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The influence of highly-purified olive polyphenols and special diets on cognition and brain mitochondrial function during the physiological aging process in mice

Dissertation

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Contents

List of abbreviations

AA ascorbic acid

ACh acetylcholine

AD Alzheimer's Disease

ADP adenosine diphosphate

AMPK adenosine monophosphate-activated protein kinase

ARD's age-related diseases

ATP adenosine triphosphate

BDNF brain-derived nerotrophic factor

b.w. body weight

Cat catalase

cAMP cyclic adenosine monophosphate

CD's cardiovascular diseases

CREB cAMP response element binding protein

CS citrate synthase

DBC's dissociated brain cells

DNA deoxyribonucleic acid

Drp1 dynamin-related protein 1

EVOO extra virgin olive oil

fis1 fission 1

FOXO3 forkhead box O3

GPx-1 glutathione peroxidase 1

IMM inner mitochondrial membrane

IU international units

H_2O_2 hydrogen peroxide

MCI mild cognitive impairment

MD mitochondrial dysfunction

MedDiet mediterranean diet

Mfn1 mitofusin 1

Mfn2 mitofusin 2

MMP mitochondrial membrane potential

mtDNA mitochondrial DNA

mTOR mammalian target of rapamycin

NA noradrenaline

NMDA *N*-methyl-D-aspartate

NMRI Naval Medical Research Institute

Nrf1/2 nuclear respiratory factor 1/2

OMM outer mitochondrial membrane

Opa1 optic atrophy 1

OPP olive polyphenols

PARL presenilins-associated rhomboid-like protein

PGC1-α peroxisome proliferator-activated receptor gamma coactivator 1-α

PINK1 PTEN-induced kinase 1

PPARγ peroxisome proliferator-activated receptor γ

ppm parts per million

Prx peroxiredoxin

ROS reactive oxygen species

SOD1/2 superoxide dismutase 1/2

Tfam mitochondrial transcription factor A

Scientific issue

How a person feeds himself has a decisive influence on his development from birth. Beginning with birth, the human being begins to age. Since humans are individuals with a relatively high life expectancy, rodents are a more suitable model to study the effects of different forms of nutrition on the physiological brain aging process. In this work, the question was addressed how the physiological aging process takes place at the cognitive and molecular biological level in mice and whether different diets (MedDiet, HF diets, antioxidant-reduced diets) may have an influence on this multifactorial process, which can lead to several chronic and neurological diseases, with focus on brain mitochondrial function.

1. Zusammenfassung

In einer Gesellschaft, die durch eine immer besser werdende medizinische Versorgung zunehmend älter wird, nehmen altersbedingte Begleiterscheinungen wie demenzartige Erkrankungen (z.B. Morbus Alzheimer), Krebs, kardiovaskuläre Erkrankungen und Erkrankungen, die durch eine lange Zeit der Fehlernährung (z.B. Diabetes Mellitus Typ II) entstehen, stetig zu [1–3]. Die Alzheimer-Krankheit, eine multifaktorielle, chronische Erkrankung, stellt mit 60-80 % eine der häufigsten Ursachen für Demenzen dar, und ist hierbei eine besondere Herausforderung und Kostenursache für unser Gesundheitssystem, da die Lebenserwartung dieser Patienten nach Diagnosestellung zwischen 4 und 8 Jahren liegt. In Einzelfällen können Menschen mit der Diagnose AD noch bis zu 20 Jahre weiterleben [4]. Für die aktuell ca. 40 Millionen Menschen, welche von AD betroffen sind, wird prognostiziert, dass diese sich alle 20 Jahre verdoppeln werden, womit im Jahre 2050 bereits mit 115 Millionen Patienten weltweit zu rechnen ist [5].

Die bisherigen Therapieoptionen bei AD sind Acetylcholinesterase-Inhibitoren, NMDA-Rezeptor Antagonisten und Ginko-Biloba Extrakt EGb 761® [6,7]. Alle diese zugelassenen Arzneimittel können den Verlauf der Krankheit allerdings nur verlangsamen und sind nicht in der Lage, die Progression der Erkrankung zu stoppen. Auf den Hinblick, dass eine kurative Therapie derzeit nicht möglich ist, gelangt die frühzeitige Prävention der AD immer stärker in den Fokus der Forschung [8]. Hierbei spielen das Erreichen eines hohen Lebensalters mit einem

gleichzeitigen Erhalt der Lebensqualität eine große Rolle. Somit werden in der Präventionsforschung zunehmend Wege gesucht, welche den physiologischen Alterungsprozess positiv beeinflussen können.

Um die Entstehung von neurologischen Erkrankungen wie AD zu verstehen, muss als erstes der physiologische Alterungsprozess auf molekularer Ebene betrachtet werden, denn er selbst ist der wichtigste Risikofaktor für die Entstehung dieser irreversiblen Erkrankung. Der physiologische Alterungsprozess ist nicht nur durch eine moderate Abnahme der kognitiven Fähigkeiten, sonder auch durch die Veränderungen verschiedener zellulärer Prozesse gekennzeichnet [9,10]. Der Alterungsprozess auf molekularer Ebene zeigt hierbei eine mitochondriale Dysfunktion, welche durch den Rückgang von zellulären ATP-Spiegeln, einer reduzierten mitochondrialen Respiration und einer Abnahme der mitochondrialen Biogenese gekennzeichnet ist [11]. Hierbei spielt die Zunahme der Bildung von reaktiven Sauerstoffspezies (ROS) eine große Rolle bei der Entstehung der MD [12]. Substanzen, die einen positiven Einfluss auf die mitochondriale Funktion zeigen, sind somit ebenfalls potenzielle Stoffe gegen altersbedingte, neurodegenerative Erkrankungen [13].

Im ersten Teil dieser Arbeit sollte der physiologische Alterungsprozess auf molekularer Ebene untersucht werden. Hierzu wurde eine Kohorte von 3 Wochen alten, weiblichen NMRI Mäusen über einen Zeitraum von 24 Monaten im Abstand von 6 Monaten auf verschiedene Parameter untersucht, die einen Aufschluss über die Veränderungen während des physiologischen Alterungsvorgangs geben sollten. Die umfassende Charakterisierung dieses Mausstammes ergab, dass bereits im Alter von 12 Monaten erste messbare Defizite im kognitiven Bereich auftraten, welche im Y-Maze-Test, ein Marker für das räumliche Gedächtnis und die Motorik, beobachtet werden konnten. Das Langzeitgedächtnis scheint allerdings erst im Alter von 18 Monaten signifikante Defizite im Vergleich zu 3 Monate alten Kontrollen zu erleiden. Die Energiespiegel im Gehirn waren ebenfalls bis zu einem Alter von 18 Monaten stabil, während die mitochondriale Respiration bereits im Alter von 12 Monaten erste Defizite der Atmungskettenkomplexe I und IV aufwies. Das mitochondriale Membranpotential (MMP), die Triebkraft für die ATP Synthese, zeigte erst im Alter von 24 Monaten einen signifikanten Rückgang im Vergleich zu jungen Kontrollmäusen. Auf molekularer Ebene der mRNA-Expression zeigten sich bei einigen Genen eine signifikante Hochregulation im Alter von 6 Monaten (Cytochrome-c-Oxidase (CIV), cAMP response element-binding protein (CREB-1), AMP-aktivierte Proteinkinase (AMPK), mitochondrialer Transkriptionsfaktor A (TFAM),

brain-derived neurotrophic factor (BDNF), was u.a. für eine Zunahme der mitochochondrialen Biogenese im jungen Erwachsenenalter der Tiere sprechen könnte. Einen Rückgang der meisten untersuchten Gene der Mitogenese, physischen Aktivität und der antioxidativen Abwehr zeigte sich im Alter von 18-24 Monaten [14].

Mit diesen Ergebnissen wurde im weiteren Verlauf dieser Arbeit der Effekt von einer wässrigen Mixtur aus hochreinen Olivenpolyphenolen und zwei Secoridiod-Derivaten als Reinsubstanzen im Rahmen eines BMBF geförderten Projektes auf den physiologischen Alterungsprozess in NMRI Mäusen untersucht. Der wässrige Extrakt aus Olivenpolyphenolen zeigte hierbei nach einer 6 Monate langen Fütterung positive Effekte auf die kognitive Leistung in 18 Monate alten NMRI Mäusen und die basalen ATP-Level im Gehirn, während keine Effekte durch die Fütterung auf die Genexpressionsmuster im Gehirn zu beobachten waren. Die Erhöhung der basalen ATP-Level konnte darüber hinaus ebenfalls in einer neuronalen Zelllinie (SH-SY5Y Mock) bestätigt werden [15]. Diese Beobachtungen lassen darauf schließen, dass hochreine Olivenpolyphenole sich positiv auf den Energiemetabolismus im Gehirn auszuwirken scheinen, was in einer zweiten Studie unserer Gruppe im Rahmen des BMBF Projektes in einer Langzeitfütterung von Oleocanthal und Ligstrosid in gealterten NMRI Mäusen bestätigt werden konnte [16].

Im nächsten Schritt der Arbeit wurde der Effekt von verschiedenen Standarddiäten in der Nagerhaltung auf den Energiemetabolismus im Gehirn und Kognition während des physiologischen Alterungsprozesses untersucht. Hierzu wurden eine antioxidantien-reduzierte und zwei verschiedene Hochfett-Diäten bezüglich der Kognition und mitochondrialer Einflüsse in Mäusen untersucht. Hierbei zeigte sich, dass eine von Vitamin C freie und gleichzeitig mit Vitamin E reduzierte Diät moderate Effekte im Y-Maze-Test nach 6 Monaten Fütterung in gealterten NMRI Mäusen hatte und zu einem Anstieg der basalen ATP-Spiegel in dissoziierten Gehirnzellen (DBC's) der Mäuse führte. Die 70 % Hochfett-Diät in C57Bl/6J Mäusen zeigte ebenfalls einen signifikanten Anstieg der ATP-Spiegel im Gehirn. Eine anschließende Umstellung auf das Kontrollfutter mit 10 % Fett zeigte einen Rückgang auf das Kontrollniveau. Zusätzlich wurde ein Rückgang der mRNA Expression von Komplex IV, Citratsynthase und Glutathion-Peroxidase im Gehirn beobachtet. Weiterhin wurde eine Diät in C57Bl/6J Mäusen getestet, welche auf Basis von Fruktose und Schweineschmalz basiert und zur Erzeugung einer nichtalkoholischen Fettleber dient. Diese Diät zeigte keine Effekte auf die mitochondriale Funktion nach einer 7-wöchigen Fütterungsperiode.

Zusammenfassend zeigte sich im Rahmen dieser Arbeit, dass der physiologische Alterungsprozess in Verbindung mit diversen Veränderungen auf molekularer Ebene einhergeht, welche durch die longitudinale Studie über 2 Jahren in NMRI Mäusen umfassend charakterisiert werden konnte. Es zeigte sich weiterhin, dass die Wahl des Standardfutters zu Veränderungen der Bioenergetik im Gehirn führen kann und dieses bei der Auswahl für Studien, die den Energiestoffwechsel im Gehirn im Alter untersuchen sollen, mit Bedacht ausgewählt werden muss. Die Supplementation von hochreinen Olivenpolyphenolen konnte hierbei den Alterungsprozess im NMRI Mausmodell positiv beeinflussen und stellt somit ein potenzielles Nutrazeutikum für den gesunden Alterungsprozess und der Prävention von neurodegenerativen Erkrankungen dar.

1.1. Summary

In a society that is getting older and older due to ever-improving medical care, age-related diseases such as dementia (e.g. Alzheimer’s disease), cancer, cardiovascular diseases and diseases caused by long periods of malnutrition (diabetes mellitus type 2) are steadily increasing [1–3]. Alzheimer's disease (AD), a multifactorial chronic disease, is one of the most common causes of dementia, accounting for 60-80 % of all cases. It is a particular challenge and cost source for our health care system, as the life expectancy of these patients after diagnosis is between 4 and 8 years. In individual cases, people diagnosed with AD can live for up to 20 years [4]. For the approximately 40 million people currently affected by AD, it is predicted that this number will double every 20 years, which means that 115 million AD patients worldwide are expected in 2050 [5].

The current treatment options for AD are acetylcholinesterase inhibitors, NMDA receptor antagonists and ginkgo biloba extract EGb 761® [6,7]. However, all these approved drugs can only slow down the progression of the disease and are not able to cure it. Due to the fact that a curative therapy is currently not possible, research is increasingly focusing on the early prevention of AD [8]. In this context, reaching an advanced age with a simultaneous preservation of a high quality of life plays a major role. Thus, research is increasingly focussing on preventive aspects to positively influence the physiological aging process.

In order to understand the development of neurological diseases such as AD, the first step is to look at the physiological aging process at the molecular level, since it is itself one of the main causes of the development of this irreversible disease. The physiological aging process is characterized not only by a moderate decrease in cognitive abilities, but also by changes in various cellular processes [9,10]. The aging process at the molecular level includes a mitochondrial dysfunction (MD), which is characterized by a decrease in cellular ATP levels, reduced mitochondrial respiration and a decrease in mitochondrial biogenesis [11]. Here, the increase in the formation of ROS plays a major role in the development of MD [12]. Substances that have a positive influence on mitochondrial function are thus also potential substances against age-related, neurodegenerative diseases [13].

The first part of this work was to investigate the physiological brain aging process at the molecular level. For this purpose, a cohort of 3-week-old female NMRI mice was examined over a period of 24 months at 6-month intervals for various parameters concerning mitochondrial function and cognitive performance.The characterization of this mouse strain showed that already at the age of 12 months the first measurable cognitive deficits occur, which could be observed in the Y-maze test, a marker for spatial memory and motor skills. However, long-term memory seems to decrease at the age of 18 months compared to 3 months old control animals. Energy levels in the brain were also stable up to the age of 18 months, while mitochondrial respiration showed initial deficits in respiratory chain complexes I and IV starting at the age of 12 months. The mitochondrial membrane potential, as the driving force for ATP synthesis, did not show a significant decrease in comparison to young control animals until the age of 24 months. At the molecular level of mRNA expression, a significant upregulation of genes was shown at the age of 6 months (CIV, Creb1, β-AMPK, TFAM, BDNF), which could indicate, among other things, an increase in mitochondrial biogenesis in the young adulthood of the animals. A decrease in most of the investigated genes of mitogenesis, physical activity and antioxidant defense was found at the age of 18-24 months [14].

With these results, the effect of an aqueous mixture of highly pure olive polyphenols and two secoridiod derivatives as pure substances were investigated in a BMBF funded project on the physiological aging process in NMRI mice. The aqueous extract of olive polyphenols showed positive effects on cognitive performance in 18-month-old NMRI mice and basal ATP levels in the brain after 6 months of feeding, while no effects of feeding on gene expression patterns in the brain were observed. Furthermore, the increase in basal ATP levels was also shown in a

neuronal cell model (SH-SY5Y mock) [15]. These observations suggest that high-purity olivepolyphenols seem to have a positive effect on the energy metabolism in the brain, which could be confirmed in the second study of the BMBF project in a long-term feeding study of oleocanthal and ligstroside in aged NMRI mice [5].

Following, the effects of different standard- and special diets in rodent husbandry were investigated on cognition and mitochondrial function during aging. It was shown that a vitamin C free and vitamin E reduced diet showed moderate effects in the Y-maze test after 6 months of feeding in aged NMRI mice with a concurrent increase of basal ATP levels in DBC‘s. The 70% high-fat diet in C57Bl/6J mice also showed a significant increase in brain ATP levels. A switch to the 10 % fat diet showed an adjustment to the control ATP levels. Additionally, a decrease in mRNA expression of complex IV, cs and GPx-1 in the brain was observed. Furthermore, a diet in C57Bl/6J mice based on fructose and lard, which is usually used to produce a non-alcoholic fatty liver in mice, was examined with regard to mitochondrial brain function. This diet showed no effects on mitochondrial function after a 7-week feeding period.

In conclusion, this work showed that the physiological aging process is associated with various changes at the molecular level, which could be characterized comprehensively at the molecular level by the longitudinal study over 24 months in NMRI mice. It was further shown that the choice of the standard diet can lead to changes in bioenergetics in the brain and must be carefully selected for studies investigating the energy metabolism in the brain during the aging process. The supplementation of highly purified olive polyphenols was able to influence the aging process in the NMRI mouse model in a positive way and thus represents a potential nutraceutical for the healthy aging process and the prevention of neurodegenerative diseases.

2. Introduction

2.1. An aging society

We live in a society that is increasingly subject to demographic change, which has dramatic consequences for our healthcare systems [17]. As a result of longer average lifespans, lower birth rates and better medical care into old age, we are getting older and older. At the same time, age-related diseases (ARD's) like cancer, AD and cardiovascular diseases (CD's) are constantly increasing [18,19]. According to the Federal Statistical Office, 1/3 of the german population will be over 65 years old in 2030 [20]. AD, which has a prevalence of 30 % at the age of 85, as well as other forms of dementias associated with the physiological aging process of the brain, will therefore dramatically become more prevalent and pose major challenges in the treatment of this progressive disease [21]. By now, specialists in the field of gerontology postulate that aging itself must be considered as a disease and so it should be possible to treate or even prevent it [22,23]. This hypothesis therefore makes it essential to understand the brain aging process on the molecular level in order to be able to interact with preventive or, if possible, curative therapeutic options.

2.2. What is aging?

Until now, at least 300 theories of aging have been proposed and none of them can be regarded as the true theory. With the advancement of science and cellular understanding, more and more theories are added, which still can't complete the complex puzzle of aging [24]. However, there are some very meaningful and often discussed theories that can give at least some clues as to what happens during the multifactorial aging process. A very popular and often discussed theorie of aging is the „mitochondrial free radical theorie of aging" which was postulated in 1956 by Denman Harman which states, that cellular aging is a direct consequence of free radicals (ROS) that are generated mainly by the mitochondrial metabolism which in consequence results in a progressive accumulation of cellular damages [25,26]. Mitochondria, small ATP producing cell organelles, produce reactive oxygen species (ROS) during the oxidative phosphorylation process (OXPHOS) that are on the one hand known to damage cellular structures, nucleic acids and proteins and on the other hand are important signaling

molecules in many cellular processes, such as immune response, inflammation, synaptic plasticity as well as learning and cognition processes [27–29]. In an intact cellular system, there is a very efficient elimination system keeping ROS at a physiological low level. Especially complex I and III of the respiratory chain form large amounts of ROS with superoxide anions, hydroxyl radicals, and hydrogen peroxide being the predominant forms of ROS [30]. The antioxidant molecules include superoxide dismutases (SOD's), which can reduce superoxide anions to H_20_2. In the further course glutathione peroxidase (Gpx), catalase (Cat) and peroxireductase (Prx) which can eliminate hydrogen peroxide to H_20 [31].

However, as the aging process progresses, the antioxidant defense system becomes more and more susceptible to errors, leading to increasing ROS levels and cellular damage and accumulation of toxic ROS [32]. These observations suggest that mitochondria seem to be a promising starting point for research on healthy aging. Nevertheless, there are further cellular changes during the (mitochondrial-) aging process. In the following chapters, the structure and function of mitochondria concernsing the physiological aging process will be described in more detail.

2.3. Mitochondria

Mitochondria are cell organelles, which occur in almost all eukaryotic cells with a diameter of about 1 µM, have their own genetic material (mtDNA) and developed from bacteria about 2000 million years ago [33]. Mitochondria were first described in the literature around 1900. From that time on, they were intensively studied and became more and more the focus of molecular biology. The role of mitochondria as a central point for the urea and citrate cycle was finally postulated around 1930. Although mitochondria were not a sustainable priority at the beginning of cell research due to the lack of molecular biological imaging methods, they have since become a popular research focus. In 1980 at the latest, with the decoding of the mitochondrial genome, new interest in mitochondria was achieved among researchers, because without the central organelles, which were first described in 1886 as the "power stations of the cell" and which represent the main site for the synthesis of ATP in every cell, life as we know would not be possible [34,35]. Mitochondria consist of an inner and an outer membrane, whereby the inner mitochondrial membrane (cristae) has multiple invaginations. Both membranes, consisting of a phospholipid bilayer, can be used to describe four different localities in mitochondria: The

inner and outer mitochondrial membrane, the intermembrane space and the matrix. The outer mitochondrial membrane surrounds the mitochondrion and serves the mass transfer of molecules and ions with the cytosol via integral protein complexes. This process is essential for the function, biogenesis and communication between the mitochondrion and the different cell compartments. The inner mitochondrial membrane is the main location of cellular ATP synthesis [36]. The mitochondrial respiratory chain, which generates a proton gradient through a chain of oxidation and reduction reactions and the resulting electron flow, is located in the inner mitochondrial membrane. Respiratory chain complexes include NADH dehydrogenase (complex I), cytochrome c-reductase (complex III), succinate dehydrogenase (complex II), cytochrome c-oxidase (complex IV) and ATP synthase (complex V). The protein complexes CI, CIII and CIV ensure the proton flow along the cristae and the resulting proton gradient is used by complex V for the synthesis of ATP [37]. According to the literature, oxidative phosphorylation in mitochondrial cristae provides between 38 and 30 molecules of ATP for the cell, whereby the stoichiometry and complexity of this metabolic reaction tend to indicate 30 molecules of ATP per molecule of glucose [38].

2.4. Aging on the molecular level

Aging can be considered as a multifactorial loss of essential metabolic processes, which takes place in every tissue. In particular, the brain is susceptible to age-related deficits, resulting in a progressive loss of memory, motor coordination and sensory perception [39,40]. During the physiological aging process, the brain loses mass which is accompanied by the loss of the gray and white matter and an enlargement of ventricles which can be monitored by magnetic resonance imaging (MRI) [41]. However, the environment and lifestyle can influence the rate of the decline of the brain mass and it has been reported that exercise training can increase hippocampal volume by 2 % [42]. Furthermore, caloric restriction and intermittent fasting can slow down brain atrophy in mice and monkey [43,44], whereas a high body mass index (BMI) was negatively associated with hippocampal volume [45]. Additionally, there are more characteristic hallmarks of the brain aging process on the molecular level, including (a) mitochondrial dysfunction (MD), (b) impaired mitochondrial DNA repair, (c) increase of oxidative damage, (d) increased inflammation, (e) deficits in calcium homeostasis, (f) impairments during the cell to cell connection, (g) deficits in the molecular waste disposal

and (f) reduced mitochondrial biogenesis and mass [46]. Especially mitochondrial dysfunction has been reported as an early hallmark of the brain aging process since a reduced mitochondrial membrane potential, a reduced function of the respiratory chain complexes, reduced cellular ATP-level as well as impaired mitophagy are common age-related mitochondrial changes during aging [15,47,48]. As mentioned before, mitochondria are the main source for the formation of ROS, resulting in damage to proteins, lipid and DNA [49]. However, the aging process at the molecular level involves further changes that need to be discussed.

Mitophagy, a selective degradation of damaged mitochondria by autophagy which is mainly regulated by the mitochondrial kinase PINK1 and the cytosolic E3 ligase Parkin was described by Dr. Lemasters in 2005 [50] and is closely connected to the physiological aging process [51]. As long as mitochondria are healthy and functional, the level of PINK1 is kept relatively low by being imported to the inner mitochondrial membrane (IMM). Afterwards, PINK1 interacts with the mitochondrial protease presenilin-associated rhomboid-like protein (PARL) resulting in degradation of PINK1 [52]. In case of mitochondrial damage, the MMP decreases and PINK1 accumulates at the outer mitochondrial membrane (OMM) which finally induces mitophagy [53]. It has been shown that brains of young (3 months) mt-Keima transgenic mice show a high level of mitophagy while a decrease could be observed in aged animals (21 months) [54]. Mitochondria, as highly dynamic cell organelles, are able to replicate themselves and thus increase their mass. This process, which is called mitochondrial biogenesis, is also reported to be reduced during the physiological aging process [55,56]. While most mitochondrial proteins are encoded in the nucleus and subsequently enter the mitochondrial membrane via specific transporter proteins, mitochondria encode their own DNA (mtDNA) for 13 subunits of the oxidative phosphorylation system (OXPHOS), 2 rRNAs and 22 tRNAs of the mitochondrial translation machinery [57]. However, during aging several important signaling pathways may be affected and one of them is called the PGC1-α signaling cascade. PGC1-α, a nuclear encoded protein of 90 kDa and a master regulator of mitochondrial biogenesis, can be activated by environmental changes like cold or a energy depletion by stimulating the sympathic nerve activity and the release of noradrenalin (NA). NA binds to specific β-adrenergic receptors which results in an increase of the intracellular second messenger cAMP which finally induces the expression of PGC1-α. Besides the expression of various subunits of the respiratory chain complexes, PGC1-α induces the up-regulation of NRF1 and NRF2, which subsequently leads to an increase of mTFAM. mTFAM finally enters into the mitochondrion and induces mitochondrial replication of mtDNA [58]. Besides the activation of PGC1-α via the increase of cAMP, this important transcription factor can also be activated via other proteins. AMPK and

SIRT1 are two other major cellular sensors to induce mitochondrial biogenesis through the AMPK-SIRT1-PGC1-α axis. AMPK can be activated by the decrease or increase of cellular ATP levels and the resulting AMP/ATP ratio. An increase in this ratio leads to AMPK activation, which phosphorylates and activates PGC1-α. Additionally, SIRT1 as a NAD^+ dependent deacetylase can induce mitochondrial biogenesis as a result of the increased NAD+/NADH ratio and a deactetylation of PGC1-α [59,60]. Furthermore, phosphorylated cAMP response element-binding protein (CREB) can induce gene expression of PGC1-α and accordingly the amplification of mtDNA [61].

The multifactorial aging process is associated to an unbalanced mitochondrial quality control which includes an impaired fusion/fission activity [62]. In order to eliminate the resulting damage to proteins and DNA and to break down defective mitochondria, various proteins are necessary. Mitochondrial fission is mainly induced via Fis1 and Drp1 and is essential for the proliferation of mitochondria. The opposite process, called mitochondrial fusion, is an important process for the communication between individual mitochondria and is controlled by Mfn1/2 and OPA1. A balance of fission and fusion processes is of great importance for normal cell function and the physiological aging process is associated with an imbalance between these two processes. Obviously, increased fusion appears to be associated with prolonged lifespan in *Drosophila Melanogaster* and *Caenorhabditis elegans* [63–65].

If we now ask ourselves why the brain in particular is very susceptible to age-related damage (lipid oxidations, DNA damage, etc.), it must be noted that the brain is dependent on the supply of glucose and uses a high level of oxidative phosphorylation to synthesize sufficient energy in form of ATP. Since neurons are post-mitotic cells, damage can easily accumulate and lead to irreparable damage, resulting in several changes on the molecular level and cognitive deficits [66].

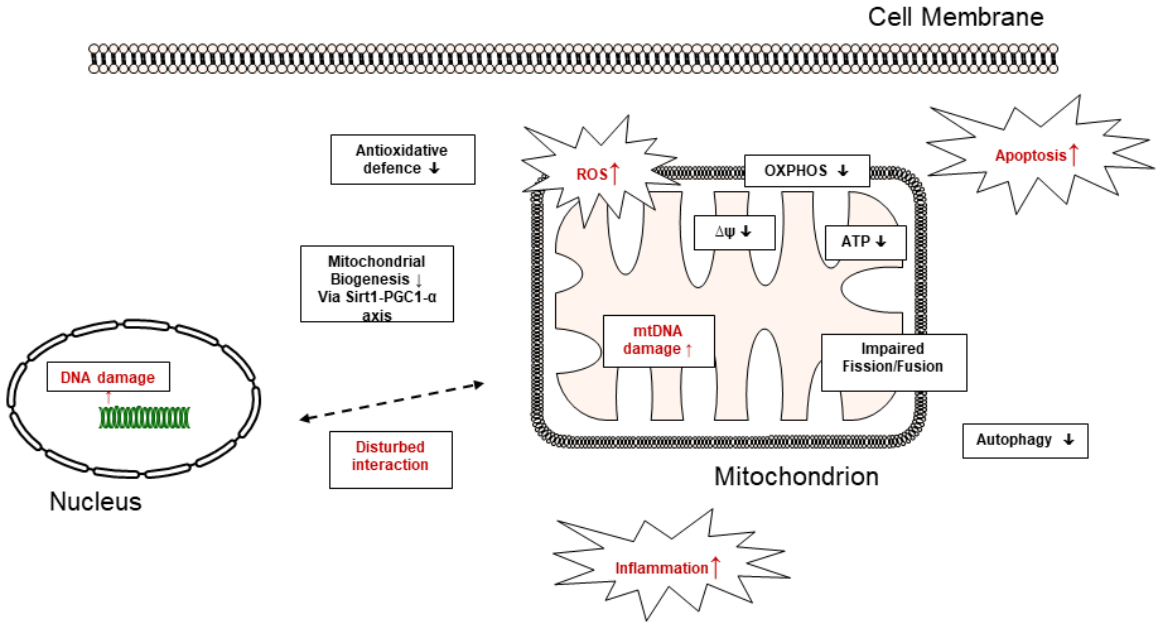

Fig 1. Multifactorial cellular changes during the physiological aging process with several cellular changes resulting in mitochondrial dysfuntion (MD) as an early event during the physiological aging process. However, the mitochondrial pathway of aging involves many different changes in the cellular domain that affect the overall cell-to-cell interaction which finally leads to increased apoptosis and cell death.

