenergetics and cell viability in two cell lines

In order to examine the effect of the extract itself, a screening was conducted using the ATP production assay and the cell viability (MTT) assay which is an indicator of in-creased mitochondrial metabolic activity. Regarding SH-SY5Y cells, all tested concentra-tions (1, 5, 10, 100 ng/ml) significantly increased the ATP levels up to 8.3% (Figure 3A). The same effect was observed in the cell viability assay in which all the concentrations in-creased the MTT values up to 15.1%. The highest increase amounted to 15.1% and 11.5% and was caused by treatment with 5 and 10 ng/ml respectively (Figure 3B). Upon Pearson correlation of the ATP production with the cell viability assay, a significant positive linear correlation was found in SH-SY5Y cells ($R2 = 0.9007$, $p = 0.0137$) with the most effective RRE concentrations being 5 and 10 ng/ml. The resulting plot allows clear visualization of the most effective concentrations of RRE (Figure 3C). This suggests that the improvement in ATP production was preferentially linked to an increase in metabolic activity of the cells. These results were confirmed in the HT22 cells in which the effect of RRE was simi-lar to that observed in SH-SY5Y cells. In detail, all tested concentrations (1-100 ng/ml) in-creased both bioenergetics, in the form of ATP production, and metabolic activity, in the form of cell viability of up to 18.8% and 19.7% respectively (Figure 3D and 3E). The RRE concentration with the most prominent effect was 5 ng/ml in both assays which is also apparent in the Pearson correlation plot in Figure 3F. Again, a significant positive linear correlation was found between the ATP production and the cell survival assays in HT22 cells ($R2 = 0.8616$, $p = 0.0228$) (Figure 3F).

3.4. RRE promotes neurite outgrowth in differentiated SH-SY5Y cells

The capacity of RRE in promoting neurite outgrowth was assessed in differentiated SH-SY5Y cells in a 2D cell culture model [48]. The two most promising concentrations from the previous experiments, 5 and 10 ng/ml, were tested in the subsequent experiments on neurite outgrowth (Figure 4). The neurotrophins nerve growth factor (NGF) and brain-derived growth factor (BDNF), both at 50 ng/ml, which are known inducers of neu-rite outgrowth and promoters or cell survival and synaptic plasticity, were used as posi-tive controls. The cells were treated for three days. After immunostaining and microscopy, it was shown that RRE promoted a significant neurite outgrowth which was visually comparable to the effect caused by the positive controls (Figure 4). In order to confirm and quantify the effect, the ImageJ (Neurophology plugin) software was used for analysis which allows the measurement of specific neurite outgrowth parameters such as soma count, neurite count, total neurite length, attachment points of neurons and endpoints of neurons (Figure 5). The extract conditions as well as the positive controls were normalized and compared to the untreated control cells. As expected, the positive controls caused a considerable increase in all the parameters. Interestingly, treatment of the cells with the RRE at 5 and 10 ng/ml had a similar effect as the positive controls. Specifically, the con-centrations 5 and 10 ng/ml caused an increase of 70.1% and 53.2% respectively in soma count, an increase of 104% and 64.3% respectively in neurite count, an increase of 90.1% and 61.8% respectively in total neurite length, an increase of 101% and 67.8% respectively in the number of attachment points and an increase of 98.7% and 61.6% respectively in the number of endpoints (Figure 5).

3.5. RRE increased the BDNF mRNA expression in SH-SY5Y cells

Determination of the effect of the RRE on the BDNF gene expression was conducted via quantitative real-time PCR in SH-SY5Y cells. Cells were treated either with plain me-dium (DMEM) or with RRE 5 ng/ml (the most promising concentration based on the pre-vious experiments) and cell lysates were produced from 1h up to 24h of treatment. RRE had an upregulating effect on BDNF gene expression (Figure 6A). The effect peaked after 3h of treatment (Figure 6A) and decreased afterwards, but still exhibited a significant in-crease of mRNA expression levels after 4h and 12h of treatment. A trend was still observ-able at 24h ($p = 0.0648$).

3.6. RRE increased the mature BDNF protein level in SH-SY5Y cells

Determination of the effect of RRE on the mature BDNF protein level was conducted via an enzyme immunoassay specifically detecting the mature form of BDNF protein. Cells were treated with either plain medium (DMEM) or RRE at a concentration of 5 ng/ml. Cell lysates were obtained after 4h, 24h and 48h of treatment. Mature BDNF con-centration was quantified and is illustrated in Figure 6B. Treatment with RRE had a sig-nificantly upregulating effect on the protein level of mature BDNF at 4h, which was less pronounced at 24h and disappeared at 48h.

4. Discussion

In this study, we initially hypothesized that RRE WS®1375 of the adaptogenic plant *Rhodiola rosea* would be able to reduce the detrimental effects of corticosteroid stress. The effect of RRE was examined at a concentration range of 1-100 ng/ml. In order to test our theory we employed two cell lines: the human neuroblastoma cells SH-SY5Y which is a known and well-characterized neuronal model and the murine hippocampal cells HT22. The latter hippocampal cell line was selected in order to confirm our results in cells gen-erated from a brain structure that is at the forefront of the impact of corticosteroid stress. Dexa, a synthetic corticosteroid which mimics the effect of the endogenous stress hormone cortisol, was used to simulate a stressful situation. Mitochondria are the main generators of ROS and are directly affected by their overproduction. At the same time, neurons are highly dependent on mitochondria for their function and survival as they demand high amounts of energy while they are more vulnerable to oxidative stress because they con-sume a lot of oxygen [2, 10, 51]. Corticosteroids such cortisol have been shown to increase ROS production leading to an oxidative stress state [21-23] with increased oxidation markers such as 8-hydroxyguanosine (8-oxoG) and 8-iso-prostaglandin F2α (IsoP) as demonstrated in the saliva of chronically stressed women [21]. Consistently, it has been shown that a non-genomic glucocorticoid receptor-mediated pathway can also lead to in-creased ROS production with concomitant activation of the ERK/CREB/PGC1-α signaling as a stress compensation mechanism in the rainbow trout [23]. In line with those findings, Dexa significantly increased the mitochondrial ROS, cytosolic ROS and mitochondrial superoxide anion levels in both cell lines in our study. Moreover, Dexa induced a decrease in cell viability in SH-SY5Y and HT22 cells that might be related to the Dexa-induced in-crease in ROS production. Of note, RRE partially rescued the cell survival and normalized ROS levels after Dexa stress in both cell lines, with the 5 ng/ml concentration being the most effective.

Of note, the protective effect of RRE on the Dexa-induced increase in the generation of different types of ROS (cytosolic, mitochondrial and mitochondrial superoxide anion) displays U-shaped dose response curves as illustrated in Figure 2 (SH-SY5Y cells: A,B,C; HT22 cells: E,F,G). Accordingly, inverted U-shaped dose response curves showing the ef-fects of RRE on the impaired cell viability induced by Dexa have been found respectively (see Figure 2, D and H). Those biphasic response curves are usually characteristic for bo-tanicals, especially plant adaptogens [37, 52-54]. Typically, low doses of an adaptogen induce a dose-dependent increase in biological response until a maximum response is reached, after which there is a gradual decrease in the response until it might even reach a non observable effect level, a phenomenon also called hormesis [52]. Thus, a biphasic re-sponse curve could reflect activation of the «adaptive cellular stress response». Consist-ently, our findings indicate that the herbal RRE WS®1375 exhibits the typical features of an adaptogen with regard to the modulation of adaptive homeostasis and stress response.

Furthermore, our findings are in line with existing literature supporting the antioxi-dant effect of RRE and especially of its compound salidroside [43, 55-60]. Thus, an etha-nolic RRE containing high concentrations of phenolic compounds, particularly salidro-side, showed a strong antioxidant activity in the 1,1-Diphenyl-2-picrylhydrazyl scaveng-ing capacity assay (DPPH), in the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) scavenging capacity assay (ABTS) and in the ferric reducing antioxidant power assay (FRAP) [57]. Additionally, salidroside protected PC12 cells from H2O2-induced apoptosis through inactivation of the caspase cascade and through prevention of cytochrome c re-lease [58]. Both, salidroside and its metabolite tyrosol, abolished the H2O2-induced oxida-tive damage in rat cortical neurons by decreasing the expression of Bax (a pro-apoptotic protein) with tyrosol being more effective possibly due to its position of the glycosyl group [59]. Moreover, in SH-SY5Y cells, salidroside was able to rescue the H2O2-induced cell via-bility loss and apoptotic cell death by inducing antioxidant enzymes such as thioredoxin, heme oxygenase-1 and peroxiredoxin-I, by downregulating the pro-apoptotic gene Bax as well as by upregulating the anti-apoptotic gene Bcl-2, mechanisms that might also be in-volved in the observed effects of the RRE WS®1375 extract [43].

The majority of endogenous cellular ROS are produced by mitochondria as by-products of OXPHOS which takes place at the electron transport chain (ETC) on the inner mitochondrial membrane. ROS are mainly generated by complexes I and III of the ETC when leaking electrons that are provided by NADH or FADH2 react with oxygen [61, 62]. Since our findings indicated that RRE neutralized mitochondrial and cellular ROS, we aimed to examine the potential effect of the extract on increasing mitochondrial func-tions such as ATP production and metabolic activity. As mentioned before, mitochondria are at the epicenter of neuronal health and survival mainly because they are the principal energy producers and suppliers (in the form of ATP). The main mechanism leading to the production of ATP is OXPHOS that takes place in the ETC of mitochondria [2, 10]. The same extract concentration spectrum as in the previous experiments (1-100 ng/ml) had a positive effect on both mitochondrial functions after treatment of the two cells lines for 24h. The RRE concentrations that increased the ATP production and the metabolic activity the most were the 5 and 10 ng/ml. Again, we can confirm that the effects of RRE follow the typical biphasic dose response curves of adaptogens (see Figure 3).

Apart from one study conducted two decades ago showing that RRE increased ATP levels in mitochondria of skeletal muscles in rats, there are no further specific studies evaluating the effect of the RRE or its compounds on bioenergetics [63]. Another study in-dicated the beneficial effect of the extract of another Rhodiola species, that of *Rhodiola crenulata*, which increased the ATP production and cell viability as well as reduced the loss of neurons in the hippocampus of rats with hypobaric hypoxia [64]. However, different Rhodiola species contain different bioactive compounds. For example, rosavin is a characteris-tic marker of RRE and is not present in many other Rhodiola species such as *Rhodiola crenulata* [65, 66]. Therefore, it can be speculated that rosavin does not contribute to the ATP-increasing effect of RRE in neuronal cells.

Healthier mitochondria lead to healthier neurons which can fulfil their functions most efficiently. One of the main functions of neurons is to form inter-connections with nearby neurons, namely synapses. The ability to form synapses in response to neuronal needs is called synaptic plasticity and it represents the cellular mechanisms of learning and memory [67]. Mitochondria are playing a major role in synaptic plasticity as the main energy suppliers and calcium concentration regulators [67]. Since the RRE had a positive effect on mitochondrial functions, we further examined its effect on neurite outgrowth. Neurite outgrowth is the growth of axons and dendrites of neurons that subsequently leads to the formation of synapses and synaptic plasticity [68]. A commonly used model to study neurite outgrowth is differentiated SH-SY5Y cells. The efficacy of the RRE con-centrations 5 and 10 ng/ml were tested in this context as they were the most promising in the previous experiments while the neurotrophins NGF and BDNF were used as positive controls to induce neurite outgrowth. Upon analysis and quantification of neurite out-growth parameters, it was revealed that RRE caused a significant increase in the number of neurites, in the total neurite length, in the attachment points of neurons and in the number of endpoints of neurons.

BDNF belongs to the protein family of neurotrophins which are mediating synaptic processes. BDNF is the main neurotrophin that regulates synaptic plasticity and neurite outgrowth as it is highly expressed in many brain regions including the hippocampus and cortex. Therefore, its expression plays a critical role in learning and memory [69, 70]. Since RRE promoted neurite outgrowth, we aimed at examining if the BDNF pathway is possibly involved by determining its potential upregulating effect on the BDNF mRNA expression. Indeed, treatment with the extract caused a fast upregulating effect on the BDNF mRNA expression peaking at 3h. Our findings are in line with previous research showing that salidroside, one of the lead compounds in RRE, has an upregulating effect on mRNA BDNF levels [71]. At the same time, this is the first study evaluating the effect of the whole extract on mRNA BDNF levels.

However, increased BDNF mRNA expression does not necessarily lead to increased BDNF protein levels because the mRNA could possibly not be translated into the respec-tive protein that is necessary for the induction of neurite outgrowth and synaptic plastici-ty. Therefore, we further evaluated the effect of RRE on the mature BDNF protein level. BDNF protein is synthetized as a pre-form that includes a pro-segment and the mature BDNF. The pro-segment is removed by proteases and the secreted mature BDNF binds to its receptor TrkB inducing long-term potentiation, while the pro-BDNF binds to the recep-tor p75 causing long-term depression [70]. Therefore, the production of mature BDNF is a post-translational process. Several studies demonstrated that the BDNF system is a fast-reacting one. Thus, mRNA expression started already to increase after 15 min of chal-lenge peaking at 2 h in cortical neurons from the rat brain [72] and is accompanied by el-evated levels of BDNF protein [73]. In line with these findings, RRE increased the mature BDNF protein level after a few hours of treatment. Consistent with our findings, in-tra-hippocampal infusion of spermidine, a natural polyamine exhibiting anti-oxidant properties, increased the mature BDNF protein level in a similar fast time frame as did RRE, namely after 3 h of treatment [74]. We can speculate that the effects of RRE on the BDNF system finding is related to its beneficial effect on neurite outgrowth and network connectivity. Synaptic plasticity is not only important in learning and memory in healthy subjects but also in stress-associated disorders and mental diseases. In these conditions, synaptic plasticity mechanisms become dysregulated and the connectivity between brain regions is obstructed resulting in pathological states [67, 75-77]. The limbic system is a group of brain regions, including hippocampus, hypothalamus and amygdala that are responsible for emotions and memory and are highly affected during stressful conditions [75, 77]. Glucocorticoids bind to glucocorticoid and mineralocorticoid receptors in order to exert their action. The hippocampus contains a high level of glucocorticoid receptors making it more vulnerable to stress upon hyper-stimulation of the HPA axis which in turn contributes to the onset and development of depression [77]. Stress causes alterations in the morphology and the functionality of hippocampal excitatory synapses [75]. Moreover, reduced BDNF mRNA as well as protein levels seem to be involved in the stress-induced decrease in synaptic plasticity in several brain regions [78]. On the basis of our results, RRE could therefore constitute an

adaptogenic candidate with the potential to combat stress-induced impairment of synaptic connectivity.

5. Conclusions

Overall, in this study we showed that the RRE WS®1375 is able to neutralize the harmful effect of corticosteroid stress, regulate neuronal ROS levels and improve neuronal survival in stressful situations. Of note, RRE WS®1375 exhibited therein biphasic dose-response curves that are typical for adaptogens constituting the activation of the «adaptive cellular stress response». In addition, we demonstrated that the RRE increased mitochondrial bioenergetics as well as promoted neurite outgrowth in neuronal cells. The mechanism underlying the promotion of neurite outgrowth might be the BDNF pathway as RRE upregulated both the BDNF mRNA and the mature BDNF protein levels. Although there are numerous studies demonstrating the anti-oxidant and neuroprotective effect of salidroside, one of the postulated lead compounds of RRE, there are very few studies ex-amining the effect of a whole extract. More research is needed to investigate the effect of different compounds of RRE such as rosavin, rosarin and tyrosol. This is particularly interesting since the efficacy of the RRE could be a result of antagonistic, synergistic or allo-steric effects of its constituents. Finally, our findings indicate that RRE could constitute a candidate for combatting stress-induced ailments and stress-associated mental disorders with the involvement of oxidative stress and could potentially lead to the establishment of a condition-specific supplement.

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Abbreviations

ABTS: 2:2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) scavenging capacity assay

ACTH: Adrenocorticotropic hormone

ATP: Adenosine triphosphate

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2 protein

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

CRH: Corticotropic-releasing-hormone

cDNA: Complementary DNA

CXR: Carboxy-X-Rhodamine reference dye

DAPI: 4′,6-Diamidino-2-phenylindol

DCF: 2',7'-dichlorodihydrofluorescein diacetate

Dexa: Dexamethasone

DHR: Dihydrorhodamine 123

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 1,1-Diphenyl-2-picrylhydrazyl scavenging capacity assay

ELISA: Enzyme-linked immunosorbent assay

ERK/CREB/PGC1-α: Extracellular signal-regulated kinase/ cAMP Response Element-binding Protein/ Peroxisome proliferator-activated receptor-gamma coactiva-tor-1alpha

ETC: Electron transport chain

FADH2: Reduced form of flavin adenine dinucleotide (FAD)

FCS: Fetal calf serum

FRAP: Ferric reducing antioxidant power assay

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase gene

GSH: Glutathione(γ-glutamylcysteinylglycine)

HBSS: Hanks' Balanced Salt Solution

HPA: Hypothalamic–pituitary–adrenal axis

HT22: Immortalized mouse hippocampal cell line

IsoP: 8-iso-prostaglandin F2α

MitoSOX: Mitochondrial superoxide anion dye

mRNA: Messenger RNA

MTT: 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl-tetrazolium bromide

NADH: Reduced form of nicotinamide adenine dinucleotide

NGF: Nerve growth factor

OXPHOS: Oxidative phosphorylation

PBS: Phosphate-buffered saline

PC12: Phaeochromocytoma cells from rat adrenal gland

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

RA : Retinoic acid

RRE: *Rhodiola rosea* extract

RNA: Ribonucleic acid

ROS: Reactive oxygen species

S.E.M.: Standard error of the mean

SH-SY5Y: human neuroblastoma cell line

Trkβ: Tyrosine receptor kinase β

WS®1375: specific Rhodiola rosea extract (Dr. Willmar Schwabe GmbH & Co KG, Karlsruhe, Germany) used in this study

8-oxoG: 8-hydroxyguanosine

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3. Mangiferin exerts beneficial effects on bioenergetics and neurite outgrowth in a cellular model of tauopathy (in preparation).

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Abstract

Objectives: Tau is a microtubule-associated protein mainly expressed in neurons and takes part in important processes such as stabilization and assembly of microtubule and axonal transport of cargoes. Tauopathies are a group of neurodegenerative diseases characterized by an abnormal accumulation of tau within neurons, excessive tau hyperphosphorylation and formation of tangles. Previous studies showed that the abnormal Tau protein (P301L) impairs mitochondrial function. Mangiferin is a xanthone present in *Mangifera indica* and in South African *Cyclopia* species and possesses antioxidant and anti-dementia properties. This study aims to test the potential capacity of mangiferin in enhancing the mitochondrial bioenergetics and in promoting neurite outgrowth in a cellular model of tauopathy. Methods: The human neuroblastoma cell line bearing the human tau P301L mutation was used in this study and was compared to the respective Mock cells (neuroblastoma cells bearing the empty vector). The capacity of mangiferin in increasing adenosine triphosphate (ATP) production and mitochondrial membrane potential (MMP) and in promoting neurite outgrowth was investigated. For neurite outgrowth evaluation, cells were differentiated with retinoic acid and nerve growth factor (NGF) was used as positive control. After treatment with mangiferin for 3 days and immunostaining, pictures were taken after using the Cytation 3 digital microscope. The soma and neurite count, the total neurite length, the attachment points and the endpoints of neurites were quantified using the ImageJ software, Neurophology plugin. Results: Mangiferin (especially at 5 uM) was found to increase the ATP production and the MMP in the mutant tau P301L cells after 24 h of treatment. Mangiferin (especially at 5 uM) promoted neurite outgrowth as it increased all the neurite outgrowth parameters. Conclusions: Mangiferin is a promising agent in increasing bioenergetics and promoting neurite outgrowth in tauopathies such as Alzheimer`s disease.

Keywords: mangiferin, neurite outgrowth, mitochondria, tau, tauopathy

Introduction

Mitochondria are essential yet independent organelles contained in the cytosol of eukaryotic cells and they are responsible predominantly for the energy production, as adenosine triphosphate (ATP). Apart from the production of energy, mitochondria are the key modulators of brain cell survival, differentiation and death by controlling calcium and reduction-oxidation (redox) equilibrium (which in turn affects neurotransmitter release and neuronal plasticity), by producing reactive oxygen species (ROS), by affecting synaptic plasticity and by controlling cell apoptosis [1-3]. The brain consumes a considerable amount of oxygen and neurons have significant energy demands. Oxidative phosphorylation (OXPHOS), taking place in mitochondria, is the main energy provider and neurons depend almost solely on this procedure in order to satisfy their energy needs. This is why mitochondria play a vital role in the nervous system [4]. Neurodegenerative diseases are characterized by mitochondrial dysfunction and often by over-accumulation of intracellular insoluble proteins in neurons (e.g. amyloid-beta aggregates in Alzheimer`s disease, alpha-synuclein clumps in Parkinson`s disease and huntingtin aggregates in Huntington's disease) [5, 6]. Tau is a microtubule-associated protein mainly expressed in neurons and in physiological conditions has an essential role in microtubule dynamics as it binds to and stabilizes the microtubules. Free and bound tau are in a dynamic equilibrium that is modulated via phosphorylation (executed by kinases) and de-phosphorylation (executed by phosphatases) [7, 8]. Tau is primarily found in the cytosol and is mainly localized in axons of neurons while it is implicated in the anterograde axonal transport (the transportation of proteins, lipids , nucleic acids, organelles and synaptic vesicles along microtubules [7, 9]. In many neurodegenerative diseases, such as Alzheimer's disease, tau metabolism is modified [7, 10]. Tauopathies are a group of neurodegenerative diseases characterized by an abnormal accumulation of tau within neurons, excessive tau hyperphosphorylation (and subsequent detachment from microtubules) and formation into neurotoxic neurofibrillary tangles [8]. There are two types of tauopathies. In primary tauopathies such as sporadic multiple system tauopathy, tau is the principal contributing element of the neurodegenerative process and mutant variants of tau proteins have been identified [8, 11]. Secondary tauopathies such as Alzheimer`s disease (AD) are diseases with diverse causes in which tau has also a major role but other important factors are involved in the

pathogenesis of the disease (e.g. amyloid-beta plaques in AD) [8, 12]. Emerging evidence underpins the impairment of mitochondria in tauopathies, including decreased bioenergetics, abnormal mitochondrial dynamics and morphology and hindered axonal transfer of mitochondria [13-17]. Tau has been shown to damage the bioenergetics in cellular and animal models. In detail, tau reduced ATP and MMP levels, impaired respiration and damaged complex I of the electron transport chain as well as increased ROS in pR5 mice (mice transfected with the human P301L mutant tau) [13, 18]. The same findings were confirmed in human neuroblastoma cells SH-SY5Y transfected with the P301L mutant tau [7, 19, 20].

