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Influence of carnitine isoforms and curcumin in models of Alzheimer's disease - Role of encapsulation in a nanoformulation

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"It's not how you fall, it's how you get up" – Lewis Hamilton

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ABSTRACT

Alzheimer´s disease (AD) is the leading cause of dementia and the most prevalent neurodegenerative disease around the globe. This pathology is characterized by two pathological hallmarks, the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and extracellular senile plaques formed by amyloid-beta peptide (Aβ) aggregates. AD predominantly affects hippocampal and cortical neurons. AD is a multifactorial disease associated with several biological dysregulations, including mitochondrial dysfunction, synaptic failure, and neuronal loss, as well as dysregulation of microglia and astrocytes, linked to neuroinflammatory processes. Specifically, $A\beta_{1-42}$ oligomers (AβO) have been described to increase mitochondrial calcium levels and depolarize the mitochondria. In addition, AβO can interact with mitochondria, with proteins involved in mitochondrial dynamics and function, exacerbating synaptic and neuronal damage in AD. Despite the identification of several pathological mechanisms, there is still no cure or effective neuroprotective therapies for AD patients. One molecule that has shown beneficial effects in rescuing mitochondrial dysfunction is L-Carnitine (LC) and its different forms, namely acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC). ALC has demonstrated good outcomes in rescuing mitochondrial abnormalities in rat hippocampal primary neurons exposed to AβO. Besides carnitines, curcumin (CUR) is another neuroprotective molecule due to its role in the clearance of AβO, having anti-inflammatory, and antioxidant properties. Based on these observations, this work aimed to evaluate the protective effects of LC, ALC, PLC and CUR, alone or in combination, in primary hippocampal and cortical neurons derived from APP/PS1 *versus* wild-type (WT) mice and Wistar rat neurons exposed to $A\beta O_{1-42}$, and further produce and characterize a liposomal formulation containing LC. We showed that, both ALC and PLC presented beneficial effects in ameliorating mitochondrial dysfunction, as determined by changes in mitochondrial membrane potential, and cell reducing capacity, an index of cell viability, in APP/PS1 mouse cortical neurons. Similar results were observed for, and LC and LC combined with CUR in rat cortical neurons exposed to $A\beta O_{1-42}$ The protective effects of CUR were associated with increased cellular levels of Nrf2. Furthermore, a liposomal formulation retaining <10% LC was produced

and characterized, however its neuroprotective efficacy still need to be further analyzed. Taken all together, carnitine forms individually or combined with curcumin might be a promising treatment for AD.

RESUMO

A doença de Alzheimer (DA) é a principal causa de demência e a doença neurodegenerativa mais prevalente em todo o mundo. Esta patologia é caracterizada por duas características patológicas, a presença de tranças neurofibrilares intracelulares, compostas por tau hiperfosforilada, e placas senis extracelulares, formadas por agregados do péptido beta-amilóide (Aβ). A DA afeta predominantemente os neurónios hipocampais e corticais. A DA é uma doença multifatorial associada a várias desregulações biológicas, incluindo a disfunção mitocondrial, perda sináptica e morte neuronal, bem como a desregulação da microglia e astrócitos, associada a processos neuroinflamatórios. Oligómeros $Aβ_{1-42}$ (AβO) foram descritos como responsáveis por aumentar os níveis de cálcio mitocondrial e despolarizar as mitocôndrias. Além disso, AβO pode associar-se à mitocôndria interagindo com proteínas envolvidas na dinâmica e função mitocondrial, exacerbando os danos sinápticos e neuronais na DA. Apesar da identificação de vários mecanismos patológicos, ainda não há cura ou terapias neuroprotetoras eficazes para os doentes de Alzheimer. Uma molécula que tem mostrado efeitos benéficos na recuperação da função mitocondrial é a L-carnitina (LC) e as suas diferentes formas, acetil-L-carnitina (ALC) e propionil-L-carnitina (PLC). A ALC demonstrou bons resultados na recuperação de alterações mitocondriais observadas em neurónios primários do hipocampo de rato expostos a AβO. Para além das carnitinas, a curcumina (CUR) é uma outra molécula neuroprotetora, devido ao seu papel na 'clearance' de AβO, propriedades anti-inflamatórias e antioxidantes. Com base nessas observações, este trabalho teve como objetivo avaliar os efeitos protetores de LC, ALC, PLC e CUR, isolados ou em combinação em neurónios primários do hipocampo e corticais derivados de murganho APP/PS1 *versus* tipo selvagem (WT) e em neurónios de rato Wistar expostos a $AβO_{1-42}$, e ainda produzir e caracterizar uma formulação de lipossomas contendo LC. Os resultados apresentados permitem mostrar que ALC e PLC apresentam efeitos benéficos na recuperação da função mitocondrial, avaliada através da análise do potencial de membrana mitocondrial, e da capacidade redutora celular, um índice de viabilidade celular, em neurónios corticais APP/PS1. Resultados semelhantes foram observados na presença de LC isolada ou LC combinada com CUR

em neurónios corticais de rato expostos a $A\beta O_{1-42}$ Os efeitos protetores observados na presença de CUR foram associados a um aumento dos níveis celulares de Nrf2. Adicionalmente, produzimos e caracterizámos uma formulação lipossomal contendo uma percentagem <10% de LC, contudo ainda será necessário analisar a sua eficácia neuroprotetora. Em suma, diferentes formas de carnitina individualmente ou em combinação com curcumina poderão constituir um tratamento promissor para a DA.

Palavras-Chave: Doença de Alzheimer, péptido beta-amilóide, carnitinas, curcumina, lipossomas, peróxido de hidrogénio, mitocôndria.

ABBREVIATIONS

ABAD – Amyloid binding alcohol dehydrogenase

Ac-CoA – Acetyl coenzyme A

ACh - Acetylcholine

AD – Alzheimer's disease

AICD – APP intracellular domain

AKT - Protein kinase B

ALC – Acetyl-L-carnitine

AMPAR - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Aph-1 – Anterior pharynx-defective 1

APOE – Apolipoprotein E

APP – Amyloid precursor protein

APP/PS1 – Amyloid precursor protein/ Presenilin-1 mouse model

ATP - Adenosine 5'-triphosphate

AβO – Amyloid beta oligomers

BACE1 - Beta-secretase 1 [also known as 'beta-site amyloid precursor protein cleaving enzyme 1' or 'beta-site APP cleaving enzyme 1']

BAG2 - BCL2 associated athanogene 2

BBB – Blood -brain barrier

CaMKII - Calcium/calmodulin-dependent protein kinase II

ChAT – Choline acetyltransferase

COX - Cyclooxygenase

CREB - cAMP response element-binding protein

CSF – Cerebrospinal fluid

CUR - Curcumin

DNMT - DNA methyltransferase

Drp-1 – Dynamin-related protein 1

DSM-5 – Diagnostic and statistical manual of mental disorders

EAOD – Early-onset Alzheimer's disease

ER – Endoplasmic reticulum

fAD – familial AD

FOXO - Forkhead box protein O

GA - Glyceraldehyde

GABA - Gamma-aminobutyric acid

Glu - Glutamate

GLUT – Glucose transporters

GPx – Glutathione peroxidase

GSH – Glutathione (reduced form)

GSK-3β - Glycogen synthase kinase-3 beta

GST – Glutathione S-transferase

H_2O_2 – Hydrogen peroxide

HDAC - Histone deacetylases

HNE - 4-Hydroxy-2-trans-nonenal

HO-1 – Heme oxygenase 1

HSP – Heat shock protein

JNK - c-Jun N-terminal kinase

LC – L-carnitine

LOAD – Late-onset Alzheimer's disease

LTD – Long term depression

LTP – Long term potentiation

MAPT – Microtubule associated protein Tau

MCI – Mild cognitive impairment

MIM – Mitochondrial inner membrane

MIS – Mitochondrial intermembrane space

MOM – Mitochondrial outer membrane

MRI – Magnetic resonance imaging

mtDNA – Mitochondrial DNA

mTOR - Mammalian target of rapamycin

NF-kβ –Nuclear factor kappa B

NFT – Neurofibrillary tangles

NGF - Nerve growth factor

NIR – Near-infrared

NMDAR - *N*-methyl-D-aspartate receptors

Nrf2 - Nuclear factor erythroid 2–related factor 2

OCTN2 - Organic cation/carnitine transporter novel type 2

OGD – Oxygen/glucose deprivation

p300/CBP - E1A binding protein p300/CREB binding protein

p38 MAPK – p38 Mitogen-activated protein kinases

Pen-2 – Presenilin enhancer 2

PET – Positron emission tomography

PKR-eIF2α – Protein kinase R- Eukaryotic translation initiation factor 2A

PLC – Propionyl-L-carnitine

PSEN1 – Presenilin-1

PSEN2 – Presenilin-2

PTEN - Phosphatase and tensin homolog

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

SOD – Superoxide dismutase

SPECT – Single-photon emission computed tomography

TBK1 - TANK binding kinase 1

TCA – Tricarboxylic acid cycle

TOM - Translocase of the outer membrane

TOMM40 - Translocase of outer mitochondrial membrane 40

ULK1 - Unc-51 like autophagy activating kinase

VDAC1 – Voltage-dependent anion channel 1

WT – Wild type

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CHAPTER I - INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease and the main cause of dementia worldwide. There are approximately 27 million people affected by AD, corresponding to 60-70% of dementia cases worldwide, and these numbers are expected to increase in the near years due to the increase in lifespan and better life conditions (e.g. Silva et al., 2019). AD is predominant in older people, being aging the major risk factor. The disease is characterized by a reduced ability to perform daily tasks, a decline in episodic memory and cognition, and language problems (Silva et al., 2019). These deficiencies are accompanied by mood alterations, namely depression, apathy and aggressiveness. AD is a disease in constant development. It's progression occurs in five stages, as shown in Figure1: preclinical AD, mild cognitive impairment (MCI), a prodromal stage of AD, and mild, moderate, and severe AD. The progression of the disease is related to the development of AD hallmarks besides the aggravation of cognitive abilities and skills (Ferris et al., 2013).

Fig. 1 – Alzheimer's disease development stages. Adapted from *2020 Alzheimer's disease facts and figures (*Alzheimer's & Dementia – Alzheimer's Association journal)

AD is marked by a loss of synapses and neurons in several brain regions, namely the cortex, hippocampus and limbic system, culminating in the degeneration and atrophy of the brain, in general. The principal AD hallmarks are the extracellular accumulation of beta-amyloid peptides (Aβ), which aggregate, forming the senile plaques, and the presence of intracellular neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein (Selkoe and Hardy, 2016). Tau protein is a microtubule-associated protein, a product of *MAPT* gene alternative splicing, located at chromosome 17q21 (Pîrşcoveanu et al., 2017). Its function is related to microtubule stabilization and polymerization, requited for axonal transport of vesicles and organelles along the microtubules (Pîrşcoveanu et al., 2017). According to the literature, when the tau protein is phosphorylated, its function is affected, detaching from microtubules

(being even mislocalized to dendrites), resulting in neuronal damage (Khan et al. 2016). In monomers, Aβ appears to have neuroprotective effects associated with synaptic function (Giuffrida et al., 2009). However, oligomeric species, particularly of Aβ$_{1-42}$ are known to induce several cellular disturbances, including neuronal degeneration. These effects are a consequence of interactions between Aβ and cell membranes and their membrane receptors, resulting in the deregulation of several intracellular processes. These events result in the impairment of neuronal signaling pathways, namely glutamatergic, GABAergic, dopaminergic/noradrenergic, serotonergic and cholinergic pathways, which potentiate the uncontrolled amyloidogenic processing, promoting the formation of senile plaques. (Silva et al., 2019). The presence of both hallmarks, senile plaques and NFT, leads to a neurodegenerative cascade, culminating in several cellular changes, namely mitochondrial and synaptic dysfunction, dysregulated axonal transport, and excitotoxicity, leading to neuronal loss (Lambert and Amouyel, 2011) (Figure 2).
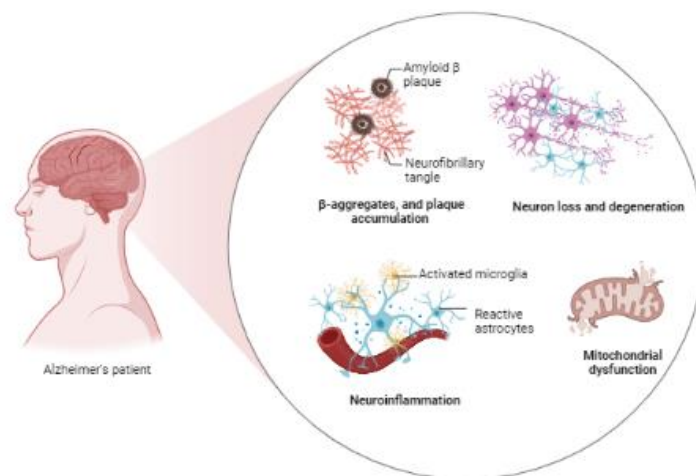
Fig. 2 – Neuropathological features of Alzheimer's disease. AD present several neurological disturbances, being characterized by the presence of extracellular senile plaques, composed by Aβ peptides, and neurofibrillary tangles, formed by hyperphosphorylated Tau protein. The disease affects several intracellular processes, including mitochondrial dysfunction, and defective calcium homeostasis, resulting in neurodegeneration. *Created with BioRender.com*

AD is a multifactorial disease, being the result of several factors, including environmental, genetic, and epigenetic factors. As described before in this work, the most important intrinsic risk factor is age, being 65 years-old is the reference age used to define modifications occurring in AD familial or sporadic forms. There are two forms of AD, early-onset (EOAD) and late-onset AD (LOAD) (Cacace et al., 2016) (Figure 3).

EOAD or familial form of AD is classified when the patient presents AD symptoms before 65 years old and is related to several rare autosomal dominant mutations. The mutations include the amyloid precursor protein gene (*APP*, located at chromosome 21), the presenilin-1 (*PSEN1*, at chromosome 14), and presenilin-2 (*PSEN2*, at chromosome 1) (Lambert and Amouyel, 2011). LOAD or sporadic form of AD most often occurs in people with more than 65 years old, associated to aging. Although several studies demonstrated that LOAD is linked to apolipoprotein E (*APOE*) gene, namely the E4 allele polymorphism, there are several other genetic and epigenetic risk factors (Corder et al., 1993). APOE is the major cholesterol carrier in the brain and plays a role in cellular processes, namely growth and repair of neurons, synaptogenesis, membrane modulation, neuroinflammation, and clearance and degradation of Aβ peptides. There are three allelic variants, E2, E3, and E4, where *APOE4* is the main genetic risk factor for AD, while *APOE2* expression seems to be neuroprotective (Lanfranco et al., 2020).
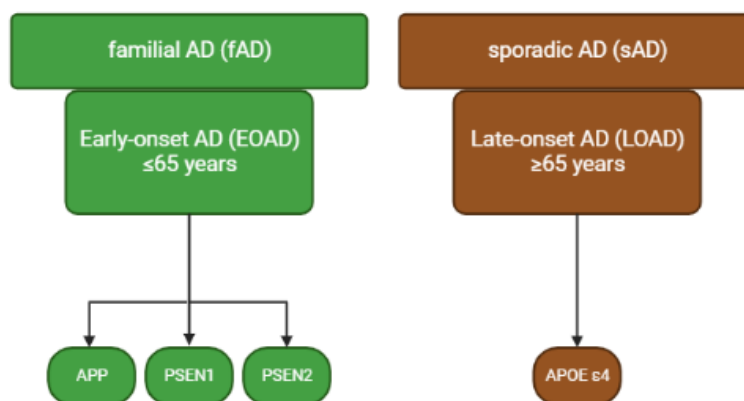
Fig. 3 – AD subtypes. AD can be classified in two types, familial (fAD) and sporadic (sAD). This classification is based on the age at which the disease begins, as well as the type of factors that cause it. fAD is also known as early-onset AD (EOAD), and is associated to mutations in *APP*, *PSEN1*, and *PSEN2* genes. sAD is also named late-onset AD (LOAD), and it is linked to *APOE* ε4 polymorphism. *Created with BioRender.com*

AD diagnosis is made through several tools, by following the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and supplementary biomarkers evidence, such as imaging, cerebrospinal fluid (CSF) and/or serum (Weller et al., 2018). In recent years several imaging techniques have been developed to help diagnose AD. Some of those techniques are positron emission tomography (PET), magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT). Detection of

AD hallmarks also include the analysis of CSF or serum biomarkers (Ballard et al., 2011); AD and MCI patients present lower levels of $A\beta_{1-42}$ due to its aggregation, but higher content of total tau protein and hyperphosphorylated tau protein, namely at threonines 181 and 231, providing evidence for senile plaque formation and neurodegeneration (Ballard et al., 2011). These specific biomarkers allow the distinction of other types of dementias.