2.5. Nutrition and the (physiological) -aging process: Focus on the Mediterranean diet (MedDiet) and the main phenolic compounds in olives

A well balanced diet is regarded to promote and maintain physiological and mental health [67,68]. In this context, the question directly arises as to what is meant by a balanced diet. The German Nutrition society recommends five portions of fruit and vegetables a day, carbohydrates should preferably consist of whole grains and dairy products and should also be consumed daily. In addition, 1-2 portions of fatty sea fish should be consumed to cover the need for omega-3 fatty acids and iodine, while processed meat should be limited to a maximum of 600 g per week. In addition, high-quality oils and fats (rapeseed oil, olive oil) should be preferred [69]. In particular, especially the herbal components of a diet seem to have positive effects on human health [70,71]. While primary plant constituents such as proteins, fats and carbohydrates are involved in cellular energy metabolism, secondary plant components serve primarily to protect plants against predators and are suitable for the intensive colouring of fruits and vegetables. Furthermore, they show antifungal, antibacterical and antiviral activity, [72] which makes them interesting as potentially health-promoting substances [73–75]. The secondary plant ingredients include flavonoids, terpenes, carotenoids and phytoestrogens and especially the mediterranean diet (MedDiet), a predominantly plant-based diet with a high consumption of high quality olive oil, has caused great interest in the prevention of several age-related and chronic diseases like AD, cardiovascular disease and cancer [76–78].

One characteristic hallmark of the MedDiet is the high intake of extra virgine olive oil (EVOO) (25-50 ml/day) of high quality [79]. EVOO contains plenty of bioactive substances, particularly olive polyphenols (OPP) and secoiridoid derivatives which seem to be responsible for the health-promoting effects due to their anti-oxidative and anti-inflammatory properties [80–82]. Additionally, a high intake of OPP is associated with an improvement of cognitive functions in mice and humans [83,84] but only a few studies with antioxidants were able to increase lifespan significantly until now [85]. Particularly, the interest in bioactive substances in leaves and fruits from *olea europaea* has increased over the last years. Olive flesh contains about 2-3 % of phenolic substances, primary glucosides and esters. An EVOO of high quality comprises about 50-60 mg/100 g of polyphenols in total [75,86]. Secoiridoids, Iridoids, where the cyclopentane ring is interrupted, are another special class of biofunctional substances with potentially beneficial effects on the physiological aging process. The main secoiridoids found in olives

include oleuropein, dimethyl-oleuropein, ligstroside and their derivatives oleuropein-aglycone, oleocanthal, hydroxytyrosol, tyrosol and oleacein [87].

However, it should be noted that not all secoiridoids are equally included at every stage of the olives development and that the cultivation and the geographical origin influences the composition of secoiridoids in olive tissue on a large scale. Due to the enzymatic structural transformation of olive secoirdidoids, not all bioactive substances are present in olives at the same time. Most secoiridoids are present in young olives and are altered by different transformations during the ripening process. In unripe olives, mainly oleuropein is found which is transformed into the aglycone during the ripening process of the olives. Ligstroside, a secoiridoid found in olives, has been little studied to date and is mainly found in the leaves, pulp and stone of the olive tree but can be hardly found in olive oil [88,89].

The potential health benefits of olive polyphenols are limited due to the reduced bioavailability and ongoing biotransformation in the gastrointestinal tract. Several studies have shown that polyphenols, in particular, are modified by intestinal microbiotics and then taken in as metabolites in reduced amounts. [90] Consequentially other studies demonstrated a low absorption of untransformed olive polyphenols and their ability to cross the blood-brain barrier [91–93]. Polyphenols, especially olive polyphenols, are described in the literature as health-promoting substances. They are said to have a positive influence on cardiovascular diseases and are able to positively influence the aging process [94]. If we now consider the aging process as multifactorial, which is associated with mitochondrial and nuclear DNA damage, increased ROS levels and a resulting MD, olive polyphenols have been described to protect against most of those age-related hallmarks [95].

Olive secoiridoids are therefore able to intervene at different points of cellular death. In particular, the anti-inflammatory and antioxidative properties of olive polyphenols play a decisive role for the positive influence on the aging process [87]. For example, olive polyphenols were able to counteract H_20_2-induced DNA damage in the cellular model of PBMCs and it was shown that increased gene expression leads to increased antioxidative defence and reduced ROS levels [96,97]. If we look at the aging process in particular based on the accumulation of ROS and an increase in cellular protein and DNA damage, olive polyphenols are potential substances which can influence the aging process in a positive way.

2.6. Special diets and the effects on mitochondrial bioenergetics and (brain-) aging

Besides polyphenols, there are other important nutritional parameters that can influence our cognitive abilities and the health status of mitochondria. For this reason, we carried out a study in female NMRI mice which received three different diets for six months. One group was fed to an antioxidant-reduced diet whereas the two remaining groups were fed two different standard control diets. The effect of antioxidants like ascorbic acid (AA) and vitamin E on the aging process are strongly discussed in the literature. Vitamin E, a bioactive natural compound, consisting of four tocotrienols and four tocopherols, is associated with numerous positive effects on the progression of the brain aging process [98]. Some studies have shown an effect of vitamin E supplementation on the development of MCI and AD, while other studies have questioned the benefit of vitamin E supplementation in this regard [99,100]. Ascorbic acid, a water-soluble vitamin, has the highest concentrations in the brain compared to the other organs of the body [101]. Since the "free radical theory of aging" was postulated, ROS were blamed as a major cause of many age-related processes. This theory directly put the focus on antioxidants, such as AA and vitamin E, in the prevention of cardiovascular diseases and neurodegeneration. In addition of being a free radical scavenger, AA has also been described as a potential remedy for arteriosclerosis, cardiovascular diseases and cancer [102–104]. Furthermore, the positive and protective effects of vitamin E supplementation are evident, with most studies focusing on α-tocopherol. It remains to be said, in a meta-analysis, administration of high doses (> 400 IU / d) of vitamin E were associated with an increased risk of mortality, although this claim could not be confirmed in 2009 [105,106]. In particular, the combination of vitamin E and AA is often described as particularly advantageous, since AA is able to reduce vitamin E and the two antioxidants can therefore act together in a recycling mechanism [107]. Summing up, until now the effects of vitamin E an AA alone have not been fully clarified in the literature. However, the tendency of the studies suggests that the supplementation of one antioxidant alone does not appear to have a major effect on the brain aging process [108].

Another form of nutrition known especially in the western society is a diet that contains a large amount of fat and saturated fatty acids. These high-fat diets are considered risk factors for developing illnesses such as type 2 diabetes mellitus, cardiovascular diseases or neurological disorders [109–112]. However, a high intake of fat does not only seem to have a promoting effect on the classical widespread diseases, but also seems to have an effect on the physiological aging process and the course of AD [113,114]. Not only chronic intake of high amounts of

saturated fatty acids has a negative influence on health, but even short episodes of excessive consumption of a few days lead to an increase in neuroinflammation and a decrease in cognitive abilities in aged rats [115]. Besides to the induction of insulin resistance, an increase in inflammation and oxidative stress in the brain, HFD's have been reported to have direct effects on mitochondrial function. It has already been shown that HFD's can induce MD in skeletal muscles, which is associated with a reduced ADP sensitivity [116]. The effect of HFD's on brain mitochondria has been relatively little studied until now [117], while the focus has often been on mitochondria of the non-central nervous system. In 2015, it has been shown that HFD's have a negative effect on the function of mitochondria in skeletal muscles in rats, but brain mitochondria were not affected simultaneously in this study [118]. The poor data of HFD's regarding brain mitochondrial function was further investigated in this work by two different diets fed to C57Bl/6J mice.

2.7. Studies, Cell and animal models

This work dealt with the monitoring of the longitudinal physiological aging process in NMRI mice (longitudinal aging study), as well as with the long-term feeding of high-purity olive secoiridoids in aged NMRI mice. Additionally, the effect of highly purified olive secoiridoids on basal ATP levels in SH-SY5Y-Mock cells were investigated. In further studies, the effects of different standard diets in rodent husbandry as well an antioxidant-reduced diet and two different HFD's in C57Bl/6J mice were investigated with respect to mitochondrial function.

2.8. SH-SY5Y-Cell model

SH-SY5Y cells are a human cell line obtained from a bone marrow biopsy of a patient with neuroblastoma in 1970 and containing neuroblastoma cells and epithelial cells [119]. The karyotype of this cell line has 47 chromosomes and the cell line can be differentiated into neurons via different differentiation factors such as retinoic acid or BDNF [120,121], depending

on the desired cell type (cholinergic, adrenergic, dopaminergic). The SH-SY5Y cell line is a common cell line for different indications in neuroscience [122].

2.9. NMRI mice

NMRI mice come from an outbred mouse line whose name is derived from the Naval Medical Research Institute where the strain originates. Since these mice are an outbred strain, they possess a very heterogeneous gene pool, which makes them a suitable model for research into aging and general pharmacological issues [16,123,124]. NMRI mice can thus be regarded as a cross-section of the total population. The median lifespan i.e. the age corresponding to the survival of 50% of the population is 17 months [124]. NMRI mice were bred in the institute of pharmacology in Frankfurt a.M. and kept under standard conditions (23 °C, 55 % humidity, 12 h light/dark cycle) and aged until they reached the desired age (3, 6, 12, 18 and 24 months) for the long-term study. For the long-term feeding study with highly purified olive secoiridoids and the C1000 study, mice were born in the institute of pharmacology and were housed until they reached the age of 12 months. At this time point, mice were randomly divided into three groups on the basis of the performance level in the Y-Maze-spontaneous alternation test. Young mice were purchased with 3 weeks and were fed to the appropriate control diet until the age of 3 months.

2.10. C57Bl/6 mice

The C57BL/6J mouse was developed by C.C. Little in 1921, after crossing the female N.57 with the male N.52 from a commercial breeding centre in the United States (Miss Abby Lathrop). C57BL/6J is the most widely used inbred strain and the first to have its genome sequenced. This mouse strain belongs to the long-lived mouse models and survival assessments by The Jackson Laboratory revealed a median life span of 117–128 weeks [125]. C57Bl/6J mice for the HFD study were purchased from Charles River at an age of 5 weeks (Sulzfeld, Germany) and housed in individually ventilated cages in groups of five mice at a temperature

of 22 ± 2 °C, 55 ± 10 % relative humidity, and a 14:10 h light/dark cycle. Water was provided ad libitum as well as two commercially available diets from Altromin (Lage, Germany). Male mice for the WSD study (Janvier SAS, LE Genest-Saint-Isle, France) were fed for 7 weeks to the liquid control diet or the lard and fructose rich diet (ssniff, Soest, Germany).

3. Publications and Manuscripts

The following publications are integral parts of this thesis:

1. Cerebral Mitochondrial Function and Cognitive Performance during Aging: A Longitudinal Study in NMRI Mice. (IF=5.076)

Reutzel M[1], Grewal R[1], Dilberger B[1], Silaidos C[1], Joppe A[2], Eckert GP[1]*.

Oxid Med Cell Longev. 2020 Apr 13;2020:4060769. doi: 10.1155/2020/4060769

2. Effects of Long-Term Treatment with a Blend of Highly Purified Olive Secoiridoids on Cognition and Brain ATP Levels in Aged NMRI Mice. (IF=5.076)

Reutzel M[1], Grewal R[1], Silaidos C[1], Zotzel J[2], Marx S[2], Tretzel J[2], Eckert GP[1]*.

Oxid Med Cell Longev. 2018 Oct 30;2018:4070935. doi: 10.1155/2018/4070935

3. Purified oleocanthal and ligstroside protect against mitochondrial dysfunction in models of early Alzheimer's disease and brain ageing. (IF=4.691)

Grewal R[1], Reutzel M[1], Dilberger B[1], Hein H[1], Zotzel J[2], Marx S[2], Tretzel J[2], Sarafeddinov A[2], Fuchs C[2], Eckert GP[1]*.

Exp Neurol. 2020 Jun;328:113248. doi: 10.1016/j.expneurol.2020.113248

4. Effects of different standard- and special diets on cognition and brain mitochondrial function in mice. (IF=4.028)

Martina Reutzel[1], Rekha Grewal[1], Carsten Esselun[1], Sebastian Friedrich Petry[2], Thomas Linn[2], Annette Brandt[3], Ina Bergheim[3], Gunter P. Eckert[1]* Nutritional Neuroscience

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3.1. Cerebral Mitochondrial Function and Cognitive Performance during Aging: A Longitudinal Study in NMRI Mice. (IF=5.076)

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Research Article

Cerebral Mitochondrial Function and Cognitive Performance during Aging: A Longitudinal Study in NMRI Mice

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Brain aging is one of the major risk factors for the development of several neurodegenerative diseases. Therefore, mitochondrial dysfunction plays an important role in processes of both, brain aging and neurodegeneration. Aged mice including NMRI mice are established model organisms to study physiological and molecular mechanisms of brain aging. However, longitudinal data evaluated in one cohort are rare but are important to understand the aging process of the brain throughout life, especially since pathological changes early in life might pave the way to neurodegeneration in advanced age. To assess the longitudinal course of brain aging, we used a cohort of female NMRI mice and measured brain mitochondrial function, cognitive performance, and molecular markers every 6 months until mice reached the age of 24 months. Furthermore, we measured citrate synthase activity and respiration of isolated brain mitochondria. Mice at the age of three months served as young controls. At six months of age, mitochondria-related genes (complex IV, creb-1, β-AMPK, and Tfam) were significantly elevated. Brain ATP levels were significantly reduced at an age of 18 months while mitochondria respiration was already reduced in middle-aged mice which is in accordance with the monitored impairments in cognitive tests. mRNA expression of genes involved in mitochondrial biogenesis (cAMP response element-binding protein 1 (creb-1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), nuclear respiratory factor-1 (Nrf-1), mitochondrial transcription factor A (Tfam), growth-associated protein 43 (GAP43), and synaptophysin 1 (SYP1)) and the antioxidative defense system (catalase (Cat) and superoxide dismutase 2 (SOD2)) was measured and showed significantly decreased expression patterns in the brain starting at an age of 18 months. BDNF expression reached, a maximum after 6 months. On the basis of longitudinal data, our results demonstrate a close connection between the age-related decline of cognitive performance, energy metabolism, and mitochondrial biogenesis during the physiological brain aging process.

1. Introduction

The average life expectancy has increased considerably to over 80 years in developed countries [1], and the multifactorial aging process is characterized by several changes on the cellular level [2, 3]. Mitochondria are cell organelles with central functions such as energy metabolism, including ATP production and generation of reactive oxygen species (ROS); however, mitochondrial dysfunction has been identified as an important hallmark of aging [4–9]. In addition, there are many studies that describe the close relationship between various age-related diseases and impaired mitochondrial function, which makes mitochondria interesting as a potential target for the treatment and prevention of neurodegenerative diseases [10, 11]. Mitochondrial dysfunction is characterized by a reduced efficiency of the respiratory chain system diminishing the synthesis of high-energy molecules such as ATP and the expression of genes involved in mitochondrial biogenesis, cellular longevity, and the antioxidant defense systems [12]. Evidences point out that the

activity of complex I and complex IV of the respiratory chain system is impaired in aged brains which leads to a reduced capability to produce ATP [13, 14]. In 1956, the "free radical, theory of aging" was postulated by Harman which states that cellular aging is a direct consequence of free radicals, especially the superoxide anion radical ($O_2^{\cdot-}$), attacking cells and tissue [3, 15–17]. This framework has been refined over the last years. In 1979, mitochondria were identified as the key producer of ROS that significantly contribute to aging processes [18–21]. However, low "physiological" ROS levels are known to have important functions for signaling mechanisms in the cell [22]. Under physiological conditions, the antioxidative defense system, including superoxide dismutase (SOD) and the glutathione (GSH) system, is able to eliminate highly reactive molecules [23]. However, if there is an imbalance between the generation of ROS and the cellular defense system, oxidative damage occurs which can initiate apoptosis and trigger neurodegenerative diseases. Furthermore, aging is characterized by changes in mitochondrial dynamics [24]: these organelles are able to fuse and to divide. The later process, the so-called fission, is part of the cellular quality control and results in fragments of different sizes that are cleared by mitophagy [25, 26]. The fission processes also help to regulate the cellular ATP levels. Fusion leads to enrichment of mtDNA and finally reduces mutations, [24].

Most of the aging studies in rodents conducted so far compared aged animals to young ones but did not collect longitudinal data over the entire lifetime. Thus, studies on cognitive performance and bioenergetic parameters in the brain covering the lifespan are rare. Therefore, we measured the development of the energy metabolism and mRNA expression of genes involved in mitochondrial biogenesis, antioxidant capacity, and synaptic plasticity in the brain as well as the cognitive performance every six months in the same cohort of female NMRI mice. Female NMRI mice are a well-described outbred mouse model for the physiological, "normal" aging process which reflects a high variability of the genome [27–29]. This model has been described as most suitable for studies on physiological aging compared to inbred or genetically modified mouse models with accelerated aging or reduced lifespans [30, 31].

2. Material and Methods

2.1. Animals and Treatment. Female NMRI mice (Navar Medical Research Institute) were purchased at the age of 3 weeks from Charles River (Sulzbach, Germany) and kept in the animal station until they reached the ages of 3, 6, 12, 18, and 24 months. All mice had ad libitum access to a standard pelleted diet (cat. no.1324; Altromin, Lage, Germany) and drinking water. Behavioral testing was performed before all time points. Mice were sacrificed by decapitation. The brain was quickly dissected on ice after the removal of the cerebellum, the brain stem, and the olfactory bulb. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regierungspraesidium Darmstadt; #V54–19 c 20/15–FU/1062).

2.2. Passive Avoidance Test. The test was carried out using a passive avoidance step-through system (cat. no. 40533/mice; Ugo Basile, Germonio, Italy) and a protocol similar to the protocol published by Shiga et al. [32]. On day one of the experiment, the mouse was put into the light chamber (light intensity 75%). The door toward the dark chamber was opened after a 30 s delay, and time was recorded until the mouse enters into the dark chamber. In the dark chamber, the mouse received an electric shock (0.5 mA, 1 s duration). If the mouse did not enter the dark chamber after 180 s, the test was stopped. The same test was repeated 24 h later. This time, the door toward the dark chamber was already opened after 5 s and time was measured until the mouse entered the dark chamber but the electric shock was turned off. The test was aborted after 300 s.

2.3. One-Trial Y-Maze Test. A one-trial Y-maze test was conducted using a custom-made Y-maze (material: polyvinyl chloride; length of arms: 36 cm; height of arms: 7 cm; width of arms: 5 cm; and angle between arms: 120°). At the beginning of the test, the mouse was put into one of the three arms of the Y-maze, and the sequence of the entries was recorded for 5 min. Spontaneous alternation was determined using the formula (number of alternations/number of entries)/2 [33].

2.4. Preparation of Dissociated Brain Cells. One hemisphere of the brain was used to prepare dissociated brain cells (DBCs) for ex vivo studies. The brain was washed once in medium 1 (138 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose $* H_2O$, and 58.4 mM sucrose; pH = 7.35). Afterwards, it was cut into small pieces in 2 ml of medium 1 using a scalpel. The chopped brain was then pressed through a 200 μm nylon mesh into a beaker containing 16 ml of medium 1 using a plastic Pasteur pipette with a wide opening. In the last step, the brain homogenate was filtered through a 102 μm nylon mesh. The resulting brain homogenate was centrifuged (2000 rpm, 5 min, and 4°C) before the pellet was redissolved in 20 ml of medium 2 (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2 * 2\,H_2O$, 1 mM $MgCl_2 * 6\,H_2O$, 25 mM Glucose $* H_2O$, 70 mM sucrose, and 20 mM HEPES). The centrifugation step was repeated twice; after the last centrifugation, the pellet was redissolved in 4.5 ml of Dulbecco's modified without supplements. DBCs were seeded in 250 μl aliquots in 12 replicates into a 24-well plate for the measurement of the mitochondrial membrane potential. For the measurement of the ATP level, DBCs were seeded in 50 μl aliquots into a 96-well plate. Cells were incubated for 3 h in a humidified incubator (5% CO_2). Respectively, 6 wells were incubated for 3 h with sodium nitroprusside (0.5 mM for ATP measurement; 2 mM for the measurement of the mitochondrial membrane potential) in DMEM. The remaining cell suspension was reserved for protein determination and stored at -80°C.

2.5. Measurement of ATP Concentrations in DBCs. The ViaLight Plus bioluminescence kit (Lonza, Walkersville, USA)

was used for assessing ATP concentrations in DBC. At the end of the incubation, the 96-well plate was removed from the incubator and allowed to cool to room temperature for 10 min. Afterwards, all wells were incubated with 25 μl lysis buffer in the dark for 10 min. In the next step, wells were incubated with 50 μl monitoring reagent. The emitted light (bioluminescence) was recorded using a luminometer (Victor X3 multilabel counter). The ATP concentrations in the wells were determined using a standard curve; ATP concentrations of DBC were normalized to protein content.

2.6. Measurement of Mitochondrial Membrane Potential. MMP was measured in DBC using the fluorescence dye Rhodamine123 (R123). DBCs were incubated in an incubator (37°C, 5% CO_2) for 15 min with 0.4 μM R123. Afterwards, the reaction was stopped by adding Hank's Balanced Salt Solution (HBSS) into the wells. DBCs were centrifuged (914 g, 5 min, room temperature), the medium was aspirated, and DBCs were supplemented with new HBSS. DBCs were triturated to obtain a homogenous sample. Subsequently, MMP was assessed by reading the R123 fluorescence at an excitation wavelength of 490 nm and an emission wavelength of 535 nm (Victor X3 multilabel counter). The fluorescence in each well was read in four consecutive runs. The fluorescence values were then normalized to protein content.

2.7. Isolation of Brain Mitochondria and High-Resolution Respirometry. Half a brain hemisphere (the frontal part) was used to isolate brain mitochondria. The protocol is described in Hagl et al. [34]. The pellet obtained from the last centrifugation step was dissolved in 250 μl MIRO5 (0.5 mM EGTA, 3 mM $MgCl_2 * 6 H_2O$, 60 mM K-lactobionat, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 100 mM sucrose, 1 g/l BSA). Subsequently, 80 μl of the resulting cell suspension was injected into an Oxygraph 2k-chamber. A complex protocol was used to investigate the function of the respiratory chain complexes. The capacity of the oxidative phosphorylation (OXPHOS) was determined using complex I-related substrates pyruvate (5 mM), malate (2 mM), and ADP (2 mM) followed by the addition of succinate (10 mM). Mitochondrial integrity was measured by addition of cytochrome c (10 μM). Oligomycin (2 μg/ml) was added to determine leak respiration (leak (omy)), and afterwards, uncoupling was achieved by carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP, injected stepwise up to 1-1.5 μM). Complex II respiration was measured after the addition of rotenone (0.5 μM). Complex III inhibition was achieved by the addition of antimycin A (2.5 μM) and was subtracted from all respiratory parameters. COX activity was measured after ROX determination by applying 0.5 mM tetramethylphenylenediamine (TMPD) as an artificial substrate of complex IV and 2 mM ascorbate to keep TMPD in the reduced state. Autoxidation rate was determined after the addition of sodium azide (>100 mM), and COX respiration was additionally corrected for autoxidation.

2.8. Citrate Synthase Activity. Citrate synthase activity was measured photometrically in isolated brain mitochondria as described in Hagl et al. [34].

2.9. Protein Quantification. Protein content was determined according to the BCA method using a Pierce™ Protein Assay Kit (Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.10. Transcription Analysis by Quantitative Real-Time PCR (qRT-PCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using ~20 mg RNAlater stabilized samples (Qiagen, Hilden, Germany). RNA was quantified measuring the absorbance at 260 and 280 nm using a NanoDrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed using the ratio of absorbance 260/280 and 260/230. To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 250 ng total RNA using the iScript cDNA Synthesis Kit (BioRad, Munich, Germany) according to the manufacturer's instructions and was stored at -80°C. qRT-PCR was conducted using a CfX 96 Connect™ system (BioRad, Munich Germany). Oligonucleotide primer sequences, primer concentrations, and product sizes are listed in Table 1. All primers were received from Biomol (Hamburg, Germany) or Biomers (Ulm, Germany). cDNA for qRT-PCR was diluted 1 : 5 with RNase-free water (Qiagen, Hilden, Germany), and all samples were performed in triplicates. PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 58°C for 45 s, and 72°C for 29 s. Gene expression was analyzed using the $-(2\Delta\Delta C_q)$ method using BioRad CfX manager software and was normalized to the expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

2.11. Statistics. Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Tukey's multiple comparison post-test (Prism 8.0 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for p values of < 0.05.

3. Results

Female NMRI mice of one cohort were investigated at 3, 6, 12, 18, and 24 months of age. Mice aged 24 months had to be excluded from both of the cognitive tests, since they were too impaired in their mobility.

3.1. Effect of Aging on Cognitive Performance. Spatial learning memory and locomotor activity were determined using the Y-maze spontaneous alternation test in 3-, 6-, 12-, and 18-month-old mice [29, 35, 36].

Mice, aged 6 or 12 months, showed slightly but not significantly decreased changes in alternation rates compared to young controls during a five-minute testing phase (Figure 1(a)). Significant changes were observed at an age of 18 months (-21%) (Figure 1(a)). Regarding the number of alternations, 6- and 12-month-old NMRI mice showed numerically but not significantly reduced performance

Table 1: Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR.

Primer	Sequence	Product size (bp)	Conc. (μM)
AMPK (β-subunit)	5′-agtatcacggtggttgctgt-3′ 5′-caaatactgtgcctgcctct-3′	190	0.1
B2M	5′-ggcctgtatgctatccagaa-3′ 5′-gaaagaccagtccttgctga-3′	198	0.4
BDNF	5′-gatgccagttgctttgtctt-3′ 5′-atgtgagaagttcggctttg-3′	137	0.1
CI (NADH-ubiquinone oxidoreductase 51 kDa subunit)	5′-acctgtaaggaccgagaga-3′ 5′-gcaccacaaacacatcaaaa-3′	227	0.1
CIV (cytochrome c oxidase subunit 5A)	5′-ctgttccattcgctgctatt-3′ 5′-gcgaacagcactagcaaaat-3′	217	0.1
Creb-1	5′-tagctgtgacttggcattca-3′ 5′-ttgttctgtttgggacctgt-3′	184	0.5
CS	5′-aacaagccagacattgatgc-3′ 5′-atgaggtcctgctttgtcct-3′	184	0.1
GAP43	5′-agggagatggctctgctact-3′ 5′-gaggacggggagttatcagt-3′	190	0.15
Nrf-1	5′-tcggagcacttactggagtc-3′ 5′-ctagaaaacgctgccatgat-3′	228	0.5
PGC1-α	5′-tgtcaccaccgaaatcct-3′ 5′-cctggggaccttgatctt-3′	124	0.05
PGK1	5′-gcagattgtttggaatggtc-3′ 5′-tgctcacatggctgacttta-3′	185	0.4
SOD2	5′-acagcgcatactctgtgtga-3′ 5′-gggggaacaactcaactttt-3′	183	0.1
SYP1	5′-tttgtggttgttgagttcct-3′ 5′-gcatttcctccccaaagtat-3′	204	0.1
Tfam	5′-agccaggtccagctcactaa-3′ 5′-aaacccaagaaagcatgtgg-3′	166	0.5

bp: base pairs; conc.: concentration.

compared to young animals (Figure 1(b)). Significantly reduced changes in the number of alternations were recorded starting at an age of 12 months (-28%) (Figure 1(b)).

The fear-based passive avoidance test was used to assess the cognitive performance in 3-, 6-, 12-, and 18-month-old mice [37, 38]. Young mice remembered the foot shock they received when they entered the dark chamber 24 hours before quite well as indicted by an increased latency time on day two (Figure 2). On day two, old animals reentered the dark chamber faster than young control animals resulting in significantly lower latency times (Figure 2) indicating a lack in memory in mice aged 12 (-60%) and 18 months (-65%). At the age of 24 months, mice showed severe impairments in mobility. Thus, they were no longer usable for the test and had to be excluded.