Mangiferin (2-C-b-D-glucopyranosyl 1,3,6,7-tetrahydroxyxanthone) is a xanthonoid polyphenol (class of secondary metabolites) found mainly in the fruit, leaves and bark of *Mangifera indica* and has exhibited promising therapeutic or preventing effects against various diseases. It has been found to possess antioxidant, neuroprotective, anti-inflammatory, anti-diabetic and cardioprotective properties among others [21]. The therapeutic potential of mangiferin can be explained due to the many functional groups embedded in its structure. The compound consists of one glycosidic hydroxyl group, two aromatic rings, one lactonic carbonyl group and nonaromatic secondary hydroxyl groups. The catechol moiety of mangiferin forms a complex with iron and is responsible for the antioxidant action of the compound [22]. Mangiferin is also present in South African Honeybush plant extracts (*Cyclopia* species) and especially in the aqueous-ethanol extracts of *Cyclopia genistoides* and *Cyclopia longifolia* [23-25]. Recently our group showed that honeybush extracts increased mitochondrial functions in human neuroblastoma SH-SY5Y cells and rescued oxidative stress-induced deficits by eliminating ROS, increasing bioenergetics as well as ATP and MMP levels [26]. Mangiferin has been described to exert antioxidant properties in rotenone-treated SK-N-SH neuroblastoma cells [27] and in doxorubicin-treated rats [28]. It has also exerted neuroprotective and anti-dementia properties in scopolamine-treated mice [29], in $AlCl_3$-treated mice [30] as well as in healthy rats [31]. However, there are limited studies in cellular models of neurodegeneration and especially of tauopathies in order to understand the mechanisms by which mangiferin might act. Therefore, in this study we aimed to examine the potential effects of mangiferin on a cellular model of tauopathy. We used human neuroblastoma cells stably transfected with the human mutant tau P301L, a widely used cellular model of tauopathy and we tested the effect

of mangiferin on cellular bioenergetics, ATP and MMP levels and its effect on the extension and outgrowth of neurites.

Materials and Methods

Chemicals and reagents

Dulbecco's-modified Eagle medium (DMEM), phosphate buffered saline (PBS), fetal calf serum (FCS), Hanks' Balanced Salt solution (HBSS), penicillin/streptomycin, tetramethylrhodamine methyl ester (TMRM), neurobasal medium, retinoic acid, paraformaldehyde, bovine serum albumin (BSA), 4′,6-Diamidino-2-phenylindol (DAPI), dimethyl sulfoxide (DMSO) and Triton X-100were from Sigma-Aldrich (St. Louis, MO, USA). Glutamax and B27 supplement were from Gibco Invitrogen (Waltham, MA, USA), ATPlite1step kit from PerkinElmer (Waltham, Massachusetts, USA), NGF from Lubio (Zürich, Switzerland) and horse serum (HS) from Amimed, Bioconcept (Allschwil, Switzerland). Alexa fluor 488 and βIII-tubulin antibodies were from R&D Systems (Minnesota, USA), rat tail collagen type I from Corning (Amsterdam, Netherlands) and blasticidin from Invivogen (San Diego, California, United States).

Cell culture

Human neuroblastoma SH-SY5Y carrying the empty vector cell line (Mock cells) as well as human neuroblastoma cells stably transfected with the pathogenic mutant tau P301L (P301L cells) were used in this study. P301L cells is a common cellular model of tauopathy. Both cell lines were kept and grown at 37°C in a humidified incubator chamber under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 5% HS, 2 mM glutamax and 1% (v/v) penicillin/streptomycin. Cells were selected with a final concentration of 3 ug/ml blasticidin which was added in their culture medium. Cells were passaged 1-2 times per week and the cells used

for the experiments did not exceed passage 20. The cells were plated when they reached 80–90% confluence.

Treatment of cells

Evaluation of ATP production was conducted to determine the concentrations of mangiferin that increased the ATP levels the most. Mangiferin was initially screened at a concentration range of 0.1 uM to 50 uM (data not shown). Of note, mangiferin was dissolved in DMSO for our experiments (final concentration of DMSO<0.005%, no effect of the vehicle solution alone compared to the untreated condition). The screening was conducted by using an ATP detection assay (ATPlite 1step kit was from PerkinElmer). Mangiferin was not toxic for the cells for concentrations up to 10 uM. For the experiments, cells were plated and treated 1 day after plating for 24 h either with DMEM (untreated cells) or with a final concentration of 0.1 uM to 5 uM of mangiferin. Because vehicle treatment was without any effect in our assays, we evaluated the effects of mangiferin different concentrations in comparison to the untreated condition in the following experiments.

ATP levels

Total ATP content was determined using a bioluminescence assay (ATPlite 1step) according to the instructions of the manufacturer and as previously described [19, 32, 33]. Cells were plated in 6 replicates into white 96-well cell culture plates at a density of 1×10^4 cells/well. Blasticidin 3 ug/ml was added to the medium of cells. The ATP was extracted from the cells upon lysis and it was transformed into light. The method measures the formation of light from ATP and luciferin catalyzed by the enzyme luciferase. The emitted light was linearly correlated to the ATP concentration and was measured using the multi-mode plate reader Cytation 3 (BioTek instruments, Winooski, Vermont, United States).

Determination of mitochondrial membrane potential (MMP)

The MMP was measured using the fluorescent dye TMRM, since its transmembrane distribution depends on the MMP. As previously described [20, 34], the cells were plated in 6 replicates into black 96-well cell culture plates at a density of 1×10^4 cells/well and were incubated with the dye at a concentration of 0.4 μM for 30 min. Blasticidin 3 ug/ml was added to the medium of cells. After washing two times with HBSS, fluorescence was measured at 548 nm (excitation)/574 nm (emission), using a Cytation 3 multi-mode plate reader (BioTek instruments).

Neurite outgrowth determination

P301L cells were plated in black 96-well plates that were pre-coated with collagen 0.05 mg/ml. 24h post plating, cell differentiation was induced by adding neurobasal medium containing 2% B27 supplement, 1% penicillin-streptomycin, 1% glutaMax and 10 μM retinoic acid (RA) for 3 days as described previously [35]. Then, P301L cells were treated either with mangiferin at the concentrations 1 and 5 uM or with 50 ng/ml of NGF (positive control). Differentiated untreated Mock cells were also used as a reference cell line. After 3 days of treatment, cells were fixed with 2% paraformaldehyde.

Immunostaining

Immunolabeling of neurites was performed using an anti βIII-tubulin primary antibody and Alexa Fluor 488-conjugated secondary antibody (fluorescence emission in the green wavelength). Immunolabeling of the nucleus was conducted using DAPI (4′,6-diamidino-2-phenylindole) which emits blue fluorescence upon binding to adenine and thymine-rich regions of DNA.

Microscopy and analysis (software)

Images were taken using Cytation 3, a digital cell imaging multi-mode reader with a 20x objective. Analysis was performed using ImageJ (Neurophology plugin) software to evaluate parameters of neuroplasticity as described before [35]:

Soma count

Neurite count

Total neurite length

Number of branching points = attachment point

Number of contact points = endpoint

Statistical analysis

Data are given as the mean ± S.E.M. Statistical analyses were performed using Graph Pad Prism software (version 5.02 for Windows, San Diego, California, USA). For statistical comparisons of more than two groups, one-way ANOVA was used, followed by a Dunnett's multiple comparison tests versus the untreated P301L cells. $P<0.05$ were considered statistically significant.

Results

Mangiferin increased ATP production in tau P301L cells

ATP is the end product of primarily oxidative phosphorylation but additionally of glycolysis and is thus an indicator of mitochondrial and cellular viability and proper functioning. We assessed the effect of mangiferin on the ATP production in tau P301L cells and initially compared it to untreated Mock cells (Figure 1A). The ATP levels in untreated Mock cells was increased by 23.4% compared to untreated P301L cells. The mangiferin concentration range of 0.1-5 uM was tested in the P301L cells. All mangiferin concentrations increased the ATP levels with 5 uM being the most effective as it significantly increased ATP by 22.1% compared to the untreated P301L cells after 24h of treatment (Figure 1A).

Mangiferin increased mitochondrial membrane potential (MMP) in tau P301L cells

We further evaluated the effect of mangiferin on the MMP levels in tau P301L cells and initially compared it to untreated Mock cells (Figure 1B). MMP in untreated Mock cells was increased by 25.4% compared to untreated P301L cells. Treatment with all mangiferin concentrations for 24h caused an increase in the MMP levels (Figure 1B). However, only mangiferin at 5 uM enhanced MMP significantly by 26.6% in P301L cells.

Mangiferin promoted neurite outgrowth in differentiated P301L cells

The capacity of mangiferin in inducing neurite outgrowth was evaluated in differentiated tau P301L cells in a 2D cell culture model [35]. The two most beneficial concentrations from the previous experiments, namely 1 and 5 uM, were tested in the subsequent experiments of neurite outgrowth. The neurotrophin nerve growth factor (NGF) at 50 ng/ml, which is a known inducer of neurite outgrowth and promoter of synaptic plasticity and neuronal survival, was used as positive control. Differentiated untreated P301L cells were initially compared to differentiated untreated Mock cells. Untreated Mock cells visually demonstrated increased numbers of neurites compared to untreated P301L cells. After differentiation, P301L cells were treated with mangiferin for three days. After immunostaining and microscopy, it was shown that mangiferin induced significant neurite outgrowth compared to untreated P301L cells which was visually comparable to the effect caused by the positive control (Figure 2). In order to confirm and quantify the effect, the ImageJ software (Neurophology plugin) was used for analysis which allows the quantification of specific neurite outgrowth parameters such as soma count, neurite count, total neurite length, attachment points of neurons and endpoints of neurons (Figure 3). Mangiferin conditions as well as the positive control were normalized and compared to the untreated P301L cells. As anticipated, untreated Mock cells showed a considerable increase in all the parameters compared to untreated P301L cells. Also, NGF-treated P301L cells showed significantly enhanced neurite outgrowth parameters compared to the untreated P301L cells. Interestingly, treatment of the cells with mangiferin and at 1 and 5 uM promoted significantly the development and extension of neurites. Both concentrations caused

an increase of 22.1% and 28.6% respectively in soma count and an increase of 75.7% and 79.4% respectively in neurite count. Additionally, mangiferin at 1 and 5 caused an increase of 93.8% and 105.1% respectively in total neurite length, an increase of 76.2% and 88.9% respectively in the number of attachment points and an increase of 81.7% and 102.9% respectively in the number of endpoints (Figure 3). Overall, mangiferin at 5 uM was the most effective in increasing all neurite outgrowth parameters.

Discussion

In this study we investigated the effect of mangiferin, a coumpound included in honeybush extracts (*Cyclopia* species) as well as in mango fruit (*Mangifera indica*), on ATP production, MMP and neurite outgrowth using the widely used tau cellular model of P301L cells as well as the respective neuroblastoma cells bearing the empty vector (Mock cells) as a reference cell line. P301L cells are neuroblastoma cells stably transfected with the pathogenic mutant tau P301L and have been shown to have impaired mitochondrial functions compared to Mock cells and to neuroblastoma cells transfected with the wild type tau (Htau40) [34]. Since previous studies have demonstrated that neuroblastoma cells transfected with the wild type tau show protective effects on mitochondrial functions [7], in this study we focused solely on the P301L and we compared them to the Mock cells. In line with Schulz et al [7] we showed that P301L cells demonstrate significantly lower ATP and MMP levels compared to the Mock cells. Moreover, mangiferin (especially at 5 uM) was found to increase the ATP levels and MMP in P301L cells compared to the untreated cells. Interestingly, mangiferin-treated P301L cells approach the ATP and MMP levels of the untreated healthy Mock cells showing that mangiferin might be able to rescue the tau-induced deficits. During oxidative phosphorylation at the electron transport chain (ETC) of mitochondria, electrons provided by the electron carriers NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide) are transferred through complexes I-IV. This movement of electrons triggers the complexes I, III and IV to pump protons directly into the intermembrane space where

they are finally used by ATP synthase (complex V) to generate ATP. MMP is an indicator for polarized mitochondrial membranes and therefore an indicator that the pumping of protons in the intermembrane space is not deterred so that they can drive the ATP production by complex V. Improvement of ATP production by mangiferin could be as a result of its capacity to increase the MMP levels and supports this interdependence of MMP and ATP production [26, 36, 37]. In line with our results, mangiferin increased the isoproterenol-induced reduction in the energy production in myocardial infarcted rats [38] as well as repaired the rotenone-induced mitochondrial impairment in SK-N-SH neuroblastoma cells by increasing the ATP levels as well as the MMP [27]. Mangiferin has been recently shown to rescue the fluoride-induced impairment in mitochondrial dynamics and to reduce oxidative stress markers in SH-SY5Y cells by inactivating the c-Jun N-terminal Kinase (JNK) and by activating the nuclear factor erythroid 2-like 2/ Heme oxygenase-1 (Nrf2/HO-1) pathways [39]. There are some recently published studies indicating the neuroprotective effects of mangiferin via the Nrf2/HO-1 pathways in animal [40] as well as in cellular models [41]. Mangiferin seems to possess substantial antioxidant properties in neuronal cellular models which is expected due to its structural characteristics and especially the free hydroxyl groups and a catechol moiety [42]. In detail, mangiferin decreased the rotenone-induced increase in levels of thiobarbituric acid reactive substances (TBARS) and restored glutathione (GSH) levels significantly as well as enhanced the activities of catalase, manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPx) in SK-N-SH human neuroblastoma cells [27]. Mangiferin also reduced oxidative stress by increasing superoxide dismutase levels and by decreasing malondialdehyde level in a cognitive impairment rat model [28] as well as inhibited methylmercury-induced oxidative stress by increasing the GSH and glutathione-S-transferase (GST) levels, by decreasing malondialdehyde (MDA) and by rescuing MMP in IMR-32 human neuroblastoma cells [43]. Finally, mangiferin restored the 1-methyl-4-phenylpyridinium-impaired GSH levels in N2A cells [44]. Oxidative stress has been an intrinsic characteristic of many chronic diseases and especially neurodegenerative and age-related disorders. As mangiferin was shown to have significant antioxidant properties, it was expected to possess protective effects in neurodegeneration models which was recently thoroughly reviewed [45, 46]. Indeed, mangiferin enhanced the learning ability and retention of memory in mice while it also

restored the scopolamine-provoked increase in brain lipid peroxidation and acetylcholinesterase levels and the decrease in GSH [29]. Mangiferin acted via a similar way in another study as it decreased the aluminium chloride-provoked increase in acetylcholinesterase level, it alleviated the cognitive impairment and reduced the oxidative and nitrosative stress in the hippocampus of mice [30]. In a normal rat model, long-term object recognition memory was improved by mangiferin [31] while in accelerated-senescence mice, the compound improved learning and memory, reduced the expression of amyloid-β as well as the injuries in hippocampal neurons and mitochondria [47]. Although our results on the tau P301L cells are in agreement with the general promising effect of mangiferin against neurodegenerative disease models, this is the first study to evaluate the effects of the compound in a tauopathy cellular model and the first study to evaluate its effect on neurite outgrowth. According to our results, mangiferin significantly increased all the neurite outgrowth parameters in tau P301L cells. Also, we showed that, both morphologically but also quantitatively, untreated Mock cells present a much healthier phenotype than untreated P301L cells. Interestingly, mangiferin-treated P301L cells showed similar development of neurites to that of the untreated Mock cells indicating that mangiferin is able to bring the tau cells close to the state of the healthy Mock cells both morphologically and in terms of quantification of neurite outgrowth parameters. Neurite outgrowth is essential for the establishment of a functional network of neurons mainly during development but also during adulthood. It is also important for neuronal synaptic plasticity and regeneration [48]. There is a family of proteins which play a paramount role in neuronal plasticity and outgrowth, namely the neurotrophins. Brain-derived neurotrophic factor (BDNF) is the main neurotrophin that mediates synaptic plasticity and neurite outgrowth as it is highly expressed in many brain regions including the hippocampus and cortex [49, 50]. Nerve growth factor (NGF) is another neurotrophin that is generated in the hippocampus throughout life and provides cholinergic inputs to cholinergic neurons and, thus, promotes hippocampal plasticity [51, 52]. NGF is also crucial for the functioning and the healthy development of the basal forebrain. In detail, it mediates the formation and branching of dendrites and regulates the activities of acetylcholinesterase and choline acetyltransferase in basal forebrain neurons [53]. Mangiferin was shown to induce not only cell proliferation but also a significant increase in NGF levels in the supernatant of human U138-MG glioblastoma cells [31].

This finding supports our result about mangiferin promoting neurite outgrowth. We postulate that mangiferin induced neurite outgrowth by upregulating neurotrophin levels such as NGF.

There are some studies on the bioavailability of mangiferin and its ability to cross the blood-brain barrier (BBB). Although mangiferin is a poor lipophilic compound, its Log P value was measured + 2.73 which indicates its BBB permeability [54]. On the one hand, trace amounts of mangiferin were detected in the brain of rats after an acute oral treatment with a single dose of a plant extract containing mangiferin. This indicates that the compound can cross the BBB [55, 56]. On the other hand, in another study, mangiferin was not found in the rat brain after a single dose via intraperitoneal administration [57]. This is in line with another study in which mangiferin was detected via liquid chromatography and mass spectrometry (LC-MS/MS) in multiple tissues apart from the brain after a single intragastric administration of mangiferin monomer [58]. However, different assays of different sensitivity were used in these studies. Li et al 2008 developed and applied a validated and highly sensitive HPLC method in order to detect mangiferin after a single oral dose of Rhizoma Anemarrhenae extract while in the study of Zajac et al 2013, a simple TLC method was used which is much less sensitive detection method. Recently, another study claimed that mangiferin and/or its metabolite norathyriol is able to traverse the BBB in concentrations sufficient enough to change the brain electrical activity via alterations in neurotransmitter signalling. The study was conducted in rats which received 25 mg/kg of mangiferin via oral gavage administration and the researchers measured changes in electrical activity in the rat brains through quantitative electroencephalogram (EEG). Those results were also confirmed *in vitro* and in a clinical trial in the same study [59]. Bioavailability in the brain could be affected by whether the pure compound is administered or contained in a plant extract and by the route of administration. There are indications that mangiferin exerts its neuroprotective effects directly on the brain as well as on peripheral neurons.

Conclusion

Overall, we showed that mangiferin exerts beneficial effects on the bioenergetics, mitochondrial functions and neurite outgrowth in tau P301L cells. In detail, mangiferin at 1 and 5 uM increased ATP and MMP levels and promoted neurite outgrowth and in a similar way in both cell lines. Our data are in line with existing literature reporting the neuroprotective effects of mangiferin. However, the effects of mangiferin on a tauopathy cellular model as well as its effects on neurite outgrowth have been investigated here for the first time. Further research is ongoing by our team in order to study more in depth the mechanisms underlying the effect of mangiferin in enhancing neuronal functions. These findings support the use of mangiferin as a preventive agent against tauopathies and other neurodegenerative diseases.