1.2 Amyloid beta peptide

1.2.1 APP processing

The *APP* gene is located at chromosome 21q21 and codifies for the amyloid precursor protein. This gene is capable of generating 8 to 11 different APP proteins through alternative splicing, being the APP695 the most expressed in neurons (Wilkins et al., 2017). Its localization varies; APP is initially located at the endoplasmatic reticulum (ER), being transported to the plasma membrane, and the majority to the trans-Golgi network (Wilkins et al., 2017). APP is a type 1 membrane glycoprotein that plays a pivotal role in several biological processes, specifically neuronal development, signaling, homeostasis and intracellular transport (Chen et al., 2017). This protein plays a pivotal role in brain homeostasis and is also responsible for the formation of $A\beta$. The $A\beta$ peptide is a product of APP enzymatic proteolysis. The cleavage of APP can be made by two pathways, the non-amyloidogenic pathway involving the activity of α-secretase or similarly enzymes, as the ADAM (a disintegrin and metalloproteinase) family enzymes, where three ADAM enzymes have been suggested as the α-secretase, namely, ADAM 9, ADAM 10, and ADAM 17 (Zhang et al., 2011), or by the amyloidogenic pathway defined by the initial and intracellular processing by β-secretase or BACE 1 (Gupta et al., 2016; Zhang et al., 2011; Chen et al., 2017) (Figure 4). These two pathways have different functions, being the α-secretase-mediated pathway a neuroprotective pathway due to the production of sAPPα, a soluble molecule that plays roles in protection against excitotoxicity, and in neurons' plasticity and survival (Gupta et al., 2016; Chen et al., 2017). In the non-amyloidogenic pathway, there is also the production of a C-terminal

fragment of APP with 83 amino acids (CTF83). The cleavage of this fragment, by the γ-secretase complex, a heavy molecular weight complex composed of presenilin, nicastrin, anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN-2), leads to the release of the p3 fragment to the extracellular space, while the APP intracellular domain (AICD) is released to the cytoplasm (Zhao et al., 2020). In the other pathway, the β-secretase-mediated, two products are formed: sAPPβ (a molecule linked to neuronal death), and a C-terminal complex (CTF99) associated with the cell membrane. The second product, CTF99, is degraded by a γ-secretase complex to produce the Aβ peptide in the extracellular space and the AICD to the cytoplasm (Wilkins et al., 2017). Aβ may contain 38 to 43 amino acids, being the 40 amino acids form ($A\beta_{1-40}$) generated in higher amounts when compared to the 42 amino acids forms ($A\beta_{1-42}$). Of these two forms, the $A\beta_{42}$ is more subject to aggregate and its oligomers are more toxic (Yan et al., 2006). Moreover, it was established that mutations in *APP, PSEN1* and *PSEN2* genes alter the APP cleavage, increasing the production of Aβ peptides (Barage et al., 2015). In the case of mutations in *PSEN1* and *PSEN2* genes, enhanced APP processing is linked with the composition of the γ-secretase complex formed by nicastrin, anterior-pharnyx-defective-1 (Aph-1), PSEN1 or PSEN2, and the presenilin enhancer 2 (Pen-2) (Haass et al., 2007).
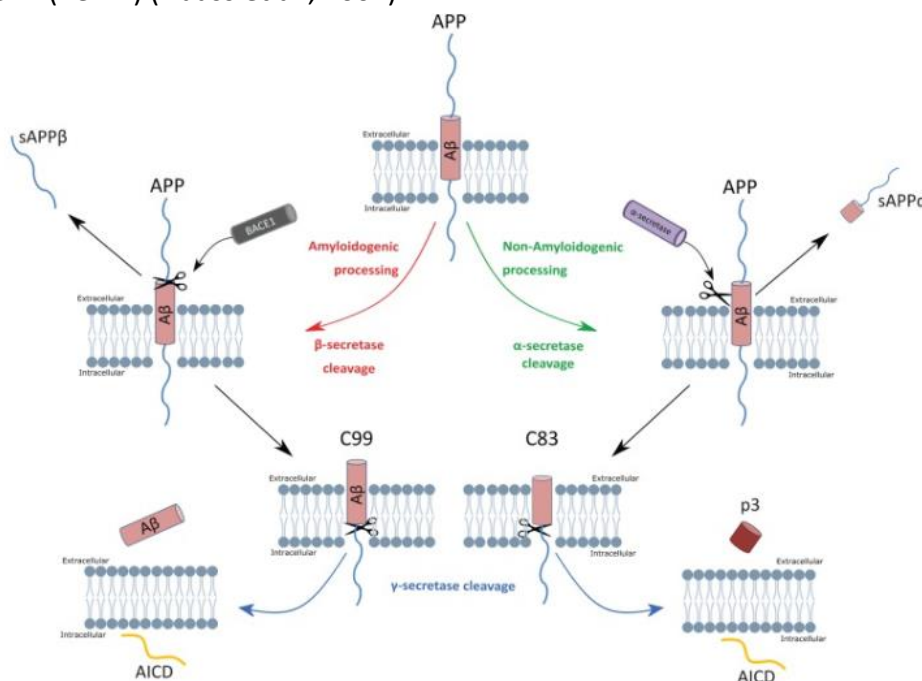

Fig. 4 – Amyloidogenic and non-amyloidogenic pathways of APP processing. In the non-amyloidogenic pathway, α and γ secretases cleave APP, generating p3 and AICD. On the other hand, β and γ secretases, in amyloidogenic pathway, are responsible for APP degradation, resulting Aβ and AICD. Adapted Wirths et al. (2019)

1.2.2 Amyloid-beta cascade Hypothesis

Several hypotheses have been proposed to explain AD pathogenesis, although there is one that prevail at least as an initial pathway, the amyloid cascade hypothesis. This hypothesis affirms that the primary trigger for AD development is the accumulation, and consequent, aggregation of Aβ peptides (Pimplikar et al., 2009). It is expected that an increased production or a decreased clearance of Aβ peptides is responsible for the progression of the disease. To affirm this hypothesis, not only AD and increased generation of Aβ is linked to the APP gene mutations (as described before in this work), but increased levels of Aβ are observed in Down Syndrome, resulting from the trisomy in the 21 chromosome, where the *APP* gene locus is located. Furthermore, increased copy number of the *APP* gene was associated with enhanced prevalence of AD (Sleegers et al., 2009). Relatively to sporadic AD cases, it has suggested that there is an inefficient Aβ clearance, being this a result of deficient degradation, higher accumulation/aggregation of Aβ peptides, and defective Aβ elimination/ transport through the blood-brain-barrier (BBB) (Sagare et al., 2012).

There is also several evidence concerning the synaptogenic effects exerted by Aβ peptides in the brain, being described as cytoskeletal defects, changes in neurotransmitters uptake/release ratios, altered receptor cellular localization, and altered synaptic plasticity due to increased long-term depression (LTD), and decreased long-term potentiation (LTP). All these effects represent causes of cognitive dysfunction, being memory deficits the most characteristic one (Ricciarelli et al., 2017). Besides these dysfunctional pathway, Aβ oligomers (AβO) also presents interactions with molecular targets, namely with, cellular prion protein and α7-nicotinic receptors, favoring neurotoxic mechanism cascades. These can further be detected as mitochondrial damage and oxidative stress, dysregulated calcium levels, defective axonal transport, and activation of pro-inflammatory glial cells (Ferreira et al., 2015; Garwood et al., 2017). According to figure 5, the amyloid cascade can be also responsible for triggering the tau pathology. Some evidence explains amyloid cascade-induced tau pathology (Blurton-Jones et al., 2006):

- Aβ induces the activation of kinases, such as glycogen synthase kinase-3 beta (GSK3β), which leads to hyperphosphorylation of tau protein, changing its structure conformation and allowing NFT formation;
- Deposition of Aβ promotes a process of neuroinflammation, increasing the synthesis of inflammatory cytokines, and enhancing tau protein phosphorylation;
- Aβ affects proteasome function, compromising its clearance role;
- Aβ disturbs the axonal transport, provoking defects in tau protein localization and of its mRNA, leading to tau hyperphosphorylation and NFT formation.
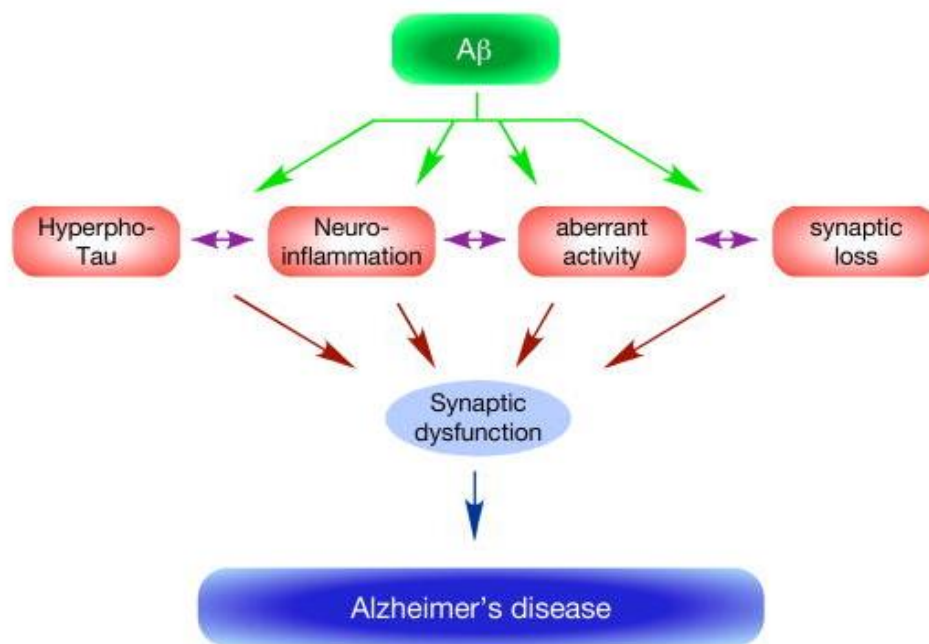
Fig. 5 – Amyloid cascade hypothesis in AD. Aβ accumulates, due to higher production and/or decreased clearance, triggering several events, namely neurofibrillary tangle formation, synaptic dysfunction, neuroinflammation and neuronal death. Adapted from Pimplikar et al. (2009)

So, taking all this evidence into account, au pathology has been suggested to be triggered by AβO formation, which appears to be a primary molecular event for AD progression.

1.3 Synaptic dysfunction in AD - Altered synaptic dynamics and transmission

AD presents several disturbances as result of synaptic dysfunction, triggered by Aβ plaques and NFTs. Most of these disturbances consist of defective synaptic function and transmission and synaptic loss, resulting in altered synaptic plasticity. Of relevance these effects have been linked to cognitive decline, a prominent feature in AD (Guo et al., 2017). Neurotransmitters have a crucial role in the normal function of synapses. In AD, neurotransmitter dysregulation largely account for by changes in the release of acetylcholine (ACh) and glutamate (Glu), which compromise the normal communication in the brain, affecting mainly the entorhinal cortex and the hippocampus, two areas involved in memory, and further the cerebral cortex, responsible for language, reasoning and social behavior (Renvoize et al., 1979).

A dysfunction in the cholinergic system was shown in AD human *postmortem* brains, including a decrease in ACh levels, lower ACh synthesis due to reduced levels of choline and in the activity of choline acyltransferase (ChAT), and also lower density of muscarinic ACh receptors. Furthermore, reduced levels of ACh were observed in the CSF of AD patients, being this proportional to the seriousness of cognitive decline in AD spectrum (Kumar et al., 1989; Karami et al., 2021).

Another very important neurotransmitter is Glu, the main excitatory messenger in the brain that plays a pivotal role in the cortex and hippocampus. Two ionotropic Glu receptors exert a key role in synaptic function, namely α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptor (NMDAR) (Liu et al., 2014). NMDARs play a role in diverse cognitive processes, such as memory and learning (Montes de Oca Balderas et al., 2018; Wang et al., 2017). As NMDARs are activated by Glu, these receptors are extremely important in order to maintain the normal cognitive function and neuronal circuits, although when there is a disturbance in Glu release and, consequently, there is an extrasynaptic activation of NMDAR causing an excitotoxic process, the neuronal circuits in the hippocampus and entorhinal cortex become compromised, allowing the development of AD. NMDARs are

located in the postsynaptic membrane; they enable the exit of K^+, allowing the entrance of Ca^{2+} (predominantly) and Na^+ into the postsynaptic neuron. NMDARs are tetramers composed of a mandatory GluN1 subunit (an obligatory subunit), linked to auxiliary subunits that can be GluN2 (A-D) or GluN3 (A-B). GluN1 is crucial for NMDAR assembly. The major NMDAR subunits present in hippocampal and cortical neurons are the GluN2A and GluN2B subunits. Postsynaptic NMDAR (mainly Ca^{2+}-permeable) activation is dependent on the release of Mg^{2+} blockade of NMDAR channel, which is dependent on AMPAR (largely Na^+ permeable)-dependent membrane depolarization. AMPAR have a higher affinity for Glu as compared to NMDAR, and thus are initially activated. Furthermore, AMPAR rapidly desensitize, whereas NMDAR exhibit a slow desensitization when compared to AMPAR, implicating a maintained postsynaptic Ca^{2+} entry, for about 20-30 more milliseconds (Forsythe et al., 1988; Stern et al., 1992). The glutamatergic system is essential for a normal and balanced cognitive function, regulating both long-term potentiation (LTP) and long-term depression (LTD). However excessive Glu levels in the synaptic cleft causes exaggerated glutamatergic receptors, leading to higher intracellular Ca^{2+} levels, which not only activate several Ca^{2+}-dependent enzymes (as proteases, lipases, endonucleases), but also compromise mitochondrial function, evokes increased generation of reactive oxygen species (ROS) and activates cell death processes. This "excitotoxic" executing programme is present in AD brains, leading to irreversible brain lesion (Butterfield et al., 2003; Conway et al., 2020). Although Glu levels did not vary significantly between a control brain and an AD patient's brain, as determined through magnetic resonance spectroscopy (MRS) in the bilateral posterior cingulate gyrus, CSF analysis revealed an increase in Glu levels in AD brains, when compared to age-matched controls (Kaiser et al., 2010). Apart from this change in Glu levels, there is evidence of hypersensitivity of NMDAR to Glu in AD. This disturbance in NMDAR function appears to be cause of AβO, as they induce increased intracellular Ca^{2+} levels through NMDAR activation after immediate AβO exposure (1 μM for 180 seconds) can also downregulate NMDAR-linked LTP (Ferreira et al., 2012). In addition, mitochondrial dysfunction is promoted by AβO, as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and cytochrome *c* oxidase activities are reduced by AβO. Alongside, also APP interacts with translocase of the outer membrane (TOM) and inner membrane translocase (TIM) occurring mitochondrial accumulation of

APP, resultant of decreased activity of cytochrome *c* oxidase, which promotes Ca^{2+} disturbances and neuronal oxidative stress, further contributing to excitotoxicity in AD (Ankarcrona et al., 2009; Ferreira et al., 2015). This interaction between GluN2B-NMDAR and AβO was demonstrated, being further observed that AβO induce endoplasmic reticulum (ER) stress in cultured neurons, by inducing NADPH oxidase (NOX)-mediated superoxide production downstream of GluN2B (Costa et al., 2012); moreover, a drastic decrease in NMDAR density was observed in AD *postmortem* hippocampus and cortex, where Aβ plaques are common (Jacob et al., 2007; Kravitz et al., 2013).

NMDAR-mediated entrance of Ca^{2+} to the post-synaptic neuron triggers a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated signaling cascade to enforce the strength of the synapse. On the other side, when LTD is promoted, a moderate NMDAR activation occurs, causing a mild intracellular Ca^{2+} in the post-synaptic neurons. Synaptic NMDAR intervenes in the activation of pro-survival transcription factors and apoptosis inhibition. Indeed, NMDARs are responsible for the activation/phosphorylation of cyclic-AMP response element binding protein (CREB), a Ca^{2+}-dependent transcription factor, and the blockade of apoptotic pathways (e.g. effector caspases and Puma) through the inhibition of transcription factor FOXO (forehead box protein O) (Montes de Oca Balderas et al., 2018; Wang et al., 2017). AβO significantly decrease the expression of presynaptic proteins, such as syntaxin, synaptophysin, and synaptotagmin, actively involved in neurotransmitter release machinery, through caspase-dependent and proteasome-dependent mechanisms (Jang et al., 2017). In concordance with this, it was shown that AβO directly modulate NMDAR electrophysiological function, by increasing synaptic current mediated by NMDAR and their toxicity (Wang et al., 2017). Reduced levels of vesicular glutamate transporters plasma membrane glutamate transporters were reported in AD brain (Kirvell et al., 2006). These disturbances cause a dysfunction in glutamate uptake and recycle system, and synaptic vesicle cycling. In contrast to synaptic NMDARs, activation of extrasynaptic NMDARs has been linked to excitotoxicity and, consequently, neuronal death. AβO may activate extrasynaptic NMDAR directly, or inhibit synaptic NMDAR (Snyder et al., 2005). Exposure of cortical neurons to AβO provokes a decrease in NMDAR-mediated currents and an internalization of synaptic NMDAR (Snyder et al., 2005). Moreover, and according to Figure 6, activation of extrasynaptic NMDAR leads to a suppression of pro-

survival signaling pathways activated by the synaptic NMDAR, namely CREB, and promotes mitochondrial dysfunction, oxidative stress and pro-death signaling cascades (Parsons et al., 2014).

Another neurotransmitter altered in AD is gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter. According to several studies, there is a reduction in GABA levels in AD brains, which compromises the crosstalk between cholinergic, glutamatergic, and GABAergic synapses, due to a decreased inhibitory function, exacerbating the excitotoxic process (Mohr et al., 1986; Solas et al., 2015).
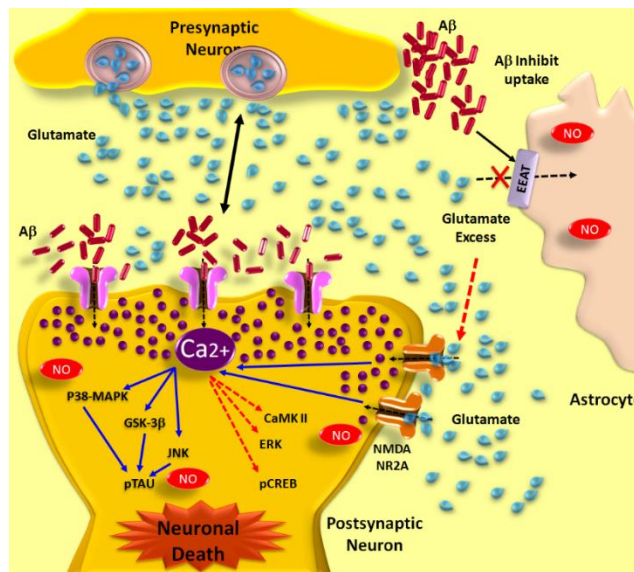
Fig. 6 – Glutamatergic transmission in AD. AβO promote the release of glutamate from presynaptic vesicles and inhibits its uptake by the astrocytes through blockade of excitatory amino acids transporter (EAAT). This leads to the activation of synaptic and extrasynaptic NMDAR, increasing the influx of calcium, and consequently, activation of signaling pathways responsible for neuronal death, such as p38-MAPK, GSK-3β and JNK. Besides this exagerous influx of calcium, AβO also leads to inhibition of protective pathways, as CaMKII, ERK, and pCREB. From Campos-Peña et al. (2014).