3.2. Effect of Aging on Gene Expression. The expression of genes involved in longevity, mitochondrial biogenesis and function, synaptic plasticity, and antioxidative properties was determined in brains of 3-, 6-, 12-, 18-, and 24-month-old mice. All genes considered showed significant changes in the expression pattern during the observed aging process in the brain. It was observed that the mRNA expression of creb-1, Tfam, complex IV, BDNF, and β-AMPK, which are mainly involved in mitochondrial biogenesis and physical activity, significantly increased during the young adulthood (6 months) and showed a significantly reduced expression with 24 months compared to that of young control animals (Figures 3(a)–3(e)). Furthermore, PGC1-α and Nrf-1, two other important transcription factors involved in mitochondrial biogenesis, showed an approximately constant

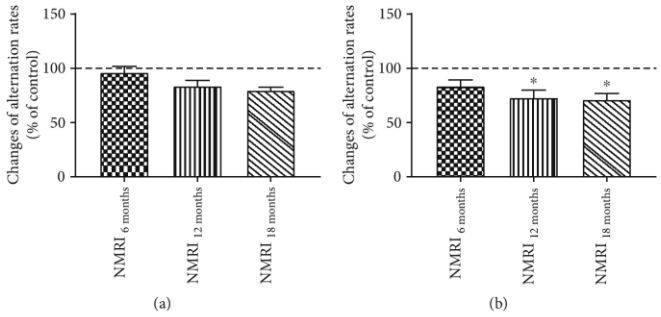

FIGURE 1: Y-Maze spontaneous alternation test of 3-, 6-, 12-, and 18-month-old mice during a five-minute period time of testing. Changes of alternation rates (% of control) (a) and changes of number of alternations (% of control) (b); $n = 12$, mean ± SEM, and one-way ANOVA with Tukey's post hoc test; $^{*}p < 0.05$. Performance of young control mice (3 months) is defined as 100%.

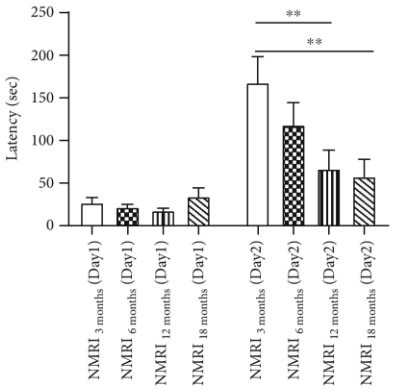

FIGURE 2: Passive avoidance test with 3-, 6-, 12-, and 18-month-old NMRI mice. On day one, mice receive a mild electric shock (0.5 mA) and time that the mouse needs to enter into the dark chamber is recorded; 24 h after the first testing period, the test is repeated and time that the mouse needs to reenter the dark chamber is recorded; $n = 15$, mean ± SEM, and one-way ANOVA with Tukey's post hoc test; $^{*}p < 0.05$ and $^{**}p < 0.01$.

expression level until the age of 24 months where mRNA expression decreased significantly in comparison to young mice (Table 2). Additionally, gene expression of the mitochondrial mass marker citrate synthase was significantly reduced starting at the age of 18 months. mRNA expression of SOD2 and Cat, enzymes responsible for the antioxidative cellular properties, significantly started to decline with 18 months. However, complex I mRNA expression was approximately constant until the age of 24 months. Expression of the synaptosomal markers SYP1 and GAP43 decreased at an age of 6 months. Unexpectedly, SYP1 mRNA expression was unchanged in 18-month-old mice only (Table 2).

3.3. Effect of Aging on Brain ATP Levels and Mitochondrial Membrane Potential (MMP). ATP and MMP levels were measured in dissociated brain cells (DBCs) of 3-, 6-, 12-, 18-, and 24-month-old NMRI mice [28, 39]. ATP levels of 6- and 12-month-old mice showed no significant changes compared to those of young control animals. ATP levels were significantly reduced in DBC isolated from 18-month-old NMRI mice (-33%). However, ATP levels showed a numerical increase with 24-month-old compared to 3-month-old mice (+25%). MMP levels were significantly reduced in brains of 24-month-old NMRI mice compared to 18-month-old animals (-28%, Table 3).

3.4. High-Resolution Respirometry in Isolated Mitochondria. The complexes of the inner mitochondria membrane (complex I, NADH: ubiquinone oxidoreductase (CI); complex II, succinate-coenzyme Q reductase (CII); complex III, cytochrome c oxidoreductase (CIII); and complex IV, cytochrome c oxidase (CIV)) are essential for building up the mitochondrial membrane potential (MMP) which is the driving force for complex V of the mitochondrial respiration chain (F_1/F_0-ATPase (CV)) that produces ATP [40]. Adult mice, aged 6 months, did not show any differences concerning mitochondrial respiration compared to 3-month-old control animals (Table 3). However, mitochondrial respiration of complexes I (-30.3%) and IV (-20.4%) was significantly reduced starting at an age of 12 months compared to that of young animals (Table 3). Respiration of CI+CII (-25.6%) and CII_{ETS} (-32%) in isolated mitochondria gradually declines during aging starting at an age of 12 months (Table 4).

4. Discussion

In order to understand the physiological brain aging process, longitudinal aging studies are superior to single point studies since they provide a good indication of when the first cognitive deficits occurred during lifetime [41, 42]. The special characteristic of our study is that we used a single cohort of NMRI mice to examine the effects of the physiological aging

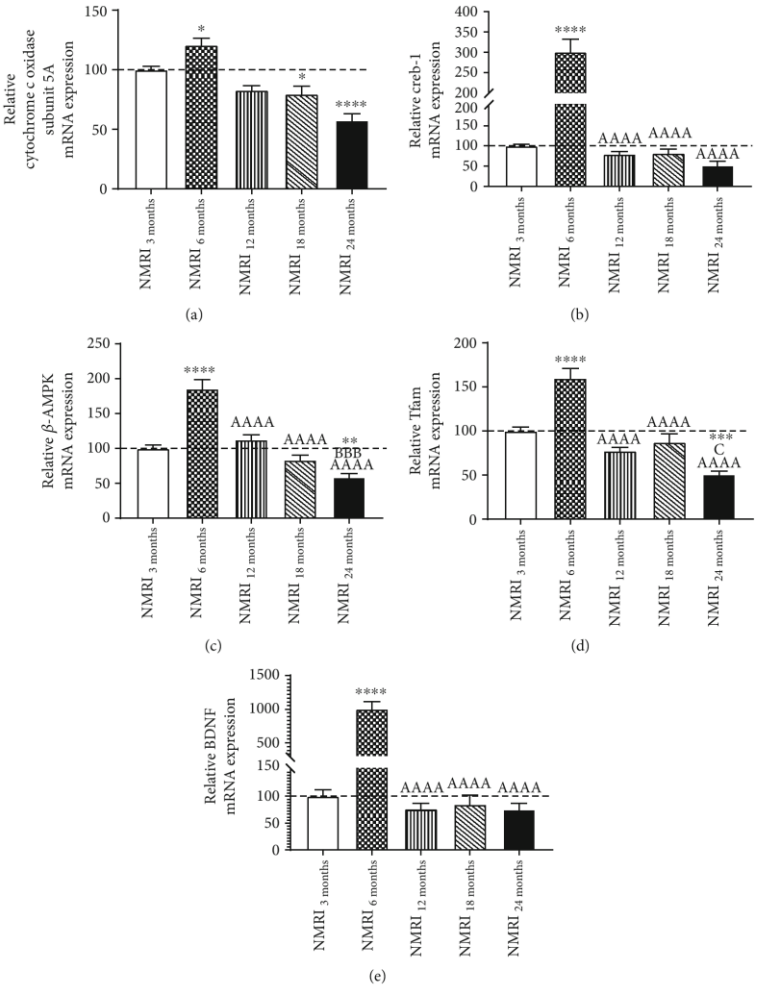

FIGURE 3: Relative normalized mRNA expression levels of cytochrome c oxidase subunit 5A (a), cAMP response element-binding protein (creb-1) (b), AMP-activated protein kinase (β-AMPK) (c), mitochondrial transcription factor A (Tfam) (d), and brain-derived neurotrophic factor (BDNF) (e) in brain homogenate of 3-, 6-, 12-, 18-, and 24-month-old mice. mRNA expression of 3-month-old control mice is 100%. $n = 9$, mean ± SEM with one-way ANOVA and Tukey's post hoc test with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ against 3-month-old control animals. "A" indicates one-way ANOVA and Tukey's post hoc test against 6-month-old mice, "B" against 12-month-old mice, and "C" against 18-month-old animals. Results are normalized to the mRNA expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

process in the brain while most previous studies focused on one time point only to examine the brain aging process (Table 5). To our knowledge, we are the first who used NMRI mice of 3, 6, 12, 18, and 24 months of age to explore changes during the aging process in one cohort. In the end, these data allow a more detailed picture of mitochondrial and cognitive functions during the aging process. Overall, our data show an early decline of cognitive functions in middle-aged NMRI mice, which do not seem to go along simultaneously with the identified impairments on the molecular level and the bioenergetics of the brain. These findings are potentially important for the prevention of neurodegenerative diseases, for which aging processes are an important risk factor and can start early, well before the first symptoms appear.

Table 2: Relative normalized mRNA expression levels in brain homogenate from 3-, 6-, 12-, 18-, and 24-month-old mice determined using quantitative real-time PCR in comparison to 3-month-old control animals. mRNA expression of 3-month-old control mice is 100%; $n = 9$; mean ± SEM with one-way ANOVA and Tukey's post hoc test with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. "A" indicates one-way ANOVA and Tukey's post hoc test against 6-month-old mice, "B" against 12-month-old mice, and "C" against 18-month-old animals. Results are normalized to the mRNA expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

Gene	6 months	12 months	18 months	24 months
Complex I (CI)	95.0 ± 3.3	102.1 ± 6.8	89.3 ± 7.0	$60.3\pm5.4^{***/AA/BBB/CC}$
Citrate synthase (CS)	83.1 ± 4.7	86.4 ± 7.7	$69.9 \pm 6.0^{*}$	$50.5\pm5.1^{***/A/B}$
Growth-associated protein (GAP43)	$66.5\pm2.8^{****}$	$66.6\pm4.8^{****}$	$80.2 \pm 4.8^{*}$	$58.7\pm4.6^{****/C}$
Synaptophysin 1 (SYP1)	$81.4 \pm 4.4^{*}$	86.6 ± 2.4	103.8 ± 6.0^{AA}	$66.9\pm4.8^{****/B/CCCC}$
Superoxide dismutase 2 (SOD2)	109.0 ± 5.3	81.6 ± 8.2^{A}	$72.7 \pm 6.9^{*/AAA}$	$50.1\pm4.6^{****/AAAA/B}$
Catalase (Cat1)	104.5 ± 3.7	89.5 ± 5.4	$66.4\pm6.3^{****/AAAA/B}$	$64.2\pm6.8^{****/AAAA/B}$
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α)	83.3 ± 3.9	82.7 ± 5.9	88.3 ± 8.5	$62.5 \pm 9.9^{*}$
Nuclear respiratory factor-1 (NRF-1)	93.9 ± 7.8	85.7 ± 6.9	86.8 ± 9.6	$51.2\pm4.8^{****/AA/B/CC}$

Table 3: Basal ATP and MMP levels of dissociated brain cells (DBCs) as well as protein-normalized respiration of complexes I and IV in isolated mitochondria from 3-, 12-, 18-, and 24-month-old mice; $n = 10$; mean ± SEM; with one-way ANOVA and Tukey's post hoc test with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ compared to young control animals. # indicates significant changes against 18-month-old mice with $p < 0.05$.

	ATP level (nmol/mg protein)	MMP level (AU/mg protein)	CI (pmol/(s∗mg protein))	CIV (pmol/(s∗mg protein))
3 mo	1.2 ± 0.1	79323 ± 3401	1906 ± 133	8060 ± 660
6 mo	1.4 ± 0.1	86166 ± 6272	1799 ± 131	8048 ± 495
12 mo	1 ± 0.1	83357 ± 5612	$1327\pm84^{**}$	$6412 \pm 202^{*}$
18 mo	$0.8 \pm 0.1^{*}$	104125 ± 11331	$1199\pm131^{**}$	$5591\pm313^{**}$
24 mo	1.5 ± 0.2	$74770 \pm 4217^{\#}$	$1232\pm145^{**}$	$4814\pm489^{****}$

Table 4: Protein-normalized respiration of CI+CII and CII_{ETS} in isolated brain mitochondria from 3-, 12-, 18-, and 24-month-old mice; $n = 10$; mean ± SEM; with one-way ANOVA and Tukey's post hoc test with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to young control animals.

	CI+II (pmol/(s∗mg protein))	CII_{ETS} (pmol/(s∗mg protein))
3 mo	3416 ± 240	1904 ± 140
6 mo	3222 ± 158	1827 ± 79
12 mo	$2539\pm119^{**}$	$1292\pm90^{**}$
18 mo	$2149\pm107^{***}$	$1000\pm104^{****}$
24 mo	$1605\pm163^{****}$	$995\pm109^{****}$

One important finding in our cohort study was that mice at the age of 6 months showed a significantly different phenotype in comparison to young but also to older mice. It seems that extensive changes take place in the brain of animals at the age of 6 months, which need to be investigated in future studies. Interestingly, most aging studies use 3-month-old NMRI mice as young controls [35, 43]. Moreover, these observations demonstrate the importance of longitudinal aging studies, investigating more than one time point to describe the brain aging process and cognitive decline. Specific aspects of our study, such as study type, behavioral tests, and the role of mitochondria in brain aging, are discussed in the following sections.

4.1. Longitudinal Study. Longitudinal studies (Table 5, A) of the brain aging process, which provide information on mitochondrial bioenergetics in combination with cognitive function, are rare. Most longitudinal studies only consider behavioral tests or mitochondrial parameters during lifetime. More importantly, most studies did not examine mice from a cohort but reported differences at individual points in time (Table 5, B), which makes it difficult to give a clear picture of the development and course of the physiological aging process in the brain.

4.2. Behavioral Testing. Monitoring the following aging process, 18-month-old mice had a significantly reduced alternation rate and number of alternations compared to young control animals, indicating a reduced spatial learning memory and mobility in 18-month-old NMRI mice (Figures 1(a) and 1(b)) which is in agreement with previous studies [29, 36]. In comparison, other studies already showed a significantly decreased performance in the Y-maze test in middle-aged mice [43, 49]. Furthermore, our findings indicate that long-term memory seems to be already impaired in middle-aged mice during the physiological aging process whereas spatial learning memory

Table 5: Results from longitudinal studies (A) and single point studies (B) focused on the results of mitochondrial bioenergetics and cognitive functions. Unless otherwise stated, 3-month-old animals served as young controls. Arrows indicate the age at which significant effects were first observed (↑ increase, ↓ decrease, and ↔ no significant effect).

	MMP	ATP	CI	CIV	Cognitive function	Mouse strain	Lit.
(A) Longitudinal studies							
Current study	↓ (24 m)	↓ (18 m)	↓ (12 m)	↓ (12 m)	↓ (12 m)	NMRI	
Navarro et al. 2005	n.d.	↔ (13 m) ↔ (19 m)	↑ (13 m) ↓ (19 m)	↓ (13 m) ↓ (19 m)	n.d.	CD-1	[44]
Kwong et al. 2000	n.d.	n.d.	↔ (13 m) ↔ (29 m)	↔ (13 m) ↔ (29 m)	n.d.	C57Bl/6	[45]
Lamberty et al. 1990	n.d.	n.d.	n.d.	n.d.	↓ (9 m) ↓ (12 m)	NMRI	[43]
Gower et al.1993	n.d.	n.d.	n.d.	n.d.	↓ (9 m) ↓ (12 m)	NMRI	[36]
(B) Single point studies							
Reutzel et al. 2018	↔	↓ (18 m)	↔	↔	↓ (18 m)	NMRI	[28]
Hagl et al. 2016	↔	↓ (18 m)	↔	↓ (18 m)	↓ (18 m)	NMRI	[27]
Afshordel et al. 2015	n.d.	↓ (24 m)	↔ (24 m)	↔ (24 m)	n.d.	NMRI	[46]
Hagl et al. 2015	↔ (18 m)	↔	↓ (18 m)	↔ (18 m)	n.d.	NMRI	[47]
Stoll et al. 1996	n.d.	n.d.	n.d.	n.d.	↔ (12 m) ↔ (22 m)	NMRI	[48]

n.d.: not determined.

seems to be more or less unaffected until the age of 18 months. The signaling molecule BDNF has several functions in brain aging and plasticity. It is an important component in biochemical pathways and is a key player in energy metabolism, neuronal survival, and neurogenesis [50–52] and has been shown to play a crucial role in hippocampus-dependent learning behavior [53]. However, during the physiological aging process, we have measured a strong decrease in gene expression of this important neurotrophic factor starting at 12 months of age. During the young adulthood of mice (6 months), BDNF mRNA expression reaches a maximum and maintains at a constant level throughout aging which is in agreement with previous findings from Webster et al. who reported a significant rise in the prefrontal cortex of BNDF in young adults [54]. Furthermore, mice lacking SYP1 show significantly reduced learning behavior [55], and an enriched environment has been reported to have positive effects on the SYP1 brain level [56]. In accordance with these findings, we detected numerically decreased SYP1 mRNA expression levels during the brain aging process starting in young adulthood which is in agreement with the observed deficits on cognition. Surprisingly, SYP1 mRNA expression increased numerically at an age of 18 months compared to 12-month-old animals. Synaptophysin is reported to be a component of neurotransmitter-containing presynaptic vesicle membranes, and its increase is closely connected to an improved neurotransmission and cognitive performance [56, 57]. However, data availability and the connection between SYP1 mRNA levels in the brain are not consistent. According to this, other studies did not show any age-related changes in SYP1 brain levels [58, 59], while others reported significantly decreased SYP1 with age [60–62]. GAP43, another nervous tissue-specific protein, is mainly involved in neurite outgrowth and elongation during the neuronal development and is also regarded as a marker for neural plasticity [63–65]. In our study, GAP43 was highly expressed in brains of 3-month-old animals which is in accordance with findings from Rosskothen-Kuhl and Illing who found the highest expression of GAP43 during the early development of the nervous system [66]. Recently, we reported that mRNA levels of all three proteins involved in neuronal plasticity were significantly decreased in brains of aged NMRI mice [39], which is confirmed by our recent data. The reduced expression of those genes indicates less synaptic plasticity and neuronal remodelling in brains of aged NMRI mice which might be one possible reason for the cognitive impairments in memory and motor performance during the aging process [67, 68].

4.3. Mitochondrial Bioenergetics during the Physiological Aging Process. In accordance with most of the previous studies from our group, we measured significantly reduced ATP levels in dissociated brain cells isolated from brains of 18-month-old NMRI mice in comparison to young control animals. In accordance with the finding of reduced ATP levels in brains of aged NMRI mice, we found reduced mRNA expression levels of complex IV in the brain of 18-month-old mice, which is one of the most important protein complexes involved in the oxidative phosphorylation process consistent with previous studies [28, 29, 39]. A reduced complex IV expression was associated with an increase in apoptosis [69, 70]. Bowling et al. showed an age-associated progressive decline of the respiratory chain complexes I and IV in cortices of primates [71], and Petrosillo et al. measured a reduced complex I activity in brain mitochondria of 24-month-old rats [72], whereas other studies could not confirm those

findings in brains of aged mice [45]. The mitochondrial membrane potential (MMP) reflects the mitochondrial functional status and is mainly produced by complex I, complex III, and complex IV that transport protons from the mitochondrial matrix into the intermembrane space [73]. However, at the age of 24 months, we detected a small, but only numerical, increase of ATP brain levels which is in contrast to the results from Afshordel et al. who reported significantly reduced brain ATP levels in 24-month-old mice [46]. Furthermore, the group of Navarro et al. did not show any significant changes of ATP levels in brains of 13- and 19-month-old CD-1 mice [44]. Thus, age-dependent changes in ATP levels seem to depend on the strain of mice that was investigated.

Regarding the mitochondrial membrane potential, we detected reduced MMP in brains of 24-month-old mice compared to young animals which is in accordance with the reduced complex I and IV mRNA expression in brains of aged mice. Based on these data, one would expect a reduced respiration of the respiratory chain complexes [74, 75]. Accordingly, in the current study, activity of respiratory chain complexes I and IV as well as CI+CII and CII_{ETS} was significantly reduced starting at 12 months of age compared to that of young animals (Tables 3 and 4) which is in accordance with previous studies of our group detecting reduced complex activity in brains of 18-month-old animals [28, 29]. Surprisingly, our longitudinal study shows that complex activity seems to be reduced already in middle-aged animals while brain ATP and MMP levels stay constant until mice reach the age of 18 and 24 months.

4.4. Mitochondrial Biogenesis and the Antioxidative Defense System during the Aging Process. Mitochondrial biogenesis is a process in which new mitochondria are formed from existing mitochondria and is regulated by peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC1-α). PGC1-α as a master regulator of mitochondrial biogenesis is activated by AMPK-activated kinase and Sirt-1 which are the two major pathways to induce mitochondrial biogenesis. AMPK is able to phosphorylate PGC1-α, or it activates Sirt-1 through increasing the NAD+ level. Alternatively, the transcription factor creb-1 pathway is induced which finally leads to the generation of new mitochondria [76]. During the brain aging process, we detected a significantly reduced mRNA expression of PGC1-α, Nrf-1, Tfam, creb-1, AMPK, and citrate synthase (CS), which is a key enzyme in mitochondria involved in the citric acid cycle (TCA) and thus provides information on mitochondrial mass. However, we did not find reduced citrate synthase activity, which is a common marker for mitochondrial mass (data not shown). In accordance with other studies, we were able to confirm that during the aging process, mitochondrial biogenesis seems to be impaired. For example, Picca et al. described an impaired protein expression of PGC1-α and Nrf-1 in liver tissue of aged rats [77]. Furthermore, overexpression of Tfam is able to reverse age-dependent memory loss in mice which shows the close connection between the detected cognitive impairments and the reduced mRNA expression of genes involved in mitochondrial biogenesis found in aged mice [78]. In contrast to our previous work which described a reduced gene expression in brains of 18-month-old mice [28, 29], in this longitudinal study, we were only able to confirm a reduced mRNA expression of most of the considered genes in brains of mice aged 24 months. We therefore hypothesize that rodents can keep physiological mRNA expression until at least the age of 18 months, but the decrease detected at 24 months of age is finally a result of the ongoing senescence [75].

Antioxidant enzymes like SOD2 and Cat1 are involved in the antioxidative defense system of the cell and are able to protect macromolecules like DNA, lipids, and proteins from oxidative damage. Thus, our findings show an age-dependent decrease of SOD2 and Cat1 starting at an age of 18 months which gives evidence that damaging effects could occur more probably in the aged mouse brain. Thus, the data availability shows variable results concerning the antioxidative enzymes. Leutner et al. showed an increased SOD activity in brains of NMRI mice starting at an age of 10 months, while other studies could not find any changes of SOD or Cat in 24-month-old rats [29, 79].

In particular, mRNA gene expression is affected first in brains of very old animals. These observations suggest that SYP1 and GAP43 [80, 81], two genes involved in synaptic plasticity and synaptogenesis, may be one possible reason for the early decline in the passive avoidance test. Furthermore, we hypothesize that the brain, as an energy-demanding organ, is able to maintain stable ATP levels until mice reach the age of 18 months, although the oxidative phosphorylation is already affected in middle-aged animals in our study. These observations suggest that glycolysis and its metabolites should be further investigated to determine the exact mechanisms behind these results since several studies showed deficits in glucose metabolism in brains of aged rats as well as healthy, old people [82, 83]. Any changes in the physiological brain glucose metabolism, mainly supported by mitochondria, affect neuronal function, cognition, learning, and memory [84].

5. Conclusion

Many clinical and neuropathological symptoms of AD occur parallel to the normal aging process which makes it difficult to keep them apart from each other. During the physiological aging process, several changes on cognitive performance, mitochondrial brain energy metabolism, and mRNA expression of genes involved in mitochondrial biogenesis were detected in a longitudinal study over 24 months. Most of the impairments on cognition and mitochondria bioenergetics were detected starting at an age of 18 months which in fact shows that aged NMRI mice are an appropriate model to study the physiological (brain-) aging process.

Data Availability

The data set generated during this study is available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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3.2. Effects of Long-Term Treatment with a Blend of Highly Purified Olive Secoiridoids on Cognition and Brain ATP Levels in Aged NMRI Mice. (IF=5.076)

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Research Article

Effects of Long-Term Treatment with a Blend of Highly Purified Olive Secoiridoids on Cognition and Brain ATP Levels in Aged NMRI Mice

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Aging represents a major risk factor for developing neurodegenerative diseases such as Alzheimer's disease (AD). As components of the Mediterranean diet, olive polyphenols may play a crucial role in the prevention of AD. Since mitochondrial dysfunction acts as a final pathway in both brain aging and AD, respectively, the effects of a mixture of highly purified olive secoiridoids were tested on cognition and ATP levels in a commonly used mouse model for brain aging. Over 6 months, female NMRI mice (12 months of age) were fed with a blend containing highly purified olive secoiridoids (POS) including oleuropein, hydroxytyrosol and oleurosid standardized for 50 mg oleuropein/kg diet (equivalent to 13.75 mg POS/kg b.w.) or the study diet without POS as control. Mice aged 3 months served as young controls. Behavioral tests showed deficits in cognition in aged mice. Levels of ATP and mRNA levels of NADH-reductase, cytochrome-c-oxidase, and citrate synthase were significantly reduced in the brains of aged mice indicating mitochondrial dysfunction. Moreover, gene expression of Sirt1, CREB, Gap43, and GPx-1 was significantly reduced in the brain tissue of aged mice. POS-fed mice showed improved spatial working memory. Furthermore, POS restored brain ATP levels in aged mice which were significantly increased. Our results show that a diet rich in purified olive polyphenols has positive long-term effects on cognition and energy metabolism in the brain of aged mice.

1. Introduction

Aging represents one of the major risk factors for developing neurodegenerative diseases such as Alzheimer's disease (AD). Currently, five million Americans are suffering from dementia, and by 2050, there will be one new case every 66 seconds [1]. The multifactorial pathology makes it difficult to develop feasible therapies, and current approved drugs attenuate symptoms but do not cure the disease. Research into AD also had several failures in terms of developing disease-modifying therapies [2]. Since AD starts many years before the first symptoms occur, new scientific approaches focus on early stages, which are discussed to be important in aging and the onset of AD. In this sense, there is growing interest in dietary patterns, stimulation of the brain, and physical activity as potential modifiable risk factors [3–6]. It has been shown that adherence to a Mediterranean diet (MedDiet) pattern significantly reduces the risk of AD [7, 8], which has been confirmed and recently summarized in reviews and meta-analysis [9–11].

One important component of the MedDiet is a high consumption of extra virgin olive oils (EVOO) [12], and a combination of MedDiet and EVOO seems to better improve cognitive function including improved performance in visual and verbal memory domains [13–15].

EVOO contains several different polyphenols [12] including secoiridoid derivatives, phenolic alcohols, and lignans as well as flavonoids which seem to have neuroprotective properties on the aging process [16–18]. Hydroxytyrosol and oleuropein are two of the main antioxidative compounds

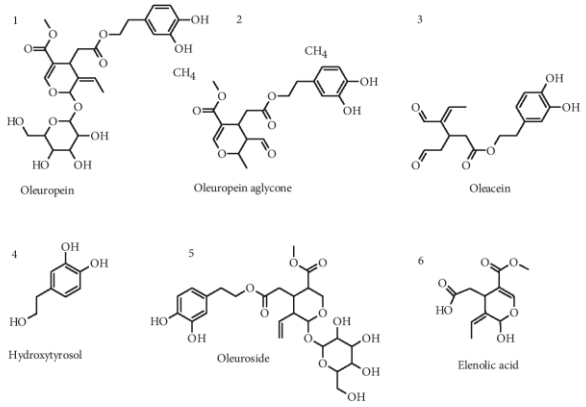

FIGURE 1: Secoiridoid derivatives (1–5) and their degradation products are the predominant phenolic compounds present in EVOO and in the tested POS. Secoiridoids are characterized by the presence in their molecules of elenolic acid (6) or its derivatives.

present in olives [19–22] providing neuroprotection [22–26]. Thus, olive polyphenols are proposed as new promising agents to combat aging-associated neurodegeneration [27].

Using a recently developed technology, olive polyphenols were isolated and highly purified from olive leaves that represent a rich source of bioactive ingredients [28]. We investigated the effects of a mixture of six highly purified secoiridoid polyphenols (Figure 1) on cognition and brain energy metabolism in aged NMRI mice. Expression of genes involved in longevity, mitochondrial biogenesis and function, synaptic plasticity, and antioxidative properties was determined to elaborate molecular mechanisms. Effects on ATP levels were confirmed in neuronal SH-SY5Y cells.