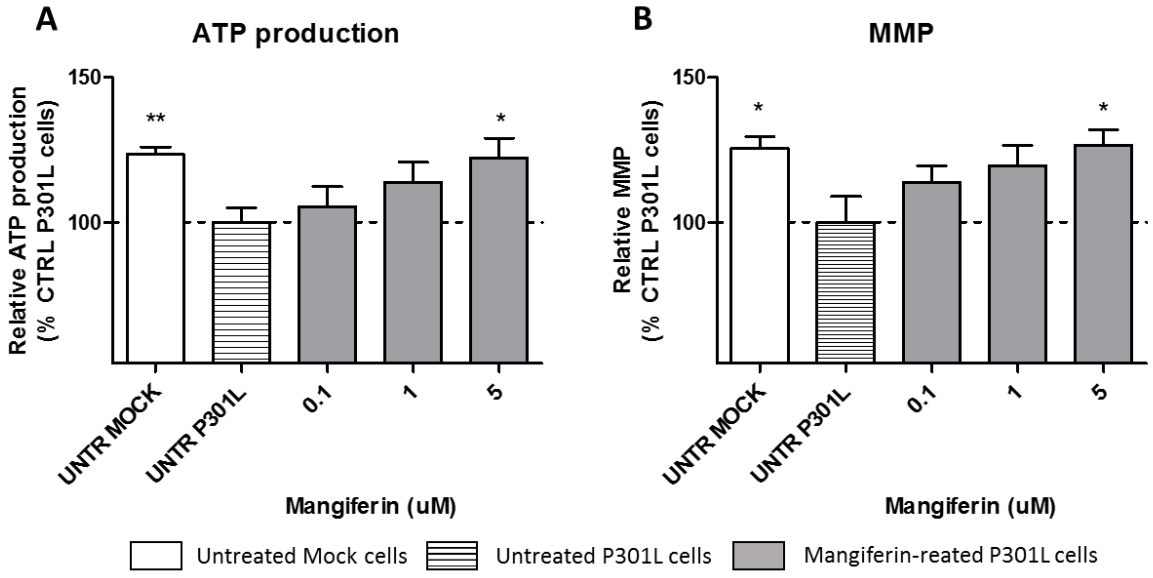

Figure 1. Effect of mangiferin on A) ATP production and B) MMP levels. Mangiferin 5 uM significantly increased ATP levels and MMP in P301L cells after 24h of treatment. Values represent the mean ± SEM of three independent experiments. For statistics, one way ANOVA and post hoc Dunnett's multiple comparison test versus untreated P301L was used, *P < 0.05, **P < 0.01, ***P < 0.001

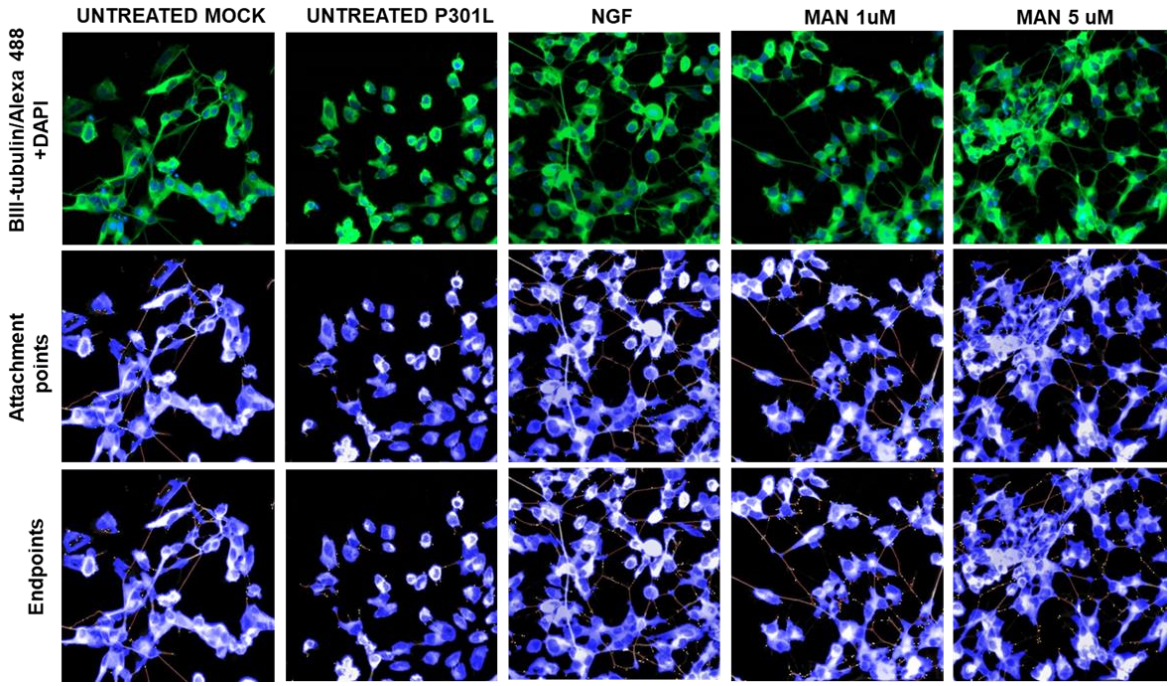

Figure 2. Neurite outgrowth in differentiated P301L cells compared to untreated neuroblastoma cells transfected with the empty vector (MOCK cells) demonstrated in the first vertical column. Mangiferin at 1, 5 and 10 uM increased the neurite extension of P301L cells after 3 days of treatment in a 2D cell culture. Pictures were taken using a digital microscope of a multi-mode plate reader (x20). Immunostaining was performed with βIII-tubulin/Alexa fluor-488 for the soma and neurites. The nucleus was stained using DAPI. Quantification of the neurite outgrowth parameters such as the attachment points (middle panels) and the endpoint numbers (lower panels), after NGF (50 ng/ml) or mangiferin at 1, 5 and 10 uM treatment are illustrated (Blue and gray: soma, red: neurites, green points: attachment points, yellow points: endpoints).

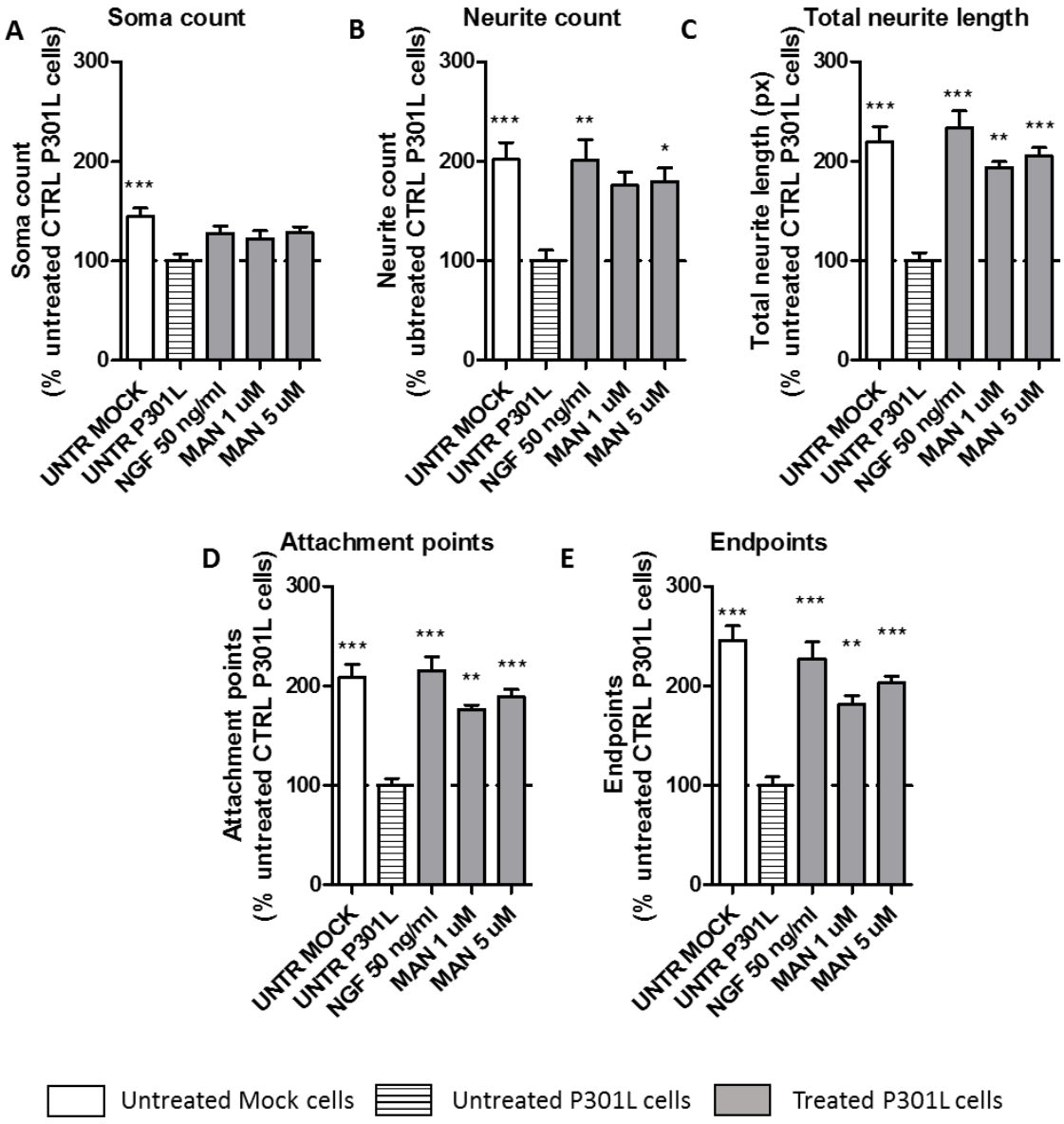

Figure 3. Neurite outgrowth parameters in P301L cells. Mangiferin increased the neurite outgrowth of P301L cells after 3 days of treatment in a 2D cell culture. Quantification was performed using NeurophologyJ plugin on the ImageJ software. At 1, 5 and 10 uM mangiferin increased: A) cell number (soma count), B) number of neurites (neurite count), C) neurite length, D) number of attachment points, E) number of endpoints. The effect of mangiferin and that of the positive control NGF was normalized to the untreated cells P301L. Values represent the mean ± SEM of three independent experiments. For statistics, one way ANOVA and post hoc Dunnett's

multiple comparison test versus untreated P301L was used, *P < 0.05, **P < 0.01, ***P < 0.001.

Abbreviations

AD: Alzheimer’s disease

ATP: Adenosine triphosphate

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

DAPI: 4′,6-Diamidino-2-phenylindol

DMEM: Dulbecco’s-modified Eagle medium

DMSO: Dimethyl sulfoxide

EEG: Electroencephalogram

ETC: Electron transport chain

FCS: Fetal calf serum

FADH2: Flavin adenine dinucleotide

GPx: Glutathione peroxidase

GSH: Glutathione

GST: Glutathione-S-transferase

HBSS: Hank's Balanced Salt Solution

HS: Horse serum

HPLC: High performance liquid chromatography

IMR-32: Human neuroblastoma cells

JNK: c-Jun N-terminal Kinase

LC-MS: Liquid chromatography–mass spectrometry

MDA: Malondialdehyde

MMP: Mitochondrial membrane potential

Mn-SOD: Manganese superoxide dismutase

NGF: Nerve growth factor

NADH: Nicotinamide adenine dinucleotide

Nrf2/HO-1: Nuclear factor erythroid 2-like 2/ Heme oxygenase-1

N2A: Neuro-2A cells

OXPHOS : Oxidative phosphorylation

P301L : Neuroblastoma cells stably transfected with the mutant tau P301L

PBS: Phosphate buffered saline

RA: Retinoic acid

ROS : Reactive oxygen species

SEM : Standard error of mean

SH-SY5Y: Human neuroblastoma cell line

SK-H-SH: Human neuroblastoma cell line

TBARS: Thiobarbituric acid reactive substances

TLC: Thin layer chromatography

TMRM: Tetramethylrhodamine methyl ester

U138-MG: Glioblastoma cell line

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C. Conclusion

Mitochondria have been proven to be essential for cellular and organismal health because of their diverse functions while they play pivotal roles not only in health but also in the pathogenesis of a plethora of diseases. Their role is remarkably prominent in the brain since neurons conduct a number of complex processes which require a great amount of energy. This energy is provided mainly by mitochondria in the form of ATP and therefore neurons highly depend on these organelles. Mitochondrial dysfunction, impaired energy metabolism, oxidative stress and synaptic dysregulation have been identified as features of many neurodegenerative diseases such as AD but also of stress-related brain disorders [1-3]. The interest on mitochondria-targeting preventive or therapeutic strategies is constantly increasing and there is a particular focus on substances that increase bioenergetics (respiration and ATP levels) and decrease ROS.

Nature has always been a source of valuable therapeutic plants and phytochemicals. However, nature's potential to further provide us with more disease-preventive solutions has been largely untapped. Current pharmacological strategies for neurodegenerative and stress-related mental disorders fail to cure or stop the progression of the disease while they treat symptoms but often with unbearable or severe adverse effects. Therefore, plant extracts and phytochemicals derived from plants could be employed in order to alleviate symptoms as they do not present severe adverse effects while they possess polyvalent and multi-targeted functions. *Rhodiola rosea* extract is an excellent example of a plant extract employed against stress-related diseases as it reduced stress markers in animal stress-models as well as it demonstrated beneficial effects in clinical trials in subjects under stress or with chronic anxiety/burnout symptoms [4, 5]. However, its effects on mitochondria and bioenergetics have not been fully resolved. At the same time, more phytochemicals need to be identified and evaluated in the battle against stress-related and neurodegenerative diseases through enhancing mitochondrial functions [2, 6, 7].

In this PhD thesis we showed the beneficial effects of *Rhodiola rosea* and Honeybush extracts (*Cyclopia* species) as well as of the natural compound mangiferin on mitochondrial functions as all three phytochemicals enhanced bioenergetics including ATP production as well as other mitochondrial activities such as MMP and respiration. Our aim was to attest the mitochondria-improving capacity of these phytochemicals in

intrinsic health status but also in different stress situations. For this matter, we employed three different types of stress *in vitro*: oxidative stress, glucocorticoid stress and abnormal tau-induced stress. We used hydrogen peroxide (H_2O_2), the main ROS species, to simulate oxidative stress situations, dexamethasone that mimics the effects of cortisol to simulate glucocorticoid stress and finally abnormally hyperphosphorylated tau overexpression to mimic tauopathies such as AD. Interestingly, all three stress agents (H_2O_2, cortisol and tau) have physiological roles within cells with ROS acting as signaling molecules, cortisol inducing eustress and increasing overall functions through promotion of cellular adaptation and tau promoting microtubule assembly, neuronal cytoskeleton organization and distribution of cargoes across axons. However, when these agents are overproduced they lead to the opposite cellular effects and become neurotoxic and disease-inducing [3, 8-10].

Honeybush (*Cyclopia* species) is a South African plant and certain species are consumed as herbal tea. Increased global demand for honeybush tea led to a scientific interest for the bioactivity of its extracts [11]. Honeybush extracts have exhibited antioxidant activity in *in vitro* antioxidant assays such as DPPH, ORAC, TEAC and ABTS while a few *in vivo* studies have demonstrated its ability to increase antioxidant defense system activity such as GSH and GSH/GSSG ratio, CAT, SOD, GST, glutathione reductase while it also decreased lipid peroxidation (TBARS and MDA) [12-14]. Although, the aforementioned *in vitro* systems measure the radical scavenging capacity of phytochemicals easily and rapidly, they are indirect methods based on the reduction of certain radicals (e.g. DPPH and TEAC assays) or of oxidizing species of inorganic nature (e.g. FRAP assay) or are based on the bleaching of a dye (e.g. ORAC assay) and do not reflect the real activity of phytochemicals in cells or in animals while they also present limitations [15]. For example, DPPH is highly impacted by the solvent of the extract and its radical can interact with other radicals as well as interfere with compounds in the extract while the method cannot be performed in protein-containing samples (e.g. plasma) due to protein precipitation in alcohol [16, 17]. Additionally, the results of ABTS and ORAC assays have been found non-reproducible [16]. Based mainly on the demonstrated *in vivo* antioxidant capacity of honeybush, we hypothesized that honeybush extracts would be able to protect mitochondrial functions from oxidative stress in neuronal cells since mitochondria are both the producers and primary targets of ROS. Indeed honeybush extracts per se

increased ATP production, MMP, respiration and glycolysis in neuroblastoma SH-SY5Y cells while they protected the cells from H_2O_2-induced mitochondrial impairment by completely rescuing the MMP levels (keeping the membranes polarized) and the respiration, by partially improving ATP production and by reducing 4 types of ROS levels. The most potent extracts were found to be the aqueous extracts of *C. subternata* and *C. genistoides*. Our results are in line with existing research showing that honeybush extracts decreased ROS in diesel exhaust particles-treated human umbilical vein endothelial cells (HUVECs) by increasing GSH levels and the GSH/GSSG ratio [18]. Therefore, we can speculate that honebush extracts, apart from enhancing mitochondrial functions, protected neuronal cells in our study by increasing the antioxidant defence system activities or by regulating the expression of oxidative stress-related genes as was proven for the extracts of *C. subternata* and *C. genistoides* in the rat liver [19]. This is the first study showing the potential neuroprotective effects of honeybush extracts in an oxidative stress cellular model and sets the basis for further research which is needed in order to resolve the exact mechanisms of action of the extracts. For the production of honeybush tea often several species are blended and therefore it would be interesting to test the effect of a combination of extracts on neuronal and mitochondrial functions both *in vitro* and *in vivo* as well as their effect on oxidative stress markers in the blood and plasma of humans.

Rhodiola rosea is a perennial plant growing in mountainous areas of Central Asia and Europe and is known for its adaptogenic properties and its use by Vikings and Russian army to enhance performance during battles. Rhodiola has been used in clinical studies to alleviate symptoms of stress, chronic stress and burnout [4, 20]. These conditions lead to the dysregulation of the HPA axis with overproduction of cortisol as an end-product. Although lower doses of glucocorticoids have been shown to enhance mitochondrial, cellular and organismal functions leading to an alert state of adaptation, overstimulation of the HPA axis results in overaccumulation of glucocorticoids which has been shown to cause various detrimental effects [8]. Of note, cortisol greatly affects mitochondria as it is able to modify MMP in genomic and non-genomic ways [21]. Regarding the non-genomic pathway, in high concentrations cortisol binds to and activate its receptor GR. The activated GR binds to mitochondrial membranes and

modifies MMP. Regarding the genomic pathway, activated GR interact with biomolecules including Hsp70/90/40 and translocate into the nucleus where they attach to DNA and cause transcriptional changes [21]. Additionally, it has been shown that GR activation causes Bax (pro-apoptotic protein) upregulation and its translocation into mitochondria where it binds to MAP-1 (modulator of apoptosis-1) and disrupts MMP. Both genomic and non-genomic pathways cause a disturbance of MMP, release of cytochrome c into the cytosol and further induction of apoptotic cascades which lead to neuronal death [21]. *Rhodiola rosea* extracts have been demonstrated to reduce stress markers such as cortisol, CRH and NO and their detrimental effects mainly by upregulation of heat shock proteins (Hsp70) and transcriptional factors (FOXO/DAF-16), by increasing the Bcl-2/Bax ratio and by preventing phosphorylation and activation of the JNK and ERK1/2 pathways. Both JNK and ERK1/2 are kinases that control several processes such as gene expression, cell proliferation and differentiation, neuronal plasticity, apoptosis and senescence. Both kinases are expressed in much higher extend in the CNS and neurons than in peripheral organs while their phosphorylation leads to their activation which has been linked to neurodegenerative diseases such as Parkinson`s disease (PD) [22].

In our study we used dexamethasone, a synthetic glucocorticoid which mimics the effects of cortisol, in order to create an *in vitro* cellular model of hormonal/glucocorticoid stress. Firstly, we showed that treatment with *Rhodiola rosea* extract was able to counteract the dexamethasone-induced increase in ROS and to rescue the cell viability in a U-shape and inverted U-shape manner respectively. Those effects were confirmed in two cells lines: the commonly used neuronal model of neuroblastoma SH-SY5Y cells as well as the murine hippocampal cells HT22. The effects of dexamethasone on the HT22 cells were more prominent than those in the SH-SY5Y cells. This is because the hippocampus is at the forefront of the glucocorticoid effects and it highly expresses GR while GR bind synthetic glucocorticoids such as dexamethasone with a high affinity. Additionally*, Rhodiola rosea* extract significantly increased ATP production and metabolic activity in an inverted U-shape manner. The biphasic response curves exhibited by Rhodiola in our study are usually characteristic for botanicals, especially plant adaptogens [23-25]. Typically, low doses of an adaptogen induce a dose-dependent increase in biological response until a maximum response is reached, after which there is a gradual

decrease in the response until it might even reach a non-observable effect level, a phenomenon also called hormesis [23]. Thus, a biphasic response curve could reflect activation of the «adaptive cellular stress response». Consistently, our findings indicate that the *Rhodiola rosea* extract exhibits the typical features of an adaptogen with regard to the modulation of adaptive homeostasis and stress response. Our results indicated that *Rhodiola rosea* extract per se enhanced mitochondrial functions as well as rescued cell viability and counteracted ROS elevation under glucocorticoid stress. These results are in line with evidence showing the ROS neutralizing and antioxidant-enhancing effects of *Rhodiola rosea* [26-30]. There is a single study conducted almost 2 decades ago showing the effects of Rhodiola on increasing ATP levels in rat muscle cells [31] and our results are in agreement with this finding. In addition, for the first time we showed *in vitro* in differentiated SH-SY5Y cells that *Rhodiola rosea* is able to promote neurite outgrowth by increasing the number and length of neurites as well as the attachment and endpoints of the newly formed neurites. The neurite outgrowth was accompanied by a fast upregulation in the mRNA and protein level of the neurotrophin BDNF which plays major roles in neuronal maturation and synaptic plasticity [32]. Therefore, the Rhodiola-induced neurite outgrowth could be mediated by the BDNF pathway. Interestingly, BDNF has been found to exert mitochondria-enhancing properties. BDNF increased mitochondrial ATP synthesis through an increase in respiratory coupling enabling neuroprotective mechanisms correlated to neuroplasticity [33]. BDNF caused a dose-dependent enhancement in the respiratory control index (RCI), a respiratory coupling efficiency measure, in ATP production and integrity of rat brain mitochondria through a MAP kinase pathway which was largely specific for mitochondrial complex I [34]. BDNF increased phosphorylated CREB in the matrix and on the mitochondrial inner membrane as well as induced mitochondrial complex V synthesis in cultured neurons under mechanical injury [35]. BDNF decreased the mPTP opening, preserved MMP and calcium content, increased mitochondrial biogenesis markers (PGC-1α, NRF-1, NRF-2) and stimulated mitophagy via the HIF-1α/BNIP3 (hypoxia-inducible factor-1α/ BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) signaling pathway in a hyperglycaemia cellular model (human brain microvascular endothelial cells) [36]. BDNF was able to inhibit neuronal apoptosis by stimulating anti-apoptotic Bcl-2 family members expression and caspase inhibitors as well as by obstructing pro-apoptotic

proteins including Bax and Bad. Finally, BDNF also enhances antioxidant enzymes expression and promoted repair of neuronal damaged DNA [37]. Taking into consideration these findings, *Rhodiola rosea* extract could exert its mitochondria enhancing properties by acting directly on mitochondria via increasing ATP levels and reducing ROS, as shown in our study, but also by acting indirectly via upregulating BDNF which in turn acts on mitochondria. Overall, our findings are in line with the use of Rhodiola against stress and stress-related disorders as it increased the neuronal energy, normalized the dexamethasone-induced increase in ROS, rescued cell viability as well as promoted neurite outgrowth possibly by upregulating BDNF mRNA and protein level. This is the first study that shows the effect of *Rhodiola rosea* extract on neuronal bioenergetics, neurite outgrowth and BDNF upregulation. Of course, more research is needed to establish these functions. Next research steps include the evaluation of *Rhodiola rosea* extract's ability to promote neurite outgrowth and other mitochondrial functions such as respiration under dexamethasone-induced stress.