1.4 Cellular dysfunction in Alzheimer's disease - oxidative stress and antioxidants defenses

Oxidative stress is a perturbation in the balance between antioxidant defenses and pro-oxidant compounds, namely an increase in the production of ROS and reactive nitrogen species (RNS) and/or a decrease in antioxidants (enzymatic or non-enzymatic) levels of activity (Kim et al., 2015). ROS and RNS are a group of oxidant molecules that include both free radicals and non-free radicals; they have a short lifetime and contain

unpaired valence electron. ROS include the hydroxyl radical ($^{\bullet}$OH) and superoxide anion ($O_2^{\bullet -}$) radicals, and other non-radicals, as hydrogen peroxide (H_2O_2) (Kim et al., 2015). When ROS are produced at a large scale, they cause the oxidation and resulting damage of several cellular proteins, lipids, or DNA. Redox regulation is a pivotal process for the protection of the organism against oxidative stress, by managing the redox status (Dröge et al., 2002). In a healthy situation, during oxidative phosphorylation, there is a production of ROS and RNS, although the antioxidant enzymes function as free radical scavengers, clearing the excessive ROS. Those scavengers include superoxide dismutase (SOD), glutathione peroxidase (GPX), thioredoxins, glutaredoxins, and catalase (Kim et al., 2015).

Many of these enzymes are a result of activation of nuclear factor erythroid-2-related factor 2 (Nrf2). Nrf2 is a transcription factor bound to the cytoplasmic repressor and stress sensor Kelch-like ECH-associated protein 1 (KEAP1), which acts as a substrate adaptor, mediating Nrf2 ubiquitination by E3 ubiquitin ligase Cullin-3 and its subsequent degradation by the proteassome. In the presence of oxidants, KEAP1 cysteines oxidize, releasing Nrf2 with its subsequent phosphorylation and translocation to the nucleus, where it activates the transcription of cytoprotective genes via promoter sequences containing conserved antioxidant response elements (AREs), allowing the transcription of antioxidant and detoxifying enzymes (Tönnies et al., 2017) (Figure 7).

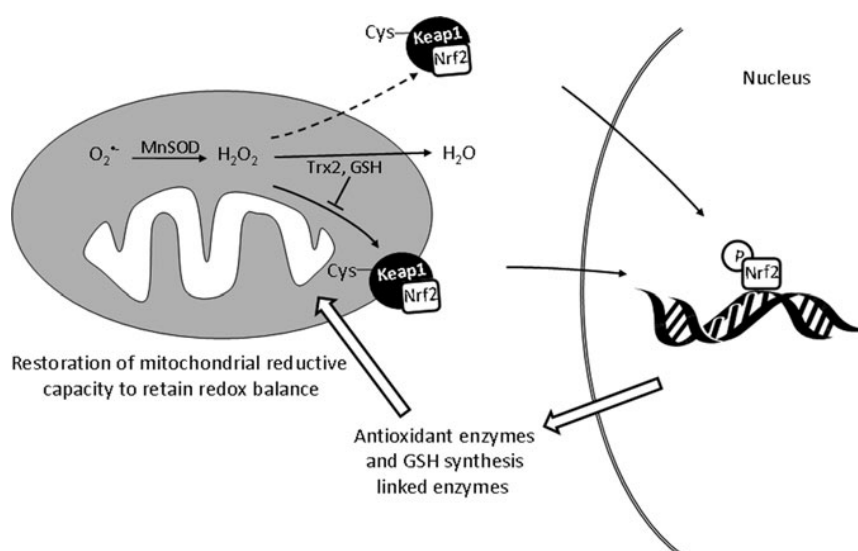Fig. 7 – Nrf2 regulation by mitochondria. Under an oxidative stress status, mitochondria contain sensors that monitories the redox homeostasis, triggering the activation of Keap1-Nrf2 antioxidant pathway. Nuclear translocation of Nrf2 leads to upregulation of ARE-linked antioxidant enzymes (e,g. superoxide dismutase, glutathione peroxidase aiming to restore the redox balance in cytosol and mitochondria. From Cheng et al. (2011).

In AD several endogenous processes including calcium homeostasis, mitochondrial function and dynamics, and DNA repair impairment, are altered, which might constitute the trigger for the development of the disease. This occurs concomitantly with a disturbance in antioxidant enzyme activities and an increase in ROS production (Tönnies et al., 2017). Relatively to antioxidant enzymes disturbances, it was demonstrated that hippocampal neurons of human AD brains presented reduced Nrf2 levels, indicative of lower Nrf2-mediated transcription of detoxifying enzymes and antioxidant (Mota et al., 2015). In addition, it was reported that despite an increase in Nrf2 activation, resulting from elevated nuclear levels of Nrf2 in the cerebral cortex of 3xTg-AD males, there was no activation of the Nrf2 pathway through the transcription of target genes, namely SOD1 and the glutamate-cysteine ligase catalytic subunit (GCLc) (Mota et al., 2015).

AβO can induce an increase in oxidative stress and lipid peroxidation. According to Butterfield and co-authors, brain regions presenting high levels of oxidative stress are characterized by increased $AβO_{1-42}$, while brain regions retaining low levels of $AβO_{1-42}$ do not present significant oxidative stress (Butterfield et al., 2018). Moreover, Aβ peptides were shown to promote lipid peroxidation, indexed by increased HNE (protein-bound 4-hydroxy-2-trans-nonenal). In mid AD patients and prodromal MCI, $AβO_{1-42}$ were shown to correlate with enhanced oxidative stress, provoking an activation of mTOR by increased ROS, changing proteostasis network through the ubiquitin proteasome system, autophagy and ER-unfolded protein response, an inefficient glucose metabolism, and dysregulated protein phosphorylation, culminating in neuronal death (Butterfield et al., 2018).

The brain is highly dependent on energy, thus having a high oxygen consumption rate by mitochondria and requiring a constant glucose "feeding"; indeed the brain uses 25% of total body glucose, to produce adenosine triphosphate (ATP) (Yin et al., 2016). Most of this ATP comes from mitochondrial oxidative phosphorylation. During aging there is a decline in neuronal glucose uptake, increased ROS synthesis, and decreased mitochondrial respiration through the electron transport chain (Yin et al., 2016). In postmortem AD patients there is a disruption in tricarboxylic acid cycle (TCA), electron

transport, and oxidative phosphorylation . More specifically, neuronal glucose metabolism consists of brain glucose uptake through the glucose transporters (GLUT), glycolytic pathway, and the entrance of pyruvate in mitochondria to serve as substrate for ATP production through oxidative phosphorylation (Yin et al., 2016). All these processes are dependent on a controlled mitochondrial function, being the mitochondrial H_2O_2 an important in sensing signals of redox-sensitive signaling, participating in c-Jun N-terminal (JNK) signaling, AMP-activated protein kinase (AMPK) signaling, and insulin/insulin-like growth factor 1 (IGF1) signaling. There is a constant interaction between cytosol and mitochondria, to maintain the adequate energy supply cable of supporting neuronal function (Yin et al., 2016). Insulin acts in the neurons via the activation of membrane receptors and their AKT and MAPK signaling pathways. Insulin receptors are present in pre- and post-synaptic compartments (Sedzikowska et al., 2021). According to the literature, insulin promotes the outgrowth of neurons, modulates the metabolism of catecholamines, and also regulates the expression and localization of GABA. On the other side, acts in excitatory synapses, being responsible for its development and maintenance. This modulation occurs through AKT and GSK3β. Through regulation of the AKT pathway, insulin promotes neuronal survival due to the block of apoptosis, although stimulation of FOXO transcription factor allows the
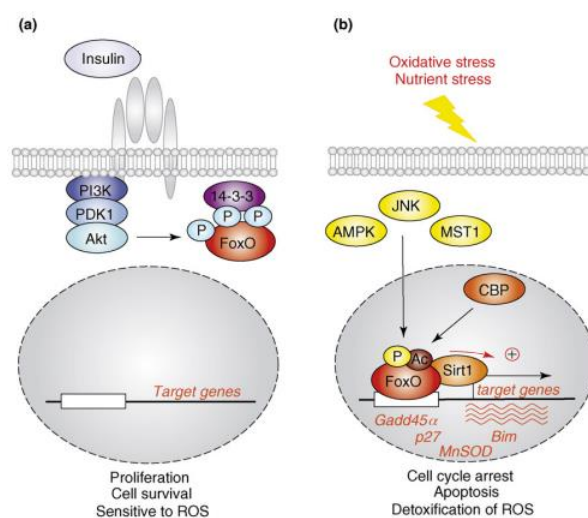
Fig. 8 – FOXO transcription factor regulation by insulin signaling pathway and stress status. (a) Binding of Insulin to its receptor leads to activation of AKT, that phosphorylate FOXO, promoting the export of FOXO from nucleus through its binding to the chaperone 14-3-3. This export allows cellular proliferation and survival, but also promotes oxidative damage. **(b)** A stress condition, through activation of AMPK (AMP-dependent kinase), JNK (Jun-N-terminal kinase), MST1 (mammalian Ste20-like kinase), and CBP (CREB-binding protein), leads to FOXO phosphorylation, acetylation, and monoubiquitination, allowing its nuclear translocation. FOXO nuclear translocation, and binding to deacetylase SIRT1 enhancing the transcription of several genes involved in cell cycle arrest, apoptosis, and ROS clearance. Adapted from Salih et al. (2008).

expression of apoptotic components, leading to cell death (Sedzikowska et al., 2021) (Figure 8).

Oxidative stress can not only induce higher production and accumulation of AβO, but also promote tau protein polymerization and phosphorylation (Tamagno et al., 2012; Kamat et al., 2016). Indeed high levels of ROS, through activation of cell death machinery and promoting AβO, intervene in several signaling pathways and transcription and epigenetic factors, namely, double stranded RNA dependent protein kinase eukaryotic translation initiation factor-2 alpha (PKR-eIF2α), nuclear factor kappa-light-chain-enhacer of activated B cells (NF-κB), p38 mitogen-activated protein kinase (p38MAPK), JNK, DNA methyltransferase (DNMT), histone deacetylase complex (HDAC), and p300/CREB-binding protein (p300/CBP), or molecules that play an important role in amyloidogenic regulation, as BACE1, APP and PS1, by regulating its transcription and its activation (Zuo et al., 2015).

1.5 Synaptic dysfunction in Alzheimer's disease - Involvement of mitochondria

One of the organelles affected in AD is mitochondria. Mitochondria is a pivotal neuronal organelle and the main source of energy in neurons. Furthermore, they constantly produce ROS and regulate Ca^{2+} levels and intrinsic apoptosis, apart from other relevant cellular processes (Yan et al., 2019). Mitochondria are composed by the mitochondrial matrix, mitochondrial inner membrane (MIM), mitochondrial inter membrane space (MIS), and the mitochondrial outer membrane (MOM). The mitochondrial matrix contains the mitochondrial DNA, which encodes some proteins involved in mitochondrial respiratory chain (Yan et al., 2019). The mitochondrial respiratory chain is linked to oxidative phosphorylation, requiring five complexes (I, II, III, IV and V, the later ATP synthase) and two mobile electron carriers (coenzyme Q and cytochrome C). These components work together by transferring electrons across these complexes (I to IV) and H^+ from the matrix to the MIS through intrinsic mitochondrial complexes, namely I, III and IV (Fernandez – Vizarra et al., 2021). The protonic gradient generated in the MIS is used by ATP synthase to ensure the final ATP production,

through phosphorylation of ADP in the matrix. The difference is electric charges across the MIM determines the mitochondrial transmembrane potential or MMP, which is highly negative at matrix site (-150 mV to -180 mV).

The role of mitochondria in neuroplasticity is very demanded as they provide energy for the release of neurotransmitters and maintenance of transmembrane ionic fluxes (Cheng et al., 2010). AD patient's cells exhibit decreased mitochondrial membrane potential (MMP) and in the activity of complex IV. Moreover, Aβ was recognized to directly interact with mitochondrial components. However, there are still some unclear links regarding mitochondrial dysfunction and Aβ. One of the first evidence for a major role of mitochondria in AD was defined by Cardoso and co-authors. These authors observed that human NT2 neural cells incubated with Aβ exhibited cell death, whereas in p0 cells, not retaining a functional mitochondria, due to the damage to mitochondrial DNA (mtDNA, exposure to Aβ did not cause a major effect in these cells. Considering that's P0 cells did not produce key respiratory chain subunits, the damage caused by Aβ was apparently due to the interaction between the peptide and mitochondrial subunits (Cardoso et al., 2001). Indeed, AβPP and Aβ were shown to colocalize with mitochondria components (Ankarcrona et al., 2000).

Apart from this evidence, it was also found that the Aβ peptide favors mitochondrial fission when compared to fusion. Moreover, Aβ binds to alcohol dehydrogenase (ABAD) and cyclophilin D, a component of mitochondrial transition pore. Thus, blocking ABAD-Aβ interaction, through a cell-membrane transduction domain of the human immunodeficiency virus–1 (HIV-1) Tat protein to ABAD-DP and ABAD-RP, which enables the peptides to cross cell membranes, and CypD deficiency inhibits oxidative stress and apoptosis (Lustbader et al., 2004; Du et al.,2008).

More findings were discovered concerning the interaction between mitochondria and Aβ peptides. It was reported that AβPP contains a mitochondrial targeting motif, more specifically, when AβPP enters mitochondria, it partly passes the mitochondrial protein import apparatus, namely TOMM40 (translocase of the outer mitochondrial membrane 40 kDa protein pore), although due to an acidic domain present in AβPP inhibits the import infrastructure protrudes from the mitochondria and into the cytoplasm. The arresting of AβPP in mitochondria leads to a reduction in cytochrome oxidase (COX) activity (Swerdlow et al., 2018).
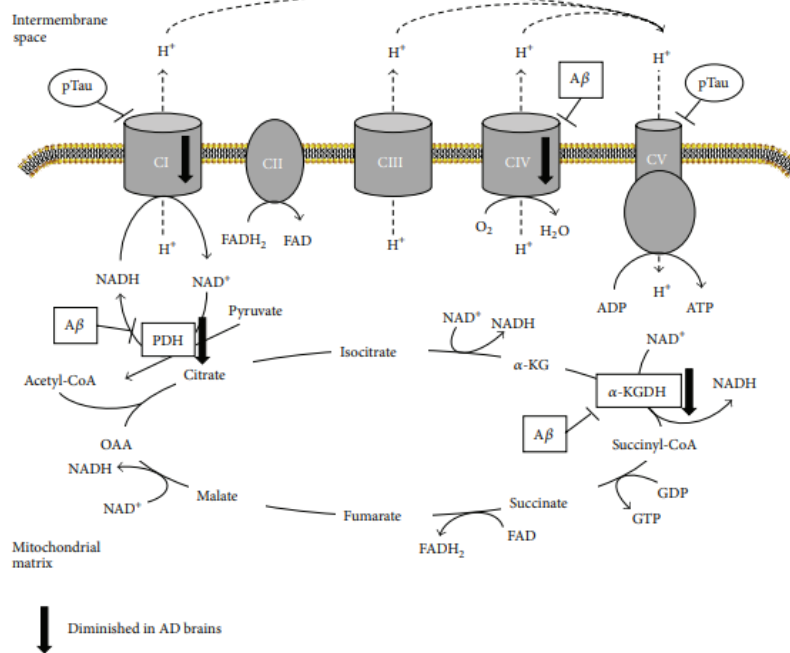
Fig. 9 – Mitochondrial respiratory chain (MRC) and tricarboxylic acid cycle (TCA) changes in AD. Alterations induced by Aβ peptide and phosphorylated Tau in specific targets of MRC and TCA cycle. MRC complexes affected by Aβ include CI and CIV. Pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH) complexes are also affected in AD. α-KG: α-ketoglutarate; OAA: oxaloacetate. From García-Escudero et al. (2013)

Moreover, mitochondria from AD patients exhibited several changes in structure and enzyme content. Relative to structure a reduction in mitochondrial surface area, higher variability in mitochondrial shape, alterations of cristae structure, a disturbance in mitochondrial fusion/fission ratio and changes in mitochondrial localization were observed in AD neurons. Also, COX activity (or complex IV), alpha-ketoglutarate dehydrogenase, and pyruvate dehydrogenase were shown to be affected, presenting reduced activities very early in AD (Figure 9) (Weidling et al., 2020).