2. Materials and Methods

2.1. Cell Culture. SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal calf serum, 0.3 mg/mL hygromycin, 60 units/mL penicillin, 60 μg/mL streptomycin, 4500 mg/L D-glucose, MEM Vitamin solution, MEM Nonessential Amino Acids, and 1 mM sodium pyruvate at 37°C in a humidified incubator containing 5% CO_2.

Two days prior to incubation, cells were seeded into 96-well plates (20,000 cells/well). Cells were incubated with the respective POS concentrations (0.001 nM–10 μM dissolved in DMEM) for 24 h (basal ATP levels) or preincubated with POS for 1 h and insulted with rotenone (25 μM) for 24 h (ATP levels after insult). The emitted light is linear to the ATP (Lonza, Switzerland) concentration and was measured with a VICTOR™ X2 Multilabel Plate Reader (Perkin Elmer).

2.2. Animals and Treatment. Female NMRI (Navar Medical Research Institute) mice, a commonly used aging model, were purchased from Charles River (Sulzbach, Germany) and were housed according to the German guidelines for animal care with access to water and food ad libitum. Mice were maintained on a 12 h light/dark cycle until they reached the age of 12 months. Young (3 months old) NMRI mice served as the control group. Mice were fed with a well-proven C1000 standardized diet (Altromin, Lage, Germany), C1000 containing vitamin A (2500 IU/kg), vitamin E (20 mg/kg), and selenium (150 mcg/kg) [29, 30]. The verum group received the identical diet supplemented with a blend of highly purified secoiridoid polyphenols for 6 months. Based on the average food consumption, a daily intake of 13.75 mg POS/kg b.w. was calculated. Feeding studies in mice reporting biological effects applied olive polyphenols in a dose range of 1 to 10 mg/kg b.w. [26, 31–33]. The estimated daily POS dose (13.75 mg/kg b.w.) in the current study equals a single human dose of approximately 1.1 mg/kg b.w/day [34]. The feeding period of the young control mice started 3 months later than the feeding period of aged mice to ensure that both of them end at the same time point. Behavioral testing was performed before the starting points and at the end of the feeding period. On the basis of behavioral testing at the beginning, mice were divided into 2 groups of the same performance level. Mice were killed by cervical dislocation and decapitation. Brains were quickly dissected on ice after the removal of the cerebellum, the brain stem, and the olfactory bulb. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regierungspraesidium Darmstadt; #V54–19 c 20/15–FU/1062).

2.3. Composition of the POS. The POS was provided from N-Zyme Biotec GmbH, Darmstadt, Germany. The composition is listed in Table 1.

2.4. Passive Avoidance Test. The test was conducted using a passive avoidance step-through system (cat. no. 40533/mice Ugo Basile, Gemonio, Italy) and a protocol similar to the

Table 1: Composition of the highly purified secoiridoid-rich extract (POS), manufactured by N-Zyme Biotec GmbH, Darmstadt, Germany.

Secoiridoid derivative	Content (%)
Oleuropein aglycone	36.7
Hydroxytyrosol	24.7
Oleacein	14.6
Elenolic acid derivatives	10.5
Oleuropein	7.2
Oleurosid	6.2

protocol published by Shiga et al. [35]. On the first day of the experiment, the mouse was put into the light chamber (light intensity of 1350 lux). After 30 s, the door toward the dark chamber was opened, and time till entering the dark chamber was measured. In the dark chamber, the mouse received an electric shock (0.5 mA, 1 s duration). The test was stopped if the mouse did not enter the dark chamber after 180 s. The test was repeated after 24 h with the door opening towards the dark chamber after only 5 s. Again, time till entering the dark chamber was recorded. This time no electric shock was applied after crossing the door. The test was stopped after 300 s.

2.5. One-Trial Y-Maze Test. One-trial Y-Maze test was conducted using a custom-made Y-Maze (material: polyvinyl chloride, length of arms: 36 cm, height of arms: 7 cm, width of arms: 5 cm, and angle between arms: 120°). At the beginning of the test, the mouse was put into one of the three arms of the Y-Maze and the sequence of the entries was recorded for 5 min. Spontaneous alternation was determined using the formula (number of alternations/number of entries)/2 [36].

2.6. Preparation of Dissociated Brain Cells for the Measurement of the Mitochondrial Membrane Potential and Determination of ATP Level. One hemisphere was used to prepare dissociated brain cells (DBCs) for ex vivo studies according to the method of [30]. DBCs were resuspended in 4.5 mL DMEM without supplements. For the measurement of ATP levels, DBCs were seeded in 50 μL aliquots into a 96-well plate. Cells were incubated for 3 h in a humidified incubator (5% CO_2). Respectively, 6 wells were incubated for 3 h with sodium nitroprusside (0.5 mM for ATP measurement) in DMEM. The remaining cell suspension was reserved for protein determination (stored at −80°C).

2.7. Protein Quantification. Protein content was determined using Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Instructions were followed as given by the manufacturer.

2.8. Glutathione Peroxidase Activity. Glutathione peroxidase activity was determined using a commercially available assay kit (kit number: ab102530; Abcam Plc., Cambridge, UK). 50 mg of frozen brain tissue was used as described in the manufacturer's manual. Glutathione peroxidase generates GSSG from GSH during H_2O_2 reduction, and the generated GSSG is reduced back to GSH by glutathione reductase during consumption of NADPH. The reduction of NADPH is proportional to glutathione peroxidase activity; thus, it can be measured calorimetrically at 340 nm.

2.9. Citrate Synthase Activity. Citrate synthase activity was determined photometrically in isolated brain mitochondria as recently described in Hagl et al. [37].

2.10. Isolation of Brain Mitochondria and Measurement of Complex I and IV Activities. Half a brain hemisphere (the frontal part) was used to isolate brain mitochondria. The protocol is described in Hagl et al. [37]. The pellet obtained from the last centrifugation step was dissolved in 250 μL MIRO5. 80 μL of the resulting cell suspension was injected into the Oxygraph 2k-chamber. A complex protocol (elaborated by Prof. Dr. Erich Gnaiger) was used to investigate the function of the respiratory chain complexes. The capacity of the oxidative phosphorylation (OXPHOS) was determined using complex I-related substrates pyruvate (5 mM) and malate (2 mM) and ADP (2 mM) followed by the addition of succinate (10 mM). Mitochondrial integrity was measured by the addition of cyctochrome c (10 μM). Oligomycin (2 μg/mL) was added to determine leak respiration (leak (omy)), and afterwards, uncoupling was achieved by carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, injected stepwise up to 1–1.5 μM). Complex II respiration was measured after the addition of rotenone (0.5 μM). Complex III inhibition was achieved by the addition of antimycin A (2.5 μM) and was subtracted from all respiratory parameters. COX activity was measured after ROX determination by applying 0.5 mM tetramethylphenylenediamine (TMPD) as an artificial substrate of complex IV and 2 mM ascorbate to keep TMPD in the reduced state. Autoxidation rate was determined after the addition of sodium azide (>100 mM), and COX respiration was additionally corrected for autoxidation.

2.11. Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using ~20 mg RNAlater stabilized samples (Qiagen, Hilden, Germany). RNA was quantified measuring the absorbance at 260 and 280 nm using the NanoDrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed using the ratio of absorbance 260/280 and 260/230. To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 250 ng total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions and was stored at −80°C. qRT-PCR was conducted using a CfX 96 Connect™ system (Bio-Rad, Munich, Germany). Oligonucleotide primer sequences, primer concentrations, and product sizes are listed in Table 2. All primers were received from Biomol. cDNA for qRT-PCR was diluted 1 : 10 with RNase-free water (Qiagen, Hilden, Germany), and all samples were performed in

Table 2: Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR. bp: base pairs; conc: concentration.

Primer	Sequence	Manufacturer	Product size (bp)	Conc. (μM)
AMPK (beta subunit)	5′-agtatcacggtggttgctgt-3′ 5′-caaatactgtgcctgcctct-3′	Biomol Hamburg, Germany	190	0.1
B2M	5′-ggcctgtatgctatccagaa-3′ 5′-gaaagaccagtccttgctga-3′	Biomol, Hamburg, Germany	198	0.4
CI	5′acctgtaaggaccgagaga-3′ 5′-gcaccacaaacacatcaaaa-3′	Biomol, Hamburg, Germany	227	0.1
CIV	5′-ctgttccattcgctgctatt-3′ 5′-gcgaacagcactagcaaaat-3′	Biomol, Hamburg, Germany	217	0.1
CS	5′-aacaagccagacattgatgc-3′ 5′-atgaggtcctgctttgtcct-3′	Biomol, Hamburg, Germany	184	0.1
GAP43	5′ agggagatggctctgctact-3′ 5′ gaggacggggagttatcagt-3′	Biomol Hamburg, Germany	190	0.15
GPx-1	5′-gtccagcgtgtatgccttct-3′ 5′-ctcctggtgtccgaactgat-3′	Biomol, Hamburg, Germany	217	0.1
PGK1	5′-gcagattgtttggaatggtc-3′ 5′-tgctcacatggctgacttta-3′	Biomol, Hamburg, Germany	185	0.4
Sirt1	5′-gtgagaaaatgctggcctaa-3′ 5′-ctgccacaggaactagagga-3′	Biomol, Hamburg, Germany	161	1
SOD2	5′-acagcgcatactctgtgtga-3′ 5′-gggggaacaactcaacttttt-3′	Biomol, Hamburg, Germany	183	0.1
Synaptophysin 1	5′-tttgtggttgttgagttcct-3′ 5′-gcatttcctccccaaagtat-3′	Biomol, Hamburg, Germany	204	0.1

triplicate. PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 58°C for 45 s, and 72°C for 29 s. Gene expression was analyzed using the $-(2\Delta\Delta C_q)$ method using BioRad CfX manager software and was normalized to the expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

2.12. Statistics. Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Bonferroni's multiple comparison posttest (Prism 7.0 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for p values of <0.05.

3. Results

Female NMRI mice were fed over 6 months with a standardized pelleted diet (aged control) or diet supplemented with a blend of highly purified secoiridoid polyphenols (13.75 mg POS/kg b.w.) (aged + POS) for 6 months. Young control mice (young control) received a standardized pelleted diet for 3 months. There was no significant difference in body weight and life span between the aged control and the aged intervention group mice. At the end of the feeding period, cognitive function and brain mitochondrial function were assessed.

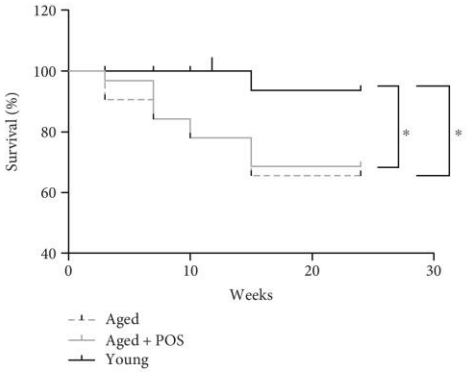

Figure 2: Survival rates of NMRI mice after feeding with or without POS. Aged (12 months old) mice were fed with a standardized pelleted diet (aged) or pelleted diet containing POS (13.75 mg POS/kg b.w., aged + POS) for 6 months. As further control, young mice (3 months old, starting point of the analysis: 12 weeks) were fed with a pelleted standard diet for 3 months (young); $n = 15 - 27$; mean without SEM; log-rank (Mantel-cox) test; $^*P < 0.05$.

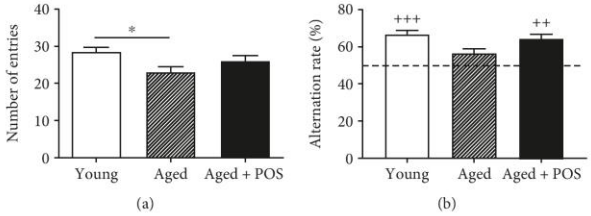

FIGURE 3: Y-Maze spontaneous alternation of young, aged, and POS-treated mice (13.75 mg/kg b.w.) during a 5 min period time of testing. Number of entries (a) and alternation rate (b); $n = 16$ mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Alternation rate (c) was compared to a theoretical value of 50% using a univariate t-test with $^{++}P < 0.01$ and $^{+++}P < 0.001$.

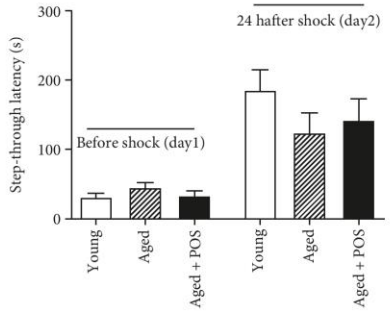

FIGURE 4: Passive avoidance test with young, aged, and long-term POS-treated mice. On day one, mice receive an electric shock (0.5 mA) and time is recorded when the mouse needs to enter into the dark chamber; 24 h after the first testing period, the test is repeated and time is recorded when the mouse needs to reenter the dark chamber; $n = 16$; mean ± SEM.

3.1. Survival. Survival rates of young and aged control mice were 93 and 66% ($P < 0.05$), while the survival rate of aged mice administrated with POS was 69% ($P < 0.05$). Thus, POS treatment did not increase the survival rate over a 6-month feeding period (Figure 2).

3.1.1. Behavioral Testing. In the Y-Maze test, aged control mice showed a significantly decreased alternation rate (56.7 ± 2%) and number of entries (23 ± 1.5) during a 5 min testing phase compared to young controls (Figure 3(b)). POS administration for 6 months significantly increased the alternation rate (64 ± 2%) and slightly but not significantly increased the number of entries (26 ± 1) (Figure 3(a)).

On day one, aged control mice showed a slightly but not significant longer latency time to enter into the dark chamber (45 ± 7 s) compared to young control animals (31 ± 6 s) in the passive avoidance test. POS-treated mice showed almost the same latency time as young mice (33 ± 7 s). On day two, aged mice showed a numerically shorter time to reenter the dark chamber (123 ± 29 s) compared to young control mice (185 ± 29 s). POS treatment for 6 months led to a slightly but not significant increased step-through latency time (142 ± 31 s) (Figure 4).

3.1.2. Effect of Long-Term POS Treatment on Brain ATP Levels. Basal ATP levels were measured in dissociated brain cells (DBCs) of young, aged, and POS-treated mice. Aged control mice showed significantly lower ATP level (1.41 ± 0.05 nmol/mg protein) in contrast to young animals (1.75 ± 0.01 nmol/mg protein) which were restored after long-term treatment with POS (1.74 ± 0.1 nmol/mg protein; Figure 5(a)). Furthermore, DBCs were incubated for 3 h with sodium nitroprusside (SNP) to examine the resistance against nitrosative stress. However, no differences were detected after SNP incubation between young (74.4 ± 2.2%), aged (74.7 ± 1.3%), and POS-treated aged mice (81.3 ± 2.2%). Long-term POS treatment resulted in a slight increase of ATP concentrations after SNP insult which did not reach a level of significance (Figure 5(b)). To confirm the effects of POS on ATP levels in vitro, SH-SY5Y cells were incubated with different concentrations of POS. A POS concentration of already 0.1 nM significantly enhanced basal ATP levels (Figure 6(a)). At this concentration, SH-SY5Y cells were also protected from nitrosative stress induced by SNP (Figure 6(b) and Table 3).

Expression of genes involved in longevity, mitochondrial biogenesis and function, synaptic plasticity, and antioxidative properties was determined in young, aged, and POS-treated mice to elaborate molecular mechanisms. All considered genes showed significantly decreased mRNA levels after aging with the exception of SOD2. Long-term POS treatment did not show any significant effects on mRNA expression levels between aged and POS-treated mice (Table 4).

3.2. Activities of Complex I, Complex IV, GPx-1, and CS. In comparison to young control animals, activities of the respiratory chain complexes I and IV and CS activity were unaffected during the aging process and after long-term POS treatment in isolated brain mitochondria. Furthermore, we measured the activities of the antioxidative enzyme GPx-1 in total brain homogenate. The activity of GPx-1 was numerically but not significantly reduced in aged control animals compared to young mice (Table 5).

4. Discussion

In the current study, the effects of long-term feeding of a blend with highly purified olive secoiridoids on cognition

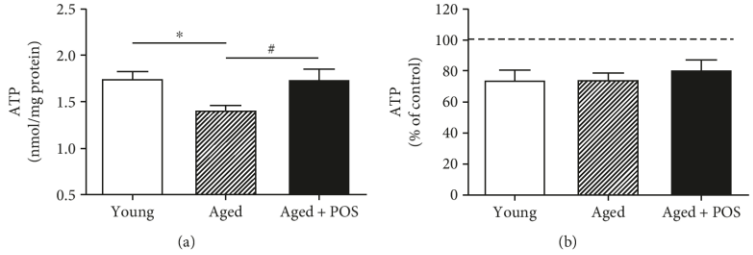

Figure 5: Basal ATP level (a) and ATP concentrations after insult with sodium nitroprusside (SNP, 3 h, 0.1 mM) (b) of dissociated brain cells (DBCs) from young, aged, and POS-treated mice; basal ATP concentrations served as control for normalization in (b); $n = 10$; mean ± SEM; one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$ vs. young; $^{\#}$one-way ANOVA aged vs. aged + POS with $^{\#}P < 0.05$.

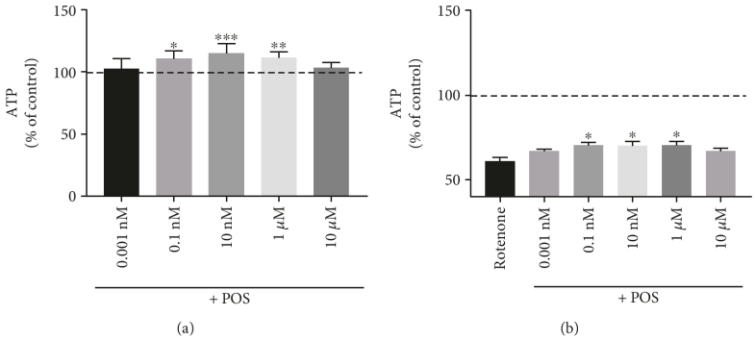

Figure 6: Basal ATP level (a) and ATP concentrations after incubation for 24 h in SY5Y-Mock cells with POS of different concentrations (0.001 nM–10 μM) and insult with rotenone 250 nm (b) in SH-SY5Y control cells; basal ATP concentrations served as control, $n = 7$, mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3: Basal ATP level and ATP concentrations after incubation for 24 h in SY5Y-Mock cells with POS of different concentrations (0.001 nM–10 μM) and after insult with rotenone (250 nM) in SH-SY5Y-Mock cells; basal ATP concentrations served as control, $n = 7$, mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Correlated values	ATP (% of control)	ATP after insult (% of control)
0.001 nM vs. control	102.6 ± 2.9	66.9 ± 1.2
0.1 nM vs. control	110.6* ± 2.0	70.6* ± 1.5
10 nM vs. control	114.9*** ± 3.3	70.3* ± 2.56.1
1 μM vs. control	111.3** ± 1.8	70.4* ± 2.4
10 μM vs. control	103.4 ± 1.7	67.1 ± 1.6

and brain ATP levels were tested in aged female NMRI mice. This strain represents a well-established model for aging studies [30, 38–40]. The results show that a diet rich in purified olive polyphenols has positive long-term effects on cognition and energy metabolism in the brain of aged mice.

4.1. Cognitive Performance in Aged and POS-Treated NMRI Mice. Aged NMRI mice showed deficits in spatial working memory and mobility which is in agreement with earlier studies [30, 38, 41]. Our findings indicated beneficial effects of POS on spatial learning memory and mobility. In agreement with our findings, administration of olive polyphenols has been associated with the improvement of cognitive functions [26, 42, 43].

Pitozzi et al. investigated the effects of long-term dietary administration of EVOO rich in polyphenols in aged C57Bl/6J mice [43]. Comparable to our study, mice were fed from middle age to senescence (total polyphenol dose/day of 6 mg/kg b.w.), and results showed improved contextual memory and prevention of the age-related impairment in motor coordination [43]. EVOO containing different concentrations of polyphenols (e.g., tyrosol, hydroxytyrosol, verbascoside, and oleuropein di-aldehyde) induced similar beneficial effects at a comparable dose as pure oleuropein [42]. Synaptophysin 1 (Syp1) and growth-associated protein 43 (GAP43) are involved in neuronal plasticity and cognition [44, 45]. However, mice lacking SYP1 show significantly reduced learning behavior [46], and enriched environment has been reported to have positive effects on SYP1 brain

Table 4: Relative normalized mRNA expression levels in brain homogenate from aged and aged POS-treated mice determined using quantitative real-time PCR in comparison to young control animals; mRNA expression of young control mice is 100%; $n = 9$; mean ± SEM with one-way ANOVA and Bonferroni posttest with $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; results are normalized to the mRNA expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

	Aged	Aged + POS
AMP-activated protein kinase (beta subunit)	$66.19^{*} \pm 6.00$	72.63 ± 8.38
cAMP response binding protein 1 (CREB1)	$64.27^{**} \pm 4.29$	$63.28^{**} \pm 5.94$
Citrate synthase (CS)	$66.75^{*} \pm 4.92$	$61.35^{***} \pm 9.16$
Complex I (CI)	$75.16^{*} \pm 3.34$	$67.53^{**} \pm 7.60$
Complex IV (CIV)	$58.89^{**} \pm 5.80$	$60.97^{**} \pm 8.00$
Glutathione peroxidase 1 (GPx-1)	$67.65^{*} \pm 8.76$	$58.78^{**} \pm 8.18$
Growth-associated protein 43 (GAP43)	$63.43^{*} \pm 9.23$	$56.73^{**} \pm 8.13$
Sirtuin 1 (Sirt1)	$74.71^{*} \pm 4.65$	$75.36^{**} \pm 5.99$
Superoxide dismutase 2 (SOD2)	97.23 ± 5.26	88.46 ± 10.39
Synaptophysin 1 (SYP1)	83.23 ± 6.08	$72.26^{*} \pm 10.43$

levels [47]. GAP43 is a nervous tissue-specific protein and is mainly involved in development and axonal remodelling in adult brains [48]. Recently, we have reported that mRNA levels of those two proteins were significantly decreased in the brains of aged NMRI mice [49], which is confirmed by our recent data. These findings indicate less synaptic plasticity and remodelling in the brains of aged NMRI mice which might be responsible for age-related cognitive decline in memory and motor performance [50, 51].

4.2. Brain ATP Levels in Aged and POS-Treated NMRI Mice. The high-energy compound ATP is the key energy source in eukaryotic cells, which is mainly generated in the mitochondria by oxidative phosphorylation (OXPHOS). The mammalian OXPHOS system comprises five large complexes (including NADH oxidoreductase, succinate reductase, cytochrome c oxidoreductase, cytochrome c reductase, and ATP synthase) at the inner mitochondrial membrane [52]. In DBCs isolated from the brains of aged NMRI mice, significantly lower ATP level was determined compared to that of young controls. This finding is in agreement with earlier reports relating lower ATP levels to an impairment of CI and CIV of the OXPHOS system [30, 49, 53, 54]. Accordingly, our current data show significantly decreased expression levels of CI and CIV which also have been reported recently [30]. Long-term treatment with POS significantly improved ATP levels in the DBC of aged NMRI mice, an effect that has not been reported yet for olive polyphenols *in vivo*. POS also improved ATP levels in neuronal SH-SY5Y cells. Recent studies indicate that a mixture of 6 polyphenols (tannic acid, resveratrol, quercetin, rutin, gallic acid, and morin) is able to increase ATP levels during age-related hearing loss [55] in female rats and in the brains of a transgenic mouse model of AD [56]. In a previous study, we showed that short-term administration of hydroxytyrosol-rich olive mill waste water extract (HTRE) to NMRI mice significantly enhanced the mitochondrial membrane potential in DBC isolated from treated mice [23]. In the same study, DBCs were treated with HTRE *in vitro*, and a concentration-dependent improvement of the MMP was detected. In this study, ATP levels were not determined. However, the MMP is the driving force for complex V of the mitochondrial respiration chain (CV; F0/F1-ATPase) to generate ATP [52]. In a following study, HTRE was tested in PC12 cells and HTRE or purified hydroxytyrosol (HT) neither improved MMP nor ATP levels, indicating a different mode of action for POS and HTRE. However, both HTRE and HT protected MMP and ATP levels in PC12 cells from nitrosative stress in a concentration-dependent manner [57]. In the current study, we only detected a numerical increase of ATP levels after SNP insult in DBCs isolated from the brains of POS-treated mice. This result also indicates that POS did not provide antioxidative properties in our current study. Accordingly, POS did not improve mRNA levels and enzyme activity of GPx-1, which is involved in the endogenous response against oxidative stress in the central nervous system [58]. Sirt1 and AMPK are important players in mitochondrial biogenesis since they activate peroxisome proliferator receptor gamma coactivator 1-α (PGC1-α) [59]. PGC1α itself is activated by deacetylation via sirtuins (SIRT) and phosphorylation via AMP-activated protein kinases (AMPK). Furthermore, phosphorylated cAMP response element-binding protein (CREB) can induce gene expression of PGC1α. PGC1α facilitates the expression of transcription factors nuclear respiratory factor 1 (Nrf1) and mitochondrial transcription factor A (Tfam) which in turn induces mitochondrial biogenesis. POS treatment did not influence the expression levels of Sirt1 and AMPK. Additionally, we measured citrate synthase activity in isolated mitochondria which is a marker for the determination of mitochondrial content [60]. Significant lower citrate synthase (CS) mRNA expression was determined in the brains of aged and aged + POS-fed NMRI mice whereas CS in isolated mitochondria was unaffected (Table 5) indicating that other molecular mechanisms were responsible for the improvement of cognition. Possibly, the increased ATP levels and the resulting improvement of cognition are a consequence of an enhanced glycolysis which supports important functions such as neuroprotection and dramatically decreases with age [61]. Typically, glucose-6-phosphatase converts glucose into pyruvate which generates two molecules of ATP. Thus, future studies should determine the levels of glucose, lactate, glucose-6-phosphatase, and pyruvate to confirm this hypothesis.

5. Conclusion

Long-term feeding of a blend containing highly purified secoiridoid polyphenols (POS) provided beneficial effects on spatial working memory and motor coordination which were probably mediated by the increased ATP brain levels.

Table 5: Activities of the respiratory chain complexes I and IV in isolated mitochondria of young, aged, and aged mice fed with POS determined using an Oxygraph-2k; $n = 10$, mean ± SEM. GPx-1 was measured in brain homogenate using a calorimetric kit; $n = 6 \pm$ SEM. CS activity was measured in isolated mitochondria; $n = 10 \pm$ SEM.