Mangiferin is a xanthonoid polyphenol found mainly in the fruit, leaves and bark of *Mangifera indica* but also in Honeubush extracts and has exhibited antioxidant, neuroprotective and anti-AD properties. These properties were mainly mediated via upregulation of the Nrf2/HO-1 and PI3K/Akt/mTOR signaling pathways via downregulation or inactivation of the ERK1/2 pathway as well as via decrease in neuroinflammation [38]. Although there are several studies showing the effects of mangiferin against Aβ neurotoxicity [39-41], there is very limited research on its effects on tauopathy cellular models [42]. Tau has many physiological roles within neurons as it enables transfer of valuable biomolecules and organelles (protein, lipids, mitochondria) across axons as well as stabilizes the microtubules and contributes to cytoskeleton organization. However, in diseased states it might overaccumulate and form neurotoxic fibrils which further shape into tangles compromising mitochondrial and neuronal health. Of note, abnormal tau prevents the axonal transport of mitochondria leading to fewer mitochondria at the synapse and thus synaptic degeneration and neuronal death while it also reduced bioenergetics, ATP levels, MMP, respiration and complex I and V activities and increases ROS production [3]. Therefore, we aimed to evaluate the effects of mangiferin on neuroblastoma cells carrying the P301L tau mutation which causes tau overaccumulation and compared

them to neuroblastoma cells carrying the empty vector (Mock cells). We showed that P301L cells demonstrate significantly lower ATP and mitochondrial membrane potential (MMP) levels compared to the Mock cells. Moreover, mangiferin was found to increase the ATP levels and MMP in P301L cells compared to the untreated cells. Of note, mangiferin-treated P301L cells approached the ATP and MMP levels of the untreated healthy Mock cells showing that mangiferin might be able to rescue the tau-induced deficits. Our results are in agreement with existing literature showing that mangiferin rescued MMP in rotenone-treated SK-N-SH neuroblastoma cells [43]), attenuated ATP levels and MMP in Aβ-treated cortical neurons [39], increased ATP levels via increasing the activity and expression of mitochondrial complex II in the skeletal muscle of high fat diet mice [44] and enhanced ATP levels via its oxidative damage protective effect in myocardial infarcted rats [45]. Those effects are attributed to the antioxidant and scavenging capacity of mangiferin, its ability to enhance the activity of antioxidant enzymes (SOD, CAT, GST) as well as its ability to upregulate important signaling pathways including Nrf2/HO-1 and PI3K/Akt/mTOR [38]. Nrf2 is a transcription factor which regulates antioxidant pathways and the expression of antioxidant proteins downstream its activation including HO-1 which further fends off free radicals [46]. The PI3K/Akt pathway modulates a range of cellular activities including cell survival, metabolism and protein synthesis [47] and promotes anti-apoptotic and pro-survival processes via the phosphorylation of Bad (pro-apoptotic protein). Therefore, the effects we observed in our study could be mediated by these pathways. Additionally, we showed that, both morphologically but also quantitatively, untreated Mock cells present a much healthier phenotype than untreated P301L cells. Interestingly, mangiferin-treated P301L cells showed similar development of neurites to that of the untreated Mock cells indicating that mangiferin is able to bring the tau cells close to the state of the healthy Mock cells both morphologically and in terms of quantification of neurite outgrowth parameters. This is the first study indicating the effect of mangiferin on neurite outgrowth and further supports its neuroprotective properties. Of note, mangiferin has been shown to increase BDNF levels in the hippocampus of $AlCl_3$-treated rats [48]. Abnormal tau has been proven to block anterograde axonal transport of mitochondria and perinuclear mitochondrial clustering which causes mitochondrial depletion at the synapse, synapse dysregulation and inhibition of neurite outgrowth [3]. Interestingly, BDNF treatment has been shown to

recruit mitochondria at presynaptic sites in cultured hippocampal neurons. As a result, mitochondrial accumulation in presynaptic sites enhanced synaptic vesicle docking (the process of vesicle and pre-synaptic membrane alignment which enables exocytosis) [49-52]. Therefore, the mangiferin-induced neurite outgrowth we demonstrated could be mediated via the BDNF pathway as BDNF regulates synaptic plasticity and recruits mitochondria at the synapse enabling synaptic transmission. Additionally, mangiferin has been shown to upregulate the PI3K/Akt/mTOR pathway as it increased the Akt, mTOR complex 1 and mTOR complex 2 expression which subsequently enhanced cell cycle progression and cell survival in a rat model of hypoxic–ischemic brain injury. mTOR belongs to the PI3K-related kinase family and consists of two distinct complexes, mTOR complex 1 and mTOR complex 2. mTOR signaling mediates several brain functions including proliferation, differentiation, dendrite formation and neurite outgrowth while it is crucial during synaptic formation and plasticity. mTOR complexes activation highly affects all the processes of neurogenesis by promoting dendrite formation, neural stem cell differentiation and migration and neuron maturation while activation of mTORC1 results in increased dendrite length. The PI3K/Akt/mTOR signaling pathway fulfils an important role in the development and differentiation of cortical layers. Disturbance in mTOR signaling has caused dendrite modifications and has been associated with severe impairments in the development of the nervous system [53]. Therefore, activation of the PI3K/Akt/mTOR pathway together with upregulation of BDNF levels could be the mechanisms by which mangiferin caused neurite outgrowth in our study. Of course, more research in needed to confirm the molecular mechanisms of mangiferin. Of particular interest would be to evaluate the effect of mangiferin on the BDNF levels in cultured neurons and its effect on other mitochondrial functions (respiration, mitophagy, complex activity).

In this PhD thesis the mitochondria-enhancing properties of three different phytochemicals and their protective effects against three types of stress was demonstrated. Honeybush extracts protected neuronal cells against oxidative stress, *Rhodiola rosea* extract protected against glucocorticoid stress while mangiferin protected neuronal cells against abnormal tau-induced stress. We showed novel functions of these three phytochemicals and new potential mechanisms of their

actions were indicated. Of the three phytochemicals *Rhodiola rosea`s* neuroprotective properties are the most established, followed by mangiferin whose molecular mechanisms in neuroprotection have been suggested. Last but not least comes honeybush for which our study was the first one to indicate its potential neuroprotective properties. Our findings indicate that honeybush extracts, *Rhodiola rosea* extract and mangiferin could constitute candidates for the prevention of oxidative stress and mitochondrial dysfunction-related neurodegenerative and stress-related disorders potentially leading to the development of condition-specific pharmacological strategies.

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Abbreviations

143B: Human osteosarcoma-derived cell line

8-oxo-dG: 7,8-dihydro-8-oxo-deoxyguanosine

Aβ: Amyloid beta protein

$ABTS^{\bullet+}$: (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay

AChE: Acetylcholinesterase

ACTH: Adrenocorticotropic hormone

ADP: Adenosine diphosphate

AD: Alzheimer's disease

ADF: Actin-depolymerising factor

AIF: Apoptosis-inducing factor

AKT: Protein kinase B

AMP: Adenosine monophosphate

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPK: AMP-activated protein kinase

ANT: Adenine nucleotide translocator

Apaf-1: Apoptotic protease activating factor 1

APP: Amyloid precursor protein

APP/PS1: Human amyloid precursor protein/ mutant human presenilin 1

ATP: Adenosine triphosphate

Bad: Bcl-2-antagonist of cell death

Bax: Bcl-2-associated X protein

BBB: Blood-brain-barrier

Bcl: B-cell lymphoma protein

BDNF: Brain-derived neurotrophic factor

BV2: Microglial cells

CA1: Cornu Ammonis 1 hippocampal region

CA3: Cornu Ammonis 3 hippocampal region

CaMKII: Ca2+/calmodulin-dependent protein kinase II

CAT: Catalase

Cdk5: Cyclin-dependent kinase 5

ChIP: Chromatin immunoprecipitation

CNS: Central nervous system

CREB: Cyclic adenosine monophosphate (cAMP) response element binding protein

CRH: Corticotropic-releasing-hormone

Cu-SOD: Copper superoxide dismutase

CypD: Cyclophilin D

dATP: Deoxyadenosine triphosphate

Dexa: Dexamethasone

DPPH: (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay

DRP1: Dynamin-related protein 1

EGTA: Calcium binding ethylene glycol tetra-acetic acid

ELK-1: ETS transcription factor ELK1

EMA: European Medicines Agency

ERK: Extracellular signal-regulated kinase

ETC: Electron transport chain

FAD+: Oxidized form of flavin adenine dinucleotide

$FADH_2$: Reduced form of flavin adenine dinucleotide

Fancc: Fanconi anemia complementation group C

FCCP: Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone

FoxO: Forkhead box O

FRAP: Ferric reducing antioxidant power

FYN: Tyrosine-protein kinase

F_0F_1 ATPase: ATP synthase

F2-IsoPs: Isoprostanes

GABA: γ-aminobutyric acid

GBE: *Ginkgo biloba* extract

GDP: Guanosine diphosphate

GPx: Glutathione peroxidase

GR: Glucocorticoid receptor

GSH: Reduced form of glutathione

GSK: Glycogen synthase kinase

GSSG: Oxidized form of glutathione or glutathione disulfide

GST: Glutathione-S-transferase

GTP: Guanosine triphosphate

H_2O_2: Hydrogen peroxide

HCN 1-A: Human cortical cell line

HepG2: Hepatocarcinoma cell line

HIF-1α: Hypoxia-inducible factor 1α

HIF-1α/BNIP3: Hypoxia-inducible factor-1α/ BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

HNE: 4-hydroxy-2-nonenal

HO-1: Heme oxygenase-1

HPA: Hypothalamic-pituitary-adrenal axis

Hsp70: Heat shock protein 70

HT22: Murine hippocampal cell line

HUVECs: Human umbilical vein endothelial cells

IL-1α: Interleukin IL-1α

IMR-32: Human neuroblastoma cells

IMR-90: Human diploid fibroblast cell line

IsoP: 8-iso-prostaglandin F2α

JIP1: c-Jun N-terminal kinase-interacting protein 1

JNK: c-Jun N-terminal Kinase

KA: Kainic acid

Kif9: Kinesin family member 9

KI-P301L: P301L tau knock-in mice

LC/NE: Locus caeruleas/norepinepherine

LTD: Long-term depression

LTP: Long-term potentiation

MAPK: Mitogen-activated protein kinase

MAP-1: Modulator of apoptosis-1

MAPT: Microtubule-associated protein tau

MDA: Malondialdehyde

MFN2: Mitofusin 2

MMP: Mitochondrial membrane potential

Mn-SOD: Manganese superoxide dismutase

MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mPTP: Mitochondrial permeability transition pore

MR: mineralocorticoid receptor

mRNA: Messenger RNA

Msn2/Msn4: multicopy suppressor of SNF1 mutation proteins 2 and 4

mtDNA: Mitochondrial DNA

mTOR: Mammalian target of rapamycin

N_2O_4: Dinitrogen tetroxide

N2A: Neuro-2A cells

NAD+: Oxidized form of nicotinamide adenine dinucleotide

NADH: Reduced form of nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NCTC 2544: Human keratinocyte cell line

NF-κB: Nuclear factor κB

NFT: Neurofibrillary tangles

NGF: Nerve growth factor

NLRP3: NLR family pyrin domain containing 3

NMDA: N-methyl-d-aspartate

NNT: Nicotinamide nucleotide transhydrogenase

NO: Nitric oxide

NO^+: Nitrosonium cation

NO^-: Nitroxyl anion

NO_2: Nitrogen dioxide

NOS: Nitric oxide synthase

Nrf2: Nuclear factor erythroid 2-like 2

NT-3: Neurotrophin-3

NT-4/5: Neurotrophin-4/5

NT-6: Neurotrophin-6

NT-7: Neurotrophin-7

$O_2^{\bullet-}$: Superoxide anion radical

OH^-: Hydroxyl radical

OONO-: Peroxynitrile

ORAC: Oxygen radical absorbance capacity

OXPHOS: Oxidative phosphorylation

P301L: Neuroblastoma cells stably transfected with the mutant tau P301L

PAK: p21-activated kinase

PAR-1: Partitioning defective-1 protein

PC12: Pheochromocytoma cells

PD: Parkinson’s disease

PGC1-α: Peroxisome proliferator-activated receptor-gamma coactivator-1alpha

PHF: Paired helical filaments

Pi: Inorganic phosphate

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PKG: cGMP-dependent protein kinase

PLC-γ: Phospholipase Cγ

PP1: Protein phosphatase 1

pR5 mice: P301L tau mutant

PSD: Postsynaptic density

Ptgs1: Prostaglandin-endoperoxide synthase 1

PTSD: Posttraumatic stress disorder

RCI: Respiratory control index

Redox: Reduction-oxidation

Rho: Ras homologue

RMS: Rostral migratory stream

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RRE: *Rhodiola rosea* extract

rTg4510 mice :P301L tau mutation mice

SAPK: Stress-activated protein kinase

Serpinb1b: Serine peptidase inhibitor clade B member 1b

SGZ: Subgranular zone

SH-SY5Y: Human neuroblastoma cell line

snf1: sucrose non-fermenting 1

SOD: Superoxide dismutase

SSRIs: Selective serotonin reuptake inhibitors

SVZ: Subventricular zone

TBA: 2-thiobarbituric acid

TBARS: Thiobarbituric acid reactive substances

TCA: Tricarboxylic acid cycle

TCAs: Tricyclic antidepressants

TEAC: Trolox equivalent antioxidant capacity

TPC: Total phenolic content

TrkB: Tyrosine kinase receptor B

TSPO: Translocator protein

TTBK1/2: Tau-tubulin kinase 1/2

Txnip: Thioredoxin interacting protein

UV: Ultraviolet radiation

VDAC: Voltage-dependent anion channel

Xpa: Xeroderma pigmentosum complementation group A

Zn-SOD: Zinc superoxide dismutase

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Effects of polyphenols and plant extracts on stress-induced defects in bioenergetics.

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List of publications

Articles

- Booker, A., **Agapouda, A**., Frommenwiler, D. A., Scotti, F., Reich, E., & Heinrich, M. (2018). St John's wort (Hypericum perforatum) products–an assessment of their authenticity and quality. Phytomedicine, 40, 158-164. Impact factor: 4.268

- **Agapouda, A**., Butterweck, V., Hamburger, M., de Beer, D., Joubert, E., & Eckert, A. (2020). Honeybush Extracts (Cyclopia spp.) Rescue Mitochondrial Functions and Bioenergetics against Oxidative Injury. Oxidative Medicine and Cellular Longevity, 2020. Impact factor: 5.076

- **Agapouda, A**., Lejri, I., Grimm, A., & Eckert, A. Rhodiola rosea extract counteracts stress via improvement of bioenergetics, elimination of ROS and induction of neurite outgrowth. (Submitted)

- **Agapouda, A.**, Lejri, I., Grimm, A., & Eckert, A. Mangiferin exerts beneficial effects on bioenergetics and neurite outgrowth in a cellular model of tauopathy. (In preparation)

Reviews

- **Agapouda, A**., Booker, A., Kiss, T., Hohmann, J., Heinrich, M., & Csupor, D. (2019). Quality control of Hypericum perforatum L. analytical challenges and recent progress. Journal of Pharmacy and Pharmacology, 71(1), 15-37. Impact factor: 2.405

- Lejri, I., **Agapouda, A**., Grimm, A., & Eckert, A. (2019). Mitochondria-and oxidative stress-targeting substances in cognitive decline-related disorders: from molecular mechanisms to clinical evidence. Oxidative medicine and cellular longevity, 2019. Impact factor: 5.076

Abstracts

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induced deficits. Poster session at the 1st International Conference on Neuroprotection by Drugs, Neutraceuticals and Physical Activity. June 6-7, 2019

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Appendix

Mitochondria- and Oxidative Stress-Targeting Substances in Cognitive Decline-Related Disorders: From Molecular Mechanisms to Clinical Evidence

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Mitochondria and oxidative stress targeting substances in cognitive decline-related disorders: from molecular mechanisms to clinical evidence

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Abstract

Alzheimer's disease (AD) is the most common form of dementia affecting people mainly in their sixth decade of life and at a higher age. It is an extensively studied neurodegenerative disorder yet incurable to date. While its main post-mortem brain hallmarks are the presence of amyloid-β plaques and hyperphosphorylated tau tangles, the onset of the disease seems to be largely correlated to mitochondrial dysfunction, an early event in the disease pathogenesis. AD is characterized by flawed energy metabolism in the brain and excessive oxidative stress, processes that involve less adenosine triphosphate (ATP) and more reactive oxygen species (ROS) production respectively. Mitochondria are at the centre of both these processes as they are responsible for the energy and ROS generation through mainly oxidative phosphorylation. Standardized *Ginkgo biloba* extract (GBE), resveratrol, phytoestrogens as well as the neurosteroid allopregnanolone have shown some mitochondria-modulating properties but also significant antioxidant potential in in vitro and in vivo studies. According to our review of the literature, GBE, resveratrol, allopregnanolone and phytoestrogens showed promising effects on mitochondria in a descending evidence order and, notably this order pattern is in line with the existing clinical evidence level for each entity. In this review the effects of these four entities are discussed with special focus on their mitochondria-modulating effects, their mitochondria-improving and antioxidant properties across the spectrum of cognitive decline-related disorders. Evidence on preclinical and clinical studies as well as on their mechanisms of action are summarized and highlighted.