Aβ peptides were also shown to facilitate the entrance of Ca^{2+} to the cytoplasm, either directly through the formation of pores formed by the peptide or indirectly through e.g. interaction with NMDARs. Neurons restore cytosolic higher levels of Ca^{2+}

by activating Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger at the plasma membrane and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in the ER , and also through the mitochondrial Ca^{2+} uniporter (MCU). Although Ca^{2+} is required for a normal mitochondrial function, increased Ca^{2+} levels in the organelle may underlie mitochondrial deregulation, namely mitochondrial depolarization or reduced ATP production, further abrogating mitochondrial Ca^{2+}buffering (Swerdlow et al., 2018).Mitochondria are also in constant movement, being transported along microtubules in neurons. In AD neurons there is a low number of mitochondria present in synapses (Pickett et al., 2018). This effect could be explained by the presence of hyperphosphorylated tau protein, since tau protein is very important for the maintenance and stability of microtubules, leading to defects in mitochondrial transport concomitantly with microtubules disturbances (Hawking et al., 2016; Simic et al., 2016)).In order to maintain mitochondrial quality control, and reestablish a normal mitochondrial function, mitophagy can be activated. Mitophagy is a specific form of autophagy characterized by the removal of deficient mitochondria in selective vesicles named autophagosomes, which can then fuse with lysosomes for degradation (Cai et al., 2020). There are several pathways characteristic of mitophagy. One of the forms mostly recognized in neurons is PINK1-Parkin-mediated mitophagy. Under conditions associated with decreased MMP, PINK1 accumulates in mitochondria, at the outer mitochondrial membrane (OMM). Accumulation of PINK1 leads to the recruitment of Parkin, which becomes activated by phosphorylation. Parkin belongs to the family of E3 ubiquitin ligases. So, active Parkin ubiquitinates several OMM proteins, which are recognized by the autophagic machinery (Bhujabal et al., 2017). Several reports suggest that damaged mitochondria move retrogradely to the neuronal soma, for mitochondrial removal by mitophagy (Cai et al., 2012).
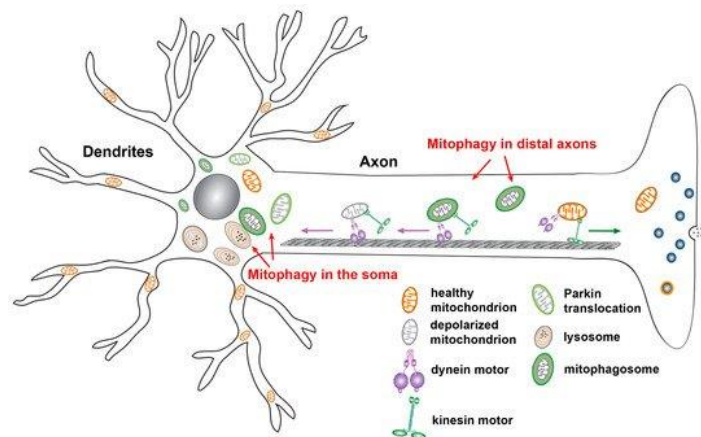
Fig. 10 - Neuronal mitophagy. Under disruption of mitochondrial membrane potential, PINK1 is not degraded in mitochondria, recruiting Parkin, further promoting mitochondrial engulfment by autophagosomes. These damaged mitochondria become accumulated at somatodendritic regions, which facilitates its removal from axons terminals and mitophagic clearance in the neuron soma, where mature lysosomes are located. From Cai et al. (2020).

In AD there is an impairment in mitophagy, and consequently, a defective removal of damaged mitochondria, enhancing the development and progression of the disease. Along with the progressive accumulation of Aβ and mitochondrial deficiency induces an up-regulation of the Parkin pathway; however, with the development of the disease, there is a depletion in cytosolic Parkin, leading to decreased mitophagy pathway and thus exacerbated mitochondrial dysfunction. So, the maintenance of a defective mitochondria pool and consequently, a deficient mitochondrial function could be provoked by mitophagy impairment (Ye et al., 2015).

Several factors can model the lysosomes' capacity and content. Some of these factors include presenilin mutations, ApoE4, Aβ, phosphorylated Tau, ROS and resulting oxidized molecules (Nixon et al., 2013; Lee et al., 2011). An important cellular compartment is the trans-Golgi apparatus, as this is responsible for vesicle maturation. As such, a deficient function of the Golgi apparatus culminates in inefficient lysosomes, leading to defective proteases. An event that can explain the low rate of basal mitophagy in AD is an impairment in the activation of Unc-51 Like Autophagy Activating Kinase 1 (ULK1) and TANK Binding Kinase 1 (TBK1), two autophagic proteins, responsible for the beginning of autophagy/mitophagy (Fang et al., 2019).

1.6 Carnitines

1.6.1 L – Carnitine

L-Carnitine (LC) is a water-soluble compound that acts in the mammalian metabolism, by promoting mitochondrial transport of long-chain fatty acids β-oxidation in mitochondrial matrix. There are several forms such as the carnitine (LC), acetyl-L-carnitine (ALC), and propionyl-L-carnitine (PLC). The abundant source of LC is acquired through diet, mainly through the consumption of milk and red meat. The body has also an intrinsic mechanism of producing LC by the endogenous biosynthesis from methionine and protein-bound lysine. The greatest storages of carnitine in the body are located in skeletal muscles and liver, although there are residual amounts of circulating carnitines (Ribas et al., 2014).

LC can also promote the recycling of coenzyme A (CoA) by facilitating the shuttling of short-chain acyl groups from the mitochondria to the cytoplasm, increasing the levels of free CoA (Evangeliou et al., 2003). This effect leads to an increase in acetyl-CoA/CoASH, interfering with several metabolic pathways, namely glycolysis, gluconeogenesis and ketone body production, and catabolism. Besides this, LC also performs clearance in mitochondria, through excretion and transesterification of acyl-CoA esters, eliminated through urine, removal of organic acids and xenobiotics, and scavenging ROS, protecting the cells from those noxious effects (Chapela et al., 2009). LC is transported to the brain by active transport, through the BBB, due to a cation transporter, the organic cation transporter novel 2 (OCTN2), accumulating itself in neuronal cells in the form of ALC (Mroczkowska et al., 1997). OCTN2 is a high-affinity carnitine transporter encoded by the *SLC22A5* gene. In the brain, OCTN2 is present in the cortex hippocampus, and cerebellum (Wawrzeńczyk et al., 2001).

Besides a role in energy metabolism, LC provides several protective characteristics in the brain, namely by having neurotrophic, neuroprotective and neuromodulatory properties. ALC is capable of transferring acetyl groups in neurons to promote ACh synthesis; increasing the expression of growth associated protein-43 (GAP-43), a molecule involved in neuronal development, neurotransmission, and neuroplasticity; protecting against oxidative stress; modulating several signal

transduction and gene expression pathways; and potentiate then regeneration of neurons (Nalecz et al., 1996; Binienda et al., 2001; Nalecz et al., 2004).

LC is very well-known for its antioxidant role, exerting ferrous metal ions chelating activity, scavenging ROS, as hydrogen peroxide and superoxide anion, and enhancing Nrf2/ARE transcriptional activity and inhibiting the NF-kB pathway (Gülçin et al., 2006).
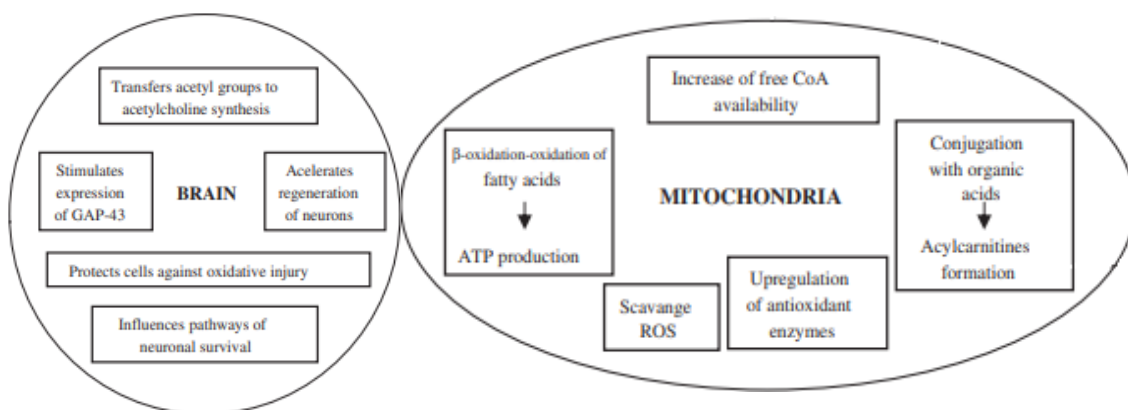
Fig. 11 – Mitochondrial and neuroprotective effects of LC. Adapted from Ribas et al. (2014).

LC protects neurons in both central and peripheral nervous systems. LC reduced ROS and cytotoxicity in rat primary cortical neurons exposed to oxygen-glucose deprivation (OGD) (Kim et al., 2012). Another study pointed out that LC is capable of inducing improvements in mitochondrial membrane and restore oligodendrocyte-mediated axonal myelination and neuronal plasticity, exerting neuroprotective effects against white matter lesions, and cognitive impairment in rat brain subjected to chronic hypoperfusion (Ueno et al., 2015).

In a model of AD-like phenotype, induced by the administration of glycolysis inhibitor glyceraldehyde (GA), LC showed several benefits, namely increased cell survival, enhanced oxygen consumption rate and ATP synthesis, lower the levels of ROS, and increased MMP. Besides this, LC also diminished the levels of pTau protein, induced by GA (Magi et al., 2021).

In models of traumatic brain injury (TBI), administration of LC decreased brain inducible nitric oxide synthase (iNOS) expression, increased MnSOD (or SOD2) levels and decreased both total and mitochondrial ROS levels, after 24 h post-injury (Chen et al., 2018).

1.6.2. Acetyl-L-carnitine (ALC)

ALC is an acetyl ester of LC, playing a similar role to LC, the transport of long-chain fatty acids from cytosol to mitochondria, and also donates acetyl groups. Similar to LC, ALC is transported to the brain by crossing the BBB thanks to OCTN2, or secondarily, via ATB0+, a Na^+/Cl^-dependent amino acid transporter.

In the brain, ALC present anti-depressive, analgesic, neurotrophic, neuromodulatory, and neuroprotective effects. Besides these effects, ALC also shows anti-apoptotic and antioxidant properties. Due to the presence of its acetyl group, ALC constitutes a supplier of acetyl groups for the synthesis of acetylcholine, as described before, and for acetyl-CoA synthesis in the mitochondria. (Traina et al., 2016). ALC also regulates dopaminergic, serotonergic, noradrenergic, and GABA transmission, contributing for the modulation of synaptic transmission, through correction of the deficiencies presented in the release of the different neurotransmitters, and the regulation of its receptors expression (MacDaniel et al., 2003; Smeland et al., 2012).

ALC also regulates several genes involved in brain metabolism and function. One of the genes altered is the prostaglandin D2 synthase gene. ALC up-regulates this gene, and the activation of a PGD2 receptor prevents neuronal injury, in an episode of acute excitotoxicity, in parallel with these findings, a product of PGD2 exerts anti-inflammatory properties (Liang et al., 2005).ALC action in cognitive function is also beneficial. As such, ALC is responsible for improving learning, attention, and spatial working memory abnormalities, but also, inhibiting of apoptotic cascade and decreasing the oxidative environment (Barhwal et al., 2007). Besides these neuromodulated effects, this carnitine form induces nerve growth factor (NGF) production and its receptor binding in vivo. The NGF is a growth factor responsible for the neuronal development and maintenance/homeostasis of central and peripheral nervous system; specifically, it can modulate nerve fiber regeneration and neuronal repair. According to the literature, ALC is responsible for enhancing the axon diameter and the density of regenerating myelinated fibers. Moreover, ALC is beneficial in primary motor neurons exposed to an excitotoxic environment or BDNF deprivation, by enhancing ALC's

neuroprotective and neurotrophic properties (Bigini et al., 2002). In this way, ALC promotes the activation of PI3K, PKC, and extracellular signal-regulated kinases (ERK)1/2 signaling cascades, allowing neuronal cell proliferation, and enhancing mitochondrial respiration in neurons, as these pathways play a pivotal role in neuronal cell survival and ERK 1/2 is an endogenous negative regulator of gamma-secretase activity. Interestingly, PKC higher levels in the rat brain cortex translated into better cognitive task performances (Abdul et al., 2007).

In addition, ALC plays several roles in normal mitochondrial function. ALC can regulate different mitochondrial mechanisms, such as mitochondrial energetics, mitochondrial respiratory chain, mitochondrial proteins synthesis, mitochondrial biogenesis, and antioxidant systems (Virmani et al., 2001). To regulate all these mechanisms, ALC promotes fatty acids beta-oxidation and ketosis for ATP production under compromised metabolic conditions (mitochondrial energetics), enhances the COX and complex bc1 gene expression (mitochondrial respiratory chain), increases the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) signaling cascade (mitochondrial biogenesis), stimulates mitochondrial DNA transcription, stability (Virmani et al., 1995). ALC further maintains membrane integrity by alleviating lipid peroxidation, by decreasing the synthesis of ROS and allowing radical scavenging activity, protecting mitochondrial enzymes, and promoting its function (Liu et al., 2004). ALC decreased levels of HNE and protein carbonyls in brains of old rats, two oxidation parameters (Poon et al., 2006). ALC induces a shift in mitochondrial and cytosol redox states, increasing the antioxidant content, and decreasing ROS formation, constituting a protective effects against oxidative stress. A reduction of inflammatory biomarkers, namely inflammatory interleukins and TNFalpha, in the blood (Barhwal et al., 2007). Relative to genetic regulation, three major mechanisms are up-regulated in the presence of ALC: heme oxygenase-1 (HO-1), Nrf2, and heat shock proteins (HSPs). HO-1 is an antioxidant gene that is mostly modulated by oxidant and inflammatory states. An up-regulation of HO-1 diminished Aβ peptide levels in cortical neurons, inhibits the decrease in complex IV activity, and protein nitration, in astrocytes under stress stimulation, and promotes an antioxidant state (Calabrese et al., 2009). Concerning the Nrf2 signaling pathway, ALC induces its acetylation, promoting Nrf2 translocation to the nucleus, and consequently antioxidant enzyme transduction. HSPs

are specific proteins, named chaperones, that protect against cell damage induced by misfiled proteins and prevent dissolution, aggregation, and refolding of accumulated proteins. These HSPs decrease apoptosis protects against oxidative stress and function as cellular protective components against aggregation of proteins. These effects result in restoration of the reduced glutathione/ oxidized glutathione (GSH/GSSG) ratio and block complex IV inhibition (Glover et al., 1998).

1.6.3 Propionyl-L-Carnitine (PLC)

PLC consists of a propionyl ester of LC, which provides enormous amounts of LC, allowing the beta-oxidation process, and consequently, ATP production. An advantage of this carnitine isoform is its bioavailability lifetime, being maintained for 72 h in the organism, while LC and ALC only remain for 24 to 36 h (Wiseman et al., 1998). As occurs for the other carnitine forms, PLC is likely transported to the brain thanks to the OCTN2 receptors or ATB0+, a Na^+/Cl^-dependent amino acid transporter, both present in the BBB. Apart from its metabolic effect, similar to the other isoforms of carnitines, PLC acts as a free radical scavenging agent and has anti-inflammatory properties (Wiseman et al., 1998; Al-Majed et al., 2006). PLC was shown to have cardiovascular-related protective effects in ischemia-perfusion lesions or vascular injuries. One of the PLC protective mechanisms is increasing the Krebs cycle efficacy under ischemia, by providing propionate, which is transformed into succinate without energy consumption (through the anaplerotic pathway) (Al-Majed et al., 2006). There is still little evidence regarding the neuronal efficacy of this compound. Previously, our group showed that PLC can prevent the decrease in cell viability, impairment in mitochondrial function impairment (MMP and mitochondrial respiration), and in mitochondrial dynamics (morphology and function) in $A\beta O_{1-42}$ treated hippocampal neurons (Mota et al., 2021). Furthermore, PLC was shown to alleviate neuronal injury induced by forebrain ischemia and prevent energy depletion and oxidative stress in the hippocampal region. In the same study, the authors showed that PLC has similar neuroprotective to ALC, namely, antioxidant and anti-inflammatory properties (Al-Majed et al., 2006).

1.7 Curcumin

Curcumin (CUR) is a dietary polyphenol that can be found in the rhizome of Curcuma longa, part of the Indian spice turmeric, and constitutes only 2 to 5% of the turmeric spice. CUR has several protective properties, namely anti-inflammatory, antioxidant, antimutagenic, antimicrobial and antioxidant. Thanks to these properties, CUR can play a preventive role in several diseases, including autoimmune, metabolic, cardiovascular, and neurological pathologies. CUR acts, mainly, through the regulation of transcription/growth factors, inflammatory cytokines, proteins, and cellular pathways, by activating or inhibiting those signaling pathways and those enzymes and proteins expression (Mandal et al., 2020).

CUR belongs to the class of curcuminoids; it has low absorption in the intestinal tract, a high metabolic rate, consequently rapid excretion, but low solubility in water. Due to this set of characteristics, CUR has low bioavailability in the body. At physiological conditions (37ºC and pH=7.2) the half-life time is less than 10 min. Curcumin is highly soluble in methanol, acetone, and ethanol, and can be degraded by solvolysis and photodegradation. Solvolysis consists in the elimination or substitution of the nucleophilic by a solvent molecule. CUR photodegradation occurs when the curcuminoid is exposed to sunlight, causing the removal of turmeric stains (Kotha et al., 2019).

Relative to the neuroprotective effects of CUR, this molecule presents several effects in different diseases. In the context of AD, CUR was shown to inhibit the production and effects of both Aβ peptides and hyperphosphorylated Tau. CUR also demonstrated protective effects in microglia modulation, ACh synthesis, regulation of signaling pathways, including Nrf2/KEAP1 (Figure 13) and MAPK/ERK, antioxidant power, and cholesterol regulation (Tang et al., 2017). According to Reddy and collaborators, CUR is capable of promoting Aβ disaggregation and even blocking Aβ aggregation, inhibit Aβ metabolism, increasing the clearance of Aβ peptides by macrophages (Reddy et al., 2018). Furthermore, by inhibiting Aβ self-assembly CUR protected against Aβ-induced oxidative stress and reduced Aβ-induced inflammation

(Reddy et al., 2018; Goozee et al., 2016). CUR was also shown to reduce the hyperphosphorylation of tau, by blocking the activity of glycogen synthase kinase 3β (GSK-3β) action (Tang et al., 2017; Goozee et al., 2016). In human neuroblastoma SHSY5Y cells, CUR inhibited tau hyperphosphorylation, by modulation of phosphatase and tensin homologue (PTEN)- protein kinase B (AKT)/ GSK-3β pathway, which raised by Aβ peptides (Tang et al., 2017). Furthermore, in neuroblastoma cells transfected with APP, CUR was shown to increase Wnt/*β*-catenin activation, through inhibition of GSK-3β (Goozee et al., 2016). GSK-3β appears to act as a negative modulator of Wnt, diminishing its activity, contributing to Tau hyperphosphorylation, and consequently, to the formation of NFTs . Moreover, in hippocampal slices, Aβ peptides block Wnt by activation of Dickkopf-1, a Wnt antagonist, being another Aβ-induced mechanism for Tau hyperphosphorylation (Goozee et al., 2016). Another beneficial evidence about CUR in Tau hyperphosphorylation is the up-regulation of BCL2 associated athanogene 2 (BAG2), a molecular chaperone responsible for the transport of Tau to the proteasome, to be degraded, as observed in rat cortical cells treated with CUR (Tang et., 2017).