Correlated values	CI activity [(pmol/s*IU CS)]	CIV activity [(pmol/s*IU CS)]	GPx-1 activity (mU/mL)	CS activity (IU/mg protein)
Young vs. aged	1545 ± 101 vs. 1625 ± 80	5487 ± 157 vs. 5673 ± 191	1139 ± 50 vs. 1032 ± 48	927 ± 108 vs. 898 ± 104
Aged vs. aged + POS	1625 ± 80 vs. 1587 ± 108	5673 ± 191 vs. 5806 ± 145	1032 ± 48 vs. 1116 ± 60	898 ± 104 vs. 843 ± 118

Therefore, POS might represent a suitable nutraceutical for age-related cognitive decline.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Supplementary Materials

The supplementary materials include 11 tables showing the complete statistical results including F value, degrees of freedom, and P value. Supplementary Table 3a: complete statistical results including F value, degrees of freedom, and P value of number of entries in Y-Maze spontaneous alternation test of young, aged, and POS-treated mice. Supplementary Table 3b: complete statistical results including F value, degrees of freedom, and P value of alternation rate in Y-Maze spontaneous alternation test of young, aged, and POS-treated mice. Supplementary Table 4: complete statistical results including F value, degrees of freedom, and P value of number of entries in Passive Avoidance test of young, aged, and POS-treated mice. Supplementary Table 5a: complete statistical results including F value, degrees of freedom, and P value of basal ATP level in DBCs of young, aged, and POS-treated mice. Supplementary Table 5b: complete statistical results including F value, degrees of freedom, and P value of ATP concentration in DBCs of young, aged, and POS-treated mice after insult with sodium nitroprusside. Supplementary Table 6a: complete statistical results including F value, degrees of freedom, and P value of basal ATP concentrations in SY5Y-Mock cells after incubation with POS. Supplementary Table 6b: complete statistical results including F value, degrees of freedom, and P value of ATP concentration in SY5Y-Mock cells after insult with rotenone. Supplementary Table 7a: complete statistical results including F value, degrees of freedom, and P value of complex I activity in isolated brain mitochondria of young, aged, and POS-treated mice. Supplementary Table 7b: complete statistical results including F value, degrees of freedom, and P value of complex IV activity in isolated brain mitochondria of young, aged, and POS-treated mice. Supplementary Table 7c: complete statistical results including F value, degrees of freedom, and P value of GPx-1 activity in brain homogenate of young, aged, and POS-treated mice. Supplementary Table 7d: complete statistical results including F value, degrees of freedom, and P value of citrate synthase activity in isolated brain mitochondria of young, aged, and POS-treated mice. *(Supplementary Materials)*

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3.3. Purified oleocanthal and ligstroside protect against mitochondrial dysfunction in models of early Alzheimer's disease and brain ageing. (IF=4.691)

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Research Paper

Purified oleocanthal and ligstroside protect against mitochondrial dysfunction in models of early Alzheimer's disease and brain ageing

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ABSTRACT

As components of the Mediterranean diet (MedDiet) olive polyphenols may play a crucial role for the prevention of Alzheimer's disease (AD). Since mitochondrial dysfunction is involved in both, brain ageing and early AD, effects of 10 different purified phenolic secoiridoids (hydroxytyrosol, tyrosol, oleacein, oleuroside, oleuroside aglycon, oleuropein, oleocanthal, ligstroside, ligstroside aglycone and ligustaloside B) and two metabolites (the plant metabolite elenolic acid and the mammalian metabolite homovanillic acid) were tested in very low doses on mitochondrial function in SH-SY5Y-APP$_{695}$ cells - a cellular model of early AD. All tested secoiridoids significantly increased basal adenosine triphosphate (ATP) levels in SY5Y-APP$_{695}$ cells. Oleacein, oleuroside, oleocanthal and ligstroside showed the highest effect on ATP levels and were additionally tested on mitochondrial respiration. Only oleocanthal and ligstroside were able to enhance the capacity of respiratory chain complexes. To investigate their underlying molecular mechanisms, the expression of genes associated with mitochondrial biogenesis, respiration and antioxidative capacity (PGC-1α, SIRT1, CREB1, NRF1, TFAM, complex I, IV and V, GPx1, SOD2, CAT) were determined using qRT-PCR. Exclusively ligstroside increased mRNA expression of SIRT1, CREB1, complex I, and GPx1. Furthermore, oleocanthal but not ligstroside decreased Aβ 1–40 levels in SH-SY5Y-APP$_{695}$ cells. To investigate the *in vivo* effects of purified secoiridoids, the two most promising compounds (oleocanthal and ligstroside) were tested in a mouse model of ageing. Female NMRI mice, aged 12 months, received a diet supplemented with 50 mg/kg oleocanthal or ligstroside for 6 months (equivalent to 6.25 mg/kg b.w.). Young (3 months) and aged (18 months) mice served as controls. Ligstroside fed mice showed improved spatial working memory. Furthermore, ligstroside restored brain ATP levels in aged mice and led to a significant life extension compared to aged control animals. Our findings indicate that purified ligstroside has outstanding performance on mitochondrial bioenergetics in models of early AD and brain ageing by mechanisms that may not interfere with Aβ production. Additionally, ligstroside expanded the lifespan in aged mice and enhanced cognitive function.

1. Introduction

To date, nearly 50 million people worldwide are suffering from dementia. Due to the increase in population and ageing, the global number of patients is expected to triple by 2050 (Gaudreault and Mousseau, 2019). Despite extensive research into the pathology, Alzheimer's disease (AD), the most common form of dementia cannot be cured yet. Currently, therapies are symptomatic and do not affect disease progression. Mitochondrial dysfunction is increasingly recognized as one of the early events in the progression of AD. Aberrant bioenergetics is likely to play an important role in AD and ageing (Stockburger et al., 2018; Friedland-Leuner et al., 2014). In particular, reduced oxygen consumption, ATP production and downregulation of mitochondrial biogenesis have been observed in cell and animal models of AD (Lin and Beal, 2006). For this reason, it may be essential to focus on preventive strategies to slow down the progression of AD.

In Mediterranean regions the prevalence of AD is low compared to those of other countries (Panza et al., 2004; Solfrizzi et al., 2003; Scarmeas et al., 2009a). Systematic reviews and meta-analysis have elaborated that the Mediterranean Diet (MedDiet) lowers the incidence of AD (Hill et al., 2019; Aridi et al., 2017; Singh et al., 2014). One integral component of the MedDiet is the high intake of extra virgin

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olive oil (EVOO), with a range of 25–50 ml consumption a day (Visioli et al., 2018). Serreli and Deinana et al. calculated if a person consumed 25–50 ml EVOO a day, the intake of polyphenols would be around 9 mg/day. It has been estimated, that 1 mg is represented by hydroytyrosol and tyrosol, whereas the remaining 8 mg are related to their elenolic esters and to oleuropein- and ligstroside-aglycones (Serreli and Deiana, 2018). It has been shown that after an intake of 25 ml/day, which is lower than the traditional daily consumption in Mediterranean areas, 98% of olive polyphenols are present in human plasma and urine as phase II metabolites, predominant as glucoronides and sulfated conjugates (Miro-Casas et al., 2003). However, it has to be considered that the contents of polyphenols in EVOO varies, depending on the area of cultivation, the time of harvest and the production process (Deiana et al., 2019). For example the amount of oleocanthal varies between 0.02% and 10% (Impellizzeri and Lin, 2006; Karkoula et al., 2012, 2014). Thus, the cited calculations only represent an approximation to the real situation. It has also to be noted that EVOO ordinarily does not contain ligstroside, since this polyphenol can only be detected in large quantities during the early ripening period of olive fruits (Deiana et al., 2019).

However, EVOO has been recently proposed as promising tool for the prevention of late-onset AD (Román et al., 2019). Beneficial health effects of EVOO are mainly attributed to olive polyphenols. Recent studies confirm that specific olive polyphenols of EVOO - in particular secoiridoid derivatives are responsible for the health-promoting effects such as antihypertensive, anti-inflammatory and antimicrobial effects (Schwingshackl et al., 2019; Gavahian et al., 2019). Polyphenols have been shown to improve mitochondrial bioenergetics and biogenesis *in vitro* and *in vivo* (Wood Dos Santos et al., 2018; Dilberger et al., 2019; Schaffer et al., 2012; Schaffer et al., 2006). Moreover, several clinical studies have shown to improve cognitive performance and slow down the progression of memory impairment (Scarmeas et al., 2006a; Valls-Pedret et al., 2015; Scarmeas et al., 2009b; Scarmeas et al., 2006b). It has been shown, that Oleocanthal is able to enhance cerebrovascular clearance of Aβ and to cross the blood-brain barrier in mice (Abuznait et al., 2013; Qosa et al., 2015).

The health promoting properties of olive polyphenols and their metabolites have been frequently investigated in mixtures and extracts. However, for a basic understanding of the cause-effect relationships it is essential to test pure compounds (Sato and Sato, 1988; Schaffer et al., 2010; Schaffer et al., 2007; Qosa et al., 2015). Therefore, we tested 10 purified olive secoiridoid derivatives and two metabolites (the plant metabolite elenolic acid and the mammalian metabolite homovanillic acid) which have been isolated from olive and privet leaves on mitochondrial function in an amyloid precursor protein (APP) transfected neuroblastoma cell line. We identified oleuroside, oleacein, oleocanthal and ligstroside (Fig. 1) as hit substances showing the highest increase of ATP production. Determination of mitochondrial respiration and citrate synthase activity was carried out after incubation with these four compounds. To elaborate molecular mechanisms, we determined the expression of genes involved in mitochondrial biogenesis and antioxidative properties of oleocanthal and ligstroside, which exclusively improved mRNA expression. Furthermore, we extended our investigation on an *in vivo* model of brain ageing and tested the effects of oleocanthal and ligstroside on cognition, brain energy metabolism, and life-span in aged female NMRI mice.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma SH-SY5Y cells were grown at 37 °C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.3 mg/ml hygromycin, 60 units/ml penicillin, 60 μg/ml streptomycin, MEM Non-Essential Amino Acids and 1 mM sodium pyruvate 1%. SH-SY5Y cells were stably transfected with DNA constructs harbouring human wild- type APP_{695} (APP_{695}) or the expression vector pCEP4 (Invitrogen, Europe) alone (control vector) and were kindly donated by A. Eckert (Basel, Switzerland). In all experiments, stably transfected SH-SY5Y cells were compared with cells overexpressing the corresponding empty or control vectors. Cells were passaged every 3–4 days and were used for experiments when they reached 80–90% confluence (Hooff et al., 2010).

2.2. Determination of cellular ATP levels

A bioluminescence assay was used to determine ATP levels, which is based on the production of light from ATP and luciferin in the presence of luciferase. After 24 h cells were incubated with the respective substance for 24 h, or preincubated with the respective substance and insulted with rotenone (25 μM) for 24 h. EtOH served as solvent control. The measurement was performed according to a previously published protocol using the ViaLightTM Plus Kit (Lonza, Basel, Switzerland) (Hagl et al., 2015).

2.3. Mitochondrial respiration

Mitochondrial respiration was monitored at 37 °C, using the Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. To analyse the function of the respiratory system, a complex protocol (elaborated by Prof. Dr. Erich Gnaiger) was applied including different substrates, uncouplers, and inhibitors. Cells were washed with PBS, scraped off into mitochondrial respiration medium (MiRO5) developed by Oroboros (Stadlmann et al., 2006), centrifuged, and resuspended in MiRO5 to get a solution containing 10^6 cells/ml. After adding 2 ml of cell suspension into the chambers of the oxygraph and stabilization of endogenous respiration, cell membranes were permeabilized with digitonin (10 μg/10^6 cells), leaving mitochondrial outer and inner membrane intact. OXPHOS respiration was determined with complex I related substrates glutamate (10 mM), malate (2 mM), and ADP (2 mM) followed by addition of succinate (10 mM). Oligomycin addition (2 μg/ml) lead to measurement of leak respiration. Stepwise addition of FCCP showed the maximum capacity of the electron transfer system. By adding the complex I inhibitor rotenone complex II non-coupled respiration was measured. Inhibition of complex III by addition of antimycin A (2.5 μM) determines residual oxygen consumption (caused by enzymes which do not belong to the electron transfer system), which was subtracted from all respiratory states. Complex IV activity was achieved by adding *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (0.5 mM) and ascorbate (2 mM). Autoxidation rate was determined using sodium azide (≥100 mM). Complex IV respiration was additionally corrected for autoxidation (Pohland et al., 2016).

2.4. Citrate synthase activity

A subsample of the cell suspension was immediately frozen in liquid nitrogen and stored at −80 °C for assessing CS activity spectrophotometrically. A detailed description was previously published (Hagl et al., 2015; Pohland et al., 2016).

2.5. Amyloid-β 1–40 levels

For the detection of Aß 1–40 levels in cell lysates, a specific solid phase sandwich enzyme-linked immune-sorbent assay (ELISA) was used. Cells were harvested and lysed after 24 h. Protein concentration was determined to normalize the samples. The ELISA was performed according to the manufacture's instructions (Elabscience Biotechnology Inc., USA).

2.6. Real time qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden,

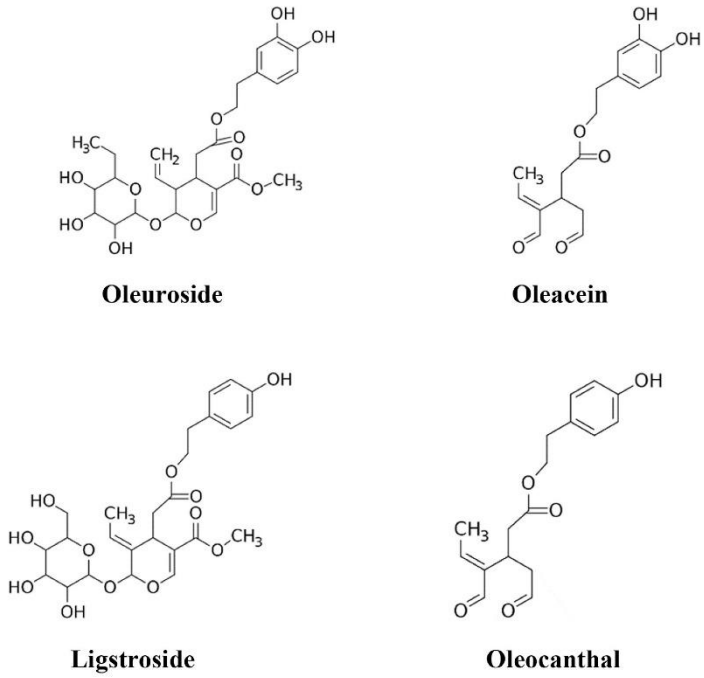

Fig. 1. Screening on ATP enhancing effects in SH-SY5Y-APP$_{695}$ cells led to the identification of four promising secoiridoid derivatives.

Germany) according to manufacturer's instructions from 500,000 cells and RNA Protect (Qiagen, Hilden, Germany) stabilized cells. RNA was quantified by measuring the absorbance at 260 and 280 nm using Nanodrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed using the ratio of absorbance 260/280 nm and 260/230 nm. To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 1 μg RNA using iScript cDNA Synthesis Kit (BioRad, Munich, Germany) according to the manufacturer's instructions and was stored at −80 °C. qRT-pcr was conducted using a CfX 96 ConnectTM system (BioRad, Munich Germany). Oligonucleotide primer sequences, primer concentrations and product sizes are listed in Table 1. All primers were received from biomol (Hamburg, Germany). We used a well-established SYBR Green based assay. According to the MIQUE guidelines, we completed a melt curve for every listed primer pair. Every primer pair resulted in one significant melt peak. Furthermore, for each primer pair we determined the efficiency of the assay, which in fact was between 90 and 110% and used the optimized concentration of each primer pair. Afterwards, all PCR products were checked on an agarose gel to ensure that the right product was amplified. cDNA for qRT-pcr was diluted 1:10 with RNase free water (Qiagen, Hilden, Germany) and all samples were analysed in triplicates. PCR cycling conditions were an initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 29 s. Gene expression was analysed using the −(2ΔΔCq) method using BioRad CfX manager software and were normalized to the expression levels of GAPDH, beta-Actin and PGK1 (Pohland et al., 2018).

2.7. Animals and treatment

Female NMRI (Navar Medical Research Institute) mice were bred in animal facilities and were maintained on a 12 h light/dark cycle until they reached the age of 12 months. At this time point mice were randomized into three groups (aged, aged + oleocanthal, aged + ligstroside). Young NMRI mice served as control group and were purchased from Charles River aged 3 weeks. Mice were fed with a well-proven C1000 standardized diet (Altromin, Lage, Germany, C1000 containing vitamin A (2500 IU/kg), vitamin E (20 mg/kg) and selenium (150 μg/kg). The verum group received the identical diet supplemented with 50 mg/kg diet oleocanthal or ligstroside for 6 months which equals a daily secoiridiod dose of 6.25 mg/kg b.w. The feeding period of the young control mice started 3 months later than the feeding period of aged mice to ensure that both of them end at the same time point. Behavioral testing was performed before the starting points and at the end of the feeding period. On the basis of the behavioral testing at the beginning, mice were divided into 3 groups of the same performance level. Mice were killed by cervical dislocation and decapitation. Brains were quickly dissected on ice after the removal of the cerebellum, the brain stem and the olfactory bulb. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regierungspraesidium Darmstadt; #V54 – 19 c 20/15 – FU/1062).

2.8. One-trial Y-Maze test

One trial Y-Maze-test was conducted using a custom-made Y-Maze

Table 1
Oligonucleotide primer sequences, product sizes and primer concentrations for quantitative real-time PCR; all primers were manufactured by Biomol (Hamburg, Germany) bp: base pairs, conc.: concentration.

Primer	Sequence	Product size (bp)	Melting point (°C)	Conc. (μM)
ACTB	5′-ggacttcgagcaagagatgg-3′ 5′-agcactgtgttggcgtacag-3′	234	84.5	200
ATP5D	5′-ggaagctcctcctcagcttt-3′ 5′-caggctttccgggtctttaat-3′	198	84.5	200
CAT	5′-actttctggagcctacgtcct-3′ 5′-cgcatcttcaacagaaaggt-3′	200	79.0	150
COX5A	5′-gcatgcagacggttaaatga-3′ 5′-agttcctccggagtggagat-3′	152	91.0	200
CREB1	5′-tggagttgttatggcatcct-3′ 5′-attttcaagcactgccactc-3	169	80.0	200
CS	5′-ccatccacagtgaccatgag-3′ 5-ctttgccaacttccttctgc-3′	186	83.0	400
GAPDH	5′-ctttgccaacttccttctgc-3′ 5′-ttgattttggagggatctcg-3′	238	81.5	200
GPx1	5′-gcttccagaccattgacatc-3′ 5′-gtgttcctcccctcgtaggtt-3′	170	84.5	400
CI	5′-cgccacctagcgtctctatc-3′ 5′-tgaaaatccggtcttcatcc-3′	213	78.5	200
NRF1	5′-gtaaccctgatggcactgtc-3′ 5′-tctggatggtcatctcacct-3′	183	78.0	200
PGC1-α	5′-tctcctggattgttgacatga-3′ 5′-tttgtcaggctgggggtagg-3′	20	76.0	200
PGK1	5′-ctgtgggggtatttgaatgg-3′ 5′-cttccaggagctccaaa-3′	198	82.5.0	200
SIRT1	5′-tgtggtagagcttgcattga-3′ 5′-gcctgttgctctcctcatta-3′	153	76.0	200
SOD2	5′-tgtcacccagtggtttttgt-3′ 5′-gccctgcaactaaacatcct-3′	152	78.5	100
TFAM	5′-tccccctcagttttgtgta-3′ 5′-atcaggaagttcectccaac-3′	189	77.5	400

(material: polyvinyl chloride, length of arms: 36 cm, height of arms: 7 cm, width of arms: 5 cm, angle between arms: 120 °). At the beginning of the test, the mouse was put into one of the three arms of the Y-Maze and the sequence of the entries was recorded for 5 min. Spontaneous alternation was determined using the formula (number of alternations/number of entries)/2 (Reutzel et al., 2018).

2.9. Preparation of dissociated brain cells for the measurement of mitochondrial membrane potential and determination of ATP level

One hemisphere was used to prepare dissociated brain cells (DBC's) for *ex vivo* studies according to the method of (Hagl et al., 2016). DBC's were re-suspended in 4.5 ml DMEM without supplements. For the measurement of ATP levels, DBC's were seeded in 50 μl aliquots into a 96 well plate. Cells were incubated for 3 h in a humified incubator (5% CO_2). Respectively 6 wells were incubated for 3 h with sodium nitroprussid (0.5 mM for ATP measurement) in DMEM. The remaining cell suspension was reserved for protein determination (stored at −80 °C) (Reutzel et al., 2018).

2.10. Protein quantification

Protein content was determined using PierceTM Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Instructions were followed as given by the manufacturer (Reutzel et al., 2018).

2.11. Manufacturing and analysis of olive polyphenols

All substances were manufactured from N-Zyme BioTec GmbH (Darmstadt, Germany) through isolation from young olives, EVOO or enzymatically/chemically transformed. Verification of the isolated olive polyphenols or metabolites were accomplished by means of mass spectrometry and NMR analysis or with reference standard (Table 2). Further information on the production and purification of the substances can currently not be provided due to an ongoing patent procedure. For the animal study, oleocanthal was isolated from olive oil and ligstroside from privet leaves. Oleocanthal concentrate in 54.9% (v/v) ethanol had a purity of 16.5%. The obtained ligstroside concentrate in 38.8% (v/v) ethanol reached a purity of 74.3%. Both, analysed by RP-HPLC.

Table 2
Purification method, purity and identification of all tested olive polyphenols.

Substance	Purification	Purity (%)	Identification
Oleuropein	FCPC, HPLC	96	Reference
Ligstroside	FCPC	96	MS, NMR
Hydroxytyrosol	FCPC	98	Reference
Tyrosol	FCPC	98	Reference
Oleuroside	FCPC, HPLC	97	MS, NMR
Ligustaloside B	FCPC, HPLC	100	MS, NMR
Oleacein	HPLC	96	MS, NMR
Oleocanthal	FCPC	96	MS, NMR
Elenolic acid	FCPC, HPLC	>92	MS, NMR
Oleuropein aglycon	FCPC	95	MS, NMR
Ligstroside aglycon	FCPC, HPLC	96	MS, NMR

FCPC (Fast Centrifugal Partition Chromatography); HPCL (High Performance Liquid Chromatography); MS (Mass-Spectrometry); NMR (nuclear magnetic resonance spectroscopy).

2.12. Statistics

Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Tukey's multiple comparison post-hoc or unpaired Student's *t*-test (Prism 8.0 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for p values of <0.05.

3. Results

To characterize the cellular model of early-onset AD, we investigated differences in mitochondrial function between SH-SY5Y-MOCK vector transfected control cells and the corresponding transgenic cell line SH-SY5Y-APP_{695} harbouring the neuronal form of human amyloid precursor protein. Additionally, we assessed effects of purified olive polyphenols (hydroxytyrosol, tyrosol, oleacein, oleuroside, oleuropein, oleuroside aglycon, oleocanthal, ligstroside, ligstroside aglycone, ligustaloside B) and two metabolites (the plant metabolite elenolic acid and the mammalian metabolite homovanillic acid) on mitochondrial function in SH-SY5Y-APP_{695} cells.

4. *In vitro* investigations

4.1. Mitochondrial dysfunction in SH-SY5Y-APP_{695} cells

A significant reduction of oxygen consumption was present across all measured complexes in SH-SY5Y-APP_{695} cells (Fig. 2a). The respiratory control ratio (RCR) indicates the coupling between oxygen consumption and oxidative phosphorylation (Hughey et al., 2011). RCR was calculated as ratio between uncoupled respiration and leak respiration after addition of oligomycin. The significant lower RCR in SH-SY5Y-APP_{695} cells indicates a reduced ability to couple mitochondrial respiration and phosphorylation – indicating mitochondrial dysfunction (Fig. 2 (c)). Consequently, SH-SY5Y-APP_{695} cells showed a significant reduced ATP production compared to control cells (Fig. 2 (b)).

To elaborate underlying molecular mechanisms, the expression of genes involved in mitochondrial biogenesis, mitochondrial function and antioxidative properties were determined in SH-SY5Y-MOCK control cells and SH-SY5Y-APP_{695} cells. Gene expression of complex I, which is

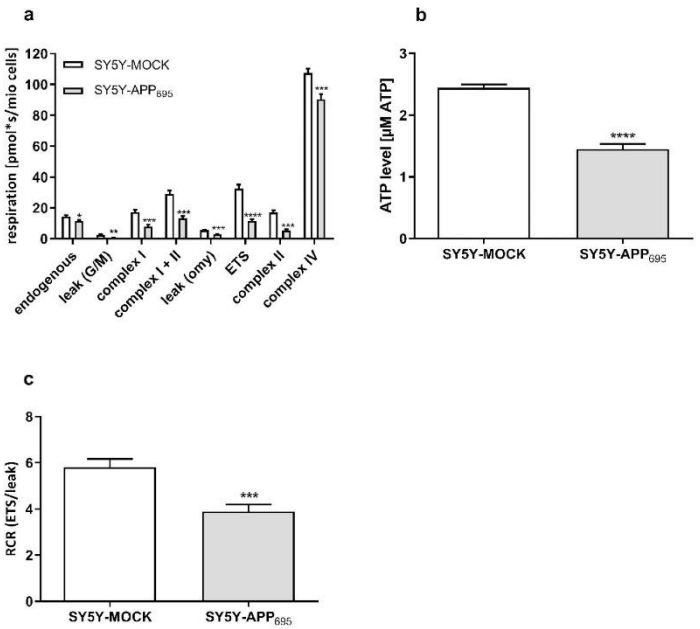

Fig. 2. (a) SH-SY5Y-APP$_{695}$ cells showed an overall reduction of all measured respiration states. For analyzing mitochondrial respiration, a solution of 10^6 cells/ml was used; mean ± SEM; student's unpaired *t*-test (*p < 0.05, **p < 0.01, ***p <0.001, ****p < 0.0001); n = 12. (b) SH-SY5Y-APP$_{695}$ cells exhibit reduced ATP levels compared to SH-SY5Y-MOCK control cells. ATP levels were determined after 24 h seeding by bioluminescence assay; mean ± SEM; student's unpaired *t*-test (****p < 0.0001); n = 6 (c) SH-SY5Y-APP$_{695}$ cells have a significant lower RCR compared to SH-SY5Y-MOCK control cells, indicating a lower capacity for substrate oxidation and ATP turnover and a higher proton leak; mean ± SEM; student´s unpaired *t*-test (***p < 0.01); n = 10.

Table 3
Relative normalized mRNA expression levels in SH-SY5Y-APP$_{695}$ cells determined using quantitative real-time PCR in comparison to SH-SY5Y-MOCK control cells (100%); n = 15 mean ± SEM with student's unpaired *t*-test (*p < 0.05); results are normalized to the mRNA expression levels of actin-ß (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1).

	SH-SY5Y-APP$_{695}$
CS	97.1 ± 5.8
CREB1	84.4(↓) ± 5.2
SIRT1	93.0(↓) ± 6.1
PGC-1α	90.5(↓) ± 7.3
NRF1	100.0 ± 10.6
TFAM	82.4(↓) ± 6.3
KI	77.2↓* ± 6.8
COX5A	100.0 ± 6.2
ATP5D	102.4 ± 6.2
GPx1	72.3↓* ± 7.0
CAT	97.6 ± 13.1
SOD2	100.0 ± 7.8

mainly associated with brain ageing and neurodegenerative diseases (Fiedorczuk and Sazanov, 2018), was significantly reduced in SH-SY5Y-APP$_{695}$ cells. Additionally, mRNA levels of the antioxidative enzyme glutathione peroxidase 1 (GPx1) was significantly lower compared to SH-SY5Y-MOCK control cells. CREB1, SIRT1, PGC-1α and TFAM which are involved in the mitochondrial biogenesis pathway, were numerically but not significantly decreased in SH-SY5Y-APP$_{695}$ cells. mRNA expression levels of NRF1, COX5A, ATP5D, CS, CAT and SOD2 were unaffected compared to SH-SY5Y-MOCK cells (Table 3).

4.2. Screening of olive polyphenols for ATP production

Olive polyphenol screening of 10 purified secoiridoids and two metabolites was carried out measuring ATP levels in SH-SY5Y-APP$_{695}$ cells in order to identify compounds with the highest beneficial effects on mitochondrial function (separately shown in Supplementary Table 1). All tested compounds led to significant elevated ATP levels with the exception of ligstroside-aglycone and oleuroside-aglycone (Supplementary Table 1). Oleuroside (OLS), oleacein (OLA), ligstroside (LIG) and oleocanthal (OLE) had a slightly higher effect on ATP levels compared to the other secoiridoids and metabolites (Supplementary Table 1). For this reason, determination of mitochondrial respiration was performed exclusively for OLS, OLA, LIG and OLE. Fig. 3 shows the ATP levels of the four hit substances in a concentration of 0.05 µM.

As brain ageing is associated with a low activity of complex I (Stockburger et al., 2014), we simulated this alteration with the complex I inhibitor rotenone in SH-SY5Y-APP$_{695}$ cells (Supplementary Table 2). The insult with rotenone (25 µM) led to a significant decrease in ATP levels. All tested substances with the exception of ligstroside-aglycon, oleacein, and oleocanthal were able to protect the cells from the rotenone insult. Hydroxytyrosol was the most active compound in this assay and showed first significant effects at 0.05 µM (Supplementary Table 2).

4.3. Effect of OLS, OLA, LIG and OLE on mitochondrial respiration in SH-SY5Y-APP$_{695}$ cells

With the exception of OLS (0.05 µM), all substances elevated endogenous respiration in SH-SY5Y-APP$_{695}$ cells (Fig. 4 (a-d)) compared to solvent controls. OLE (0.05 µM) incubation also led to an increase of complex I and coupled complex I + II respiration (Fig. 4 (d)). Additionally, LIG incubated SH-SY5Y-APP$_{695}$ cells elevated leak I respiration and the electron transfer system (ETS) (Fig. 4 (c)).

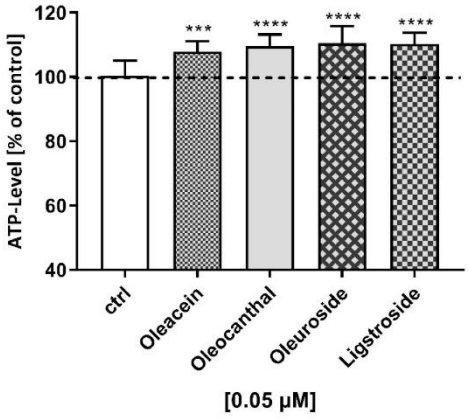

Fig. 3. ATP levels after 24 h incubation with 0.05 µM oleacein, oleocanthal, oleuroside or ligstroside increased ATP levels significantly compared to solvent control (EtOH). Cell culture medium served as control for normalization (100%). Data are represented as mean ± SEM; one-way ANOVA with Tukey's multiple comparison post-hoc test (***p < 0.001, ****p < 0.0001); n = 9.