Keywords: *Ginkgo biloba*, resveratrol, phytoestrogens, allopregnanolone, mitochondria, Alzheimer's disease

Abbreviations

17β-estradiol: estradiol
2A PP2Ac: catalytic subunit of protein phosphatase
3xTgAD: triple transgenic mouse model of AD
AD: Alzheimer’s disease
Akt: Protein kinase B
AMPK: 5' adenosine monophosphate-activated protein kinase
APP: amyloid precursor protein
ATP: Adenosine triphosphate
Aβ: beta-amyloid protein
αKGDH: α-keto glutarate dehydrogenase
Bax: apoptotic regulator
BBB: blood–brain barrier
Bcl-2: anti-B-cell lymphoma 2
BDNF: brain-derived neurotrophic factor
BDPP: bioactive dietary preparation
Ca2+: calcium
CMRglc: Cerebral Metabolic Rates of Glucose
COX-2: cyclooxygenase-2
CREB: cyclic AMP response element binding protein
CSF: cerebrospinal fluid
CTRL: untreated SH-SY5Y cells
DAT: dopamine transporters
E2: estrogen
ER: endoplasmic reticulum
ERs: estrogen receptors
ERT: estrogen replacement therapies
ETC: electron transport chain
FAD: Familial Alzheimer's disease
FOXO: Forkhead box O
GBE: Gingko biloba extract
GPx1: Glutathione peroxidase 1

GSK3β: glycogen synthase kinase 3 beta

hNSCs: human neural stem cells

HO-1: heme oxigenase 1

IA: transient potassium channel

IBO: Ibotenic acid

IKK: inhibitory kappa B kinase

IMM: inner mitochondrial membrane

IMR-32: human neuroblastoma cells

iNOS: nitric oxide synthase

JAK/ERK/STAT: janus kinases/ extracellular signal-regulated kinases/ signal transducers and activators of transcription

JNK: c-Jun N-terminal kinase

LXR: Liver-X-receptor

MCI: mild cognitive impairment

MDA: malondialdehyde

MMP: mitochondrial membrane potential

MnSOD: manganese superoxide dismutase

mPTP: mitochondrial permeability transition pore

MRI: magnetic resonance imaging

MTDLs: multitarget-directed ligands

mtDNA: mitochondrial DNA

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

Nox4: nicotinamide adenine dinucleotide phosphate (NADPH) oxidases 4

NPS: neuropsychiatric symptoms

NRF-2: nuclear factor erythroid 2–related factor 2

NRs: nuclear receptors

OVX: ovarectomized

OXPHOS: oxidative phosphorylation

p53: tumor protein

P450scc: cytochrome cholesterol side-chain cleavage enzyme

PAF-AH-1: platelet-activating factor-acetylhydrolase-1

PCG-1α: peroxisome proliferator-activated receptor γ co-activator-1α

PC12: pheochromocytoma cells cells

PDH: pyruvate dehydrogenase

PKC-δ: Protein kinase C delta

PPARγ: peroxisome proliferator-activated receptor gamma

PSD95: synapse-associated protein

PXR: pregnane xenobiotic receptor

RNS: reactive nitrogen species

ROS: reactive oxygen species

RSFC: resting-state functional connectivity

SAD: sporadic Alzheimer's disease

SAMP1: senescence-accelerated mice

SGZ: subgranular zone

SH-SY5Y: human neuroblastoma cells

SIRT1: sirtuin1

SMI: subjective memory impairment

SOD: superoxide dismutase

SOD1: superoxide dismutase 1

TH: tyrosine hydroxylase

TNF-α: tumor necrosis factor alpha

TrkA: tropomyosin receptor kinase A

TrkB: tropomyosin receptor kinase B

TSPO: the translocator protein

TyrRS-PARP1: tyrosyl tRNA synthetase-Poly [ADP-ribose] polymerase 1

VaD: vascular dementia

YY-1224: a terpene trilactone-enhanced GBE

INTRODUCTION

Alzheimer's disease- a well-known yet untreatable age-related neurodegenerative disorder

Alzheimer's disease (AD), the most common neurodegenerative disorder, as well as dementia type, is characterised by extracellular senile beta-amyloid protein (Aβ) plaques and intracellular neurofibrillary tau tangles [1]. There are two types of AD: i) the sporadic form of AD (SAD) whose onset occurs above the age of 65 and ii) the familial AD forms (FAD), that are more rare with less than 1% occurrence among the AD cases and the onset starts at younger age (<65 years). The biological system of aging is the major risk factor of SAD [2]. The familial forms (FAD) bear inheritable mutations in the amyloid precursor protein (APP), presenilin 1 and presenilin 2 genes [3, 4]. The symptoms of AD are the same in SAD and FAD [5]. There are different types of age-related cognitive diseases which differ in severity. SMI (Subjective Memory Impairment) is the condition when non-demented aged people express subjective complains related to their memory but without having any organic or identifiable condition [6]. The SMI is discussed as an early predictor of dementia [7-10] . The concept of mild cognitive impairment (MCI) defines an intermediate stage between normal aging and dementia. MCI patients show mild but measurable changes in cognitive tests and thinking abilities that are noticeable to the patients and to family members but they are able to carry out everyday activities. Approximately 15-20% of people aged 65 or older have MCI. This group of people represents a population at increased risk for developing dementia, especially MCI involving memory problems [11]. The occurrence of MCI in the population is 3.2% of which an 11.1% of cases convert to dementia within 3 years [12]. It has been indicated, yet not conclusively,

that SMI is a precursor of MCI which can then lead to dementia or AD [4, 13]. Dementia is a more severe condition than SMI and MCI which affects the aged people and interferes negatively in the performance of everyday activities. It is described as a cluster of symptoms related to mental, cognitive and memory decline [12, 14]. There are different forms of dementia such as AD, the most common type, and vascular dementia. Vascular dementia (VaD) is the second most common form of dementia and occurs as cognitive decline due to reduced blood flow in the brain (e.g. due to brain injury or stroke). However, sometimes different kinds of dementias co-exist and their discrimination is difficult due to overlapping clinical symptoms. Moreover, many of these patients also suffer from psychiatric or behavioural problems that are sometimes referred to as BPSD (Behavioural and psychological symptoms of dementia) or NPS (Neuropsychiatric symptoms), including irritability, anxiety, psychosis, and aggression [15].

Mitochondria and neuroplasticity

Mitochondria are essential yet independent organelles contained in eukaryotic cells and they are responsible for numerous functional activities within the cells. However, they were not always an intrinsic structure of eukaryotic cells. They occurred through the endosymbiosis of an alpha-proteobacterium into a prokaryotic progenitor and this is why they contain their own DNA, namely the mitochondrial DNA (mtDNA) [16]. Regarding the structural characteristics of these organelles, they contain two structurally and functionally distinct membranes, the outer and the inner membrane. The inner membrane encapsulates the matrix and also carries the electron transport chain (ETC) where the oxidative phosphorylation (OXPHOS) is taking place. The

mtDNA is located in the matrix encoding 13 proteins which are used as structural components of the ETC complexes [17].

Mitochondria have obtained the title of ‘’powerhouse of the cell’’ due to their ability of producing the energy, mainly through OXPHOS, required for the survival and functioning of the cell. Actually, they are more than just a ‘’powerhouse’’ as they are the ultimate multi-taskers which define the cell fate. Apart from the production of energy in the form of ATP, mitochondria are the key modulators of brain cell survival and death by controlling calcium (Ca^{2+}) and redox equilibrium (which in turn affects neurotransmitter release and neuronal plasticity), by producing reactive oxygen species (ROS) and by controlling cell apoptosis [17-19]. The brain is an organ which requires a considerable amount of energy in order to operate, maintain and enhance neuronal functions and plasticity. Neurons are post-mitotic polarized cells with significant energy demands. OXPHOS, taking place in mitochondria, is the main energy provider in the form of ATP and neurons depend almost solely on this procedure in order to satisfy their energy needs [20]. In particular, neurons direct this energy into formation of interconnections, the synapses. The number and strength of these neuron interconnections define synaptic plasticity which is responsible for cognitive function [21]. Synaptic plasticity is a crucial mechanism by which the neural activity generated by an experience alters synaptic transmission and therefore modifies brain function. [22]. Neurite outgrowth is a process wherein developing neurons generate new projections as they grow in response to guidance cues. Nerve growth factors (NGF), or neurotrophins, are one family of such stimuli that regulate neurite growth [23]. Brain derived neurotrophic factor (BDNF) exerts several actions on neurons ranging from acute enhancement of transmission to long-term promotion of neurite outgrowth and synaptogenesis [24, 25]. Synaptic plasticity includes the

dynamic regulation of long –term potentiation (LTP), spine density, number and length of dendrites and axons (neuritogenesis) as well as neurogenesis. Adult neurogenesis generates functional neurons from adult neural precursors in restricted brain regions throughout life [26]. These plasticity processes need a high energy requirement and this is why mitochondria play such a pivotal role in the well-being of neurons especially when neurons need to adapt to periods of pathologically reduced functions.

Mitochondria, oxidative stress, aging and AD

However, while mitochondria regulate the functions of healthy neurons, they are also largely affected during aging and pathological states such as age-related neurodegenerative diseases. Mitochondria are both the regulators of energy metabolism but also the main ROS generators. ROS are immensely reactive species which are produced in mitochondria mainly by complexes I and III of the ETC when there is a leak of electrons. They are chemical species including hydroxyl radical (·OH), superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) which can interact and damage DNA, proteins and lipids compromising cell survival and leading to aging and to vulnerability to several diseases [27, 28]. When they exist at normal levels they constitute signaling agents in many physiological processes such as redox homeostasis, cellular death, cellular senescence, cell proliferation and they can also trigger immune responses, synaptic plasticity and cognitive enhancement [20, 27]. ROS are neutralized by anti-oxidant enzymes such as superoxide dismutase, which transforms the radicals (O_2-) into H_2O_2, and by catalase, glutathione peroxidase and thioredoxin peroxidase which diffuse H_2O_2 [27]. In healthy state, there is a balance between the ROS production and neutralization. Nevertheless, when ROS are

produced in excess, e.g. during aging, they directly affect mitochondria since mitochondrial membranes consist of long polyunsaturated fatty acids which are easily oxidized. Also, the mtDNA is found in close proximity to the ROS source and is susceptible to mutations resulting in the production of faulty ETC proteins, leading back to the production of more ROS [18, 28]. It could be said that mitochondria are the main organelles in aging and neurodegeneration by being both generators and targets of ROS. It has been shown that aging is characterized by a rise in oxidative stress, a decline in anti-oxidant defense systems and impairment of the OXPHOS. So, aging is characterized by energy deprivation and a shift of the redox state towards oxidation. Mitochondria are at the center of these hallmarks [20]. Neurons, which highly depend on OXPHOS to satisfy their energy demands, are particularly susceptible to energy hypometabolism [20]. In addition, taking into account that they are non-dividing cells, neurons are almost as old as the entire organism and are not replaced during life with the exception of the hippocampus that continuously generates new neurons during adulthood [20, 29]. This means that neurons accumulate oxidative stress and therefore defective mitochondria during aging [20, 30]. This is particularly important since mitochondrial dysfunction represents an early event in AD pathogenesis [20, 28, 31].

Intense oxidative stress and decreased brain energy metabolism are common characteristics of both normal aging and AD, although to different extent [20]. Of note, mitochondrial abnormalities are observed in FAD and SAD brains [32, 33]. On one hand, recent data obtained from AD models, in which mitochondrial failure is a prominent feature, implicate tau hyperphosphorylation as well as Aβ overproduction and deposition. On the other hand, Aβ and tau target mitochondria synergistically, thereby possibly amplifying each other's toxic effects. This interrelationship of Aβ, tau

and mitochondrial function constitutes a vicious cycle [34]. The mitochondrial cascade hypothesis postulates that mitochondrial dysfunction represents the most upstream pathology in AD [28]. According to this hypothesis, arresting brain aging will prevent the development of AD [32].

Mitochondria-directed natural compounds

The current mitochondrial cascade hypothesis postulates mitochondrial dysfunction as a central pathomechanism in age-related degenerative disorders [28, 35, 36]. Taking into account their primary role in aging and in the early stages of AD, mitochondria constitute promising targets for therapeutic strategies. For this reason, pharmacological studies are directed in enhancing mitochondrial functions such as ATP production and respiration or in reducing mitochondrial harmful by-products such as ROS [36]. To date no drugs are able to cure or stop the progression of age-related neurodegenerative disorders. Most of them may be beneficial in delaying the progression of AD and only partially treat some of its symptoms (*e.g.* confusion and memory loss).

Many drugs including whole plant extracts and single compounds originate from natural and botanical sources. Two single compound AD drugs derived from plants: i) the acetylcholine-esterase inhibitor, galanthamine, derived from *Galanthus* species (*Galanthus caucasicus*, *Galanthus woronowii*) and ii) rivastigmine, a physostigmine analogue (physistigmine was isolated from Calabar bean, *Physostigma venenosum*) [37, 38]. In addition, the phytopharmacon GBE that is used as anti-dementia medicine was shown to improve mitochondrial function emphasizing the concept of targeting mitochondria as an emerging and promising therapeutic approach [35, 39]. Therefore,

we focused our search on natural compounds that possess mitochondria-enhancing properties based on our own past and ongoing research as well as on research of other groups. Standardized Gingko biloba extract (GBE), resveratrol, phytoestrogens and the natural neurosteroid allopregnanolone fulfilled our criteria. Common targets of these agents (Figure 1) have been reported such as ROS, Mitochondrial Membrane Potential (MMP), Aβ, tau protein, anti-apoptotic protein (Bcl-2), and OXPHOS (Figure 2 and 4). Accordingly, in this review we aimed to summarise molecular modes of action of these natural agents with special focus on mitochondria, their mitochondrial function-enhancing properties and their anti-oxidant properties. We discuss evidence on their mechanism of action from preclinical as well as clinical studies. Especially regarding clinical trials, there is different level of existing evidence for each phytochemical. GBE, resveratrol, phytoestrogens and allopregnanolone appear in a descending order according to their clinical evidence level. The databases Pubmed, Google Scholar as well as the database Clinical Trial Gov were used for our search with a focus on the years 2000-2018. For the clinical evidence we considered randomized, double-blind, placebo-controlled trials as well as ongoing trials, systematic reviews, meta-analyses and cochrane analyses.

PHARMACOLOGIC FEATURES OF NATURAL SUBSTANCES IN ALZHEIMER'S DISEASE

Ginkgo biloba

Gingko biloba has existed for over 250 million years and is native from Japan, Korea, and China, but can be found worldwide. Traditional Chinese clinicians originally

utilized GBE for a variety of applications [40]. There are several *Ginkgo biloba* extracts sold on the market but also used in studies including standardised and non-standardised extracts. The standardised extracts have to meet specific criteria regarding their manufacturing process, the quality of the plant material and their composition which is not the case with the non-standardised extracts. Many products have been reported on the market which are not standardised and are even adulterated. These products do not only reduce the efficacy of GBE but they can be potentially harmful [41]. GBE contains two main groups of active constituents ensuring its medicinal effects, terpenes (including bilobalide, ginkgolides A, B, C) and flavonoids (including meletin, isorhamnetin and kaempferol). Both the United States Pharmacopoeia and the European Pharmacopoeia define as standardised only extracts that contain the active components of Ginkgo in a certain and defined content. In particular, the standardised extracts should contain 5-7% triterpene lactones, 22-27% flavonoids and less than 5 ppm of ginkgolic acids, which are toxic ingredients of Ginkgo. [42]. Most toxicological, pharmacological, and clinical investigations have focused on the neuroprotective value of two main standardized extracts labeled EGb761 and LI 1370 [43-45]. The EGb761 extract consists of 24% flavone glycosides (mainly quercetin, kaempferol and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B and C, and 2.6-3.2% bilobalide while the extract LI 1370 is composed of 25% ginkgo flavone glycosides as well as 6% terpenoids. Several terpene lactones (Ginkgolides, Bilobalide) show substantial mitochondria-protecting properties while the flavonoid fraction seems to play an important role in the free radical scavenging properties [46]. In the following parts, only effects of standardized GBE will be discussed.

Mechanisms of action based on preclinical evidence

Direct effects of GBE on mitochondria

Several findings demonstrate the mitochondria-modulating effect of GBE, mainly in cellular and animal models of AD. In particular, GBE has been shown to attenuate effectively mitochondrial dysfunction through several mechanisms of action such as antioxidant effect and free radical scavenging properties, with all the evidence leading to this conclusion having been reviewed extensively [35, 47-49]. I*n vitro*, GBE was shown to ameliorate mitochondrial function by improving MMP and ATP levels at a low concentration of 0.01 mg/ml in pheochromocytoma cells (PC12) cells [46]. In amyloid precursor protein (APP) transfected human neuroblastoma cells, an AD cellular model with increased Aβ generation, GBE improved respiration of mitochondria, stimulated mitochondrial biogenesis and increased ATP production [50]. Mitochondrial-related modes of action of GBE are summarized in Figure 2.

Effects of GBE on oxidative stress, Aβ and tau toxicity related to damage of mitochondria

Aβ plaques deposition is one of the main hallmarks of AD. The overexpression of both Aβ itself and its precursor protein, the amyloid precursor protein (APP), has been used to create cellular and animal models of AD. GBE has been shown effective in reducing both Aβ deposition but also its toxicity. In detail, the pro-oxidant $A\beta_{25-35}$ peptide treatment was shown to decrease complexes I and IV activities and to increase the level of reactive oxygen/reactive nitrogen species (ROS/RNS) in SH-SY5SY cells [51]. Thus, pre-treatment with GBE was able to reduce the Aβ-related increase in ROS/RNS levels as well as to ameliorate the complex I and IV activities [51]. GBE

protected against $A\beta_{1-42}$ oligomer-induced neurotoxicity and cell damage with an indirect effect on SH-SY5Y neuroblastoma cells by improving Hsp70 protein expression, and subsequently by activating the Akt (Protein kinase B) pathways as well as ER stress [52]. GBE also attenuated $A\beta_{1-42}$ oligomer-induced cell damage and protected against Aβ toxicity and oxidative stress [53, 54], apoptosis [52], and was able to reduce Aβ production [55]. In terms of animal models, a chronic treatment with GBE improved cognitive defects in a transgenic mouse model of AD (Tg 2576), a model that overexpresses a mutant form of APP [53]. GBE was also shown to decrease Aβ oligomers and to significantly increase neuronal proliferation in the hippocampus of young (6 months) and old (22 months) mice in a double transgenic mouse model (TgAPP/PS1) [54]. A chronic daily treatment with GBE for 6 months improved the cognitive function and alleviated amyloid plaque deposition in two-month-old APP/PS1 mice. Of note, GBE treatment seems to decrease the level of insoluble Aβ while the soluble content of Aβ was unchanged [56]. GBE reduced the hyperphosphorylation of tau at AD-specific Ser262, Ser404, Ser396 and Thr231 sites, rescued the activity of tau phosphatase PP2Ac and kinase GSK3β and reduced the oxidative stress in the hippocampus and prefrontal cortex on a hyperhomocysteinemia- treated rat model of AD. Memory lesions were also restored and the expression of synapse-associated protein PSD95 and synapsin-1 protein was upregulated [57].

Effects of GBE on neuroplasticity pathways

GBE exerts its beneficial effects by acting on the Akt pathway, as aforementioned, but also by acting on cyclic AMP response element binding protein (CREB) [54, 58, 59] . CREB phosphorylation induces transcriptional activation which results in expression of BDNF and therefore in synaptic plasticity and cognitive enhancement. Conversely,

lack of CREB phosphorylation is a pathological ailment of neurodegenerative diseases such as AD [60].

In detail, administration of GBE restored CREB phosphorylation in the hippocampus of TgAPP/PS1 mice [54]. Quercetin and bilobalide are the major constituents that have contributed to GBE-induced neurogenesis [58]. Both constituents promoted dendritic processes in hippocampal neurons and restored Aβ oligomers-induced synaptic loss as well as restored CREB phosphorylation [58]. Ginkgo flavonols quercetin and kaempferol have been shown to stimulate BDNF and phosphorylation of CREB in neurons isolated from double transgenic AD mouse (TgAPPswe/PS1e9) [59]. Recently, our team could confirm the neurite outgrowth stimulating effects of GBE in a 3D cell culture model **(Figure 3)**.

Clinical evidence

Apart from the preclinical studies, the extract has been largely investigated in clinical trials in a range of age-associated cognitive conditions from SMI and MCI to dementia and AD. GBE has been suggested for both the symptomatic treatment and prevention of those cognitive decline-related diseases. The standardised GBE is considered a phytopharmacon and the dose of 240 mg/ day is recommended as the most effective by the guidelines for biological treatment of dementias [12]. There are 9 categories (A, B, C, C1, C2, C3, D, E, F) and 5 grades (1-5) of pharmaceuticals used for AD and other dementias according to the level of existing clinical evidence and the occurrence of side effects respectively. GBE belongs to category B of level of evidence (limited positive evidence from controlled studies) and to grade 3 [12].Here we are going to highlight evidence on the extract's efficacy on subgroups of age-associated cognitive conditions in an ascending severity order **(Table 1)**.

Patients with SMI and MCI

Three randomised, double-blind, placebo-controlled, parallel-group trials were conducted for patients with memory complaints, one in SMI and two in MCI patients. In total, data from 61 SMI and 460 MCI patients were evaluated. One trial conducted in healthy aged patients with SMI showed that GBE enhanced cognitive flexibility without changes in brain activation and that mildly increased prefrontal dopamine [61]. Two trials showed that GBE ameliorated neuropsychiatric symptoms (NPS) and cognitive ability in patients with MCI [62] as well as improved cognitive functioning and aspects of quality of life in subjects with very MCI [63].

Patients with dementia

GBE has been found particularly efficacious in demented people with neuropsychiatric symptoms (NPS) [64, 65]. In total, 3 original papers, 1 systemic review, 6 meta-analyses and 1 cochrane analysis involving 14974 demented patients were evaluated. In detail, the pooled analyses of 4 published trials in a systemic review, involving outpatients with mild to moderate dementia and BPSD, demonstrated the efficacy of GBE at a daily dose of 240 mg [66]. Six meta- analysis (3 trials included in these meta-analyses were conducted in 1997 [67-69]) of 32 controlled, randomized, double-bind clinical trials and one bivariate meta- analysis of 6 trials come to the conclusion that GBE is efficacious and well tolerated in patients with a diagnosis of AD, VaD, or mixed dementia in three typical domains of assessment in dementia, ie, cognition, activities of daily living (ADL), and clinical global judgment [65, 70-74]. However, there are also the studies with inconclusive or contrasting results to the efficacious effect of GBE in demented subjects [75-77].

Patients with specific dementia type: AD and vascular dementia

In total data from 1 original paper, 1 review and 3 systematic reviews and meta-analyses involving 6880 patients with AD VaD were evaluated. In detail, in an original paper, low doses of GBE administered to patients with vascular cognitive impairment in a randomized, double-blind, placebo-controlled trial, showed significant deceleration of cognitive decline versus placebo only in one (Clinical Global Impression) of the four tests conducted in the trial [78]. The systematic reviews and meta-analyses (3 trials included in these meta-analyses were conducted in 1997 [67-69]) concluded that GBE exerts potentially beneficial effects on the improvement of activities of daily living, cognitive function and on global clinical assessment in patients with MCI or AD, in mainly AD type of dementia and in aged people with VaD having NPS [79-82].