In addition to these neuroprotective properties related to AD hallmarks, CUR also has other neuroprotective effects, namely the control of neurogenesis (Figure 12).

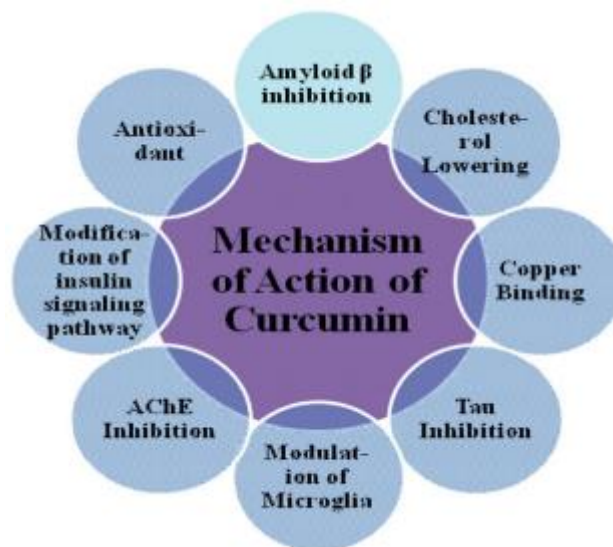Fig. 12 – **Neuroprotective effects of curcumin in AD**. From Mandal et al. (2020).

According to Li and collaborators, APP/PS1 mice treated with CUR present great improvements in learning and memory functions. These results were mainly due to a significant increase in new neural stem cells and newborn neurons, alongside a diminished number of apoptotic neurons in the hippocampus (Li et al., 2019). These

authors also identified a significant increase in Notch1 and Hes1 gene expression, and CDK4, Cyclin D1, NICD, and Hes1 proteins (Li et al., 2019). Notch 1 signaling pathway is known for allowing neurogenesis and neural stem cell development. Moreover, CUR can activate different ERK) and p38 kinases, which are part of transduction pathways related to neuronal plasticity and stress responses (Gooze et al., 2016). Cur has beneficial effects in neuronal cells, namely anti-inflammatory and antioxidant properties. CUR is capable of regulating age-related signaling pathways and decreasing oxidative stress because of its biochemical properties. Due to the presence of phenolic groups in its chemical structure, CUR exhibits a potent hydrogen-donating antioxidant activity. Furthermore, CUR was shown to stabilize the Nrf2, and increase *HO-1* gene expression (Figure 13). CUR also activates several antioxidant enzymes, as GPx, GST, SOD and increases GSH, preventing lipid peroxidation. Another pathway involved in antioxidant defenses and regulated by CUR is the PI3K/AKT and FOXO proteins. FOXO proteins are linked to different cellular processes such as glucose metabolism, cell differentiation, DNA repair, cellular detoxification, and apoptosis. The insulin/IGF-1 signaling pathway, which is mediated by the PI3K/AKT pathway, allows the phosphorylation of conserved residues of FOXO transcription factors (Zia et al., 2021). So, CUR is capable of inducing the activation of PI3K/AKT, allowing the transport of FOXO to the nucleus and activating FOXO-dependent gene expression, increasing the expression of antioxidant -FOXO-related genes, and thus enhancing the lifespan aged mouses by decreasing oxidative stress (Zia et al., 2021). CUR is also responsible for increasing lifespan through modulation of several factors involved in apoptosis and inflammation, namely NF-kB, Bad, caspase 9, Bcl-2, and Fast. According to Uğuz and collaborators, CUR prevents apoptosis by regulating ROS, mitochondrial depolarization and intracellular calcium release in neuroblastoma SH-SY5Y cells exposed to H_2O_2. This occurs in parallel with increases in GSH and GPx activity. These effects are, mainly, a result of CUR's scavenging capability (Uğuz et al., 2016). CUR analogs and curcuminoids can also be used as fluorescent imaging probes. One of these probes is the 8b. Some of its characteristics are the higher binding to the Aβ, and emission in the near-infrared (NIR) wavelength region, precisely at 667 nm.

Furthermore, it shows an increase in fluorescence (by about 20-fold) after binding with the target biomarker, and high contrast between wild-type and AD model (Park et al., 2021).
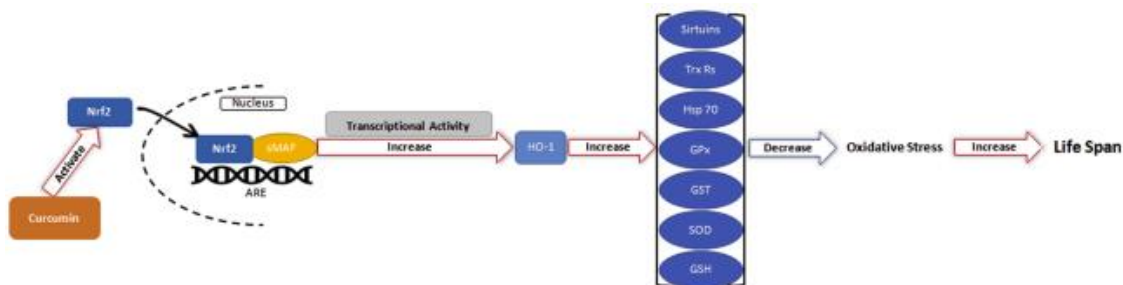

Fig.13 – Curcumin's effects on activation of antioxidant Keap1-Nrf2 pathway. Curcumin activates Nrf2, allowing it's translocation to the nucleus, promoting the transcription of antioxidant enzymes, which combat oxidative stress, enhancing the lifespan. From Zia et al. (2021).

1.8 Liposomes

Liposomes are a drug encapsulation system consisting of small spherical artificial vesicles, composed by at least one lipid bilayer that may contain selective amphipathic lipid composition, as cholesterol and phospholipids (Akbarzadeh et al., 2013). This, together with their biocompatibility and easy degradation in the organism, make this pharmaceutical nanotechnology system a promising approach for drug delivery (Abu Lila et al., 2017). Liposomes may have a wide spectrum of formulations; their composition is based on different properties, namely the lipid composition of the bilayer, the surface charge of the liposome, size, and method of preparation (Akbarzadeh et al., 2013). During liposomal synthesis, the components of the lipid bilayer determine the cargo present in the system, as well as its rigidity or structural fluidity. For example, if the objective is the synthesis of a more flexible and permeable system, the use of unsaturated phosphatidylcholine is preferred, whereas if a rigid structure is desired, the use of saturated phospholipids is promoted (Akbarzadeh et al., 2013; Abu Lila et al., 2017). Moreover, this kind of pharmaceutical nanotechnology system is also resistant, to prevent either oxidation or degradation of liposome content, protecting from digestible enzymes, and free radicals. Different methods can be used to produce liposomes, all of which comprise four common steps: elimination of the organic solvent,

dispersion of the lipid in an aqueous medium, purification of the liposome, and analysis of the final product (Akbarzadeh et al., 2013; Abu Lila et al., 2017). Methods of liposome preparation can be divided into two broad groups: passive and active loading techniques. The most used are passive loading techniques, and these include three different methods: mechanical dispersion, solvent dispersion, and detergent removal. In the present study, three mechanical dispersion techniques were used: sonication, extrusion, and hydration of the lipid film (Akbarzadeh et al., 2013). Sonication is a process where the energy of sound waves is used, usually ultrasound. This procedure has some disadvantages, which can lead to low encapsulation efficiency, and degradation of phospholipids and drugs, among others. Extrusion is a process where the liposome sample passes through an extrusion column to homogenize the size of the nanoparticles. Hydration of the lipid film consists in the addition of a solution containing the drug to the lipid film formed after evaporation of the organic solvent (Akbarzadeh et al., 2013). Considering their biocompatibility, biodegradability, low excitotoxicity, and ability to associate both hydrophilic and lipophilic compounds, as well as the envisage of site-specific drug delivery, liposomes can be used as DNA or drug-delivery systems.

1.9 Objectives:

According to previous studies, both CUR and the different forms of carnitine, LC, ALC and PLC, play neuroprotective effects in neurodegenerative diseases, particularly in AD. These neuroprotective outcomes are based on the regulation of several endogenous molecular mechanisms underlying AD, such as mitochondrial and neuronal dysfunction, oxidative stress, altered Aβ metabolism/clearance, or synaptic loss, among other deregulated pathways. Although both therapeutic molecules may have beneficial properties from the pharmacological point of view, some of their physicochemical properties are obstacles for their use Therefore, a pharmaceutical nanotechnology is a smart approach to drug encapsulation, to target the drug to specific areas of the brain, circumventing the physicochemical characteristics of the therapeutic agents and increasing their effects on target brain areas. Therefore, the aim of the present study was to determine the therapeutical capacity of CUR and carnitine forms in *in vitro* AD

models, and also the design of a nanoformulation containing LC. To reach these purposes, the following specific objectives have been traced:

I. **To evaluate the influence of different carnitine forms (LC, ALC, and PLC) on mitochondrial function and cell reducing capacity in APP/PS1 mouse hippocampal and cortical neurons.**

II. **To analyze the effect of curcumin and L-carnitine, individually and in combination, on mitochondrial function and cell reducing capacity in APP/PS1 mouse cortical neurons and Wistar rat cortical neurons exposed to A$\beta_{1\text{-}42}$ oligomers**

III. **To produce and characterize L-carnitine encapsulated liposomes, as an aim to create a pharmacological nanoformulation for potential AD therapeutic application**

CHAPTER II – MATERIALS AND METHODS

2.1 <u>Materials</u>

Neurobasal medium, B27 supplement, fetal bovine serum (FBS), and all antibiotics were purchased from GIBCO (Paisley, UK). Fura – 2/AM, Amplex Red, Tetramethylrhodamine Methyl Ester Perchlorate (TMRM), and Hoechst 33342 nucleic acid stain were purchased from Invitrogen/Molecular Probes (Life Technologies Corporation, Carlsbad, CA, USA). Trypsin, trypsin inhibitor, fatty acid-free bovine serum albumin (BSA), Hydrogen peroxide (H_2O_2), MitoPY, Thiazolyl blue tetrazolium bromide (MTT), and other analytical grade reagents were obtained from Sigma Chemical and Co. (St. Louis, MO, USA). All the primary and secondary antibodies, and probes used for the immunocytochemistry technique are described in Table 1.

Table 1 – Antibody information.

Primary Antibody	Host	Dilution	Reference
Nrf2	Rabbit	1:1000 (ICC)	Abcam #31163-500
SIRT 3	Rabbit	5 μg/mL (ICC)	Abcam ab86671
TFAM	Goat	1:200 (ICC)	Santa Cruz sc-30965
Secondary antibody/ Probes			
Alexa Fluor 488	Donkey (Anti-rabbit)	1:300 (ICC)	#R37118 (Molecular Probes-Invitrogen)
Alexa Fluor 568	Donkey (Anti-goat)	1:300 (ICC)	#A11057 (Molecular Probes-Invitrogen)
MitoTracker Deep Red FM		500 nM	M22426 (Molecular Probes-Invitrogen)

2.2 **<u>Primary hippocampal and cortical culture</u>**

Hippocampal and cortical neuronal cultures were prepared according to a previous protocol performed by Ambrósio et al. 2000, with some differences.
WT female mice (obtained from Charles River; under B6C3F1 background) were previously crossed with heterozygous APP/PS1$^{+/-}$ male mice (B6C3Tg(APPswe, PS1dE9)85Dbo, CNC ORBEA approval reference ORBEA 211_2018). Pregnant WT female mice or rat Wistar females, were sacrificed by anesthesia with isofluorane followed by

cervical dislocation. The embryos (E17-18) were collected and placed in HBSS medium containing 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM NaH_2PO_4-$2H_2O$, 5 mM glucose, 5.36 mM HEPES, 5.36 mM Pyruvate, 0.001% phenol red, pH 7.3, and after the cortex and hippocampus were dissected. After genotyping was completed, the cortex and hippocampus were digested in a plating medium composed of 84 mM MEM M0268, supplemented with 43.3 mM $NaHCO_3$, 52.73 mM glucose, and 1 mM sodium pyruvate, 10% inactivated horse serum, pH 7.2, by mechanic digestion with a micropipette. The cells were plated at $2.50x10^4$ in Poly-D-Lysine coated 96 wells microplates, for 2 hours in a plating medium. After the 2 hours of incubation in the plating medium, the plating was replaced by serum-free Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 0.12 mg/mL gentamicin for cortex neurons, and serum-free Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 0.12 mg/mL gentamicin and 25 µM glutamate for hippocampal neurons. Both cells were cultured for 17 days in vitro (DIV) in 95% air and 5% CO_2, to obtain mature hippocampal and cortical cells. Both cell types were observed daily, and medium changes were performed each DIV 5-6 in cortical neurons and DIV 7 for hippocampal neurons.

All animal experiments were carried out following the guidelines of the Institutional Animal Care and Use of Committee and the European Community directive (2010/63/EU) and protocols approved by the Faculty of Medicine, University of Coimbra (ref: ORBEA_211_2018) and the Direção Geral de Alimentação e Veterinária (DGAV, ref: 0421/000/000/2019). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3 <u>Experimental conditions:</u>

The hippocampal and cortical neurons from Wild-Type (WT) and APP/PS1 mice, and rat Wistar cortical neurons were subjected to different compounds: H_2O_2, carnitines, curcumin, and AβO. The experimental conditions were described below:

- Cortical and hippocampal neurons of WT and APP/PS1 were subjected to different concentrations of H_2O_2 (1, 10, and 100 µM) for 24 hours;

- WT and APP/PS1 cortical neurons were subjected to administration of 5 mM LC, ALC and PLC for 24 hours;

- Wistar cortical neurons were exposed to 1 μM AβO for 24 hours, with a pretreatment (1 hour before the addition of AβO) of 1 mM LC, ALC, and PLC individually and in combination;

- Wistar cortical neurons were exposed to 10 μM H_2O_2 for 24 hours, with a pretreatment (1 hour before the addition of AβO) of different concentrations of curcumin (1, 5, 10, 15, 20, 25, 30 μM);

- Wistar cortical neurons were exposed to 1 μM AβO, pretreated (1 hour before the addition of AβO) with 5 mM LC, 10 μM CUR, and a combination of both.

2.4 MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay is based on the reduction of MTT by intracellular dehydrogenases (present in living cells) to the insoluble blue formazan salt, being the intensity of color produced dependent on the cell reducing activity (Mosmann 1983). MTT assay is a commonly used test to evaluate cellular metabolic activity as an indicator of cell viability. Thus, loss of MTT reduction in neurons may be used as an indicator of cellular dysfunction. After treatment, cells were washed three times with PBS and incubated with MTT (0.5 mg/mL) prepared in Na^+-based medium containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4/NaOH, in the dark, for 2 h at 37 °C. At the end of the incubation with MTT, an equal volume of isopropanol acid (0.04 M HCl in isopropanol) was added and mixed thoroughly to dissolve the formazan crystals. The mixture was then collected from the wells and the extent of MTT reduction was measured spectrophotometrically at 570 nm. The results were expressed as a percentage of control cells.

2.5 Mitochondrial membrane potential assessment

Hippocampal and cortical neurons were incubated in Na^+-based medium containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4/NaOH, with the mitochondrial membrane potential (MMP)-sensitive probe ($TMRM^+$ at 300 nM, under quench mode) for 30 min in the incubator (37 °C, 5% CO_2). After a washing step with Na^+ medium, 100 μL of Na^+ medium were added to each well and using a microplate reader SpectraMax iD3 (Molecular Devices, USA) (540 nm excitation, 590 nm emission, TMRM+ fluorescence was determined. Changes in MMP were assessed by analysis of $TMRM^+$ fluorescence dequenching after complete mitochondrial depolarization (MMP collapse) achieved by adding a protonophore [10 μM carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP)] plus oligomycin (5 μg/mL), the latter to prevent ATP synthase reversal.

2.6 Mitochondrial H_2O_2 levels quantification

MitoPY1 fluorescent probe was used to determine mitochondrial H_2O_2 produced by primary cortical neurons. Neurons were incubated with 10 μM of MitoPY1 in Na^+ medium supplemented with Ca^{2+} (C_f = 1 mM), for 30-40 minutes. After washing step to clean the non-internalized probe, MitoPY1 fluorescence was recorded using a Spectrofluorometer Gemini EM (Molecular Devices, USA) microplate reader at 503 nm excitation and 540 nm emission. The experience consists of 15 minutes period for basal condition, the addition of stimulus Myxothiazol (C_f = 3 μM), a complex III inhibitor which prevents electron transfer at the ubiquinone redox site Q0, and 30 minutes reading after stimulus addition. Fluorescence values were normalized to the baseline.

2.7 Intracellular Ca^{2+} recording

Primary cortical neurons were incubated with a Fura-2/AM radiometric fluorescent probe (2.5 μM), a high-affinity probe for intracellular calcium, for 30-40 minutes, at 37ºC, in an Mg^{2+}-free Na^+ medium. Fura-2/AM, when present in cells, crosses the cell membrane, and in the cytoplasm, the acetoxymethyl groups are cleared by cellular esterases, generating Fura-2, a pentacarboxylate calcium indicator. After

incubation, the cells were washed to remove the probe that does not cross the plasma membrane. After the washing step, Fura-2 fluorescence was analyzed using a Spectrofluorometer Gemini EM (Molecular Devices, USA) microplate reader at 340 (Ca^{2+}-bound)/380 (Ca^{2+}-free) nm excitation and 510 nm emission wavelengths. The probe was recorded for 2 minutes (basal) and for 5 minutes after the addition of stimulus (Oligomycin (C_f = 2 µg/mL)/FCCP (C_f = 2 µM)), stimulus used to evaluate the mitochondrial Ca^{2+} release to the cytosol). The fluorescence data (ratio 340/380) were normalized to the baseline.