4.4. Citrate synthase activity in OLS, OLA, LIG and OLE incubated SH-SY5Y-APP$_{695}$ cells

Citrate synthase activity (CS) is a known marker for mitochondrial content (Larsen et al., 2012). CS was significantly increased after incubation with LIG or OLE (Fig. 5 (c) and (d)). Treatment with OLA or OLS revealed no changes of CS (Fig. 5 (a) and (b)).

4.5. Analysis of mRNA in OLE and LIG incubated SH-SY5Y-APP$_{695}$ cells

The mRNA expression of the antioxidant enzymes CAT and SOD2 were significantly decreased after incubation with both, OLE and LIG in SH-SY5Y-APP$_{695}$ cells whereas LIG incubation led to a significantly increased mRNA expression of GPx1 compared to solvent control (Table 4). In contrast, CREB1 and SIRT1 were significantly increased after LIG incubation. Moreover, LIG treated cells showed numerical elevations of PGC-1α, NRF1 and TFAM gene expression compared to control cells (Table 4).

4.6. Influence of OLE and LIG on Aβ 1–40 levels in SH-SY5Y-APP$_{695}$ cells

Aβ 1–40 levels were significantly decreased after 24 h incubation with oleocanthal. Ligstrosid did not show any effect on Aβ levels (Fig. 6).

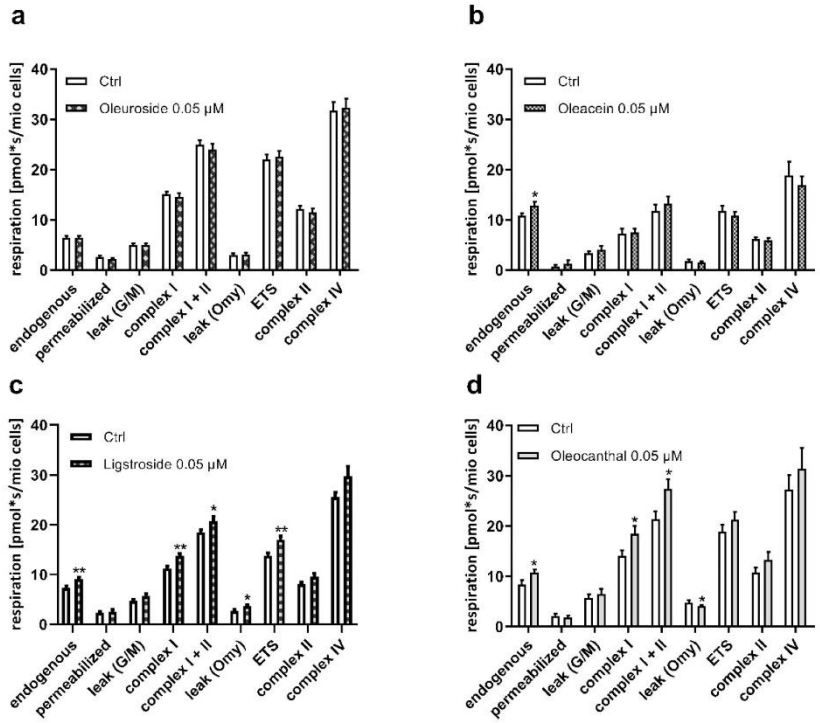

Fig. 4. Mitochondrial respiration in SH-SY5Y-APP$_{695}$ cells after 24 h incubation with the respective olive polyphenol (0.05 µM) or solvent control (EtOH). A solution containing 10^6 cells/ml was used for analyzing oxygen capacity. (a) OLS had no influence on mitochondrial respiration. (b) OLA elevated endogenous respiration. All other complexes were unaffected. (c) LIG treatment led to significant elevation of endogenous, complex I, coupled complex I + II, leak I (omy) respiration and increased capacity of the electron transfer system (ETS). (d) Incubation with OLE elevated endogenous, complex I and coupled complex I + II respiration. Data are represented as mean ± SEM; student's unpaired *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001); n = 13.

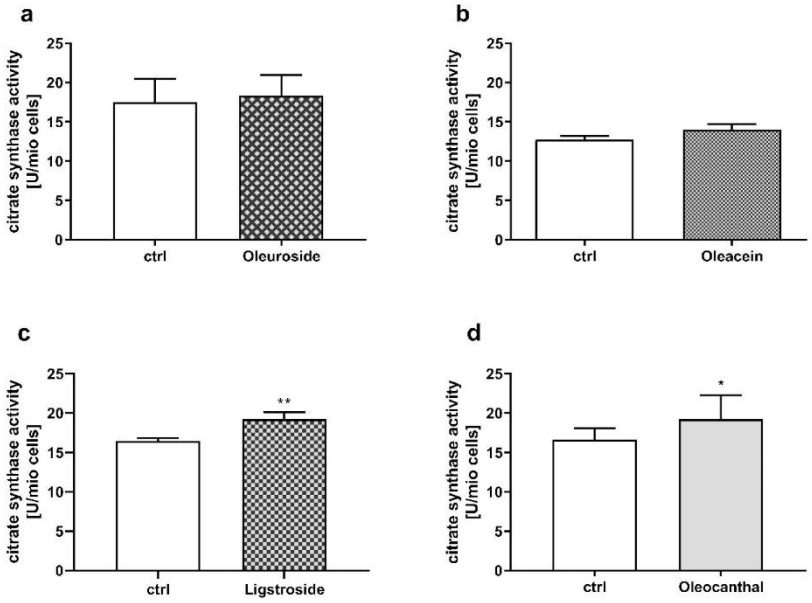

Fig. 5. Citrate synthase activity of SH-SY5Y-APP$_{695}$ cells after 24 h incubation with (a) oleuroside (b) oleacein (c) ligstroside or (d) oleocanthal and EtOH control. Data are represented as mean ± SEM; student's unpaired *t*-test (*p < 0.05, **p < 0.01); n = 13.

Table 4
Relative normalized mRNA expression levels in SH-SY5Y-APP$_{695}$ cells after oleocanthal or ligstroside treatment determined using quantitative real-time PCR in comparison to solvent control (100%); n = 7 mean ± SEM with one-way ANOVA and Tukey's multiple comparison post-hoc test, (↑/↓) trend to increase/decrease; (*p < 0.05, **p < 0.01, ***p < 0.001); results are normalized to the mRNA expression levels of actin-ß (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1).

	Ligstroside	Oleocanthal
CS	63.6 (↓) ± 13.5	99.3 ± 15.7
CREB1	137.2↑* ± 12.9	88.5 ± 15.3
SIRT1	175.2↑** ± 17.7	70.1 ± 15.5
PGC-1α	138.9(↑) ± 29.1	113.7 ± 32.6
NRF1	138.6(↑) ± 20.7	75.3 ± 13.2
TFAM	125.4(↑) ± 20.4	78.8 ± 19.3
KI	177.2↑* ± 25.2	51.4 ± 26.3
COX5A	95.4 ± 9.6	65.1 ± 16.0
ATP5D	58.8 ± 24.0	80.1 ± 17.7
GPx1	163.0↑** ± 19.7	65.8 ± 18.6
CAT	66.6↓* ± 15.2	54.1↓* ± 19.9
SOD2	50.2↓*** ± 11.3	62.3↓* ± 13.6

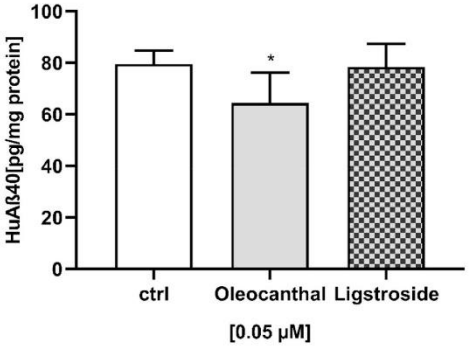

Fig. 6. Human Aβ 1–40 level in SH-SY5Y-APP$_{695}$ cells after 24 h incubation with EtOH as control, oleocanthal or ligstroside. Cell were lysed and Aß levels were determined by ELISA, and normalized to protein. Data are represented as mean ± SEM with one-way ANOVA and Tukey's multiple comparison post-hoc test; (*p < 0.05); n = 8.

5. *In vivo* investigations

The two lead substances OLE and LIG were further tested in a mouse model of ageing (Reutzel et al., 2018) regarding cognition, survival, and energetics.

5.1. Behavioral testing

Aged control mice showed a significantly reduced number of alternations (11.2 ± 0.96) during a 5 min time of testing compared to young control animals (17.96 ± 0.90) in the Y-maze test (Fig. 7 (a,b)). Aged mice fed with OLE and LIG showed a significantly increased number of alternations (15.86 ± 0.95 and 15.29 ± 1.24). However, the alternation rate was numerically but not significantly increased after 6 months of supplementation.

5.2. Life-span

Survival rates of aged mice were 67% while the survival rate of aged mice fed with OLE for 6 months showed no significant changes (70%) (Fig. 7 (c)). Mice fed with LIG showed a significantly increased life expectancy compared to aged control animals (85%; **p < 0.01).

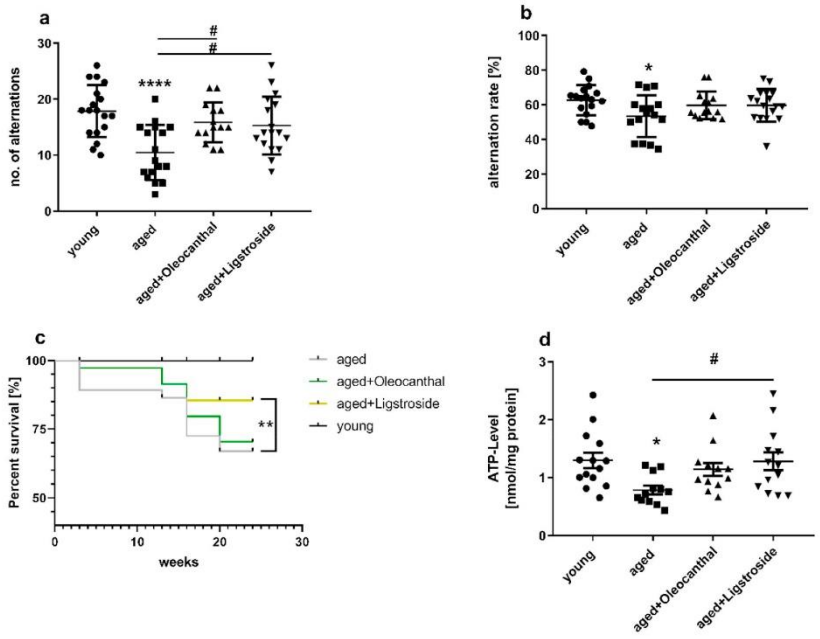

Fig. 7. (a) and (b) Y-Maze spontaneous alternation of young, aged and OLE or LIG treated mice (6.25 mg/kg b.w.) during a 5-min period time of testing. Alternation rate (b) and number of alternations (a). (c) Survival rates of NMRI mice after feeding with OLE or LIG. Aged (12 months old) mice were fed with a standardized pelleted diet containing OLE or LIG (6.25 mg/kg b.w.) for 6 months. (d) Basal ATP level of dissociated brain cells (DBC's) from young, aged and OLE or LIG treated mice; n = 13; mean ± SEM; one-way ANOVA with Tukey's multiple comparison post-hoc test; *p < 0.05 *vs.* young control animals; # one-way ANOVA aged *vs.* aged + LIG treated mice with #p < 0.05; as further control, young mice (3 months old, starting point of the analysis at week 12) were fed with a pelleted standard diet for 3 months (young); n = 18; mean without SEM; log-rank (Mantel-cox) test; *p < 0.05; n = 16 mean ± SEM, one-way ANOVA with Tukey's multiple comparison post-hoc test; *p <0.05; ****p < 0.0001 and # ANOVA against aged mice with #p < 0.05.

5.3. Cerebral ATP levels

Basal ATP levels were measured in dissociated brain cells of young, aged, and aged mice fed with OLE or LIG (6.25 mg/kg b.w) for 6 months (Fig. 7 (d)). Aged control animals showed significantly reduced brain ATP levels (0.82 ± 0.05 nmol/mg protein) compared to young control animals (1.18 ± 0.01 nmol/mg protein). OLE fed mice showed numerically increased ATP levels (1.14 ± 0.11 nmol/mg protein) whereas mice which received the LIG supplemented diet showed significantly increased brain ATP levels (1.28 ± 0.15 nmol/mg protein) compared to young control animals.

6. Discussion

In the current study, we tested purified phenolic olive secoiridioids in a cellular model for the initial phase of sporadic AD (Stockburger et al., 2014) and focused on two lead substances in a follow up study in aged female NMRI mice for 6 months, which represents a well-established model for brain ageing (Gower and Lamberty, 1993; Hagl et al., 2016; Reutzel et al., 2018).

6.1. Effects of purified olive secoiridoids on mitochondrial function in SH-SY5Y-APP$_{695}$ cells and brains of aged NMRI mice

Mitochondrial dysfunction is associated with a decline of cellular ATP levels and has been reported to be one of the characteristics of an early stage of AD (Beck et al., 2016). ATP as a high energy molecule is mainly produced in mitochondria by the oxidative phosphorylation system (OXPHOS) which is made up of five large protein complexes located in the inner mitochondrial membrane. In this study, we detected a decrease in the respiratory capacity of the respiration chain and a subsequent decline of ATP levels in SH-SY5Y-APP$_{695}$ compared to control cells which is in line with earlier published data (Stockburger et al., 2014). All investigated olive secoiridoids, except the two tested aglycones, significantly increased cellular ATP levels. Since a decline of the mitochondrial bioenergetic system is a hallmark of both, AD and brain ageing these findings may reveal a positive influence of olive secoiridoids on the cellular energy metabolism and probably in the prevention of mitochondrial dysfunction. In accordance to these results, LIG and OLE incubation led to a significant improvement of mitochondrial respiration, whereas OLS and OLA showed no changes in oxygen consumption, despite measuring an increase in ATP levels. This finding may indicate a shift of metabolism towards upregulated glycolytic ATP production to compensate for declining mitochondrial respiration and OXPHOS energy production. The conversion of energy production from OXPHOS to glycolysis was previously reported in late-onset AD (LOAD) fibroblasts and hippocampal neurons from 3xTg-AD mice (Sonntag et al., 2019; Yao et al., 2009). However, hydroxytyrosol, a well-studied olive polyphenol, improved complex I, II, III and IV activity in adipocytes (Hao et al., 2010). Furthermore, Martins et al. determined oxygen consumption of a mitochondrial suspension with olive mill waste water, which led to an increase of complex IV respiration (Martins et al., 2008) and increased the mitochondrial membrane potential in dissociated brain cells (Schaffer et al., 2007). In addition, OLE and LIG significantly increased citrate synthase activity which is a well described marker for mitochondrial content (Larsen et al., 2012)

whereas OLS and OLA had no effect on this Krebs cycle enzyme. There are hardly no data available regarding the effects of olive polyphenols on citrate synthase activity in neuronal cells. However, olive oil fed rats did not show any effects on mitochondrial mass in muscle homogenate (Bronnikov et al., 2015), which is consistent with our results of OLA and OLS. Nevertheless, incubation with LIG and OLE led to a significant increase of CS in SH-SY5Y-APP$_{695}$ cells. This could be an indication of an beneficial effect on mitochondrial biogenesis, which is known to be impaired in both, ageing and AD (Golpich et al., 2017; Srivastava, 2017; Sheng et al., 2012). Peroxisome-proliferator receptor gamma coactivator 1-α (PGC-1α) is an important key transcription factor of mitochondrial biogenesis which is activated by deacetylation by sirtuin 1 (SIRT1) or phosphorylation *via* AMP-activated proteinkinase (AMPK). PGC-1α itself activates nuclear-respiration factor 1 (NRF1) and mitochondrial transcription factor (TFAM) which induces mitochondrial biogenesis (Cantó and Auwerx, 2009; Fernandez-Marcos and Auwerx, 2011). In SH-SY5Y-APP$_{695}$ cells, genes of the mitochondrial biogenesis pathway tend to be downregulated compared to control cells (see Table 3). In order to determine the molecular mechanism of the two lead substances, mRNA expression was determined after 24 h incubation with OLE and LIG. Concerning the influence on genes of mitochondrial biogenesis, only LIG could be considered as a potential candidate to induce mitochondrial replication *via* the PGC-1α cascade, due to the significant upregulation of SIRT1, CREB1 and numerical increase of PGC-1α, TFAM and NRF1. Previously, our group reported no influence on mRNA expression in mice, which received a mixture of purified olive polyphenols (oleuropein-aglycon, hydroxytyrosol, oleacein, elenolic acid derivates, oleuropein and oleuroside) for 6 months (Reutzel et al., 2018). On the other hand, it has been shown, that mice receiving hydroxytyrosol (10 or 50 mg/kg per day) for 8 weeks, led to an improvement of protein expression of SIRT1 and PGC-1α in brain tissue (Zheng et al., 2015). Other polyphenols like resveratrol, quercetin and curcumin have been reported as potential activators of mitochondrial biogenesis (Calabriso et al., 2018; Diaz-Gerevini et al., 2016; Qiu et al., 2018). In muscle cells, oleuropein showed a significant increase of mRNA levels of TFAM and NRF1 (Kikusato et al., 2016). Nevertheless, the influence of olive polyphenols on mitochondrial mRNA expression is barely investigated. Previous studies described OLE as a potential candidate to clear Aβ levels in mice brain and reduce Aβ levels in SH-SY5Y-APP cells (Abuznait et al., 2013; Batarseh et al., 2017; Qosa et al., 2015). As ligstroside did not affect Aβ 1–40 levels, the beneficial effect on mitochondrial function might be independent of the Aβ-pathway. These findings additionally flaw the Aβ-hypothesis and focus on new mechanisms (Kuehn, 2020). In contrast to OLE, LIG proves to be a novel potential olive polyphenol that has not yet received much attention in the field probably due to the lack of data which is probably also due to the fact that the substance is not commercially available.

Previous studies have already shown direct and indirect mechanisms of antioxidative properties of olive polyphenols. Visioli et al. presented the direct binding and elimination of ROS by hydroxytyrosol and oleuropein in human neutrophils (Visioli et al., 1998). Omar et al. showed an increase in H_2O_2 treated SH-SY5Y cells for the imitation of oxidative stress (Omar et al., 2017). Oleuropein and hydroxytyrosol were able to increase the cell viability compared to non- incubated cells. Our experiments showed an elevation of GPx1 mRNA expression after LIG incubation, whereas the antioxidative enzymes CAT and SOD2 were downregulated. The influence of olive polyphenols on the antioxidant system was also observed *in vivo*. Brain tissue of 6 weeks OLE-rich EVOO treated SAMP8 mice showed a significant increase of GSH as well as a significantly increased SOD activity (Farr et al., 2012). Therefore, further investigations regarding the influence of purified secoiridoids on the antioxidative defence system should include the measurement of enzyme activities.

The lead compounds OLE and LIG were also investigated in a mouse model of ageing. Aged NMRI mice were fed with 50 mg/kg diet OLE or LIG for 6 months or a standardized pelleted diet (C1000, Altromin, Lage). Aged Mice fed with OLE did not show significant effects on longevity compared to aged control animals whereas mice which received LIG over 6 months had a significantly extended life-span. Comparable to previous studies, polyphenol rich diets seem to have a positive effect on lifespan (Stefanatos and Sanz, 2018; Asseburg et al., 2016; Kitani et al., 2007; Diomede et al., 2013).

Furthermore, OLE and LIG treated mice showed an enhanced performance in the one-trial Y-Maze test since they had significantly more numbers of alternations during a 5 min time period of testing compared to aged control mice, indicating an improved mobility of olive secoiridoid treated mice. In agreement with earlier studies, administration of olive polyphenols has been associated with positive effects on cognitive performance (Grossi et al., 2013; Pantano et al., 2017). Comparable to this study, Pitozzi et al. fed mice from middle age to senescence (total polyphenol dose/day of 6 mg/kg b.w.) led to an improved contextual memory and prevention of the age-related impairment in motor coordination (Pitozzi et al., 2012). However, since we did not measure the basal activity of mice we cannot exclude that an increase of activity contributes to the observed effects.

In accordance with the previous described results of LIG on ATP levels *in vitro*, LIG administration over 6 months resulted in a significant increase of brain ATP levels in aged mice compared to control animals. We have recently published that long-term feeding of a mixture of different purified olive polyphenols significantly increased basal ATP levels in brains of old NMRI mice (Reutzel et al., 2018). Although, LIG was not part of the applied polyphenols in this study (Reutzel et al., 2018) our current data show positive effects of LIG on energy levels in DBC's as pure confirming our *in vitro* results.

7. Conclusion

In this study, purified olive secoiridoids were tested on mitochondrial function *in vitro* and *in vivo* which has not been described yet. Although our study does not provide a causative mechanism on how secoiridoid derivatives interact in detail, the current results identified different effects on mitochondrial dysfunction and energy metabolism of the individual compounds present in EVOO. Specifically, LIG showed the highest potential to combat mitochondrial dysfunction in a cellular model of early AD and in a mouse model of ageing by mechanisms that may not interfere with Aβ production.

Data availability

The dataset generated during this study is available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2020.113248.

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3.4 . Effects of different standard- and special diets on cognition and brain mitochondrial function in mice. (IF=4.028)

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Effects of different standard and special diets on cognition and brain mitochondrial function in mice

Human nutrition plays an important role in prevention or at least slowing down the progression of age- and diet-related diseases. Thereby, mitochondrial dysfunction represents one comon underlying mechanism, which is being investigated in mouse models. However, the influence of the selected diets in preclinical studies on cognition and mitochondrial function has not yet been reported cohesively. Therefore, we present the results of three different studies that addressed this question. First, we investigated the influence of two standard control chow diets and a special diet low in antioxidants over 6 months in aged NMRI mice. Additionally, a 70% high-fat (HF) chow diet as well as a western-style diet (WSD) rich in lard and fructose were examined in C57/BL6 mice. Cognitive performance, mitochondrial function and bioenergetics in the brain were investigated. Moreover, cerebral expression of genes involved in biogenesis and antioxidant defence (citrate synthase, complex I, complex IV, SOD2, Cat1, GPx-1) were quantified. The results show that a modified, low antioxidant diet increased ATP levels in the brain of aged mice, while cognitive functions remained largely unaffected. A HF diet also showed significant effects on ATP levels and gene expression levels of relevant antioxidant markers, while the WSD had marginal effects on mitochondrial function and bioenergetics in the brain. Our results indicate that standard- and special diets have an impact on cognition and mitochondrial function in the brain. Thus, appropriate caution is warrented when selecting a suitable diet for preclinical studies in mice.

Keywords: Mitochondria, antioxidants, high-fat diet, ageing

55 **Introduction**

56 Age- and diet-related diseases, such as obesity, diabetes mellitus type 2, and neurodegenerative
57 diseases are a growing burden for healthcare systems in societies worldwide. Thereby, the impact of
58 nutrition on the progression but also on the prvention these age- and diet-related diseases is a topic of
59 great interest. In particular, mitochondria, which are the main synthesis site of energy production, are
60 potential targets for bioactive ingredients such as polyphenols and can therefore directly be
61 influenced by the diet. Several studies have shown the importance of cerebral antioxidants on the
62 activation of neuroprotective signal pathways includeing the improvement of mitochondrial function
63 [1,2]. Mitochondria, which are the main sites for the formation of reactive oxygen species (ROS)
64 ($O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$) through cell respiration itself, are protected in some ways from those highly
65 reactive molecules. Cellular mechanisms to keep ROS at a physiological level include the enzymes
66 catalase (CAT), superoxide dismutases (SOD2), glutathione peroxidases (GPX), and glutathione
67 reductase (GPR). Ascorbic acid is one of the best known antioxidants, which is involved in the
68 antioxidative defence system and can be synthesized by most mammals in the liver. However,
69 humans, other primates and guinea pigs, lack L-gulono-ɣ-lactone oxidase, so that these species are
70 dependent on an external supply. Plasma levels of 200-400 μM in adults should be maintained to
71 have a well-functioning antioxidant defence and it is known that the brain has the highest
72 concentration of ascorbic acid in the body [3]. Besides ascorbic acid, vitamin E, which consists of a
73 family of eight different chemical, lipid-soluble compounds, plays an important role in the
74 antioxidative defence system and in maintaining good cognitive performance in old age. Vitamin E is
75 comprising of four tocopherols and four tocotrienols (α-, β-, γ- and δ-isoform) whereas α-
76 tocopherol is the best bioavailable form [4]. The relevance of a sufficient supply of antioxidants for
77 normal brain- and memory functions is well described, whereby combinations of different
78 antioxidants, for example Vitamin C and E, are often reported to be limited to the desired effects
79 [5,6]. On the other hand, low antioxidant diets have already shown that they lead to an increase in
80 neuroinflammation and to deficits in cognitive abilities in mice [7]. Additionally, special diets,
81 including high fat (HF) diets, have been shown to have negative effects on mitochondrial function
82 which has been reported to be in a close connection to the induced insulin resistance, obesity and
83 several changes in the central nervous system [8]. Although animal models are extremely important
84 to study the fundamental basis of the physiological and pathophysiological role of cognitive function
85 and cerebral bioenergetics, the influence of the chosen standard or special-diet has not yet been
86 systematically reported for such kind of study. Thus, the effects of an antioxidant-reduced diet
87 compared to two standard diets on mitochondrial function and cognition in aged female NMRI mice

and of two different high fat diets on brain mitochondrial function in C57BL/6 mice were investigated.

Materials and methods

Aim of the studies

The aim of this investigation was to examine the effects of different standard- and special diets on brain bioenergetics including their effects on the ATP- and MMP-levels in dissociated brain cells of mice. In addition either mRNA data of brain homogenate or mitochondrial respiration and citrate synthase activity of isolated mitochondria were measured in order to elaborate the underlying molecular mechanisms.

Animals and Treatment

Study 1: Standard- and antioxidant reduced diets

Female NMRI mice (Navar Medical Research Institute) were born in the institute of pharmacology in Frankfurt a.M. and were kept in the animal facility until they reached the age of 12 months. At this time point, mice were randomly divided into three groups that received the following feeds for six months: two groups which received a standard diet (C1000, Acont) and one group which was fed with a vitamin C depleted and vitamin E reduced diet (C1000mod). At the beginning of the feeding period all three groups showed an equal performance in the Y-Maze-test. All mice had ad libitum access to drinking water or a pelleted diet based on their group (see diet consumption). Mice were sacrificed by decapitation at the age of 18 months. The brain was quickly dissected on ice after the removal of the cerebellum, the brain stem and the olfactory bulb. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regierungspraesidium Darmstadt; #V54 – 19 c 20/15 – FU/1062). The study design is presented as Fig. 7 in the supplements.

Diet consumptions of the tested diets:

The following table (tab 1) shows the diet consumptions of five different availaible diets from Altromin [9].