Prevention

The preventive effect of GBE was reported in 14812 patients in three original papers and one systematic review and meta-analysis. In contrast, there are 4 studies that do not support the efficacious effect of GBE in preventing the onset of AD in either healthy aged or aged with MCI people [83-86]. The outcome for the efficacy of GBE in preventing the onset of AD in healthy individuals varies among different studies. However there is also high variability in the design of the studies in terms of GBE doses, duration of the treatment, sample size, statistical tools and compliance with the medication. Therefore, there is space for criticism regarding the methodological design of studies and the interpretation of the outcome. There are two large studies which form good examples of scepticism towards their negative outcome, the GEM study and the GuidAge study [83, 84, 87]. The GEM study was conducted in healthy old

people (80 years old or more) and found no efficacy of GBE in preventing the onset of AD. In this study the compliance of subjects with the treatment was non-adequate yet this parameter was not taken into account in the interpretation of the results. In the GuidAge study, the conversion rate from memory complaints to dementia was examined in aged people with memory complaints and no difference was found between GBE and placebo. But, the statistical power for the analysis of hazards was found low. The selection of suitable statistical methods to take into account increasing hazards overtime is crucial for meaningful results and increased significance [35].

Based on the included studies, GBE has been reported in only a few studies that show no effect. The majority of the recent studies demonstrated that the treatment with doses up to 240 mg/day was safe, well-tolerated and efficacious against age-related disorders.

In summary, GBE has been proved more effective in patients with cognitive impairment at baseline than preventing the onset of cognitive impairment in healthy aged subjects. As mentioned before (see introduction), mitochondrial dysfunction is more profound in cognitive disorders than in normal aging. Similarly, GBE shows increasing promising effects with increasing cognitive impairment. This, again, represents an indicator that GBE exerts its effects clinically by acting on mitochondria [35]. Thus, we can conclude that GBE can potentially improve mitochondrial dysfunction across the aging spectrum.

RESVERATROL

Resveratrol, known as a polyphenol from white hellebore (*Veratrum grandiflorum*), was discovered by Takaoka (1939) as a component of several dietary sources such as berries, peanuts and red grape skin or wine. Siemann and Creasy discovered that resveratrol is present at high concentration in red wine [88]. Resveratrol has been reported to possess several benefits including antitumor, antioxidant, anti-aging, anti-inflammatory, cardioprotective and neuroprotective properties. This polyphenol has emerged as a novel natural agent in the prevention and possible therapy of AD [89].

Mechanisms of action based on preclinical evidence

In vitro and *in vivo*, the direct molecular targets of resveratrol are not known in detail. However there is evidence that resveratrol exerts a complex mode of actions through the protection of mitochondrial function and the activation of biogenesis, through its effect on certain signalling pathways, through its antioxidant effects, through the increase of Aβ clearance and the reduction of Aβ neurotoxicity [90] **(Figure 4)**.

Direct effects of resveratrol on mitochondria

Dietary supplementation with 0.2 % (w/w) resveratrol suppressed the aging-associated decline in physical performance in senescence-accelerated mice (SAMP1) at 18 weeks of age by improving several mitochondrial functions such as the activity of respiratory enzymes, oxygen consumption, mitochondrial biogenesis as well as the activity of lipid-oxidizing enzymes [91]**.** In 18-month-old aged mice, resveratrol

(15mg/kg/day) and/or exercise for 4 weeks were able to counteract aging-associated oxidative damage targeting mitochondrial biogenesis and function by causing over-expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) mRNA and by increasing citrate synthase enzyme activity [92]. Mitochondrial biogenesis is induced by resveratrol through SIRT1 activation and deacetylation of PCG-1α [90] (**Figure 4**).

Effects of resveratrol on oxidative stress

Damaged mitochondria activate ROS production during oxidative stress which is involved in apoptosis [95]. ROS may damage the mitochondrial and cellular proteins and nucleic acids, causing lipid peroxidation and resulting in the loss of membrane integrity [106] **(Figure 4)**. Resveratrol protects also mitochondria by increasing the expression of the ROS-inactivating enzymes glutathione peroxidase 1 (GPx1) and superoxide dismutase 1 (SOD1) and by reducing the expression of the ROS-producing enzyme NADPH oxidase 4 (Nox4) [95, 107] **(Figure 4)**. In line with this, resveratrol rescued Aβ-treated human neural stem cells (hNSCs) from oxidative stress by increasing the mRNA of antioxidant enzyme genes such as SOD-1, nuclear factor erythroid 2–related factor 2 (NRF-2), Gpx1, catalase, and heme oxigenase 1 (HO-1) [103]. In addition, resveratrol exerted antioxidant properties and attenuated oxidative damage by decreasing iNOS and COX-2 levels [95].

Effects of resveratrol on Aβ toxicity related to damage of mitochondria

Thanks to its natural antioxidant properties and/or by sirtuin1 (SIRT1) activation, resveratrol shows neuroprotective effect because it counteracts Aβ toxicity. In more details, resveratrol increases the clearance of Aβ through the activation of AMPK [90]

(**Figure 4**). This natural molecule plays an important role in reducing Aβ neurotoxicity by phosphorylating Protein kinase C delta (PKC-δ) [90] (**Figure 4**). Resveratrol influences also the Aβ-induced apoptotic signalling pathway through SIRT1 activation, including inhibiting the expression of caspase protein 3 (caspase-3), apoptotic regulator Bax, Forkhead box O (FOXO), tumor protein p53, through blocking the activation of c-Jun N-terminal kinase (JNK), restoring the decrease of B-cell lymphoma 2 (Bcl-2) expression as well as through inhibiting the increase of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) DNA binding [90](**Figure 4**). Resveratrol (20 μM) protected PC12 cells against neurotoxicity caused by $A\beta_{25-35}$ by provoking autophagy which was proved dependent on the tyrosyl tRNA synthetase-Poly [ADP-ribose] polymerase 1 (TyrRS-PARP1) and SIRT1 pathway (TyrRS-PARP1-SIRT1 pathway) [93]. A very low concentration of resveratrol (0.2 mg/L) significantly attenuated Aβ neuropathology and AD-type deterioration of spatial memory function in Tg2576 mice compared to control [94]. In a transgenic mouse model of AD (Tg19959), dietary supplementation with resveratrol (300 mg/kg) decreased amyloid plaque formation [95]. In order to translate the animal doses into ones that are relevant in humans, a scaling factor of 0.08 is used to calculate the human equivalent dose (http://www.fda.gov/cber/gdlns/dose.htm). For resveratrol this is about 24 mg/kg or 1.68 gms per day for a 70 kg individual [95]. Resveratrol is also known to act as a phytoestrogen (This mode of action of resveratrol is discussed in more detail in the phytoestrogen section later in this article).

Effects of resveratrol on metabolic and signalling pathways

Resveratrol has been suggested to regulate cellular processes by activating key metabolic proteins such as SIRT1, 5' adenosine monophosphate-activated protein

kinase (AMPK) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG-1α) [96-98]. Sirtuins and nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases are described as novel therapeutic agents against neurodegenerative disease pathogenesis [99]. In fact, the essential neuroprotective effect of resveratrol is based on the action of SIRT1 and AMPK and on the phosphorylation/acetylation status of PGC-1α that activates consequently the mitochondrial biogenesis leading to the improvement of the mitochondrial activity [100](**Figure 4**).

In a study using Aβ-treated hNSCs, the neuroprotective effect of (10 μM) resveratrol was demonstrated by the activation of AMPK-dependent pathway by rescuing the expression levels of inhibitory kappa B kinase (IKK) and by restoring iNOS and COX-2 levels [101]. In the inducible p25 transgenic mouse model of tauopathy and AD, resveratrol-mediated (5 μg/μl) SIRT1 activation reduced learning impairment and hippocampal neurodegeneration [102]. The JAK/ERK/STAT signalling pathway (janus kinases, extracellular signal-regulated kinases and signal transducers and activators of transcription) is implicated in cell survival, proliferation and differentiation while the dysregulation of the JAK/STAT pathway in neurodegenerative disorders contributes to neuronal loss, cognitive impairment and brain damage [103]. Treatment with 20 mg/kg resveratrol exerted neuroprotective effect via JAK/ERK/STAT signalling pathway in a rat model of ischemia-reperfusion injury. In detail, resveratrol attenuated the increase in phosphorylation of JAK, ERK, STAT and JNK caused by ischemia-reperfusion [103] (**Figure 4)**.

Clinical evidence

Only eight clinical trials and four ongoing trials on resveratrol aim to evaluate the effects of this compound on cognitive function in humans [104] (**Table 2**). Efficacy results of resveratrol are based only on one clinical trial in MCI and one in AD patients.

Young and old healthy subjects

Witte and group conducted a study to evaluate the effect of resveratrol (200 mg/day) supplementation in a formulation with quercetin 320 mg in 23 healthy overweight older individuals versus placebo during 26 weeks. They showed that resveratrol supplementation is able to improve memory performances, glucose metabolism and to increase hippocampal functional connectivity in older adults for the maintenance of brain health during aging [105]. No effect on cognitive function was detected in young healthy people [106, 107].

Patients with cognitive decline and MCI

Lee and collaborators examined the effects of grape consumption (which contains resveratrol) on cognitive function and metabolism in the brain of patients with mild cognitive decline and demonstrated a protective effect of the grape extract against pathologic metabolic decline [108]. In a more recent 14 weeks study carried out on 80 post-menopausal women aged 45-85 years, it was proven that a regular consumption of a modest dose of resveratrol (75 mg twice daily) is able to enhance cerebrovascular function and cognition and to reduce their heightened risk of accelerated cognitive decline [109].

Clinical studies are underway to explore the beneficial effect of resveratrol on MCI. In the ongoing trials, one four-month resveratrol supplementation study in phase 1 aims at evaluating the efficacy and safety of bioactive dietary preparation (BDPP) at low, moderate and high doses in treating mild cognitive impairment on 48 MCI subjects (55-85 years) [110]. The purpose of another study in phase 4 is to test the effect of six month administration of resveratrol on brain functions in MCI subjects (50-80 years) (National Institutes of Health, Clinical Trials.gov). Effects of Dietary Interventions on the Brain in Mild Cognitive Impairment (MCI) [111]. In a randomized, double-blind interventional study, resveratrol intake (200 mg/day; 26 weeks) reduced glycated hemoglobin A1c, preserved hippocampus volume, and improved hippocampus resting-state functional connectivity (RSFC) in 40 well-characterized patients with MCI (21 females; 50-80 years) [112].

Patients with moderate AD and dementia

Class II evidence provided by the study of Turner and colleagues on patients with AD showed that resveratrol (500 mg/day to 2 g/day, 52 weeks) is well-tolerated, safe, and was able to decrease $A\beta_{40}$ levels in Cerebrospinal Fluid (CSF) and plasma but had no significant effects on cognitive score [113]. Recently, a Phase II study was conducted investigating the effect of resveratrol (500 mg) in individuals with mild to moderate AD confirming its tolerability and safety as well as its modulation of AD biomarker pathways [114]. Currently, an ongoing study in phase 3 tests the effect of resveratrol supplementation (215 mg/day for 52 weeks) on cognitive and global functioning in Mild-to-moderate AD patients (50-90 years) [115]. A second ongoing study in phase 3 aims at evaluating the effect of resveratrol combined with glucose

and malate in slowing down the progression of AD after 12 months in Mild-to-moderate AD (50-90 year old patients) [116].

On the basis of the results from the very few clinical trials in MCI and AD, no conclusion about efficacy of resveratrol on cognition can be drawn at current time, but promising trials are underway.

NEUROSTEROIDS

Neurosteroids offer therapeutic opportunities through their pleiotropic effects on the nervous system. They are a sub-category of steroids synthetized *de novo* from cholesterol in the central nervous system independently of supply by peripheral steroidogenic glands [117, 118] and accumulate within the brain in neurons or glial cells [119, 120]. Neurosteroids derive from cholesterol which is translocated from the outside to the inside of mitochondria via the translocator protein (TSPO). In the inner mitochondrial membrane, cholesterol is then converted by the cytochrome cholesterol side-chain cleavage enzyme (P450scc) to pregnenolone, the precursor of all the neurosteroids [121]. Particularly pregnenolone and allopregnanolone, play an essential role in aging, in performance of memory and physiopathology. Indeed, age-related drop of neurosteroids give rise to neuronal degeneration and dysfunction in human and animal models owing to the loss of neurosteroid neuroregenerative and protective effects [122, 123]. Allopregnanolone is used in several studies as a plasmatic biomarker for AD because of its reduced level in the plasma of demented patients [122]. It is known to be a regenerative agent in the brain [124]. Several neurosteroids were quantified and were found decreased in post mortem brains of aged non-demented controls and aged AD patients. The transgenic mice model of AD

(APPswe+PSEN1Δ9 mice) presents a decreased ability to form allopregnanolone in the hippocampus [125].

Allopregnanolone

Mechanisms of action based on preclinical evidence

Direct effects of allopregnanolone on mitochondria

In control and APP/Aβ SH-SY5Y cells, allopregnanolone improved basal respiration and glycolysis as well as increased the bioenergetic activity and ATP production [126]. In APP-transfected cells, a pre-treatment with allopregnanolone exerted neuroprotective effect against oxidative stress-induced cell death via the amelioration of the cellular and mitochondrial energy, the reduction of ROS and the improvement of mitochondrial respiration [126]. Thereby, it exerted its beneficial effect by improving the mitochondrial redox environment such as MnSOD activity and mitochondrial ROS levels [127]. Moreover, allopregnanolone increased ATP levels and respiration in mouse primary cortical neurons [127]. In addition, *in vitro,* allopregnanolone potentiated mitochondrial respiration in both adult neural stem cells (NSCs), neurons and mixed glia [128]. *In vivo,* allopregnanolone was able to restore the ovarectomized (OVX)-induced decrease in mitochondrial respiration in both nonTg and 3xTgAD mice [128]. Moreover, allopregnanolone also improved activity of bioenergetic enzymes such as pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGDH) [128].

Effects of allopregnanolone on Aβ toxicity related to damage of mitochondria

In a recent study, allopregnanolone was shown to exert an increased neuroprotective activity against $A\beta_{42}$-induced cell death in neural stem cells [129] **(Figure 5)**. *In vivo*, the natural neurosteroid allopregnanolone appears to be a promising therapeutic tool for the development of neurogenic and/or neuroprotective strategies but diverse points have to be taken into account including the dosing regimen, the treatment regimen, bioavailability, solubility, route of administration and sex differences. Acute single administration of allopregnanolone promoted neurogenesis in the subgranular zone (SGZ) in the triple transgenic mouse model of AD (3xTgAD) at 3 months of age prior to the appearance of AD [71]. Allopregnanolone reversed memory and learning deficits in these mice. Chen and collaborators showed that allopregnanolone administration (once/week for 6 months) decreased Aβ generation and promoted survival of newly generated neurons in the brain of 3xTgAD [130]. They demonstrated also that allopregnanolone increased oligodendrocyte myelin markers and ameliorated cholesterol homeostasis and clearance from the brain by increasing the expression of PXR and Liver-X-receptor (LXR). Singh and colleagues reported that allopregnanolone is able to restore cognitive performance in the pre-plaque phase of AD as well as memory and learning in aging 3xTgAD mice [131]. All these studies demonstrated the neuroprotective effects of allopregnanolone against the Aβ toxicity in 3xTgAD mice and also its capacity to stimulate rodent and human neural progenitor cell proliferation and to compensate the cell loss [130, 132]. Continuous infusions of allopregnenanolone were anti-regenerative while intermittent administration promoted repair and renewal in a mouse model of AD [124]. The mode of action of allopregnanolone is summarized in Figure 5.

Clinical evidence

Currently, there is only one Phase I ongoing clinical trial testing the safety and the tolerability of allopregnanolone in patients with mild cognitive impairment and early AD [133] (**Table 3**). The primary aim of this phase 1 study is to evaluate the maximally tolerated dose after intravenous injection of allopregnanolone (2, 4 or 6 mg, once per week for 12 weeks). Thus, no clinical evidence is currently available.

The natural neurosteroid allopregnanolone appears to be a promising therapeutic tool with specific regard to its neurogenic properties besides its mitochondria-directed effects. However, more trials are urgently needed to prove that.

PHYTOESTROGENS

Phytoestrogens are the most bioactive molecules of soy and present structural similarity to the 17β-estradiol, which is the major circulating estrogen. Specific estrogen receptors (ER) have been shown to localize in mitochondria in the frontal lobe and the hippocampus of men and women suggesting a role of estrogen in controlling cognitive functions and memory processes via energy supply [134]. Estrogen plays a neuroprotective role during the aging process, especially through its beneficial impact upon mitochondrial metabolism by increasing glucose utilization by cells as well as by enhancing ETC activity, by stabilizing the MMP and by preventing ROS production and calcium-induced excitotoxicity [135]. Moreover, females live longer than males and this can be attributed in part to the antioxidant effect of estrogen and the up-regulation of life longevity-related genes [19, 136]. The phytoestrogens are characterized by their ability to bind to estrogen receptors ERs α and ERs β and to

exert similar responses to endogenous estrogens [137]. Isoflavones are a subclass of phytoestrogens and are contained abundantly in soy and soybeans. Soy presents estrogenic effects attributed to genistein, daidzein and glycitein. The most potent isoflavone is genistein, while daidzein and glycitein present an affinity to ER, 100-500 times lower than genistein [138]. ERs are localized in the important brain areas, including the prefrontal cortex and the hippocampus that are also known to be vulnerable to age-related decline [139-142].

Mechanisms of action based on preclinical evidence

Effects of phytoestrogens on Aβ and tau toxicity and cognitive performance related to damage of mitochondria

One of the most important phytoestrogens is resveratrol, an estrogen receptor agonist/antagonist. In particular, resveratrol acts on estrogen receptor β, whose activation is known to play a major role in cognitive processes, leading to the improvement of cognitive impairment in AD [143]. The soybean is a source of vegetable proteins and contains also other functional ingredients including phytoestrogens. The isoflavones genistein and daidzein have been shown to present protective effects against tau protein phosphorylation [144]. Animal models confirmed the neuroprotective effects of phytoestrogens. Genistein, the most active molecule of soy isoflavones, improved Aβ-induced cell death and reduced neuronal loss in rats [145-147]. In OVX female rats, dietary supplementation of soy phytoestrogens (0.4 g/kg or 1.6 g/kg) or 17β-estradiol (0.15 g/kg) for 12 weeks has been shown to increase expression of brain neurotrophic factors such as BDNF and tropomyosin receptor kinase B (TrkB) and, as a result, to ameliorate hippocampal learning [148]. In normal

and OVX transgenic AD mice, a selection of phytoestrogens in combination, composed of genistein, daidzein and equol, has been shown to improve spatial working memory performance, to reduce mortality as well as to delay neuropathological changes associated with AD [149].

Effects of phytoestrogens on oxidative stress

The phytoestrogens are also known for their neuroprotective antioxidant effects in neuronal cell models after exposure to neurotoxic substances [150-152]. Phytoestrogens are able to reduce ROS within a cell and to protect from cellular damage [153]. In aged mice, soybean supplementation has been shown to prevent cognitive deficits by decreasing free radical generation, by enhancing scavenging of free radicals and by increasing GSH levels [154]. Compared to estrogen itself, less evidence is provided for direct effects of phytoestrogens on mitochondria, but anti-oxidant properties were demonstrated [155-158]. The molecular effects of phytoestrogens are summarized in figure 6.

Clinical evidence

Until today, no clinical trials in MCI and AD were performed. Thus, currently there is no clinical evidence.

Healthy and post-menopausal women

Among five randomised controlled trials, four recent studies reported the beneficial effect of phytoestrogens on cognitive function in healthy individuals **(Table 4)**. In a study with young healthy adults of both sexes, a high soya or a low soya diet for 10 weeks had beneficial effect and showed significant improvements in short-term and

long-term memory as well as in mental flexibility [159]. In another cross-over design study, the administration of 4 capsules/day containing soya isoflavones during 6 weeks improved spatial working memory of men aged 30-80 years [160]. In post-menopausal women, 6 months of treatment duration with isoflavones supplementation provoked better learning, mental flexibility, increased attention as well as caused improvement in mood and lower depressive symptoms [161]. In a small mixed gender sample of older adults, soy supplementation ameliorated the visuospatial memory and the construction of verbal fluency and speeded dexterity [162]. All these studies demonstrated that phytoestrogens may affect human cognition. However, no clinical trials of phytoestrogens are known for the prevention or the treatment of AD.

Inconclusive findings have been also reported from randomized controlled trials and observational studies in humans. In fact, these discrepant data could have several possible reasons. Investigation in European cohorts showed that a low dietary consumption of phytoestrogens had a significant effect on the improvement of the quality of life but no effect on cognition [163].

Mediating variables in the characteristics of the study population such as gender, age, ethnicity and menopausal status appears to play an important role [164]. Phytoestrogens have been shown to have time-limited positive effects on cognition. These findings are in line with estrogen treatment which also exerts an initially positive short-term effect on cognition and a reversion after a long-term continuous use in aged women [164].

Globally, the effects of phytoestrogens can be dependent upon a window of opportunity for treatment, and can affect males differentially than females due to the diminished presence of ER-mediated protective mechanisms and the tyrosine kinase activity with a potentially deleterious outcome of the supplements [165]. An age-

dependent effect of phytoestrogen supplements is suggested in post-menopausal women [165]. In the males, the findings are equivocal and sparse, and more investigations are needed to determine whether the effects will be deleterious or beneficial [165].