2.8 <u>Immunocytochemistry</u>

Mature cortical neurons cultured in glass coverslips were washed three times with PBS (at warm temperature), then incubated with MitoTracker deep Red(500 nM) for 45 minutes. After MitoTracker staining concluded, the cells were washed once three times with PBS (at warm temperature), and then fixed with 4% paraformaldehyde (at 37ºC) for 15 minutes, and permeabilized in 0.2% Triton X-100 in PBS for 2 minutes. After fixation and permeabilization concluded, neurons were blocked for 1 hour at room temperature with 3% (w/v) BSA in PBS (blocking solution) and posterior incubation with primary antibody prepared in blocking solution, overnight, at 4ºC. After finished primary antibody staining, cells were washed three times with PBS and then incubated with the secondary antibody in a blocking solution for 1 hour at room temperature. Nuclei were marked using Hoechst 33342 in PBS (1 µg/mL) for 10 minutes and coverslips were prepared using 20 µL of DAKO. Confocal images were obtained by using a PlanApochromat/1.4NA 63x lens on an Axis Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software.

2.9 Preparation of liposomes nanoformulation

1. Standard L-Carnitine solution

LC was accurately weighed (2 mg) and then transferred to a 2 mL Eppendorf, to which 2 mL PBS (1x) was added, to dissolve the LC and obtain a homogeneous LC solution (1 mg/mL), for later addition to the lipid film.

2. Liposomes nanoformulation - neutral liposomes & cationic liposomes

A mixture of cholesterol (50%) and phosphatidylcholine (POPC, 50%) was used for the synthesis of neutral liposomes. In a round-bottomed flask, previously washed with ethanol, cholesterol (76 µL) and POPC (152 µL) were added. For the preparation of cationic liposomes, a mixture of cholesterol (50%), POPC (50%), and ethylphosphatidylcholine (EPOPC, 20%) were used. The procedure was similar to that used in neutral liposomes, so for this formulation, 76 µL of cholesterol, 92 µL of POPC, and 165 µL of EPOPC were pipetted. After preparing the mixtures of both types of liposomes, the organic solvent was then removed using a rotary evaporator. In this apparatus, the flasks were placed in turn, at a temperature of 40ºC and 150 rotations per minute, until complete evaporation of ethanol was observed. After obtaining the lipid film, proceed to its hydration. 1 mL of the standard LC solution was added to each

Table 2 – Composition of liposomal formulations

Neutral liposomes		Cationic liposomes	
Excipient	Proportion	Excipient	Proportion
Cholesterol	50%	Cholesterol	50%
Phosphatidylcholine (POPC)	50%	Phosphatidylcholine (POPC)	30%
API (L-Carnitine)	1 mg	E-Phosphatidylcholine (EPOPC)	20%
		API (L-Carnitine)	1 mg

lipid film and the lipid film was vortexed until a cloudy solution with no pieces of lipid film was observed. Then we performed the sonication process for two periods of 10 minutes, for both nanoformulations. Just the cationic liposome solution gone to the extrusion system, to homogenize the size of the particles. Finally, the cationic liposomes sample was filtered by an exclusion column, and the liposomes containing LC were quantified by a cholesterol assay.

3. L-carnitine quantification by reverse-phase HPLC

LC was quantified by a reverse-phase HPLC procedure. This reverse-phase HPLC process took place on a Shimadzu LC-20 AT HPLC system, SPD-M20A. A RP-HPLC C18 column (Waters, Milford, MA, USA, 25 cm length, 4.6 mm internal diameter, 5 µm particle size with a pore diameter of 125 A˚) was used for all analyses. A mixture phosphate buffer (0.05 mM, pH= 3.2; prepared by mixing HPLC grade methanol and buffer in the ratio of 5:95 V/V, and filtered by a 0.5 µM membrane filter), methanol and type I H_2O in the ratio of 80:10:10, respectively, was used as a mobile phase with a flow-rate of 1.0 ml/min for a maximum elution time of 30 min, at 40ºC. The sample size was 20 µl of liposomes containing LC, being the analyses performed in triplicates for each sample. LC was detected at 225 nm, being its retention time approximately 4 minutes. For the internal standard, a free LC calibration curve was prepared using final concentrations of 0.05, 0.075, 0.1, 0.125 and 0.25 mg/mL in triplicate, in order to quantify LC content in liposomes. The R^2 value was 0.9993 and the slope R.S.D. values between injected samples were lower than 8%. Besides these two analyses, third run with empty liposomes was also performed to verify the specificity of the method, and that the liposomal formulation did not interfere with LC quantification. The conditions of the third run were equally to the previous ones.

2.10 Statistical analysis

Statistical significance was obtained through one-way (ordinary or repeated measures) or two-way ANOVA for the multiple groups or by the Side's multiple comparison test, Mann-Whitney test and unpaired Student's t-test for comparison between two Gaussian populations, as identified in figure legends. Data were analyzed through the use of Excel (Microsoft, Seattle, WA, USA) or Numbers (Apple IOS, California, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) softwares. Data were expressed as the mean ± S.E.M of the number of experiments indicated in figure legends.

CHAPTER III - RESULTS

3.1 Influence of hydrogen peroxide (H2O2) on mitochondrial function and cell viability in APP/PS1 mouse cortical and hippocampal neurons

The primary task of the study was to carry out a mitochondrial characterization of APP/PS1 *versus* WT mouse, cortical and hippocampal primary neurons, to determine their susceptibility following exposure to hydrogen peroxide (at 1, 10 and 100μM). Mitochondrial function was assessed by determining the MMP and cell viability assessed by determining cellular reducing capacity (largely mitochondrial-driven), using the TMRM$^+$ probe and MTT assay, respectively.

1. Decreased mitochondrial membrane potential and cell reducing capacity in APP/PS1 cortical neurons

In Figure 14, we observed a significant decrease in MMP in APP/PS1 cortical neurons compared to WT neurons by about 50%, in the absence of H_2O_2. Following exposure to H_2O_2 in WT cortical neurons showed a large and significant reduction in MMP after exposure to 100 μM H_2O_2. In APP/PS1 cortical neurons, exposure to 1 μM H_2O_2, caused a significant and unexpected increase in MMP, suggesting a compensatory response of neurons undergoing mild oxidant stress. After incubation with 10 or 100 μM H_2O_2, APP/PS1 neurons, exhibited a similar difference in MMP as WT neurons subjected to the same H_2O_2 concentrations.

In WT mouse cortical neurons, exposure to H_2O_2 induced a concentration dependent decrease in cell reducing capacity, which was, significant in the presence of 10 and 100 μM H_2O_2. APP/PS1 neurons showed a significant decrease in cell reducing capacity when compared to WT neurons (by about 50%), indicating a decreased in cell viability linked to decreased levels of cellular reducing factors, particularly derived from

mitochondria. A significant difference in cell reducing capacity in APP/PS1 was only observed after exposure 100 µM H_2O_2, similarly as detected in WT neurons.
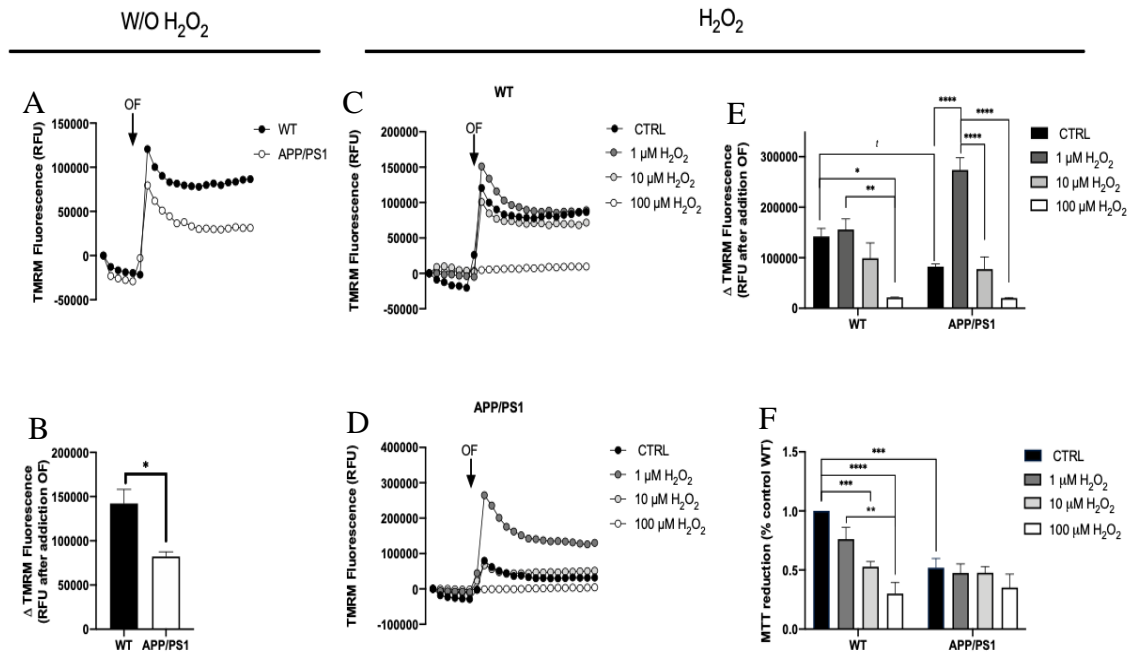

Fig. 14 - Effect of hydrogen peroxide (H_2O_2) in APP/PS1 and WT mouse cortical neurons. Mature WT and APP/PS1cortical neurons (16-18 DIV) were subjected to different concentrations of H_2O_2 (1 µM, 10 µM and 100 µM) for 24h, and both MTT reducing assay and TMRM+ fluorescence assay were performed to assess cell viability and mitochondrial membrane potential (MMP), respectively. MMP was measured after complete mitochondrial depolarization using oligomycin plus FCCP (5µg/mL and 10 µM, respectively). **A,C,D_** Representative TMRM+ fluorescent traces before and after exposure to oligomycin plus FCCP in WT and APP/PS1 neurons. **B,E_** TMRM+ florescence changes after addition of oligomycin plus FCCP **F)** MTT reduction assay. Data are expressed as the mean ± SEM of n = 3–6, independent experiments run in triplicates. In **B,E_** Statistical analysis was performed by two-way ANOVA. Two-way repeated-measure analysis of interaction (genotype × concentration) revealed a significant main effect of concentration ($F_{3,25}$= 11.79; p < 0.0001), only. Genotype ($F_{1,25}$ = 0.06194; p = 0.8055), and interaction between factors ($F_{3,25}$ = 0.7920; p = 0.5098) did not reveal any significance. In **F_** Analysis was performed by two-way ANOVA; Two-way repeated-measure analysis of interaction (genotype × concentration) revealed a significant main effect of concentration ($F_{3,22}$ = 13.47; p < 0.0001) and genotype ($F_{1,22}$ = 15.18; p = 0.0008), and a significant interaction between factors ($F_{3,22}$ = 5.953; p = 0.0039). The symbols represent statistical significant: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

2. Unchanged mitochondrial membrane potential, but decreased cell reducing capacity in APP/PS1 hippocampal neurons

Concerning the hippocampal neurons no significant changes in mitochondrial membrane potential were observed in APP/PS1 *versus* WT neurons (Figure 15). Furthermore, in both WT and APP/PS1 cells, exposure to 100 µM H_2O_2, only evoked a drastic reduction in MMP.

Analysis of cell reducing capacity showed significant decrease in APP/PS1 *versus* WT hippocampal neurons (Fig.15), suggesting reduced cell viability independently of mitochondrial dysfunction. Exposure to 100 µM H_2O_2 induced a higher decrease in cell reducing capacity in both WT and APP/PS1 neurons.
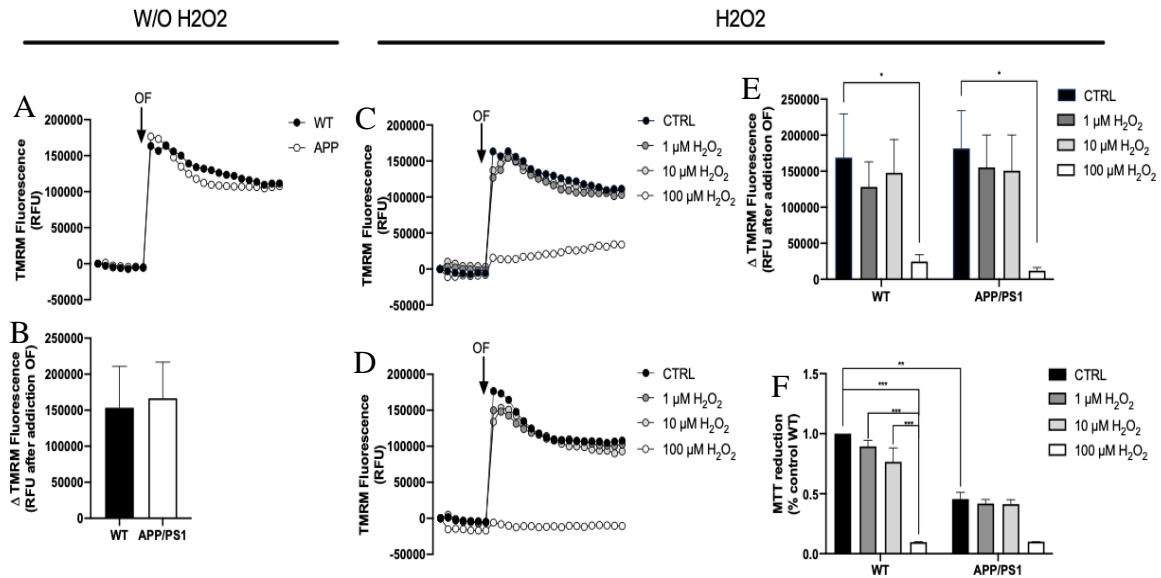

Fig.15 - Effect of Hydrogen peroxide (H2O2) in WT and APP/PS1 hippocampal neurons. Mature WT and APP/PS1 hippocampal neurons (16-18DIV) were subjected to different concentrations of H2O2 (1µM, 10µM, 100µM) for 24h, and both MTT reducing assay and TMRM + fluorescence assay were performed to access cell viability and mitochondrial membrane potential (MMP), respectively. MMP was assessed after complete mitochondrial depolarization using Oligomycin plus FCCP (5µg/ml and 10 µM, respectively). **A,C,D-** Representative TMRM+ fluorescent traces before and after exposure to oligomycin plus FCCP in WT and APP/PS1 neurons. **B,E** TMRM+ florescence changes after addition of oligomycin plus FCCP. **F)** MTT reduction assay. Data are expressed as the mean ± SEM of n = 3-4, independent experiments run in triplicates. In **E,** statistical analysis was performed by Side's multiple comparison test, showing significance in WT-CTRL vs. 100 µM H_2O_2 (p=0.0238), and, in APP/PS1-CTRL vs. 100 µM H2O2 (p=0.0309); In **F,** statistical analysis was performed by two-way ANOVA; Two-way repeated-measure analysis of interaction (genotype × concentration) revealed a significant main effect of Concentration ($F_{3,9}$ = 30.33; p < 0.0001) and Genotype ($F_{1,9}$ = 45.36; p < 0.0001), and a significant interaction between factors ($F_{3,99}$ = 5.501; p = 0.0201). The symbols represent statistical significant: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

3.2 Unaltered mitochondrial calcium or mitochondrial H_2O_2 levels in cortical and hippocampal APP/PS1 neurons

The levels of mitochondrial Ca^{2+} and H_2O_2 were further determined in both cortical and hippocampal APP/PS1 *versus* WT primary neurons (Figure 16), to further help characterizing the mitochondrial deregulation defined in APP/PS1 cortical neurons. Selective fluorescent probes, namely Fura-2/AM (which measures cytosolic Ca^{2+}) after complete mitochondrial depolarization with oligomycin plus FCCP, and MitoPY, were used to determine the changes in mitochondrial Ca^{2+} retention and organelle H_2O_2 production, respectively. According to the results depicted in Figure 16, none of the measurements revealed any significant differences between the two genotypes in either
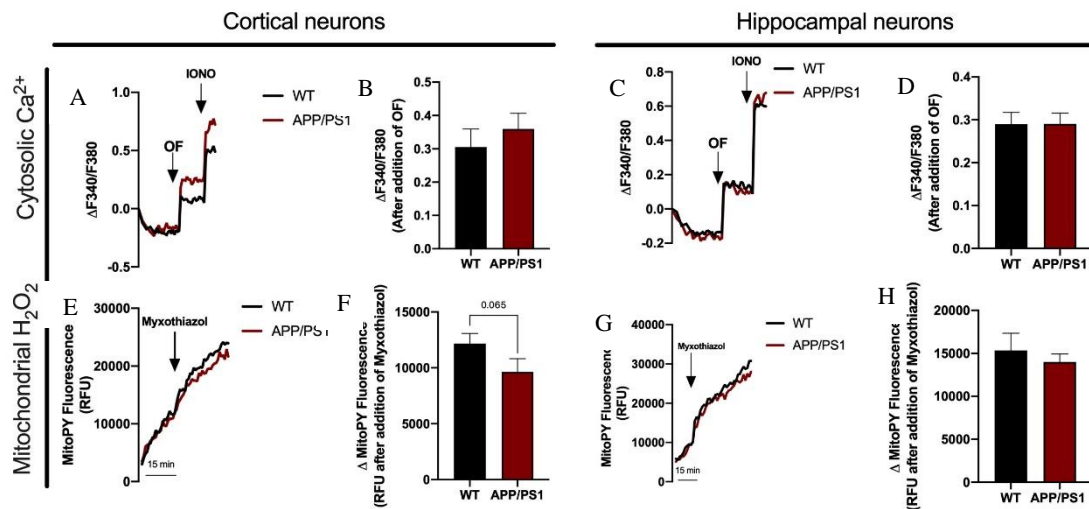

Fig. 16 – Mitochondrial calcium retention and H_2O_2 levels in WT & APP/PS1 cortical and hippocampal neurons. Mature WT and APP/PS1 cortical and hippocampal neurons (16-18 DIV) were subjected to MitoPY 1 and Fura-2/AM assays, to access mitochondrial H2O2 and calcium levels, respectively**. A) C)** Representative traces of Fura-2/AM. **B) D)** Difference between last and first value measured before and after OF addition. **E) G)** Representative traces of MitoPY1. **F) H)** Difference between last value obtained before Myxothiazol addition and the final point. Data are expressed as the mean ±SEM of n=3, independent experiments run in triplicates. **B)D)F)H)** All graphs were analyzed by Mann-Whitney Test, and non-revealed any significance.

cortical or hippocampal neurons. When assessing mitochondrial H_2O_2 levels, we observe a tendency for a decrease in mitochondrial H_2O_2 in APP/PS1 neurons, compared to WT neurons, which was unexpected.