Table 1: Diet composition of the standard (C1000, A_{cont}) and special diets (C1000mod, C1000-70 and C1000-10) according to the supplier (Altromin, Lage, Germany).

	unit	C1000	$C1000_{mod}$	A_{cont}	C1000-70	C1000-10
Content	kcal/kg					
Total Energy	kcal/kg	3506	3506	3386	5385	3469
Macronutrients						
Fat	kcal/kg	457	457	367	3784	354
Protein	kcal/kg	691	691	768	829	827
Carbonhydrates	mg/kg	2358	2358	2091	771	2289
Moisture	mg/kg	82540	82540	113426	25303	63269
Crude Ash	mg/kg	54846	54846	58553	69406	43636
Crude Fibre	mg/kg	30970	30970	60744	56293	51668
Crude Fat	mg/kg	50830	50830	40791	420441	39325
Crude Protein	mg/kg	172650	172650	192111	207280	206670
Nitrogenfree extractives	mg/kg	608164	608164	534374	221277	595432
Monosaccharides	mg/kg	0	0	0	44931	15027
Disaccharides	mg/kg	98105	98105	47779	11865	117705
Polysaccharides	mg/kg	471727	471727	391216	115020	426602
Minerals/trace elements						
Calcium	mg/kg	9311	9311	7010	9285	6711
Potassium	mg/kg	7089	7089	8800	7155	4896
Magnesium	mg/kg	684	684	2220	751	566
Sodium	mg/kg	2488	2488	2128	4672	3936
Phosphorus	mg/kg	7523	7523	5014	7411	5556
Aluminium	mg/kg	3.71	3.71	79.37	7.82	9.45
Chlorine	mg/kg	3630	3630	3448	3630	2420
Iron	mg/kg	178.6	178.6	192.51	208.4	151.13
Flourine	mg/kg	4.17	4.17	2.80	4.17	2.78
Iodine	mg/kg	0.51	0.51	1.66	0.45	0.30
Cobalt	mg/kg	0.15	0.15	0.34	0.13	0.11
Copper	mg/kg	5.75	5.75	12.81	8.74	7.12
Manganese	mg/kg	100.89	100.89	95.06	102.62	69.38
Molybdenum	mg/kg	0.20	0.20	1.10	0.20	0.13

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Sulfur	mg/kg	2791.54	2791.54	1141.22	2673.48	2470.87
Selenium	mg/kg	0.33	0.33	0.25	0.23	0.17
Zinc	IU/kg	29.30	29.30	95.18	32.03	24.82
Vitamins						
Vitamin A	IU/kg	15000	2500	15000	15000	15000
Vitamin D3	mg/kg	500	500	600	500	500
Vitamin E	mg/kg	180	20	75	150	150
Vitamin K3	mg/kg	10	10	3	10	10
Vitamin B1	mg/kg	20	20	18	20	20
Vitamin B2	mg/kg	20	20	12	20	20
Vitamin B6	µg/kg	15	15	9	15	15
Vitamin B12	mg/kg	41	41	24	30	30
Niconinic acid	mg/kg	50	50	36	50	50
Pantothenic acid	mg/kg	50	50	21	50	50
Folic acid	µg/kg	10	10	2	10	10
Biotin	mg/kg	201	201	167	200	200
Choline chloride	mg/kg	1012	1012	600	1000	1000
Vitamin C	mg/kg	20	0	36	20	20
Amino acids						
Alanine	mg/kg	2528	2528	8292	9912	9951
Arginine	mg/kg	9829	9829	11175	14274	14189
Aspartic acid	mg/kg	3583	3583	15297	24974	24790
Cystine	mg/kg	3196	3196	3182	3456	3455
Glutaminc acid	mg/kg	23675	23675	38695	51803	51525
Glycine	mg/kg	3136	3136	8028	8291	8267
Histidine	mg/kg	5276	5276	4351	5758	5743
Isoleucine	mg/kg	7223	7223	7408	10591	10534
Leucin	mg/kg	14763	14763	13214	12224	12320
Lysine	mg/kg	17401	17401	8101	6678	6654
Methionine	mg/kg	7223	7223	2709	3456	3452
Phenylalanine	mg/kg	7172	7172	8221	7834	7836
Proline	mg/kg	12763	12763	12523	9686	9755
Serine	mg/kg	5268	5268	8965	10595	10565

6

Threonine	mg/kg	7154	7154	6399	8291	8262
Tryptophan	mg/kg	1977	1977	2342	3452	3420
Tyrosine	mg/kg	9285	9285	5849	7602	7594
Valine	mg/kg	3296	3296	8634	5533	5561
Fatty acids						
Arachidic acid C-20:0	mg/kg	50	50	35	1226	93
Eicosanoic acid C-20:1	mg/kg	150	150	44	680	56
Alpha-Linolenic acid C-20:1	mg/kg	150	150	2210	7155	512
Linolenic acid C-18:3	mg/kg	28500	28500	16152	54103	4236
Palmitic acid C-16:0	mg/kg	2500	2500	3878	46668	3396
Stearic acid C-18:0	mg/kg	1350	1350	1187	27259	1944
Oleic acid C-18:1	unit	13500	13500	6823	34724	2636

Fat source:

C1000: sunflower oil

A_{cont}: soybean oil

C1000-70: lard, concentrated butter, coconut oil, soybean oil

C1000-10: lard, cream

Study 2: Western-Style Diet study

Animal experiments with male C57Bl/6 mice (Janvier SAS, Le Genest-Saint-Isle, France) were carried out in a specific-pathogen-free barrier facility of the University of Vienna, Austria. All procedures were approved by the local institutional animal (BMBWF-66.006/0019-V/3b/2018) care and use committee and animals were handled in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Experiments were approved by the regional authority. Mice had always free access to tap water and were housed under controlled conditions. After adaption to the liquid diet as described previously [10] mice were

fed a liquid control diet (15.7 MJ/kg with 19kcal% protein, 12kcal% fat and 69kcal% carbohydrates, Ssniff, Soest, Germany) or a fat- and fructose-rich diet (17.8 MJ/kg with 15kcal% protein, 25kcal% fat and 60kcal% carbohydrates with 50% wt/wt fructose, Ssniff, Soest, Germany) for seven weeks. Mice were anesthetized with a ketamine/xylazine mixture (i.p.; ketamine 100 mg/kg BW + xylazine 16 mg/kg BW) at the end of the trial and prior to killing and tissue collection, blood was collected from portal vein. The brain was dissected on ice as described previously in [11]. The study design is presented as Fig. 9 in the supplements.

Table 2: Diet consumption of the control and and the fat- and fructose-rich diet according to the supplier (Ssniff, Soest, Germany).

	unit	**Control diet**	**Fat- and fructose rich diet**
Content			
Total Energy	kcal/kg	3749	4251
Macronutrients			
Crude Protein	kcal/kg	712	637
Crude Fat	kcal/kg	449	1062
Carbohydrate	kcal/kg	2586	2550
Crude Protein	mg/kg	174000	16000
Crude Fat	mg/kg	51000	118000
Crude Fibre	mg/kg	50000	22000
Crude Ash	mg/kg	42000	42000
Starch	mg/kg	346000	50000
Sucrose	mg/kg	110000	0
Glucose	mg/kg	0	50000
Fructose	mg/kg	0	500000
Minerals/trace elements			
	IU/kg		
Vitamin A	IU/kg	15000	15000

Vitamin D	mg/kg	1500	1500
Vitamin E	mg/kg	150	150

Fat source:

C: soybean oil

fat- and fructose-rich diet: Pork Lard

Study 3: HF- study

All animal experiments were conducted with the approval of the institutional animal welfare officer and the local veterinary department and in accordance with the German Animal Welfare Law. Experiments were approved by the regional authority (codes GI 20/11 No. G3/2017 and G48/2018) We acquired 40 male C57Bl/6J mice aged 5 weeks from Charles River (Sulzfeld, Germany). They were housed in individually ventilated cages in groups of five mice at a temperature of 22 ± 2 °C, 55 ± 10 % relative humidity, and a 14:10 h light/dark cycle. Tab water was provided ad libitum. Two different commercially available diets (C1090-70 and C1090-10; Table 1) were fed ad libitum in this study. The experimental high-fat diet (HFD) consisted of 70 % fat, 14 % carbon hydrates, and 16 % protein with an energy content of approximately 5.389 kcal/kg (diet C 1090 – 70). The matching control diet (CD) consisted of 10 % fat, 66 % carbon hydrates, and 24 % protein with an energy content of approximately 3.514 kcal/kg (diet C 1090 – 10). During two weeks of adaption to our animal facility, both groups of mice were fed the control diet. Afterwards, at 7 weeks of age, the animals were divided into an experimental (E) and a control (C) group. The experimental group was fed the HFD from 13 weeks (up to 20 weeks of age), followed by 3 weeks of CD (until 23 weeks of age), and another 4 weeks of HFD feeding (27 weeks of age). Thus, the dietary impact of a HFD was separated from a standard chow. The controls underwent no diet change. Sacrifices were carried out at 7, 20, 23, and 27 weeks of age in according to the dietary switches in the experimental group. A mixture of 1 mg Ketamine (Medistar, Ascheberg, Germany) and 0.2 mg Xylazine (Ceva, Düsseldorf, Germany) per 10 g body weight dissolved in NaCl 0.9 % was injected intraperitoneally for anesthesia. The abdominal wall was excised, and the blood drained by cutting the aorta and brains were quickly dissected on ice like described previously. The study design is presented as Fig. 8 in the supplements.

Passive Avoidance Test

The test was conducted using a passive avoidance step through system (cat. no. 40533/mice Ugo Basile, Germonio, Italy) and a protocol similar to the protocol published by Shiga et al. [12]. On the

first day of the experiment, the mouse was put into the light chamber (light intensity of 1350 lux). After 30 s, the door toward the dark chamber was opened and time till entering the dark chamber was measured. In the dark chamber, the mouse received an electric shock (0.5 mA, 1s duration). The test was stopped if the mouse did not enter the dark chamber after 180 s. The test was repeated after 24 h with a door opening towards the dark chamber after only 5 s. Again, time until entering the dark chamber was recorded. This time no electric shock was applied after crossing the door. The test was stopped after 300 s.

One-Trial Y-Maze Test

One trial Y-Maze-test was conducted using a custom-made Y-Maze (material: polyvinyl chloride, length of arms: 36 cm, height of arms: 7 cm, width of arms: 5 cm, angle between arms: 120°). At the beginning of the test, the mouse was put into one of the three arms of the Y-Maze and the sequence of the entries was recorded for 5 min. Spontaneous alternation was determined using the formula (number of alternations/number of entries)/2 [13].

Preparation of dissociated brain cells for the measurement of the mitochondrial membrane potential and determination of ATP level

One hemisphere was used to prepare dissociated brain cells (DBC's) for ex vivo studies according to the method of [30]. DBC's were re-suspended in 4.5 ml DMEM without supplements. For the measurement of ATP levels, DBC's were seeded in 50 µl aliquots into well plate. Cells were incubated for 3 h in a humified incubator (5 % CO2). Respectively 6 wells were incubated for 3 h with sodium nitroprussid (0.5 mM for ATP measurement) in DMEM. The remaining cell suspension was reserved for protein determination (stored at -80 °C).

Citrate synthase activity

Citrate Synthase Activity was measured photometrically in isolated brain mitochondria as described in Hagl et al [14].

Protein Quantification

Protein content was determined using PierceTM Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Instructions were followed as given by the manufacturer.

Isolation of Brain Mitochondria and measurement of mitochondrial respiration

Half a brain hemisphere (the frontal part) was used to isolate brain mitochondria. The protocol is described in Hagl et al. [14]. The pellet obtained from the last centrifugation step was dissolved in 250 µl MIRO5. A volume of 80 µl of the resulting cell suspension was injected into the Oxygraph 2k-chamber. The capacity of the oxidative phosphorylation (OXPHOS) was determined using complex-I related substrates pyruvate (5 mM) and malate (2 mM) and ADP (2 mM) followed by the addition of succinate (10 mM). Mitochondrial integrity was measured by addition of cyctochrom c (10 µM). Oligomycin (2µg/ml) was added to determine leak respiration (leak (omy)) and afterwards uncoupling was achieved by carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, injected stepwise up to 1-1.5 µM). Complex II respiration was measured after the addition of rotenone (0.5 µM). Complex III inhibition was achieved by addition of antimycin A (2.5 µM) and was subtracted from all respiratory parameters. COX activity was measured after ROX determination by applying 0.5 mM tetramethylphenylenediamine (TMPD) as an artificial substrate of complex IV and 2 mM ascorbate to keep TMPD in the reduced state. Autoxidation rate was determined after the addition of sodium azide (> 100 mM), and COX respiration was additionally corrected for autoxidation.

Gene expression analysis by quantitative real-time pcr (qRT-pcr)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using ~20 mg RNAlater stabilized samples (Qiagen, Hilden, Germany). RNA was quantified using SYBR Green® technology as previously described in [11]. Primer sequences are listed in table 3.

Table 3 Oligonucleotide primer sequences, product sizes and primer concentrations for quantitative real-time pcr; bp: base pairs, conc. Concentration

primer	sequence	manufacturer	product size [bp]	conc. [µM]

B2M	5'ggcctgtatgctatccagaa-3' 5'gaaagaccagtccttgctga-3'	Biomol, Hamburg, Germany	198	0,4
PGK1	5'-gcagattgtttggaatggtc-3' 5'-tgctcacatggctgacttta-3'	Biomol, Hamburg, Germany	185	0,4
SOD2	5'-acagcgcatactctgtgtga-3' 5'-gggggaacaactcaacttttt-3'	Biomol, Hamburg, Germany	183	0,1
GPx-1	5'-gtccagcgtgtatgccttct-3' 5'-ctcctggtgtccgaactgat-3'	Biomol, Hamburg, Germany	217	0,1
Complex I	5'acctgtaaggaccgagaga-3' 5'-gcaccacaaacacatcaaaa-3'	Biomol, Hamburg, Germany	227	0,1
Complex IV	5'-ctgttccattcgctgctatt-3' 5'-gcgaacagcactagcaaaat-3'	Biomol, Hamburg, Germany	217	0,1
CS	5'-aacaagccagacattgatgc-3' 5'-atgaggtcctgctttgtcct-3'	Biomol, Hamburg, Germany	184	0,1
CAT	5'-acttctggagcctacgtcct-3' 5'-cgcatcttcaacagaaaggt-3'	Biomol, Hamburg, Germany	200	0,1

231

232 *Statistics*

233 Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical
234 analyses were performed by applying one-way analysis of variance (ANOVA) with Tukey's multiple
235 comparison post-hoc test (Prism 8.0 GraphPad Software, San Diego, CA, USA). Statistical
236 significance was defined for p values of < 0.05.

237

238 ***Results***

239 *Study : Standard- and antioxidant reduced diets*

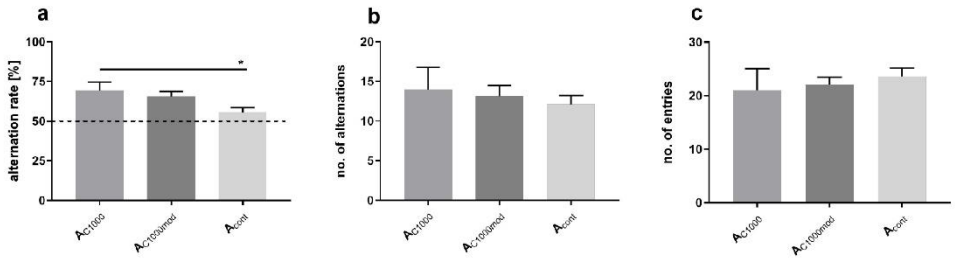
240 To assess the effect of a diet low in antioxidants, which is of great importance in the evaluation of
241 studies seeking to assess the influence of oxidative stress in mice, both cognitive abilities and
242 mitochondrial functions in the brain were investigated.

243

244 *Effects of long-term treatment with C1000, $C1000_{mod}$ and A_{cont} on spatial learning memory and*
245 *locomotor activity*

246 Behaviour was assessed using the Y-Maze spontaneous alternation test. Mice fed with the standard
247 C1000 diet showed a significant increase of the alternation rate (Fig. 1a) compared to aged mice, fed
248 with the standard A_{cont} diet indicating an improved short-term memory. Additionally, mice fed with
249 the standard A_{cont} diet had a numerically, but not significant decrease of the number of alternations
250 compared to mice fed a standard C1000 diet (Fig. 1b). Overall, the studies show that the choice of
251 standard diet may influence spatial learning memory and locomotor activity.

252



253

254 **Figure 1.** Y-Maze spontaneous alternation of mice fed with C1000, $C1000_{mod}$ or mice fed with
255 $Altromin_{cont}$ for 6 months during a 5-min period time of testing. Alternation rate [%] (a), number of
256 alternations (b), number of entries (c); n= 9 mean ± SEM, one-way ANOVA with Tukey's post-test;
257 $*p < 0.05$.

258 The fear-based passive avoidance test was used to asses the cognitive performance in mice [15]. On
259 day one, there were no significant differences in the time entering the dark chamber between all
260 groups. One day later, after mice have received a mild food shock a day before, all mice showed an
261 numerically enhanced latency to enter the dark chamber. However, the feed had no affect on the
262 long-term memory in NMRI mice (Fig. 2).

263

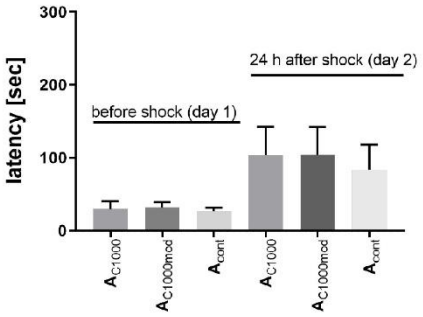


Figure 2. Passive avoidance test with mice fed a C1000, $C1000_{mod}$ or A_{cont} diet for 6 months. On day one, the mice received an electric food shock (0.5 mA) and the time was recorded that the mouse needs to enter into the dark chamber; 24 h after the first testing period the test is repeated and time is recorded that the mouse needs to re-enter the dark chamber; n= 9; mean ± SEM.

Effects of long-term treatment with C1000, $C1000_{mod}$ and A_{cont} on brain mitochondrial function

Basal ATP-levels were measured in dissociated brain cells of mice fed with C1000, $C1000_{mod}$ or A_{cont} (Fig. 3a). The $C1000_{mod}$ diet lead to a significant increase of basal ATP levels compared to C1000 fed animals (Fig. 3a). The mitochondrial membrane potential (MMP) did not show any differences between all three considered groups (Fig. 3b). Mitochondrial respiration was measured in isolated brain mitochondria from C1000, $C1000_{mod}$ or A_{cont} fed mice. (Fig. 3c). Citrate synthase activity, a well-established marker for mitochondrial mass, was determined in isolated mitochondria [16]. $C1000_{mod}$ fed mice showed a significant decrease in CS activity compared to mice which received the A_{cont} diet (Fig. 3d).

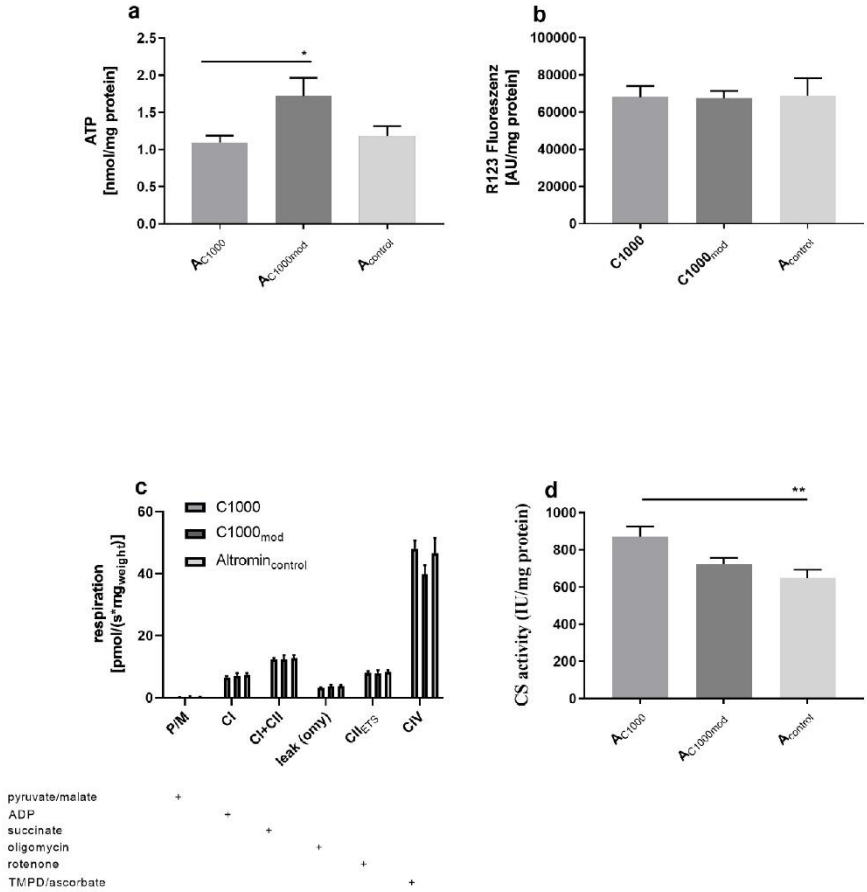


Figure. 3: Basal ATP- (a) and MMP (b)-levels in dissociated brain cells (DBCs) from C1000, $C1000_{mod}$ or A_{cont} fed mice; n =6; mean ± SEM; with one-way ANOVA and Tukey's post-test with *p < 0.05 compared to C1000 fed mice. (c) shows the brain wet-weight normalized mitochondrial respiration in isolated brain mitochondria of C1000, $C1000_{mod}$, and A_{cont} fed mice. n= 9, mean ± SEM. (d) represents citrate synthase (CS) activity in isolated mitochondria from C1000, $C1000_{mod}$, and A_{cont} fed mice. n= 8, mean ± SEM with one-way ANOVA and Tukey's post-hoc test and *p < 0.05 compared to C1000.

Effects of long-term treatment with C1000, $C1000_{mod}$ and A_{cont} on cerebral gene expression

Gene expression analysis was performed from brain homogenate of C1000, $C1000_{mod}$ and A_{cont} fed mice. Interestingly, the $C1000_{mod}$ fed mice showed a significant increase of complex I gene expression compared to young control animals (Tab. 4).

Table 4: Relative normalized mRNA expression levels in brain homogenate from C1000 fed mice, $C1000_{mod}$ fed mice or mice fed with $Altromin_{cont}$ diet determined using quantitative real time pcr in comparison to C1000 control animals; mRNA expression of C1000 control mice is 100 %; n = 9; mean ± SEM with one-way ANOVA and Tukey's post-hoc test with *p < 0.05 against C1000 control animals; results are normalized to the mRNA expression levels of beta 2 mikroglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

	$C1000_{mod}$	$Altromin_{cont}$
Complex I	172.9 ± 19.8*	84.9 ± 26.9
Complex IV	89.7 ± 6.1	138 ± 23.3
Citrate synthase (CS)	109.0 ± 16.6	78.7 ± 18.5
Catalase (CAT)	83.5± 12.0	89.4 ± 15.6
Glutathion Peroxidase 1 (GPx-1)	104.1 ± 13.3	107.7 ± 17.5
Superoxide Dismutase 2 (SOD2)	98.6 ± 11.3	94.5 ± 13.6

<u>*Study 2: HF- study*</u>

In addition to the use of low antioxidant diets, special diets designed to emulate the Western style diet play a major role in the treatment of non-communicable diseases such as obesity and type II diabetes mellitus [17], in particular a high-fat diet which was investigated in the following study in C57Bl/6 mice for mitochondrial brain function.

Effects of a special high fat diet in C57Bl/6 mice on brain mitochondrial function

Basal ATP- and MMP-levels were measured in DBC's from 7, 20, 23 and 27 weeks old C57Bl/6 mice. Until the age of 7 weeks, all mice were fed with the CD diet and did not show any difference in ATP or MMP levels (data not shown). After 13 weeks of HFD, mice exclusively showed significant elevated brain ATP levels whereas the MMP was unchanged (Fig. 4). The subsequent change of the HFD to CD diet for 3 weeks (see Fig. 7 supplements) led to an approximation of the basal ATP

levels compared to the control group and remained stable even after the second change to HFD for 4 weeks (Fig. 4).

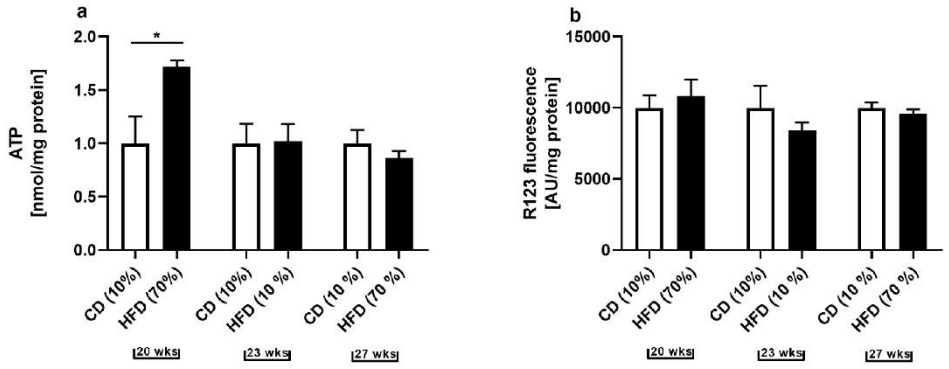


Figure 4. Basal ATP- (a) and MMP (b)-levels in dissociated brain cells (DBCs) from CD or HFD fed mice; n =6; mean ± SEM; unpaired t-test with *p < 0.05 compared to CD animals. Feeding period started at an age of 7 weeks until mice reached the age of 20 weeks. Afterwards, the HFD mice were fed with the CD for 3 weeks, followed by 4 weeks of HFD.

Gene expression analysis in C57Bl/6 mice fed with a special high fat diet

Gene expression analysis was performed in brain homogenate obtained from 20, 23 and 27 weeks old animals (for study details, see Fig 1). However, 13 weeks of HFD lead to a significant reduced mRNA expression of CIV, CS, and GPx-1. After 3 weeks of food change to CD, there was a still a significant reduction in CIV and numerically increases of CI, CS and GPX-1 compared to CD animals. After the second change to HFD for four weeks, CI and CAT mRNA gene expression were significantly increased after 27 weeks (Tab. 5)

Table 5. Relative normalized mRNA expression levels in brain homogenate from mice fed with HFD containing 70 % total fat using quantitative real time pcr in comparison to mice which received CD with 10 % total fat; mRNA expression of 10 % fat fed mice is 100 %; n = 5; mean ± SEM with unpaired t-test and *p < 0.05; **p < 0.01 against control mice; results are normalized to the mRNA expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1). mRNA expression was normalized to mice fed with the CD (100%).

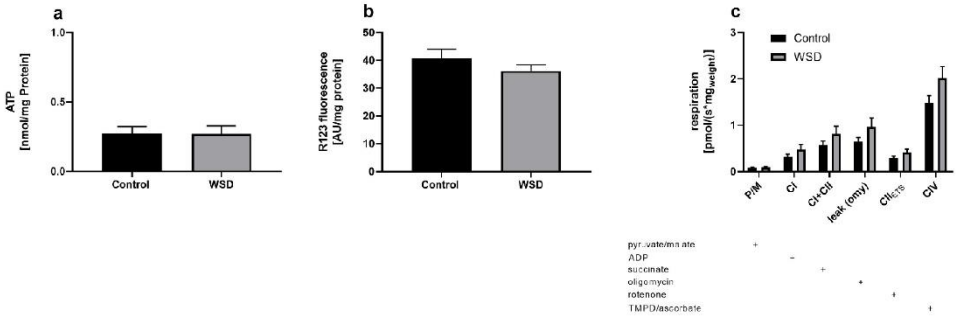
	20 weeks	23 weeks	27 weeks
Complex I	79.4 ± 24.6	145.0 ± 20.4	209.8 ± 51.1*
Complex IV	56.5 ± 5.5*	51.9 ± 8.1*	155.4 ± 38.7
Citrate synthase (CS)	45.0 ± 13.2*	103.5 ± 29.6	219.7 ± 51.6
Catalase (CAT)	88.5 ± 20.7	87.7 ± 3.0	165.4 ± 19.1*
Glutathione Peroxidase 1 (GPx-1)	56.0 ± 8.7**	93.7 ± 10.9	61.4 ± 17.2
Superoxide Dismutase 2 (SOD2)	87.1 ± 26.0	80.3 ± 9.6	114.8 ± 22.0

Study 3: Western-Style Diet study

The third study dealt with the effect of a fructose and lard based diet, which is a common means of producing the NAFL in mice [10]. This diet, which in addition tot he monosaccharide fructose also contains a high amount of saturated fatty acids, was also examined fort he effect on mitochondrial brain function in C57Bl/6 mice.

Effects of a lard and fructose rich diet in C57Bl/6 mice on brain mitochondrial function

Mice fed with a lard and fructose rich diet did not show any changes in brain ATP- and MMP-levels in DBC's (Fig. 5a; 5b). However, WSD-fed mice had a numerically increased respiration of all considered respiratory chain complexes in isolated mitochondria (Fig. 5c).



351 Fig. 5: Basal ATP- (a) and MMP (b) -levels in dissociated brain cells (DBC's) from Control or WSD
352 fed mice; n =4 for ATP and n=8 for MMP and n= 6 for mitochondrial respiration normalized to the
353 brain wet weight; mean ± SEM

354

355 **Discussion**

356 Nutrition and in particular the intake of bioactive compounds including antioxidants and its impact
357 on mitochondrial dysfunction is a current focus of research in the prevention and therapy of age- and
358 diet-related diseases. In this resprect, preclinical studies using feeding studies are an essential tool for
359 nutritional sciences. A closer look at the scientific literature revealed experimental designs in which
360 different basic diets were used in animal experiments. Up to now, the possible influence of standard
361 diets as well as of special diets, which are intended to simulate specific disease modalities, has not
362 yet been sufficiently examined regarding their effects on cognition and cerebral mitochondrial
363 function. By presenting relevant data from three different studies dealing with the above-mentioned
364 topics, we began to shed light on this poorly studied research question. Our data show that the
365 investigated parameters were affected to different degrees by the different diets.
366
367 The tested diets have different applications in research on rodents. While the C1000 and the A_{cont}
368 standard diets are established for general housing and for control, the $C1000_{mod}$ diet is a modified,
369 low antioxidant diet which is often used to test antioxidants that are incorporated into the product.
370 The 70% high fat diet is primarily used to generate obesity and for diabetes research, while the
371 lard/fructose rich diet is used to induce non alcoholic fatty liver (NAFL) in rodents [10].
372 In the context of the aging process, there is an imbalance between the formation and elimination of
373 ROS resulting in an increased damaged DNA, lipids and deficits in cellular transduction
374 mechanisms. The intake of ascorbic acid, one of the most important antioxidants in the brain, has
375 been described as positive for cognitive performance, especially in combination with other
376 antioxidants such as α-tocopherol. However, current data is controversial [18,19]. In the current
377 study, aged mice fed with a diet depleted of ascorbic acid and reduced amounts of vitamin A and C
378 only showed a marginally impairment in cognition compared to the other standard diets (Fig. 1a-c).
379 The existing data from *in vivo* and clinical studies on the effects of antioxidants on cognitive
380 functions seems to be not cleary proven yet. While some studies in vitamin E and C deficient mice
381 observed small effects on cognition [7] which in fact is in agreement with the moderate effects found
382 in our study, some clinical trials were not able to report any effects on cognitive functions. For
383 example, studies have shown an association between low vitamin C plasma levels and cognitive
384 impairments.The administration of 1000 mg/day vitamin C and 400 IU/day vitamin E in Alzheimer
385 patients was not able to slow down the progression of the cognitive impairments compared to the
386 control group which received a cholinesterase inhibitor [20].