CONCLUSION

In this article, the efficacy of standardized *Ginkgo biloba extract*, resveratrol, allopregnanolone and phytoestrogens in combatting age-related cognitive decline has been reviewed. The mechanisms of action as well as preclinical and clinical evidence for each of those entities have been discussed. The four entities share common mechanisms of action but also diverse ones. In terms of the main AD features, Aβ and tau, all four categories were able to reduce the Aβ accumulation but only GBE and phytoestrogens seem to reduce tau hyperphorphorylation. Similarly (and quite predictably due to their phenolic character) all four act as antioxidants either by reducing ROS and oxidative stress (GBE, phytoestrogens, allopregnanolone) or by enhancing the activity of antioxidant enzymes such as SOD and GPx1 (GBE, resveratrol, phytoestrogens) and by reducing lipid peroxidation (GBE) and pro-oxidant enzymes such as Nox4 (resveratrol). GBE, resveratrol and allopregnanolone target mitochondria by enhancing their functions (complexes activity, oxidative phosphorylation, oxygen consumption, respiration, mitochondrial membrane potential, ATP production) while GBE and resveratrol promote in addition mitochondrial biogenesis. This is particularly important since mitochondria play a pivotal role in synaptic plasticity that is reduced in pathological states in the brain. However, there are also some differences in the mechanisms of action of the four discussed

substances and mainly in the pathways through which they exert their beneficial effects. Based on our review of the literature, GBE rescues the Aβ neurotoxicity through the activation of the Akt pathway and through phosphorylation of CREB. Neurotrophic factors such as BDNF are stimulated both by GBE and by phytoestrogens. Resveratrol leads to Aβ clearance, enhancement of mitochondrial biogenesis and metabolism, reduction of inflammation and ROS mainly through the activation of SIRT 1 and AMPK pathways as well as through the deacetylation of PGC-1 α and the modulation of JAK/ERK/STAT pathway. Phytoestrogens act as ER receptor modulators. Resveratrol additionally can act as a phytoestrogen and bind to the ERβ receptor. In terms of *in vitro* assays, it should be taken into account that the extract and the substances should be tested in meaningful, physiologically relevant concentrations and not in irrationally high ones.

Regarding clinical trials, there is a different level of evidence for the four phytochemicals. Standardised GBE, resveratrol, allopregnanolone and phytoestrogens appear in a descending order according to the level of existing clinical evidence. According to the World Federation of Societies of Biological Psychiatry (WFSBP) Guidelines, GBE has been classified in category B and grade 3 in terms of the outcome of existing studies. Therefore there is sufficient and good clinical evidence for the efficacy of GBE. There is increasing and promising clinical evidence for resveratrol but more and of larger sample size studies are definitively needed. Lastly, there are no clinical trials indicating the beneficial effect of allopregnanolone and phytoestrogen in age-related cognitive decline disorders. There is only promising evidence from preclinical data regarding allopregnanolone and phytoestrogen. Notably, the four entities follow the same descending order regarding the existing level of clinical evidence and their mitochondria-improving properties. All in all, effect on

mitochondria goes hand in hand with clinical effect and this highlights one more time the importance of these organelles in the pathogenesis of AD but also in aging in general.

Conflict of interest

With the relevance to this review, there is no direct conflict of interest to declare. AE has received grant/research support from Schwabe and Vifor. She has served as a consultant or on advisory boards for Vifor and Schwabe.

Table 1.Clinical trials on the effects of GBE

Study design	**GBE dose/ preparation**	**Duration**	**Subjects**	**Purpose**	**Main results**	**References**
Patients with memory complaints, SMI and MCI						
R, DB, PC	240 mg once daily of GBE or placebo	56 ± 4 days	(61) Healthy aged patients with subjective memory decline (SMI)	Test the effect of GBE on cognitive functions associated with prefrontal dopamine	GBE caused mild increase in prefrontal dopamine, there were indications for enhanced cognitive flexibility and for ameliorated response	Beck *et al* 2016, [61]

					inhibition results	
R, DB, PC	240mg GBE once daily or placebo	12 weeks	(300) patients 45-65 years old with very mild cognitive impairment (MCI)	Evaluate the effects of GBE on cognition and quality of life in patients with very MCI	GBE improved cognitive ability and quality of life of patients	Grass-Kapanke 2011,[63]
R, PC, DB, MC	240 mg GBE once daily or placebo	24 weeks	(160) patients with MCI	Test the effect of GBE on NPS and cognition in patients with MCI	GBE improved NPS and cognition, the extract was safe and well-tolerated	Gavrilova *et al* 2014, [62]
Patients with dementia						
R, DB, PC	240 mg once daily of GBE	22 weeks	(400) Demented patients with NPS	Test the efficacy of GBE on NPS of dementia	GBE statistically superior to placebo in ameliorating NPS (e.g irritability, apathy, anxiety)	Scripnikov *et al* 2007, **[64]**
Systematic review	240 mg once-daily of GBE	22 weeks	(1628) Demented patients with Behavioural and Psychological	Demonstrate efficacy of GBE in dementia with BPSD	Improvements of quality of life, cognition and BPSD activities of daily living clinical global impression	Von Gunten *et al* 2016, [66] ([12, 166-168])

			Symptoms (BPSD)			
Meta-analysis and systematic review	Different dosages of GBE	Not available	Demented patients	Test the efficacy of GBE in ameliorating symptoms of demented patients	GBE improved cognitive function and activities of everyday life in patients with dementia	Brondino *et al* 2013, [72] ([12, 67-69, 79, 166, 169, 170])
Meta-analysis of randomized placebo controlled trials	GBE 120 mg or 240 mg per day or placebo	22-26 weeks	(2684) demented patients	Evaluate evidence for efficacy of GBE in dementia	Confirmation of efficacy of GBE and good tolerability	Gauthier and Schlaefke 2014, [70] ([12, 69, 166, 167, 169, 171])
Systematic review and meta-analysis of randomized controlled trials	240 mg once daily of GBE	22-26 weeks	(2561) Demented patients	Evaluate the clinical efficacy and adverse effects of GBE in dementia and cognitive decline	GBE was found more effective than placebo in decelerating cognition deficits and in improving daily life activities and NPS in dementia	Tan *et al*, 2015, [65] ([12, 62, 69, 167, 169-174])
Meta-analysis of randomized controlled	240 mg/day	22 or 24 weeks	Old patients aged over 60 years	Effects of GBE on anxiety, dementia and depressi	Improvements in dementia, anxiety and depression	Kasper 2015, [73] ([12, 166-168])

clinical trials				on in the aging		
Meta-analysis of randomized controlled trials	240 mg once daily of GBE	22 or 24 weeks	(1628) demented patients with behavioural and psychological symptoms (BPSD)	Test the effects of GBE on BPSD of demented patients	Significant superiority of GBE to placebo in improving BPSD and therefore caregiver experience	Savaskan *et al*, 2017, [74] ([12, 166-168])
Bi-variate meta-analysis	Different dosages of GBE	Approximately 6 months	Demented patients	Evaluate baseline risk on the treatment effect and assess the efficacy of GBE on cognitive symptoms of dementia	GBE was effective at improving cognitive functions in dementia after treatment of 6 months	Wang et al 2010, [71] ([12, 67, 69, 166, 169, 170])
R, DB, PC, PG, MC	160 mg or 240 mg daily of GBE	24 weeks	(214) Patients with dementia or age-related memory loss	To assess the efficacy of GBE in aged demented patients or with age-related	No beneficial effect of GBE for demented or age-related memory impaired patients	Van Dongen, 2000, [75]

				memory loss		
R, DB, PC, PG	120 mg daily of GBE	6 months	176 mildly to moderately demented patients	Assess the efficacy and safety of GBE for treating dementia in early stages	GBE not beneficial in mild to moderate dementia after 6 months treatment	McCarney *et al*, 2008, [76]
Cochrane analysis of R, DB, PC trials	Different GBE doses ranging from low to high	Different treatment periods	Aging with dementia or cognitive impairment	Assess the efficacy and safety of GBE in dementia and cognitive impairment	GBE displays unreliable and inconsistent evidence in being beneficial for demented people	Birks and Evans 2009, [77]
Patients with AD and vascular dementia						
R, DB, PC	GBE 120 mg, GBE60 mg or placebo	6 months	(90) patients with vascular dementia (VaD)	Evaluate the efficacy and safety of GBE in vascular demented patients	GBE slowed down the cognitive deterioration in vascular demented patients, effect shown in only one of the four neuropsychological tests	Demarin *et al* 2017, [78]
Review of R, PC	120 mg twice daily or 240 mg once	22 or 24 weeks	(1294) Demented patients (AD or	Test the efficacy of GBE in older patients with AD/	Confirmation of efficacy of GBE and good tolerability	Ihl 2013**,** [79] ([12, 166,

	daily of GBE		VaD) with NPS	vascular dementia with NPS		167, 175])
Systematic review and meta-analysis	GBE extract	12-52 weeks	(2372) Patients with AD or vascular or mixed dementia	Evaluate the effects of GBE in AD, vascular and mixed dementia	Superiority of GBE to placebo in improving every-day life activities in mainly AD type of dementia	Weinmann *et al* 2010, [80] ([67-69, 166, 169, 173, 175])
Systematic review and meta-analysis	240mg and 120 mg daily	24 weeks	Patients with MCI or AD	Assess the effectiveness and safety of GBE in treating MCI and AD	There is an indication for the beneficial effect of GBE in MCI and AD but the results were inconsistent	Yang *et al* 2016, [81](AD: [67, 68, 169, 170, 174-176]; MCI: [62])
Systematic review of randomized controlled trials	GBE 240 mg daily	Period ≥16 weeks	Patients with mildly to moderately severe and severe AD	Assess the beneficial effect of GBE in AD	Evidence of beneficial effects of GBE in amelioration cognition, every day activities and psychopathological symptoms but great heterogeneity among the results	Janssen *et al*, 2010, [82] ([67, 69, 166, 169])
Prevention						

R, DB, PC, PG	120 mg of GBE twice daily	5 years	Adults 70 years or older with occasional memory problems	Efficacy of long-term use of GBE for the prevention of AD in aging with memory complaints	GBE did not reduce the incidence of AD compared to placebo	GuidAge study, Vellas *et al*. 2012**,** [83]
R, DB, PC	120 mg twice daily of GBE	Every 6 months from 2000 to 2008	(3069) healthy oldpeople or with MCI aged 72 to 96 years	Test whether GBE delays or prevents global or domain-specific cognitive impairment in aging	GBE did not prevent cognitive decline in aging.	Snitz *et al*, 2009, [84]
R, DB, PC	GBE 120 mg twice per day	5 years	(3000) healthy aging over 80 years old	Assess the ability of GBE in prevention of dementia in normal aging or those with MCI	GBE does not prevent dementia	GEM study, Deksoky *et al* 2006, [87]
Systematic review and	240 mg GBE daily	Not available	Non demented patients aged 70	Evaluate the efficacy of GBE for the preventio	GBE in not able to prevent the development of dementia	Charemboon and Jaisin 2015

meta-analysis			years or older	n of dementia in non-demented adults		[86] ([83, 85],)

SMI, subjective memory impairment, MCI, mild cognitive impairments; AD, alzheimer's disease;VaD, vascular dementia; R, randomized; DB, double blind; PC, placebo controlled; MC, multicentre; PG, parallel group, BPSD, behavioural psychological symptoms, VCI: vascular cognitive impairment, the number of patients involved in the trials is indicated in parentheses

Table 2.Clinical trials on the effects of resveratrol. Ongoing trial is marked in grey.

Study design	**Resveratrol dose / preparation**	**Duration**	**Subjects**	**Purpose**	**Main results**	**References**
Young and aged healthy individuals						
R, DB, PC, CO	*Trans*-resveratrol from BiotiviaBioceuticals 250 mg or 500 mg	21 days	(24) 18-25 years healthy	Ability to increase cerebral blood flow and modulate mental function	Increase in cerebral flow, no effect in cognitive function	Kennedy *et al* 2010 [106]
R, DB, PC, CO	*Trans*-resveratrol 250 mg/ day or *trans*-resveratrol 250 mg/ day with 20 mg piperine	21 days	(23) 19-34 years healthy	Effect of piperine on the efficacy and bioavailability of resveratrol	Piperine enhances the effect of resveratrol on cerebral blood flow but no effect on bioavaility and cognition	Wightman *et al* 2014 [107]
Study in older adults	200 mg of resveratrol per day	26 weeks	(46) Healthy overweight subjects aged 50-75 years	Test whether resveratrol would improve memory performan	Resveratrol ameliorates memory performance in combination with	Witte *et al* 2014 [105]

				ce in older adults	improved glucose metabolism and increased hippocampal functional connectivity in healthy overweight old people	
Patients with cognitive decline and post-menopausal women						
R, DB, PC	72 g of active grape formulation	6 months	(10) Adults with mild cognitive decline with mean age of 72.2 years	Evaluate the effects of grapes on regional cerebral metabolism	Grapes could possess a protective effect against early pathologic metabolic decline	Lee *et al* 2017, [108]
R, PC, Intervention trial	75 mg twice daily of trans-resveratrol	14 weeks	(80) Post-menopausal women between 45 and 85 years old	Test the effects of resveratrol on cognition, mood and cerebrovascular function in post-menopausal women	Resveratrol was well tolerated and able to improve cognition which was related to the improvement of cerebrovascular function. Mood was improved but not significantly.	Evans *et al* 2017, [109]
Patients with MCI						

R, DB, interventional study	200 mg of resveratrol per day	26 weeks	(40) Old patients with MCI	Assess if resveratrol improves long-term glucose control, resting-state functional connectivity of the hippocampus, and memory function in petients with MCI	Resveratrol supplementation t decreased glycated hemoglobin A1c, preserves hippocampus volume and improves hippocampus RSFC in patients with MCI	Koebe *et al* 2017, [112]
R, DB Phase 1	Bioactive dietary polyphenol preparation (BDPP) at low, moderate, high dose	4 months	(48) 55-85 years MCI	Safety and efficacy in treating mild cognitive impairment	-	**NCT02502253 [110]**
R, DB, PC Phase 4	Resveratrol or omega-3 supplementation or caloric restriction	6 months	(330) 50-80 years MCI	Effects on brain function	-	**NCT01219244 [111]**
Patients with mild to moderate AD						
R, DB, PC, MC Phase 2	Resveratrol 500 mg/ day with excalation by 500 mg increments ending with 2 g/ day	52 weeks	(119) Over 49 years Mild-to moderate AD	Assess efficacy and safety	No effect on cognitive score, decrease of CSF and plasma	Turner *et al* 2015, [113]

					Aβ40 levels	
R, DB, PC Phase 2	Resveratrol 500 mg daily (orally) with a dose elevation by 500 mg every 13 weeks until a final dose of 1000 mg twice daily was reached for the final 13 weeks.	52 weeks	(119) Adults older than 49 years old with a diagnosis of mild to moderate dementia due to AD	Evaluation of safety and tolerability of resveratrol and its effects on AD biomarkers and also on clinical outcomes	Resveratrol was well tolerated and safe, it was detected in the cerebrospinal fluid (nM), it changed the AD biomarkers paths, it modified the CNS immune response and it maintained the BBB integrity but more research is needed	**Sawda *et al* 2017, [114]**
R, DB, PC Phase 3	Longevinex brand resveratrol supplement (resveratrol 250 mg/ day)	52 weeks	(50) 50-90 years Mild to moderate AD on standard therapy	Effects on cognitive and global functioning	-	NCT00743743 [115]
R, DB, PC Phase 3	Resveratrol with malate and glucose	12 months	(27) 50-90 years Mild to moderate AD	Ability to slow the progression of AD	-	NCT00678431 [116]

MCI, mild cognitive impairments; AD, alzheimer's disease; R, randomized; DB, double blind; PC, placebo controlled; CO, cross over; MC, multicenter; CSF, cerebrospimal fluid, the number of patients involved in the trials is indicated in parentheses

Table 3. Ongoing clinical trial on the effects of allopregnanolone in MCI and mild AD.

Study design	**Allopregnanolone dose/ preparation**	**Duration**	**Subjects**	**Purpose**	**Main results**	**References**
R, DB, Parallel assignment Phase 1	Allopregnanolone 2 or 4 or 6 mg intravenous injection once per week Or Placebo intravenous injection once per week	12 weeks	(8) For each dose group 55 years and older both genders MCI or Mild AD (6) randomized to AP (2) randomized to placebo	Determine the maximally tolerated dose, safety and tolerability, Pharmacokinetic profile and effects on cognitive function	Not available	**NCT02221622 [133]**

The number of patients involved in the trials is indicated in parentheses

Table 4.Clinical trials on the effects of phytoestrogens.

Study design	Phytoestrogens dose/ preparation	Duration	Subjects	Purpose	Main results	References
Healthy individuals and post-menopausal women						
Randomized control trial	High soya (100 mg total isoflavones/ day) or a low soya (0.5 mg total isoflavones/ day) diet	10 weeks	(27) Young adults healthy (15 men, 12 women)	Effects on memory, attention and frontal lobe function	Improvements in short-term and long-terms memory and mental flexibility	File *et al* 2015, [159]
DB, CO, PC	4 capsules/ day containing soya isoflavones (116 mg isoflavone equivalents/ day: 68 mg daidzein, 12 mg genistein, 36 mg glycitin) or placebo	6 weeks	(34) Men aged 30-80 years	Effects on cognitive function	Improvements of spatial working memory but no effect on auditory and episodic memory and executive function and visual-spatial processing	Throp *et al* 2009, [160]
18 R,DB, CO, PC	Isoflavones supplementation 60 mg/day or placebo	6 months	(78) Post-menopausal woman (Mean age 49.5 years)	Effects of soy isoflavones on mood and cognitive function in postmenopausal women	Improvements mental flexibility, attention, mood and lower depressive symptoms	Casini *et al* 2006, [161]
	100 mg/day soy		Older non-	Examination of safety, feasibility	Improvements of visual-	Gleason *et al*

R, DB, PC	isoflavones (glycoside weight) or matching placebo tablets	6 months	demented men and women (age 62-89 years)	and cognitive efficacy of soy isoflavone administration	spatial memory and construction, verbal fluency and speeded dexterity	2009, [162]
R, DB, PC	20 g of soy protein containing 160 mg of total isoflavones	12 weeks	(93) Healthy post-menopausal women (mean age 56 years)	Effect of high-dose isoflavones on cognition, quality of life, lipoproteins and androgen status in post-menopausal women	Significant improvement in the quality of life versus placebo. No significant effects in cognition. The testosterone and HDL levels were significantly lower at the end of the study.	Basaria *et al* 2009, [163]

The number of patients involved in the trials is indicated in parentheses

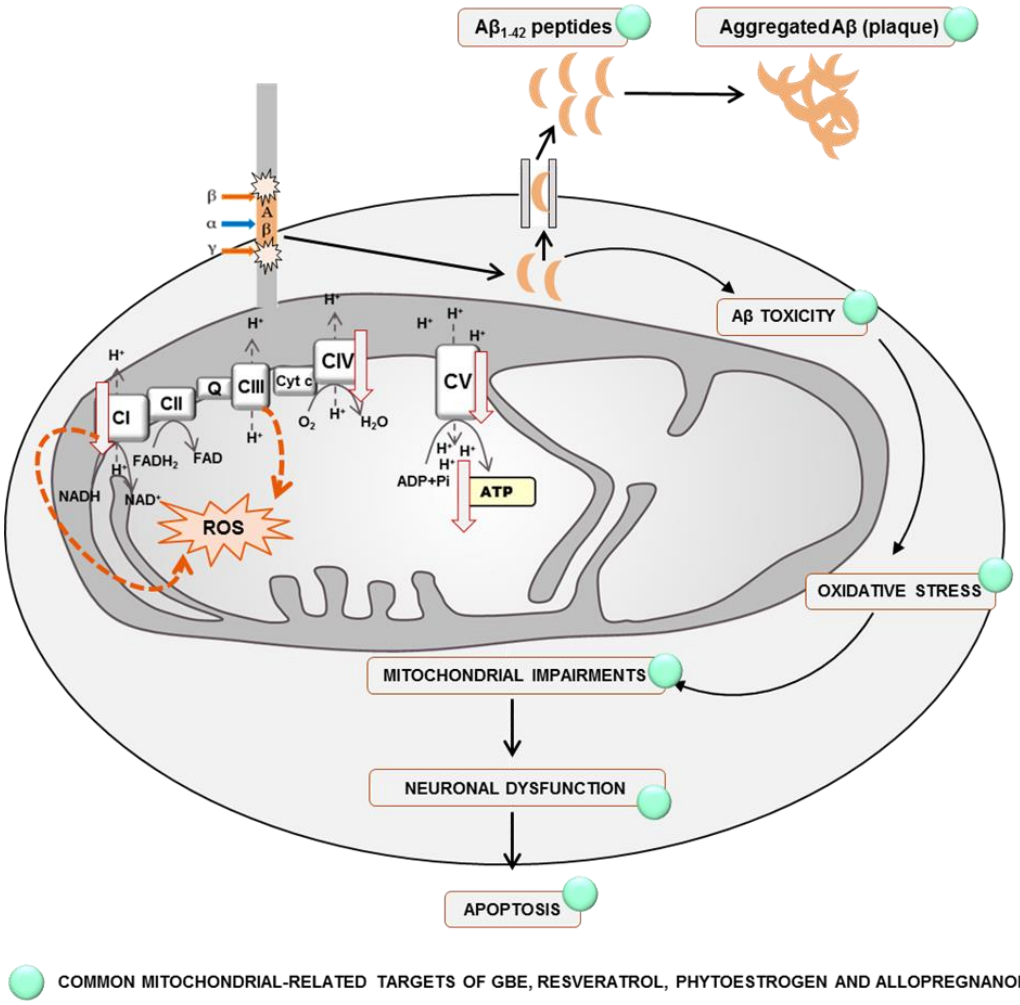

Figure 1. Common mitochondrial-related targets of natural substances in the neuroprotection. In AD, the precursor of amyloid protein APP is cleaved sequentially by β- and γ-secretases leading to the production of Aβ peptides, their aggregation and the formation of extracellular plaques. Different Aβ species exist but the A$\beta_{1\text{-}42}$ is one of the most abundant and due to its hydrophobic and fibrillogenic nature is the one that is mainly deposited in the brain. AD is associated with electron transport chain (ETC) impairments leading to decreased ATP levels, basal respiration, with a decrease of antioxidant defenses, and an increase of ROS production by complex I and complex III (orange dashed arrows). Globally, *Gingko Biloba*, resveratrol and phytoestrogens

have been shown to protect against cell death in AD through common mechanism of action by reducing abnormal aggregation of Aβ, amyloid beta (Aβ) toxicity, oxidative stress, mitochondrial impairments leading to neuronal dysfunction and apoptosis. *Gingko Biloba*, resveratrol and phytoestrogens are suggested to exert beneficial effect in AD affected neurons but their specific mechanisms of mitochondrial interaction are not fully described yet. ↓: AD-related decrease. The green circle indicates the common mitochondria-related targets of GBE, resveratrol, phytoestrogen and allopregnanolone.