3.3 Acetyl-L-carnitine and propionyl-L-carnitine induce improvements in mitochondrial membrane potential and cell reducing capacity in cortical neurons

Next, we analyzed the influence of different forms of carnitine, namely LC, ALC and PLC (at 5 mM) in, WT and APP/PS1cortical neurons (Figure 17). Of relevance, all carnitine forms presented significant improvements in cell reducing capacity in WT neurons, while only ALC and PLC significantly improved cell reducing capacity in APP/PS1 neurons. When assessing mitochondrial membrane potential in WT neurons, ALC only caused a significant increase in MMP. In APP/PS1 neurons, in turn, all forms apparently contributed for an increase in MMP, however, data was significant for PLC. These results indicate that PLC effectively promotes both mitochondrial function and cell viability in APP/PS1 neurons.
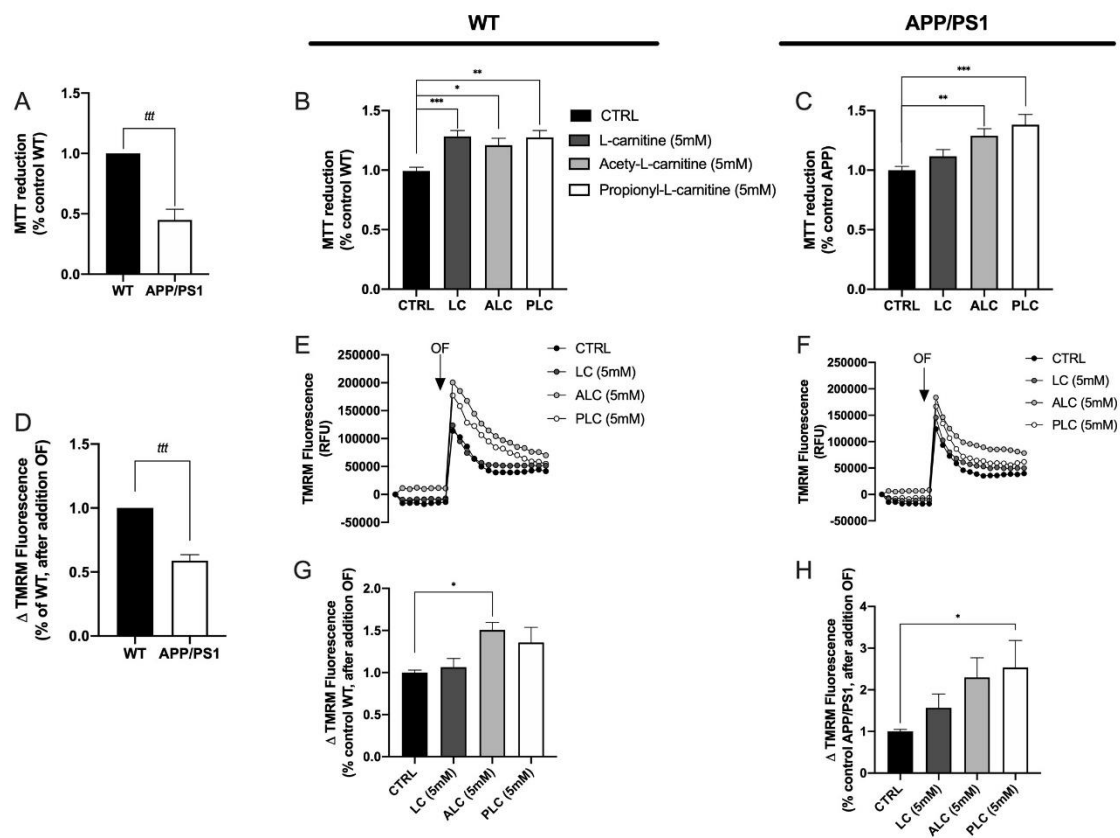

Fig. 17 - Effect of carnitine isoforms (LC, ALC and PLC) in WT and APP/PS1 cortical neurons. Mature WT and APP/PS1 cortical neurons (17-18DIV) were subjected to different forms of carnitine (5mM) and MTT reducing assay and TMRM + fluorescence assay were performed to access cell viability and mitochondrial membrane potential (MMP), respectively. MMP was assessed after complete mitochondrial depolarization using Oligomycin plus FCCP (5µg/ml and 10 µM, respectively). **E) F)** Representative TMRM+ fluorescent traces of FCCP/Oligomycin - induced mitochondrial membrane potential changes. **D)G) H)** Δ of TMRM after addiction of FCCP+Oligomycin. **A) B) C)** MTT reduction assay. Data are expressed as the mean ± SEM of n = 3-5, independent experiments run in triplicates. **A) D)** Analysis was performed by unpaired Student's t test. Graph A presents a p=0.0009 and t=6.132, df=6. Graph D presents p=0.001 and t=8.692, df=4; **B) C) G) H)** Analysis was performed by one-way ANOVA with multiple comparisons. Graph presents B=6.07 and p=0.0013. Graph C presents F=7.783 and p=0.0002. Graph G presents F=4.627 and p=0.0101. Graph H presents F=2.610 and p=0.0711. Statistical symbols: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

3.4 Low concentrations of carnitine forms do not ameliorate disturbances in mitochondrial membrane potential or cell reducing capacity in cortical neurons exposed to AβO

After determining visible protective effects induced by 5 mM of carnitine forms, we tested the effect of a lower concentration (1 mM) of carnitines forms, to further determine a possible synergistic effect when they were combined in pairs. These studies were conducted in Wistar cortical neurons due to the lack of pregnant APP/PS1 females. Figure 18 revealed no significant effects of Aβ oligomers alone or carnitine forms, either individually or combined, on MMP in Wistar cortical neurons.

When assessing cell reducing capacity by the MTT assay, we observed that Aβ oligomers caused a significant effect in Wistar cortical neurons, compromising cell-reducing capacity and thus cell viability in cortical neurons. Under these conditions, carnitine forms, individually or in pair combinations, did not prevent the changes in cell reducing capacity. These data suggest that 1 mM carnitine forms are unable to rescue the dysfunction exhibited after Aβ oligomers exposure.
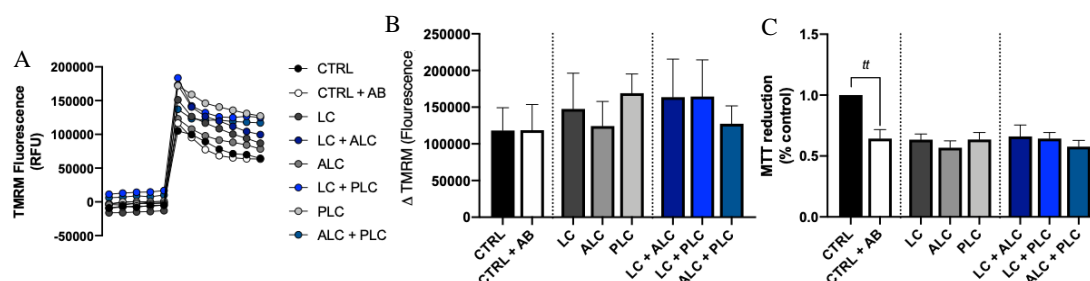
Figure 18 – Effect of carnitine forms (LC, ALC and PLC) in Wistar cortical neurons exposed to AβO. Mature Wistar cortical neurons (16-18 DIV) were exposed to AβO (1 µM) for 24h, with a prior treatment (1 h before Aβ exposure), individually or in pairs, of carnitine forms (1 mM) and TMRM+ fluorescence and MTT assay were performed, to assess mitochondrial membrane potential and cell reducing capacity, respectively. **A)** Representative TMRM+ fluorescent traces. **B)** Δ of TMRM fluorescence after addition of oligomycin plus FCCP. **C)** MTT reduction assay. Data are expressed as the mean±SEM of n=3, independent experiments, run in triplicates. **B) C)** Both graphs were analyzed by ONE-WAY ANOVA, although none of them revealed any significance. **C)** Control conditions (CTRL & CTRL + Aβ) were analyzed by Mann-Whitney test and showed significance. $t<0,05$; $tt<0.01$

3.5 Apparent protective effects of Curcumin in rat cortical neurons exposed to H_2O_2 (10 µM)

CUR was also assessed as a possible therapeutic agent in AD neuronal model. To determine the best concentration of CUR to be used, we analyzed the cell reducing capacity as an indicator of cell viability. In rat Wistar cortical neurons exposed to H_2O_2. Figure 19 shows an apparent decrease in cell reducing capacity induced by exposure to 10 μM H_2O_2. Under these conditions, pre-exposure to at 1, 5 and 10 μM increased cell reducing capacity to values almost similar to control/untreated cells., In accordance with these data, several articles indicate that the range of CUR concentrations that have beneficial effects in neurons varies between 1-10 μM (Wang et al., 2012; Huang et al., 2012; Wu et al., 2015; Qin et al., 2009).

Fig. 19 –Effect of different concentrations of curcumin in Wistar cortical neurons exposed to H_2O_2 on cell reduction capacity. Mature Wistar cortical neurons (16-18 DIV) were exposed to 10 μM H_2O_2 for 24h, and pre-treated (1 h before H_2O_2 exposure)with different concentrations of curcumin (1, 5, 10, 15, 20, 25, 30 μM). MTT reduction assay was performed to assess cell reducing capacity. Data are expressed as the mean ± SEM of n=2, independent experiments runs in triplicates.

3.6 L-carnitine and curcumin rescue cell viability in AβO-exposed Wistar rat cortical neurons

Considering that both CUR and LC might have a major protective effect if combined, next we evaluated their effect in cortical neurons exposed to AβO, by determining the changes in MMP and cell reducing capacity. Figure 20 shows that administration of LC (5 mM) per se promotes cell reducing capacity in untreated neurons, while LC alone or combined with CUR (10 μM) significantly enhances MTT reduction in neurons subjected to 1 μM AβO The effect obtained following the administration of the combined compounds seem to be mainly due to the activity of LC, since the % of MTT reduction in LC individually or CUR plus LC were very comparable.

Indeed LC promotes an improvement in cell viability, in both neurons treated or non-treated with AβO. When assessing the changes in MMP, none of compounds caused significant effects. Therefore, LC or CUR, alone or combined, are not able to reverse the mitochondrial dysfunction caused by AβO.
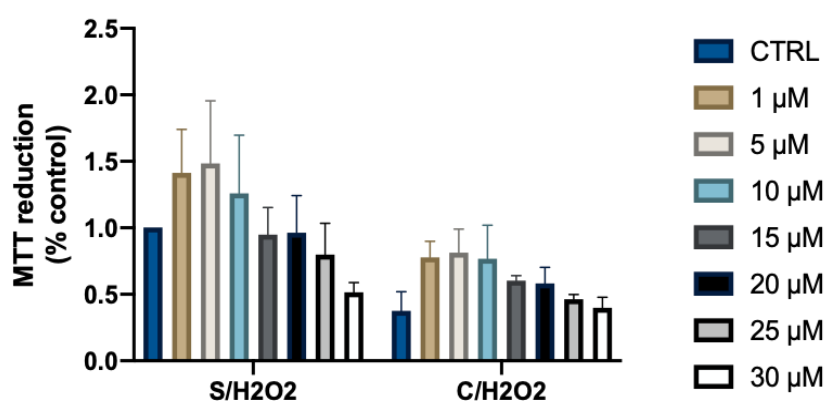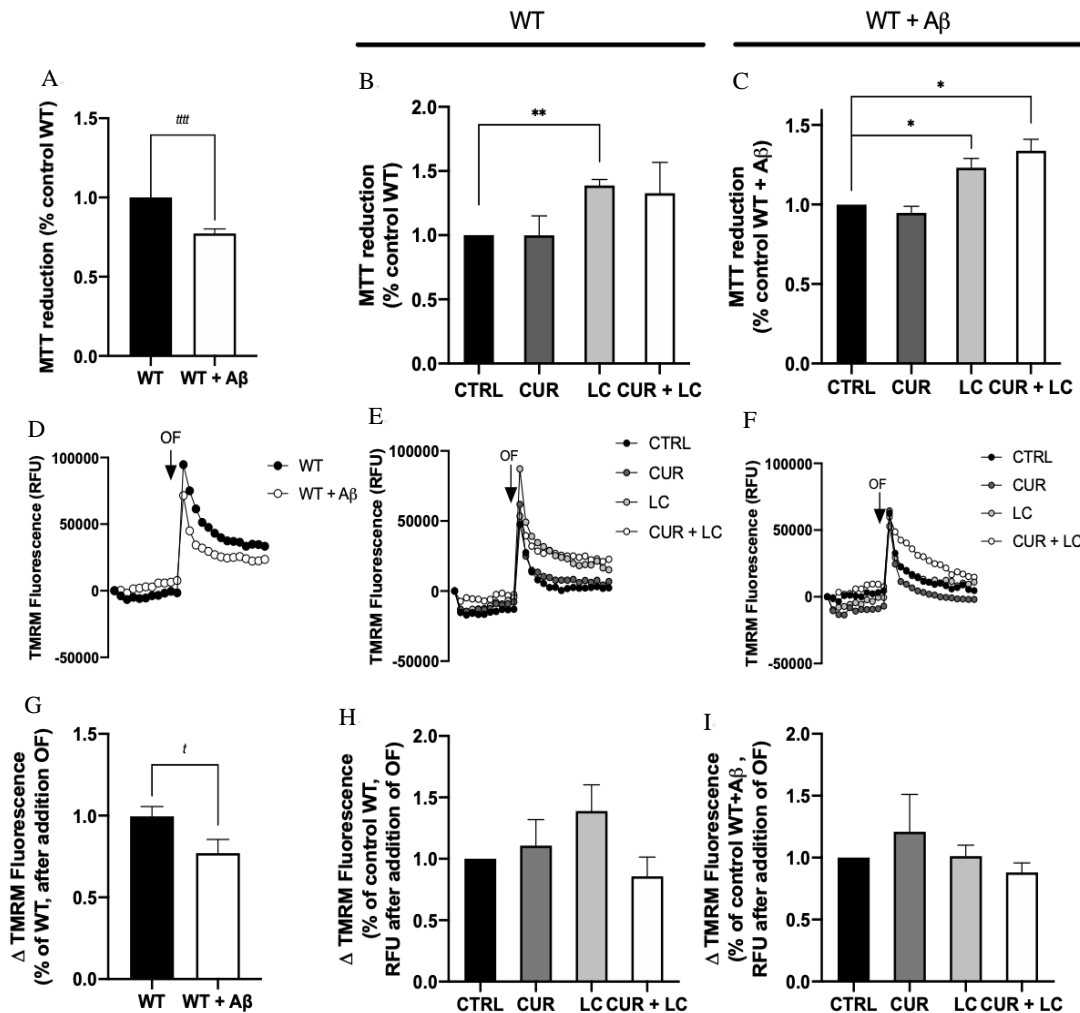

Fig. 20 - Effect of curcumin, L-carnitine, alone or combined in Wistar rat cortical neurons exposed to AβO. Mature Wistar cortical neurons (16-18 DIV) were exposed to AβO (1 μM) for 24h, following a pre-treatment (1h before Aβ exposure) with CUR (10 μM), LC (5 mM), or CUR plus LC. MTT reduction assay and TMRM+ fluorescence assay were performed to assess cell reducing capacity and mitochondrial membrane potential, respectively. **A) B) C)** MTT reduction assay. **D) E) F)** Representative TMRM+ fluorescent traces. **G) H) I)** Δ of TMRM+ fluorescence after addition of oligomycin plus FCCP. Data are expressed as the mean±SEM of n=3-5, independent experiments runs in triplicates. **A) G)** Analysis was performed by unpaired Student's t test. Graph A presents p=0,0002, t=12.18, and df=4. Graph G present p=0,0479, t=2.167, and df=14. **B) C)** Analysis was performed by one-way ANOVA. Graph B presents p=0,1502, F=3.645, and Graph C presents p=0,0021, F=17.14.Statistical symbols: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