Other studies, which exclusively investigated the effect of vitamin C on cognitive function in aged mice, could not find a consistent result. In addition, a gender-specific effect was identified in the current study, which is consistent with the missing effects on cognitive performance in this study with aged NMRI mice [21]. The vitamin E content of the modified $C1000_{mod}$ diet, which was tested in the long-term study over 6 months was reduced by 90 % compared to the C1000 standard diet. In accordance to the literature, vitamin E does not appear to have beneficial effects on cognitive functions in the Y-Maze- and the passive avoidance test [22]. Confirmatively, Farina et al. summarized in 2017 the effects of α-tocopherol on cognition in AD and mild cognitive impaired patients and were not able to find evidences of protective effects for the progression of the cognitive decline [22]. To investigate the effect of a reduced antioxidant intake on brain bioenergetics, basal ATP levels in dissociated brain cells were measured in the C1000 long-term study. However, a significant increase of ATP content was observed in the $C1000_{mod}$ group compared to the C1000 group. In our recently reported aging study, mice received $Altromin_{cont}$ feed during their entire lifetime. Due to the low antioxidant content in A_{cont}, one would expect an increase in cellular ROS levels which in fact have been reported to have inhibitory effects on the mitochondrial respiratory chain complexes [23]. However, the increased ATP levels could be a compensation mechanism to keep brain ATP levels stable and is in accordance to the monitored increase of mRNA expression of complex I in the $C1000_{mod}$ group. Another explanation would be an increase in mitochondrial biogenesis through an increased amount of antioxidants, due to their close connection in the cAMP/PKA pathway [24]. For example, it has been shown, that an increase of cellular oxidative stress can result in an increase in PGC1α expression which in fact is one of the most important regulators for mitochondrial biogenesis [11]. This hypothesis is strenghtend by the numerical increase in CS mRNA expression in the $C1000_{mod}$ group, whereby the activity of CS in isolated brain mitochondria remained unchanged at this time point (Fig. 4). The antioxidative defence system, assessed by the determination of the of SOD2, CAT and GPx mRNA expression seems not to be changed, although the supply of ascorbic acid and vitamin E was reduced for 6 months (Tab. 3). These observations have already been confirmed in feeding studies on chickens with vitamin C and E, where also only marginal effects on the antioxidative defence system have been described [25].

Additionally, we were able to examine two different HFD studies and investigated the effects on brain mitochondrial funtion. In summary, after 13 wks of a 70 % HFD mice showed significantly increased ATP, but not MMP level which can be a result of the increased energy which is provided by the citric acid cycle, and finally elevates of ketone body levels since β-hydroxybutyrate is able to produce 13 molecules ATP, whereas pyruvate only leads to 10 molecules ATP [26]. To the best of

our knowledge, the effects of a 70% HFD on mitochondrial functions have not yet been published. On the other hand, a lard and fructose rich diet did not show any significant effects on brain bioenergetics which is in accordance to the findings from Crescenzo et al. who only find marginal effects of a fructose rich diet in middle-aged rats on brain mitochondrial function [27].

In general, only a few studies have investigated the effect of high-fat diets on mitochondrial function, and the exact mechanism is not yet fully understood. Although it has been shown in mice that medium-term feeding with HF diets leads to increased neuroinflammation and decreased synaptic platicity in the brain and therefore this type of diet also appears to have a negative effect on brain mitochondrial function and the physiological aging process [27,28]. The fact that, in our studies, a fructose-rich diet did not show any significant influence on mitochondrial function is at least partly in contrast to the literature, which reported in particular on a negative influence of high fructose but not of glucose on mitochondrial function [29,30]. Since only monosaccharides were included in the high-fat diets, while disaccharides and polysaccharides were the main source of carbohydrates in all the other diets tested, this is possibly a variable which should be investigated further with regard to mitochondrial function and a possible reason for the different results of the study diets.
Taken together, a HFD seems to be able to change brain mRNA expression levels of genes which are associated with mitochondrial respiration, antioxidative defence system and mitochondrial mass. This might be an indication for inflammation, ROS production, and an impaired redox balance in brains of HFD fed mice. However, future studies have to adress this issue further.

Conclusion

When planning *in vivo* studies to investigate noncommunicable diseases, attention should be paid to the selected diet, since even different standard diets appear to have an influence on mitochondrial markers, which can influence the results of the experiment itself.

Data Availability

The dataset generated during this study is available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgement

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Supplementary

Study 1.

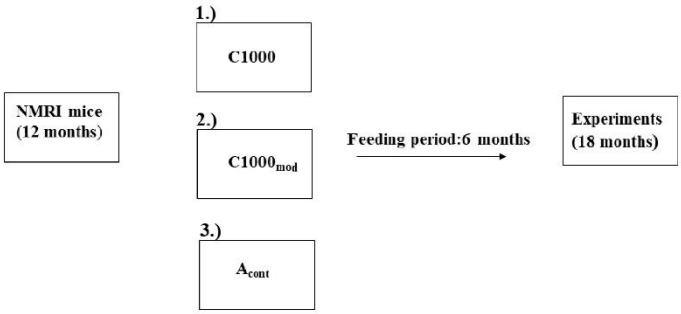

Fig 6: Animal study procedure of study 1. Female NMRI mice, aged 12 months, were divided into three groups of the same performance level in the Y-Maze spontaneous alternation test. Afterwards, mice were fed with the C1000, $C1000_{mod}$ or A_{cont} diet ad libitum until they reached the age of 18 months. All animals were sacrificed at the age of 18 months.

Study 2.

Figure 7: Animal study procedure. During the accommodation to the animal facility from 5 to 7 weeks of age, all mice were fed the control diet (CD). After that, they were divided into an experimental (E) and a control (C) group. From 7 to 20 weeks of age (13 weeks in total) the E group was fed the HFD, before being switched back to CD from 20 to 23 weeks of age (3 weeks). Eventually, it was switched back again to the HFD from 23 to 27 weeks (4 weeks). The C mice were fed the CD throughout the 27-weeks study period. Animals of both groups were sacrificed at 7, 20, 23, and 27 weeks of age.

Study 3.

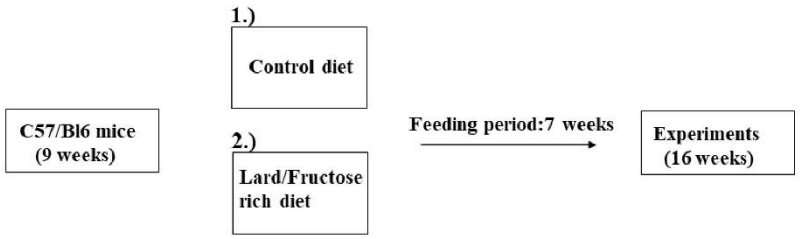

Figure 8: Animal study procedure of study 3. Male C57/Bl6 mice, aged 9 weeks, were divided into two groups. Afterwards, mice were fed to the control diet or the lard and fructose rich diet over 7 weeks. All animals were sacrificed at the age of 16 weeks.

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4. Discussion

We live in an increasingly aging society, which suffers not only from age-related diseases like AD, but increasingly also from nutrition-related diseases such as obesity, diabetes mellitus type-2 and cardiovascular diseases [126–128]. The increase in energy-dense, processed foods, which also contains a deficiency of important micronutrients, lead to obesity and diabetes, and might possibly accelerate the aging process and produce considerable changes at the cellular level. If we take the aging process as a measurable parameter, it should be possible to influence and slow down the aging process with a diet rich in bioactive substances [129]. In order to understand the influence of bioactive substances and micronutrients on the aging organism, the physiological aging process has to be characterized first at the molecular level [130,131]. Aging itself is a multifactorial process, which is characterized by many changes at the molecular level, as well as physiological and psychological changes [132,133]. To characterize the physiological aging process, the NMRI mouse is described as a well-established aging model in the literature [123,134,135]. As an outbred strain it has a gene pool that is as heterogeneous as possible and a median lifespan with 782 days, which is in fact shorter than reported from other commonly used mouse strains like the C57Bl/6J mice (901 days median lifespan) [125,136]. In this work the NMRI mouse was examined over a period of 24 months, which roughly reflects the mean life span of this mouse strain, for its cognitive abilities and the mitochondrial brain function at intervals of 6 months. In the current literature, the NMRI mouse is a common model that is used for age research [124,134,135], but so far there have been no studies that investigate the longitudinal course of cognitive and bioenergetic markers in combination. For studies that investigate the effects of bioactive substances on the aging process, mice aged 12 months are often used to start long-term feeding as they show a reliable mitochondrial dysfunction and cognitive deficits in many studies when they reach the age of 18 months [15,135,137]. If we now ask ourselves how the transferability of the aging process from mouse to human looks like, it has to be mentioned, that the maturational rate of mice does go simultaneously with humans. The aging process is up to 150 times faster, especially in the first month, which means that mice at the age of 3-6 months are considered fully grown. However, mice from 18-24 months are equal to humans from 56-69 years of age [138]. Especially substances that are able to prolong life span are therefore of great interest in preventive ageing research. While neither a long-term feeding of the aqueous extract with olive polyphenols nor the supplementation of oleocanthal

as a pure substance could lead to a life prolongation in aged NMRI mice, a feeding over 6 months with ligstroside was able to significantly extend the lifespan of the mice [16]. This observation seems to be in line with other studies that have already described life extension by polyphenol supplementation in rodents [139,140]. However, it is known that polyphenols undermine a high metabolism in the intestine by the microbiotics and the liver and that the positive, life-prolonging properties may well be due to the metabolites in the bloodstream [141–144]. With this finding, the focus could be put on ligstroside as a new nutraceutical, since it has not been described in the literature in connection with life extension yet. Especially oleuropein is often discussed to have a positive effect on the expression of genes associated with longevity via the mechanistic target of rapamycin (mTOR) pathway and subsequent Forkhead-Box-Protein O3 (FOXO3) and autophagy induction [145,146]. Although the life-prolonging effects of polyphenols are already known and partially elucidated [139,143] with ligstroside we have identified a new, promising substance for life prolongation. However, especially longitudinal data over lifetime in NMRI mice are rare and the data is focused on cognitive functions which already seem to decline between 9 and 12 months of age [124,134]. This is in agreement with the monitored deficits in the Y-Maze- and passive avoidance test in our current long-term study. Although not in the feeding study with a blend of highly purified olive secoiridoids and the C1000 study [15]. The feeding study with a mixture of olive secoiridoids over 6 months, showed significant changes in the Y-Maze test with 18 months compared to 3 months old control animals. Looking at the two studies in context, the first deficits in motor skills seem to appear from as early as 12 months of age and appear to be reliably observed with 18 months. The data suggests a limitation of cognitive abilities from the age of 12 months and there are only few studies that could not prove any effects in the passive avoidance learning at the age of 12 and 22 months [147]. Long-term feeding studies over 6 months in aged NMRI mice were carried out in order to assess the effect of special diets rich in bioactive olive polyphenols and, conversely, a low-antioxidant diet and two standard diets, on the course of cognitive abilities. It has been shown that a supplementation with a mixture of various olive secoiridoids (13.75 mg/kg b.w.) had positive effects on motor skills and short-term memory in the Y-Maze test compared to aged NMRI mice [15]. Deficits in the long-term memory could not be observed in this study. At this point, exclusively ligstroside turned out to have positive effects concerning the mobility of aged NMRI mice, as they showed a significantly higher number of alternations in the Y-maze test than old control animals. This observation is in line with previous studies, which were able to report a positive influence of olive polyphenols on cognitive abilities and

mobility [83,148,149]. A dose of 6 mg/kg b.w. polyphenols in the form of EVOO were able to achieve significant improvements in cognitive abilities in C57Bl/6J mice after a long-term feeding from middle-aged to senescence [149]. The effect of a diet containing a reduced amount of antioxidants has been investigated with regard to the influence on cognitive abilities during the physiological aging process. While some studies reported positive effects of cognitive abilities after supplementation of vitamin E or ascorbic acid, aged NMRI mice only showed marginally effects of cognitive skills if they received an antioxidant-depleted diet. The effects of vitamin E and C were often tested in combination, which did not slow the course of the disease after one year of treatment of AD patients [150]. Additionally, it is known that supplementation of vitamin C in old subjects did not lead to significant improvements in cognitive performance, which supports the monitored low effects found in the Y-Maze test in the C1000 Study [151]. The tested low antioxidant diet not only contained no vitamin C, but also showed only 10% of the vitamin E content compared to the investigated control diet. Our results from the behavioural tests cast doubt on the positive effects of vitamin E on cognitive abilities during the physiological aging process. This hypothesis is consistent with other studies that reported no or only marginal effects of α-tocopherol on cognitive abilities in MCI patients [99,152,153]. However, the administration of various tocotrienols has been reported to have potential positive effects on cognitive abilities, as they have an even higher antioxidant capacity than tocopherols [154–157]. Thus, a clinical study has already shown that the administration of 200 mg mixed tocotrienols over 2 years leads to a slower decline of the white matter in the brain, which is linked to an improvement in cognitive abilities [158,159]. In summary, it can be said that during the physiological aging process in the mouse brain, the first measurable deficits already occur at 12 months, which could significantly be improved after 6 months of feeding with a blend of highly purified olive secoiridoids and ligstroside, while the influence of a low-antioxidant diet had hardly any effects on the course of cognitive abilities during the physiological aging process.

The progress of the brain aging process was observed within the physiological framework, as well as the development after feeding with highly pure olive polyphenols and three special diets (antioxidant-depleted, high-fat and a western style diet). The focus was on the bioenergetic situation in the brain, such as the measurement of ATP and MMP levels in DBCs, as well as mitochondrial respiration in isolated brain mitochondria and citrate synthase activity. Furthermore, several markers of the antioxidative capacity (SOD2, CAT1 and GPx-1), mitochondrial biogenesis (PGC1-α, CREB1, AMPK, SIRT1, NRF1, TFAM) and cognitive

functions (SYP1, BDNF, GAP43) at the mRNA level were determined in brain homogenate of mice. It was shown that the universal energy supplier ATP in DBC's of 18 months old NMRI mice was significantly reduced compared to 3 month old control animals in the long-term study. This observation is in line with previous studies from our group [135] which monitored a decline of CI, CIV, CI+II and CII_{ETS} respiration of isolated mitochondria starting at the age of 12 months. This could also be confirmed by the group of Navarro et al. which reported in a long-term aging study of rats, in particular CI and CIV, to be susceptible to age-related deficits in hippocampal mitochondria [160]. In a further step, the effect of a mixture of different olive polyphenols as well as oleocanthal and ligstroside as pure substances in aged NMRI mice was investigated with respect to the energy levels in the brain. Both studies showed that the supplementation of olive polyphenols could significantly increase the energy levels in the brain of aged mice. The mixture of different olive polyphenols and ligstroside showed an increase in brain ATP levels compared to old control animals. This observation is consistent with the observed in vitro effects in SH-SY5Y cells after incubation with ligstroside. A significant increase in citrate synthase activity, mitochondrial respiration and upregulation of mitochondrial biogenesis was observed, which could indicate an increase in mitochondrial mass due to increased biogenesis via the SIRT1/PGC1-α axis. For olive polyphenols such as hytroxytyrosol, effects on PGC1-α and an increase in mitochondrial biogenesis have already been described in adipocytes [161]. Interestingly, a high-fat diet could significantly raise basal energy levels in the brain after 13 weeks of feeding, which may be a result of the higher energy supply. A high-fat diet, containing 70-80 % fat (ketogenic diet), imitates a long-term fasting phase and leads to an increase in ketone bodies, acetoacetate and β-hydroxybutyrate, and has been reported to be associated with an increase in ATP synthesis efficiency in muscle cells [162,163]. In the further course and the following changes to the control diet, an adjustment mechanism of the ATP levels occurred. (In order to be able)? to finally evaluate this observation, a determination of the ketone bodies in the further course would be of elementary importance. Surprisingly, MMP-levels seem to be stable during the aging process until mice reached the age of 24 months. It is known that during the physiological aging process the complexes of the respiratory chain lose activity, which is associated with a decrease in cellular energy provision. Even relatively small depolarizations of the MMP from -190 mV to -140 mV make the synthesis of ATP from ADP and P_i almost impossible [164]. However, a very mild depolarization of the inner mitochondrial membrane is essential for ATP production, but should be low enough to avoid the formation of ROS. In short-lived mice the aging process has been

shown to go along with an inactivation of depolarization mechanisms and an increase in ROS [165]. However, a decrease in MMP does not seem to occur simultaneously with the reduced ATP levels in the brain in the longitudinal aging study. The reduced brain MMP level of 24 months old NMRI mice appear to be consistent with the observed reduced complex activities, which already showed significantly deficits starting at an age of 12 months. In the following two studies with NMRI mice, no decrease in the MMP was observed and we therefore speculate that the biochemical changes in brain function with age seem to depend on the mouse strain under investigation [166,167]. Both the effect of a secoiridoid-rich, ligstroside rich diets and an antioxidant-poor diet over 6 months in NMRI mice showed no effects on the MMP. This result is in contrast to most studies with olive polyphenols regarding the effects on MMP. Studies in PC12- and PMA-stimulated endothelial cells have shown positive effects of hytroxytyrosol in a concentration of 10-50 µM on basal MMP levels [168,169]. However, the very complex metabolism and the low bioavailability of olive polyphenols often represents an obstacle for in vivo studies. However, the data suggests that hydroxytyrosol from olives also has positive effects on the MMP of isolated brain cells [170]. The missing effect on the MMP level after feeding with the low-antioxidant diet over 6 months is a result that should be further investigated. Since a diet low in antioxidants should result in an increase in cellular ROS levels and therefore lead to negative effects on the mitochondrial respiratory chain and the MMP. This seems to be an unclear mechanism. Studies with antioxidative substances like vitamin C and E or other antioxidative natural products have already shown positive effects on the MMP *in vitro* [171,172]. It has also been shown that a vitamin C deficiency leads to a decrease in the MMP level and an increase in ROS in cortices of mice, which makes antioxidative substances critical for mitochondrial health [173]. In addition, it was shown that a vitamin C and vitamin E deficient mice led to an increase in pro-inflammatory markers such as TNF-α and IL-6 in the hippocampus which is an indication of elevated oxidative stress [107,174].

The environmental and nutrition-related behaviour may influence the gene expression of relevant genes for mitochondrial biogenesis, neuronal plasticity and several markers for the antioxidative defence system [174–177]. For this reason, mRNA expression of relevant genes were determined during the physiological aging process, after long-term feeding with a polyphenol-rich diet, after feeding a vitamin C and E deficient diet and after being fed with two different high-fat diets. One of the most important findings during the physiological aging process with regard to mRNA expression in the brain of mice was, that some genes associated

with the induction of mitochondrial biogenesis, synaptic plasticity and complex IV of the mitochondrial respiratory chain does not seem to reach a maximum level at the age of 3 months. In comparison to the other considered genes, which already show first, non-significant decreases in gene expression at 6 months, a significant maximum of gene expression could be observed in CIV, CREB1, β-AMPK, TFAM and BDNF with 6 months, which decreased significantly with the ongoing aging process of the brain. While the age-related decrease in gene expression from relevant markers seems to decline with an age of 18 months [15,135], the longitudinal aging study showed for the first time that a maximum of gene expression appears to have been reached at the age of 6 months. This observation is of great relevance, since it should therefore be considered to use 6 instead of 3 month old NMRI mice as control animals in subsequent aging studies.

Contrary to the current literature, feeding NMRI aged 12 months with an extract rich in polyphenols, ligstroside or oleocanthal for 6 months could not cause a significant change in the mRNA expression pattern in the brain, while incubation experiments with ligstroside obviously showed clear effects on the gene expression of SIRT1/PGC1-α axis [16]. For example, the administration of oleuropein showed a positive effect on the gene expression of CREB1 in the brain of rats [178]. In addition to other biologically active substances, polyphenols in particular have shown that they have a positive effect in a hormetic interval and that they have no or negative effects on cellular processes in too low or too high concentrations [75,179]. For olive polyphenols, several studies have already demonstrated a hormetic dose response curve, which could possibly explain the low effects in our long-term feeding applications concerning the mRNA gene expression [180,181]. It is already known that olive polyphenols can be absorbed in the intestine, metabolised and then excreted in gluconiridated forms with the urine. In particular for hytroxytyrosol, tyrosol and some other non-polar olive polyphenols such as ligstroside-aglycone and oleuropein-aglycone, absorption studies have been published, while the exact mechanism of absorption does not seem to be fully understood [92,182,183]. The effects of a vitamin C and vitamin E deficient diet showed small effects in aged NMRI mice regarding mRNA expression in the brain. Only a significant increase of the gene expression of CI could be observed, which seems to be in agreement with the increased ATP synthesis in brains of mice fed with a vitamin C depleted and E deficient diet. A surprising result was that the antioxidant defence system also seems to be unaffected with regard to gene expression after 6 months feeding with olive polyphenols. These observations are contrary to the literature, since olive polyphenols in particular, such as hydroxytyrosol and oleuropein, are known free radical

scavengers and thus have positive effects on the mechanisms of antioxidant defence in the cell [184,185]. However, in 2019 the group of *Leskovec et al*. showed only marginal effects of olive extracts on the antioxidant stress parameters in piglets [186]. It could be speculated that the missing effects in our study on antioxidant defence are a result of hormetic response. Thus, the dose of 13.75 mg/kg b.w. polyphenols seems to be sufficient to have a positive influence on energy metabolism and cognition. However, the effects at the enzyme level can not be observed with the chosen dose, which needs to be clarified in further studies, since the amount we used was in the upper range of the usual supplementation of polyphenols in rodents [148,187–189]. In order to summarize the effects at the molecular level, it is necessary to clarify if the considered genes were regulated at the protein level, since the mRNA gene expression can only provide indications of this.

4.1. Future prospects

In this work, the aging process in NMRI mice was observed and described comprehensively on physiological and molecular levels over a period of 24 months. In this area, an analysis of other molecular mechanisms relevant to longevity, such as the mTOR pathway and the determination of telomere length, would be of interest. Based on the longitudinal data, the effects of different diets were investigated and their molecular mechanisms of action elucidated. When fed with high-purity olive polyphenols, it remains to be clarified which metabolites were responsible for the observed positive effects, since no analysis of the metabolites was possible in the context of this work. Especially at low plasma levels, an improvement of the bioavailability of these bioactive substances would be important. With regard to the tested standard and special diets, it is still necessary to clarify how basal ATP levels were increased during HFD and why there was no significant change in antioxidant enzyme complexes such as SOD2 and GPx-1 under low antioxidant diets. Finally, human studies confirming the positive effects of high-purity olive polyphenols, e.g. in PBMCs, are an indispensable option for future projects.

4.2. General Conclusion

In an ever aging society, which is increasingly suffering from age-related and diet-related diseases, it is of great importance to take a preventive approach to nutrition, which can have a positive influence on the physiological aging process. Since the physiological ageing process is accompanied by extensive changes at the mitochondrial level (MD), substances must be identified which can intervene in these extensive cellular processes. High-purity olive polyphenols, which were fed for 6 months, proved to be potential candidates that could positively influence the mitochondrial aging process. Our conclusion is that olive polyphenols may be a potential nutraceutical for the management of healthy aging that has yet to be proven in further studies. The focus should be on the bioavailability and the metabolism of these substances.The effect of HFDs and low antioxidant diets on mitochondrial brain function proved to be rather small in our studies, but it could be shown within the framework of these diets that it is essential for studies which focus on mitochondrial brain function to make a conscious choice of the diets used in order to ultimately collect meaningful data.

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„Der Weg ist es, der uns glücklich machen sollte, nicht das Ziel." (Unbekannt)

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hydroxytyrosol in rat brain slices subjected to hypoxia reoxygenation," *The Journal of nutritional biochemistry*, vol. 24, no. 12, pp. 2152–2157, 2013.

7. List of Publications

The following publications were published in peer-reviewed journals, the manuscripts are under review at peer -reviewed journals:

1. Cerebral Mitochondrial Function and Cognitive Performance during Aging: A Longitudinal Study in NMRI Mice. (IF=5.076)

Reutzel M, Grewal R, Dilberger B, Silaidos C, Joppe A, Eckert GP.

Oxid Med Cell Longev. 2020 Apr 13;2020:4060769. doi: 10.1155/2020/4060769

2. Purified oleocanthal and ligstroside protect against mitochondrial dysfunction in models of early Alzheimer's disease and brain ageing. (IF=4.691)

Grewal R, **Reutzel M**, Dilberger B, Hein H, Zotzel J, Marx S, Tretzel J, Sarafeddinov A, Fuchs C, Eckert GP. Exp Neurol. 2020 Jun;328:113248. doi: 10.1016/j.expneurol.2020.113248

3. Metabolic Fate and Distribution of 2´-Fucosyllactose: Direct Influence on Gut Microbial Activity but not on Brain.

Kuntz S, Kunz C, Borsch C, Vazquez E, Buck R, **Reutzel M**, Eckert GP, Rudloff S. Mol Nutr Food Res. 2019 May 24;63(13):e1900035. doi: 10.1002/mnfr.201900035

4. Effects of Long-Term Treatment with a Blend of Highly Purified Olive Secoiridoids on Cognition and Brain ATP Levels in Aged NMRI Mice. (IF=5.076)

Reutzel M, Grewal R, Silaidos C, Zotzel J, Marx S, Tretzel J, Eckert GP.

Oxid Med Cell Longev. 2018 Oct 30;2018:4070935. doi: 10.1155/2018/4070935

5. MH84 improves mitochondrial dysfunction in a mouse model of early Alzheimer's disease.

Pohland M, Pellowska M, Asseburg H, Hagl S, **Reutzel M**, Joppe A, Berressem D, Eckert SH, Wurglics M, Schubert-Zsilavecz M, Eckert GP. Alzheimers Res Ther. 2018 Feb 13;10(1):18. doi: 10.1186/s13195-018-0342-6

6. A diet rich in omega-3 fatty acids enhances expression of soluble epoxide hydrolase in murine brain.

Ostermann AI, **Reutzel M**, Hartung N, Franke N, Kutzner L, Schoenfeld K, Weylandt KH, Eckert GP, Schebb NH. Prostaglandins Other Lipid Mediat. 2017 Nov;133:79-87. doi: 10.1016/j.prostaglandins.2017.06.001

7. Effects of Long-Term Rice Bran Extract Supplementation on Survival, Cognition and Brain Mitochondrial Function in Aged NMRI Mice.

Hagl S, Asseburg H, **Heinrich M**, Sus N, Blumrich EM, Dringen R, Frank J, Eckert GP. Neuromolecular Med. 2016 Sep;18(3):347-63. doi: 10.1007/s12017-016-8420-z

8. Curcumin Micelles Improve Mitochondrial Function in a Mouse Model of Alzheimer's Disease.

Hagl S, **Heinrich M**, Kocher A, Schiborr C, Frank J, Eckert GP. J Prev Alzheimers Dis. 2014;1(2):80-83. doi: 10.14283/jpad.2014.2

Under review:

1. Effects of different standard- and special diets on cognition and brain mitochondrial function in mice

Martina Reutzel[1], Rekha Grewal[1], Carsten Esselun[1], Sebastian Friedrich Petry[2], Thomas Linn[2] Annette Brandt[3], Ina Bergheim[3], Gunter P. Eckert[1]* Nutritional Neuroscience (IF=4.028): Submitted: 06.07.2020

2. Metabolic fate and organ distribution of 13C-3´-sialyllactose and 13C-N-acetylneuraminic acid in wild-type mice – no preference for incorporation into the brain

Christina E. Galuskaa[b], Silvia Rudloff[c] , Sabine Kuntz[c], Christian Borsch[c], **Martina Reutzel**[c], Gunter P. Eckert[c], Sebastian P. Galuskaa[b],*, Clemens Kunz[c],* J Functional Foods