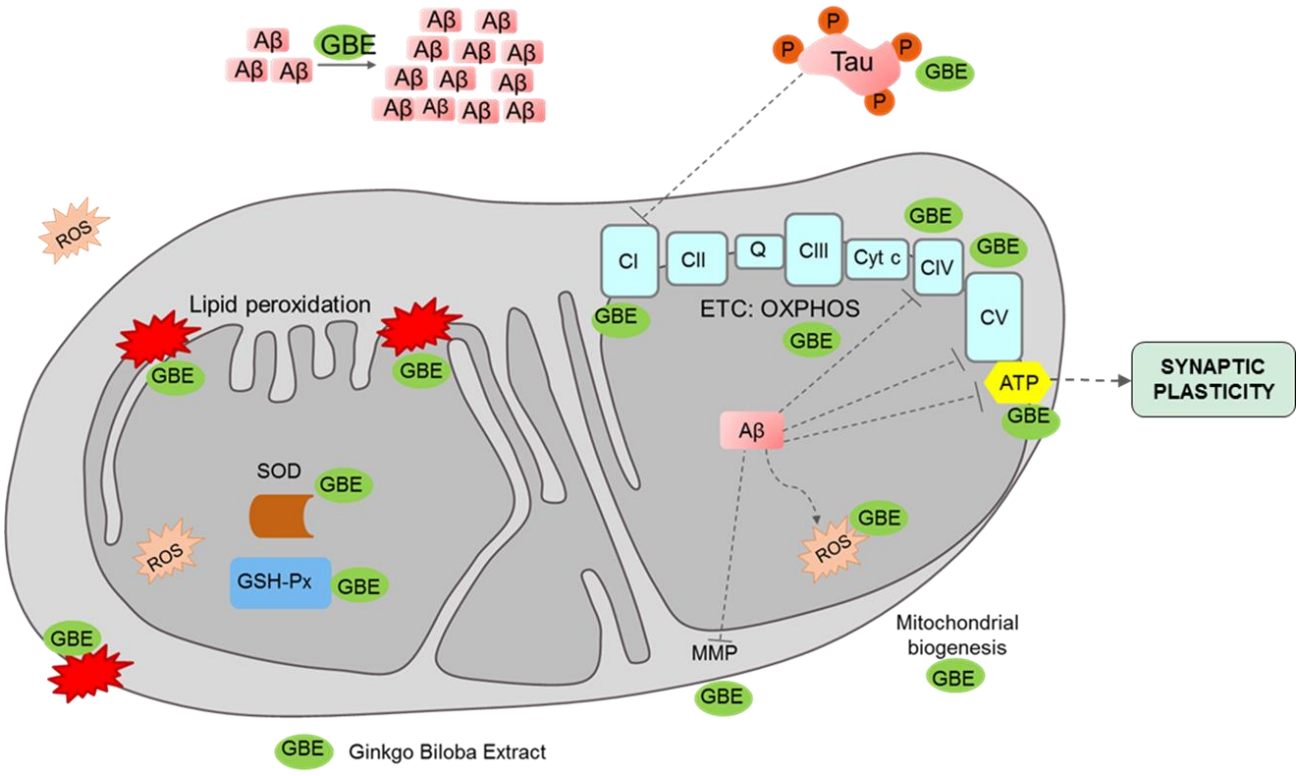

Figure 2. The effects of Aβ, hyperphosphorylated tau and Standardized *GINKGO BILOBA EXTRACT* (GBE) on mitochondrial function in AD. It has been shown that mitochondrial dysfunction is a key feature in AD and plays a pivotal role on the onset of the disease. While defining the chronologically first hallmark of the disease can be

puzzling, there is evidence about mitochondrial dysfunction being the first hallmark at the early stages of AD with Aβ occurring as a result. Aβ has been shown to cause a decline in OXPHOS, taking place at the ETC, which leads to defective complexes IV and V and decreased ATP production. Faulty OXPHOS function results in production of ROS which, when in excess, cannot be counter-balanced by the antioxidant enzymes like GSH-Px and SOD. ROS can cause membrane lipid peroxidation and instable MMP. Hyperphosphorylated tau inhibits complex I activity. However, GBE has been proven to reduce Aβ aggregation and tau hyperphosphorylation, to enhance OXPHOS, complexes activity and ATP levels as well as to restore MMP. ROS and consequently lipid peroxidation are reduced due to GBE while the extract has the ability to enhance SOD and GSH-Px activity and also induce mitochondrial biogenesis. ↓: represents increase, : represents inhibition.

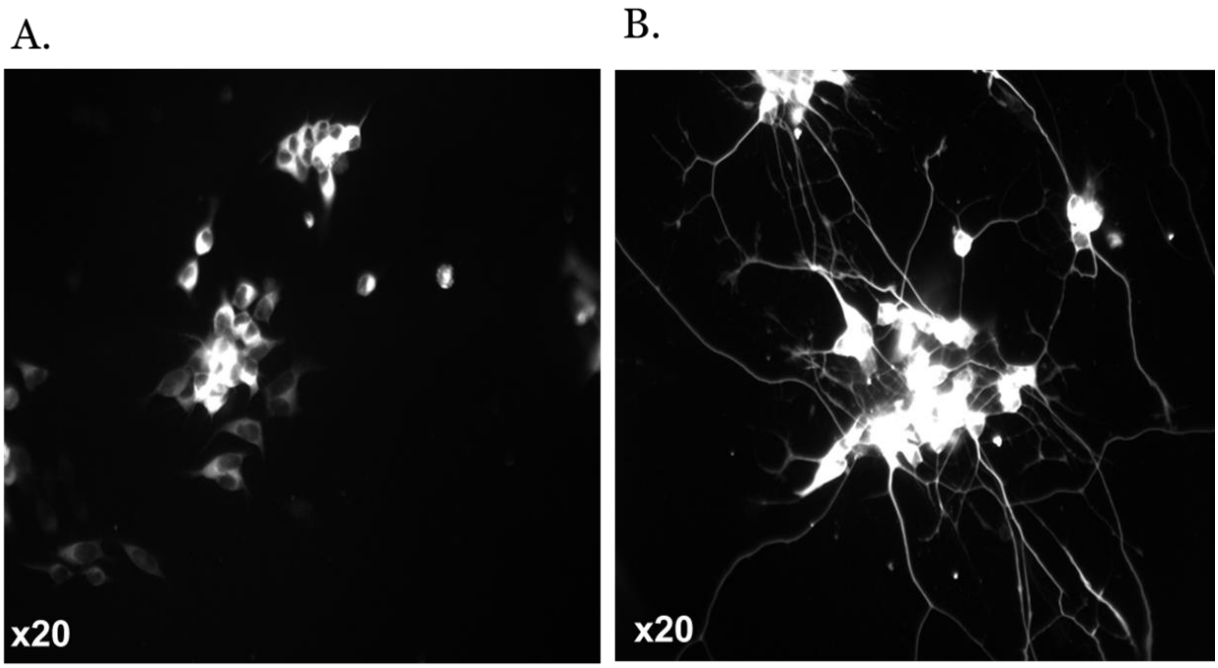

Figure 3: Standardized *GINKGO BILOBA EXTRACT* (GBE) LI 1370 (Vifor SA, Switzerland) (100 ug/ml) increased neurite outgrowth of SH-SY5Y neuroblastoma cells after 3 days of treatment in 3D cell culture.

Pictures were taken using a cell imaging multi-mode reader Cytation3 (Biotek, X20 in black and white) after immunostaining (IMS, βIII –tubuline/ Alexa488). Compared to the untreated SH-SY5Y cells (CTRL, **A**), 100 ug/ml of GBE (**B**) was efficient in increasing the formation of neurites.

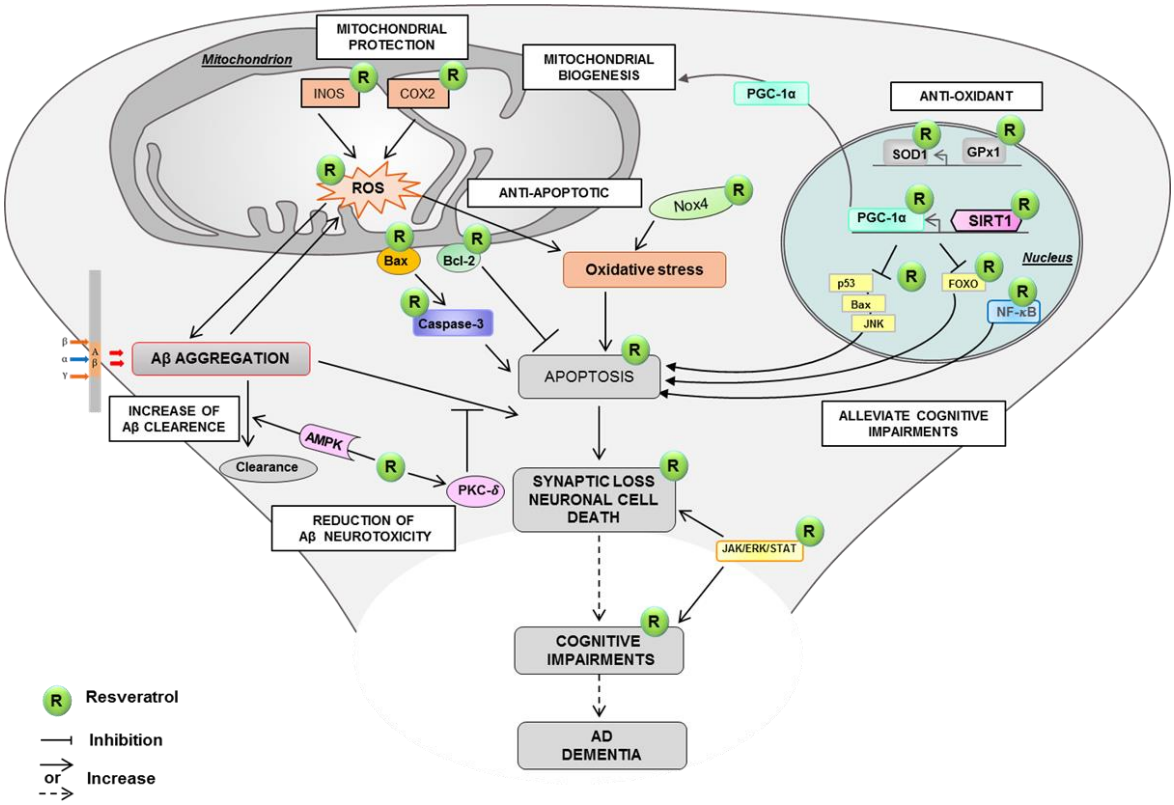

Figure 4: Neuroprotective effects of resveratrol in AD. The precursor of amyloid protein APP is cleaved sequentially by β- and γ-secretases leading to the production of Aβ and their aggregation. Resveratrol increases the clearance of Aβ peptides through the activation of AMPK. Resveratrol plays an important role in the neuroprotective properties as it reduces Aβ neurotoxicity by phosphorylating PKC-δ. Damaged mitochondria generate ROS which are implicated in apoptosis. iNOS and COX-2 also enhance the production of ROS. Resveratrol exerts antioxidant properties and attenuates oxidative damage by decreasing iNOS and COX-2 levels. Resveratrol also protects mitochondria by increasing the expression of ROS-inactivating enzymes GPx1 as well as SOD1 and by reducing the expression of the ROS-producing enzyme Nox4. Resveratrol influences also the Aβ-induced apoptotic signalling pathway by inhibiting the expression of caspace-3, Bax, FOXO, p53, by blocking the activation of

JNK, by restoring the decrease of Bcl-2 expression as well as by inhibiting the increase of NF-κB DNA binding. Mitochondrial biogenesis is induced by resveratrol through SIRT1 activation and deacetylation of PGC-1α. Resveratrol was also able to protect hippocampal neurons by alleviating cognitive impairment, reducing neuronal loss via modulating the janus kinases, extracellular signal-regulated kinases and signal transducers as well as activators of transcription (JAK/ERK/STAT) signalling pathway.

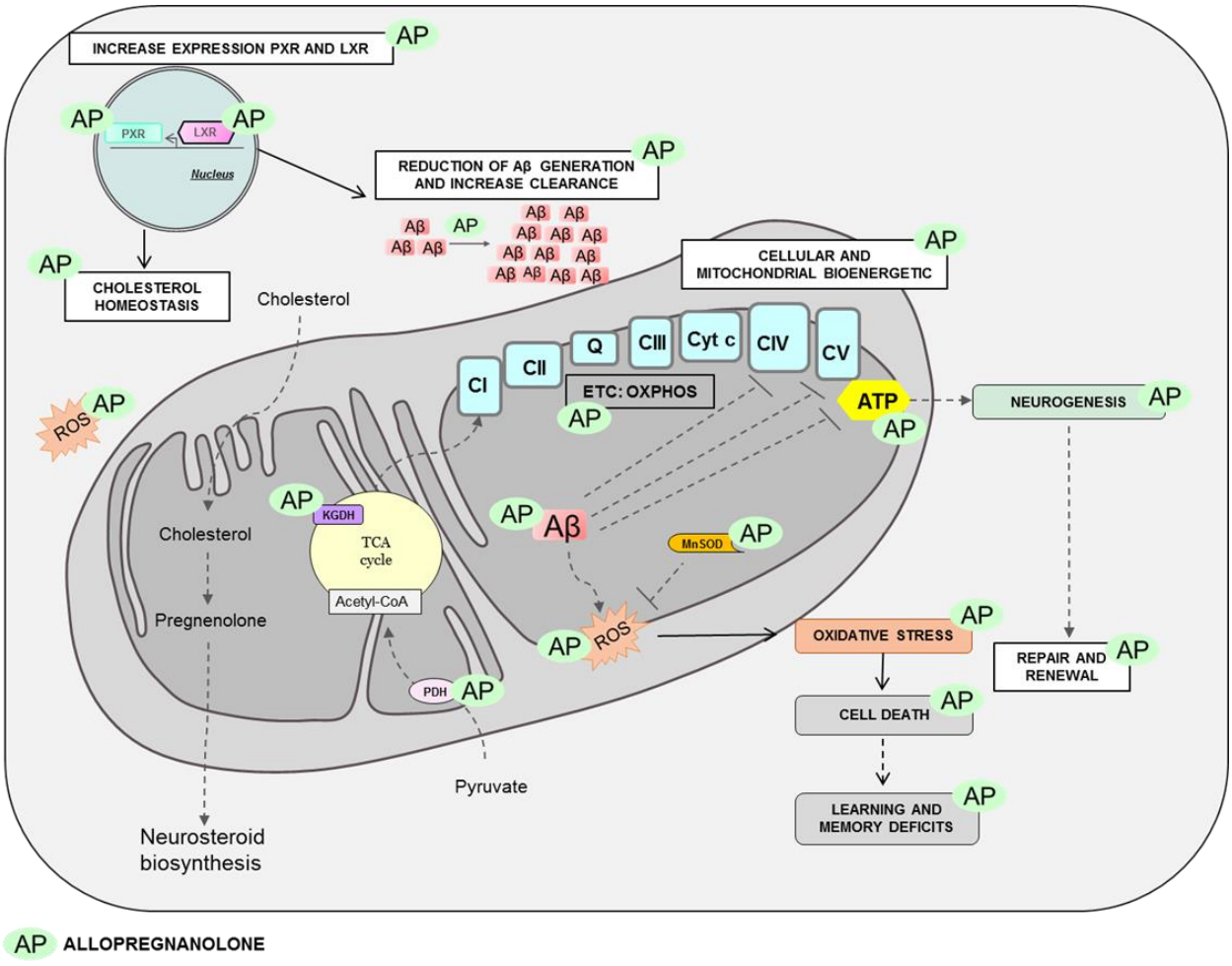

Figure 5. Neuroprotective effects of ALLOPREGNANOLONE (AP) in AD. AP has been proved to reduce Aβ aggregation-induced cell death. It exerts neuroprotective effect against oxidative stress-induced cell death via the improvement of the cellular and mitochondrial energy by enhancing the OXPHOS and ATP levels. AP ameliorates the mitochondrial redox environment by decreasing ROS and by improving the activity of the enzyme MnSOD. AP has also beneficial effects on bioenergetic enzymes such as PDH and αKGDH implicated in the TCA cycle. AP ameliorates cholesterol homeostasis and clearance for the biosynthesis of neurosteroids by raising the expression of PXR and LXR. AP promotes repairment and renewal of neurons leading to restored cognitive performances in AD.

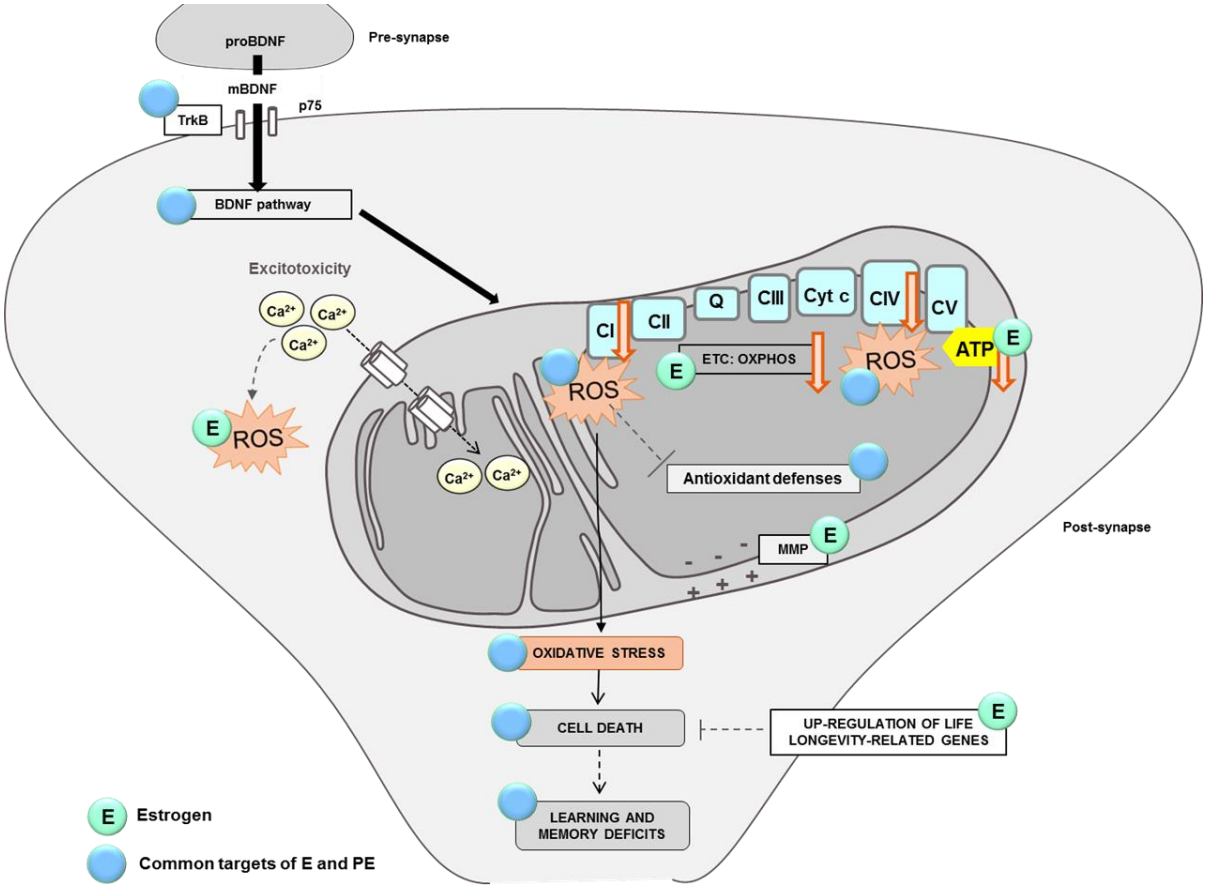

Figure 6: Modulation of mitochondrial function by *ESTROGEN* and *PHYTOESTROGEN*. Less evidence is provided for direct effects of phytoestrogen on mitochondria compared to estrogen, but anti-oxidant properties were demonstrated.

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