3.7   Influence of L-carnitine and curcumin on candidate protein levels Wistar cortical neurons exposed to Aβ oligomers

We further analyzed influence of LC and CUR treatment on the levels of potentially protective proteins, namely sirtuin 3 (SIRT3), mitochondrial transcription factor A (TFAM), and Nrf2, in Wistar rat cortical neurons exposed to AβO, by immunocytochemistry. Using this technique, nuclei and mitochondria were labeled, in addition to the proteins identified above (Figure 21).Mitochondria were labelled with MitoTracker Deep Red FM, which is sensitive to changes in MMP.CUR also and CUR plus LC combined treatments significantly increased fluorescence intensity of MitoTracker Deep Red, which is indicative of an increase in MMP, when compared to AβO exposure only, suggesting that both treatments may play a role in restore of mitochondrial function. The protein levels of SIRT3 were not significantly different in any experimental condition tested, suggesting that SIRT3 levels are not regulated by any of the compounds tested or by AβO. Concerning the protein levels of TFAM, statistical significant decreased values were observed after administration of CUR only in neurons treated with AβO, suggesting either that CUR treatment has an inhibitory effect on TFAM protein expression, and/or promotes its degradation. Finally, we determined Nrf2 protein levels; pre-treatment with CUR alone or with CUR plus LC in neurons exposed to AβO significantly increased Nrf2 protein levels relatively to neurons treated with AβO alone. These results suggest that CUR effectively increases Nrf2 cellular levels, either through increased expression and/or decreased degradation, which may potentially activate the Keap1-Nrf2 antioxidant pathway, exerting a protective effect against the exposure of AβO.
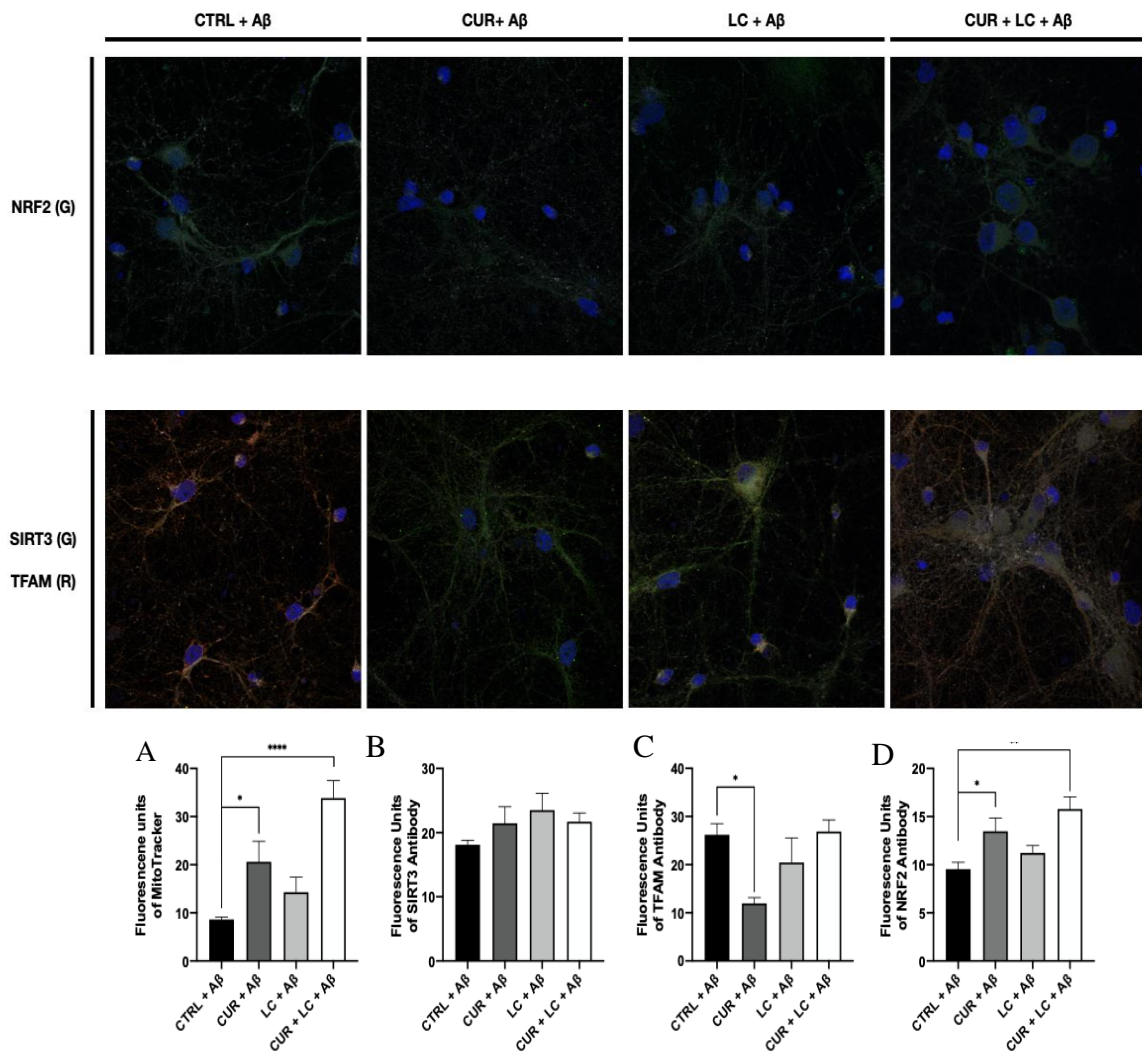
Fig. 21 – Protein levels (NRF2, SIRT3, and TFAM) in Wistar cortical neurons exposed to AβO, pretreated with CUR, LC, and CUR & LC. Nucleus was visualized by Hoechst staining (blue), mitochondria were marked with MitoTracker deep Red FM (white), TFAM was immunostained with a specific antibody (red), and both SIRT3 and NRF2 were immunostained with a specific antibody (green) in different coverslips. Images were treated with Fiji program. Confocal images were obtained using a 63x objective, NA=1.4 on a Zeiss LSM 710 inverted microscope. **A)** MitoTracker fluorescence graph. **B)** SIRT 3 fluorescence graph. **C)** TFAM fluorescence graph. **D)** NRF2 fluorescence graph. Data expressed as the mean±SEM of n=1, in quintuplicates. **A) C) D)** Analysis was performed by one-way ANOVA. Graph presents p=0.0213, F=3.655. Graph A presents p<0.0001, F=11.36. Graph C presents p=0.0123, F=5.008. Graph D presents p=0.0046, F=6.418. Statistical symbols *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

3.8   L-carnitine analysis retained in cationic liposomes

In the final part of the study, we produced a cationic liposomal formulation (constituted by cholesterol, phosphatidylcholine and ethylphosphatidylcholine) containing LC. For this purpose, we first determined LC free levels by reverse-phase HPLC (Figure 22) to further determine the encapsulated LC levels in the liposomal formulation. To guarantee the specificity of the technique of quantification of free and encapsulated LC, a third

sample containing empty liposomes was run, to verify that at the peak of detection of carnitine, in the HPLC technique, the sample of empty liposomes had no characteristic peak for the presence of carnitine; this way we could assure that the technique was specific for the quantification of carnitine. The free LC calibration curve is represented in Figure 22.
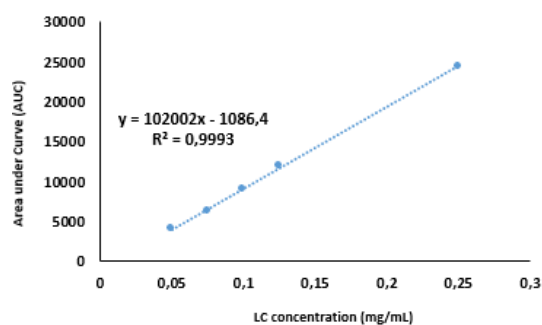
Fig. 22 – Free LC calibration curve. This calibration curve was obtained through independents runs in HPLC;

After completing the quantification of free LC, through reverse-phase HPLC, we proceeded to the characterization, both physical and chemical, of the encapsulated LC. This characterization was based on five parameters: particle size, zeta potential (which corresponds to the particle charge), drug loading, encapsulation efficiency, and pH. To verify if there were changes in the physical characterization of the formulation, with the exclusion chromatography process, we performed the physical analysis before the exclusion chromatography step and after as depicted in Table 3. In terms of particle size and zeta potential, the differences are minimal in the two procedures, as observed through the comparison between Figure 23A (the particle size from a liposomal formulation before exclusion chromatography) and Figure 23B (the particle size from a liposomal formulation after exclusion chromatography). Furthermore, the pH remained the same for the two samples, and the encapsulation efficiency of LC-containing liposomes was less than 10% (using the above calibration curve).

Table 3 - Characterization of cationic liposomes containing L-carnitine.

Parameters	Before exclusion chromatography	After exclusion chromatography
Particle Size (z-average) (nm)	110 ± 1.8	111 ± 1.6
Zeta Potential (mV)	26.6 ± 2.2	24.6 ± 1.3
Drug loading	1 mg/mL	n.d.
Encapsulation Efficiency	n.d.	< 10 %
pH	7.4	7.4

Characterization of liposomes concerns of particle size, zeta potential, the drug loading, encapsulation efficacy and pH. nm – nanometers; mV – millivolts; n.d – not determined;
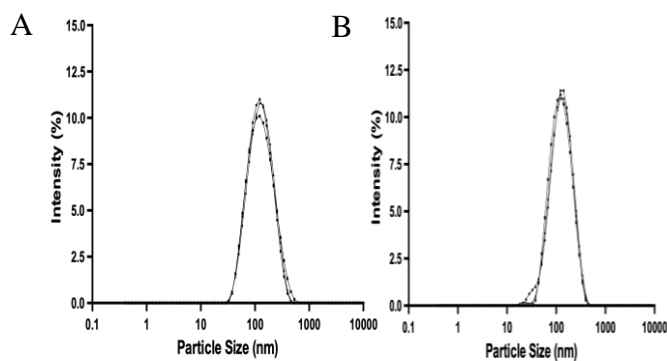

Fig. 23 – LC-containing liposomes particle size. A) Particle size before the exclusion chromatography; **B)** Particle size after exclusion chromatography; **A)** and **B)** were both obtained through the use of Zetasizer;

CHAPTER IV – DISCUSSION AND CONCLUSIONS

4.1 Discussion

AD affected areas include the hippocampus and the cerebral cortex. According to the results, cortical neurons were highly affected when compared to the hippocampus in APP/PS1 *versus* WT mice, when assessing the MMP. However, differences were observed in both brain areas cell viability, as determined by cell reducing capacity. Furthermore, hippocampal neurons were also apparently more resistant to H_2O_2 exposure, when compared to cortical neurons. Notably, changes in MMP or cell reducing capacity in cortical and hippocampal APP/PS1 neurons were apparently not accounted for by altered basal mitochondrial Ca^{2+} or mitochondrial H_2O_2 production.   AD molecular pathological have been largely described to be associated with synaptic and mitochondrial dysfunction (Barage et al., 2015; Guo et al., 2017). According to Hardy and co-authors (2006), in the pathological process of AD, the excessive accumulation of ROS leads to the subsequent collapse of the MMP and lower cell viability, due to low reducing capacity. Also Yan et al. (2013) presented that AD models demonstrate lower MMP and cell-reducing capacity. Furthermore, Sompol and co-workers (2008) observed that the MMP of APP/PS1 cortical neurons was reduced compared to WT neurons, in accordance with data shown in this study. When assessing the administration of different forms of carnitine (at 5 mM final concentration, but not at 1 mM in Aβ-treated neurons), both ALC and PLC significantly increased cell reducing capacity in APP/PS1 neurons, compared to the compound-free neurons. According to Yang and co-authors (2015), ALC induces an increase in antioxidant protein levels, also increasing Nrf2 mRNA levels. In addition, Hora et al. (2012) demonstrated that ALC administration protected hippocampal neurons from excitotoxicity, neurodegeneration and, consequently, mitochondrial dysfunction, through increased activation of the Keap1-Nrf2 signaling pathway. Concerning the effects caused by PLC, this was shown to act mainly in metabolic and vascular diseases, with little information about the effects in the brain. However, in an article recently published by the group, PLC was observed to be able to increase both the MMP and cell viability in Wistar rat neurons exposed to AβO (Mota et al., 2021). Also, in a work carried out by Al-Majed et al. (2006), in a model of transient ischemia in the rat hippocampus, PLC exhibited neuroprotective effects

similarly to ALC, by attenuating neuronal death, oxidative stress, and the loss in ATP levels. Thanks to its anti-inflammatory and antioxidant properties, CUR has been described to be an effective therapeutic molecule for AD. In our work, CUR apparently promotes an increase in the reducing ability in neurons exposed or not to H_2O_2. According to Uğuz et al. (2015), CUR attenuated cell death through a decrease in caspase 3 and 9 activities, a decrease in ROS levels and increased levels of antioxidant enzymes, as GSH and GPx in SH-SY5Y neural cells exposed to H_2O_2. In addition, Zhao et al. (2011) observed that, in Neuro-2A mouse neuroblastoma cells exposed to H_2O_2, CUR treatment significantly improved cell viability, as manifested as reduced apoptosis, decreased mitochondrial ROS and Ca^{2+} levels and stabilized the MMP. These effects caused CUR may be due to its ability to inhibit the activation of lipoxygenase, the synthesis of nitric oxide, block the production of inflammatory cytokines and transcription factors, such as TNF-alpha and NF-kB, and have an anti-aggregating effect on AβO (Morales et al., 2017).

Interestingly, both CUR and LC showed beneficial effects in "combating" the harmful effects of AβO. As described previously in this work, CUR has antioxidant, anti-Aβ aggregation, and anti-inflammatory properties, constituting a very interesting therapeutic approach. Several evidence indicate that CUR also promotes Aβ breakdown and reduces the size of accumulated protein deposits (Yang et al., 2005; Garcia-Alloza et al., 2007). CUR is also capable to enhance Aβ uptake by macrophages, as well as suppressing inflammatory damage, by inhibiting the induction of NF-kB by metals (Fiala et al., 2007; Baum et al., 2004). There are also published data on how CUR can decrease Aβ levels and deposits in APP/PS1 mouse models (Yang et al., 2005). Indeed, CUR mechanism of action appears to be due to the activation of Keap1-Nrf2 signaling pathway and a decrease in the levels of inflammatory cytokines, as discussed previously.

LC is very effective molecule in mitochondria due to its antioxidant and neurotrophic properties. LC was shown to prevent mitochondrial damage in valproic acid-exposed cortex(Salimi et al., 2020; Maldonaldo et al., 2020). Also, in a study carried out previously in the group, LC prevented the decrease in cell viability in hippocampal neurons exposed to AβO, as well as enhanced MMP (Mota et al., 2021). According to the results obtained in the present work, CUR and the combination of CUR plus LC increase cell reducing capacity and, consequently, the cell viability of cortical neurons exposed to AβO. It is important to highlight the significant differences obtained for CUR

individually and combined CUR plus LC, concerning the labeling of Nrf2 protein, largely suggesting a major activation of the Nrf2/ARE antioxidant pathway. Thus, CUR (in particular) appears to have beneficial properties capable of combating the harmful effects of exposure to oligomeric Aβ.Pharmaceutical nanotechnology is a very promising approach to transport drugs to specific locations, ensuring drug targeting and greater bioavailability and lifetime. Taking into account the hydrophilic character of LC, the best transport vector for this molecule would be a nanoparticle with amphipathic properties, ensuring that a high amount of drug could be loaded, in a very safe and practical way, being liposomes the chosen vesicles. Given the lack of definition of the isoelectric point of LC (pH= 6.5 - 8.5), two liposomal formulations were used, one neutral and the other cationic, to determine which would be the most suitable, noting that the more suitable formulation would be the cationic base. Previously, Yaşacan and co-authors (2020) synthesized a formulation of liposomes containing LC, indicating that liposomes with LC measured around 98 nm, with a zeta potential of 6.36 mV, a drug loading of 2 mg/mL, and encapsulation efficiency of approximately 14%. Characterization of our liposomal preparation carrying LC showed liposomes with a larger size, a much higher surface charge, a lower drug loading, and a much lower encapsulation efficiency, around 10%. These differences, namely the difference in the size of liposomes may originate from the liposomal synthesis protocol, as we performed an exclusion chromatography and an extrusion process, which may potentiate a larger size; the difference in surface charge may be due toa cationic lipid in the liposomal formulation; and finally, the differences observed in the encapsulation efficiency may be mainly due to the different drug loading doses, as well as to the protocol used; in our case, the liposomes were quantified based on a staining assay in which the quantification of the cholesterol content in the sample, constituting the liposome formulation, whereas in the formulation already published the liposomes underwent a single centrifugation step. Thus, a simple way to improve the formulation of liposomes with LC would be to concentrate the current formulation or increase drug loading in a new formulation. If sufficiently improved, such LC liposomal formulation could be tested both in *in vitro* and *in vivo* AD models.

4.2 Conclusions

AD is a multifactorial disease, evidencing several deregulated processes, namely synaptic dysfunction, deregulation of cytosolic, ER and mitochondrial Ca^{2+} levels, lysosomal deficiencies, mitochondrial dysfunction and oxidative stress, and a pro-inflammatory environment, among several other pathological features, eventually leading to neuronal death. A very important factor in the development of AD is the accumulation of Aβ peptide, which potentiates the entire cascade of neurodegenerative events. Exposure to H_2O_2 in cortical and hippocampal neurons from WT and APP/PS1 revealed that there are differences between these two brain regions affected in AD. A selective mitochondrial dysfunction inherent to APP/PS1 cortical neurons, was exacerbated after exposure to H_2O_2, which was not evident in hippocampal neurons, despite some cellular susceptibility (evidenced through decreased reducing capacity) of both neuronal subtypes. This is intriguing considering that the hippocampus is primarily affected in AD patients, although synaptic plasticity in this area seems to be mostly compromised in old, but not in young AD mouse model (Gengler et al., 2010). Of relevance, both ALC and PLC rescued the dysfunctional mechanisms in APP/PS1 cortical neurons. Exposure of rat cortical neurons to AβO was also shown to cause mitochondrial dysfunction and reduced cell viability, which could be prevented by CUR and/or LC alone or in combination. These data revealed a major protective effect of CUR, in particular by increasing Nrf2 levels and potentially inducing the activation of the Keap1/Nrf2/ARE pathway, a proeminent antioxidant signaling pathway. Finally, although there was an attempt to produce LC liposomal formulations, these will have to be improved, namely regarding the encapsulation efficiency. These can then be tested in both *in vitro* and *in vivo* AD models, the later potentially through intranasal administration. Moreover, it would be interesting to synthesize nanoformulations containing both CUR and LC, to evaluate their protective properties, first *in vitro* and then *in vivo*. This may also require the vectorization of liposomes, for example, with ligands capable of modulating the negative superficial charge of endothelial cells to a more positive one to ensure the easier passage through

the BBB, and with receptor-specific ligands to selectively target AD brain affected regions.

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