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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mitochondria: the common up-stream driver of Aß and Tau pathology in Alzheimer´s disease.

Diana Filipa Ferreira da Silva

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Sandra Morais Cardoso (Faculdade Medicina, Universidade de Coimbra) e supervisão do Professor Doutor Emilia Duarte (DCV, Universidade de Coimbra).

Diana Filipa Ferreira da Silva

2010

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List of abbreviations

AD, Alzheimer´s disease

ADNP, activity-dependent neuroprotective protein

ALP, autophagosome-lysosome pathway

apoE, apolipoprotein E

APP, amyloid precursor protein

ATP, adenosine triphosphate

AV, autophagic vacuole

Aβ, amyloid β-peptide

BACE, β-secretase

BSA, Bovine serum albumin

cdk5, cyclin-dependent protein kinase 5

CNS, central nervous system

COX, mitochondrial respiratory chain complex IV

cybrid, cytoplasmic hybrid

DLP1, dynamin-like protein 1

DTT, Dithiothreitol

EDTA, Ethylenediamine tetraacetic acid

EGTA, ethylene glycol tetraacetic acid

ER, endoplasmic reticulum

ETC, electric transport chain

FAD, familial forms of Alzheimer´s disease

FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone

GSK-3β, glycogen-synthase kinase-3β

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LA, lipoic acid

LC3, microtubule-associated light chain 3

MAP, microtubule-associated protein

MCI, Mild Cognitive Impairment

MgCl2, magnesium chloride

mtDNA, mitochondrial DNA

MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide

NAD^+, nicotinamide adenine dinucleotide

NFT, neurofibrillary tangles

NIC, nicotinamide

PBS, phosphate-buffered saline

PDH, pyruvate dehydrogenase

PKA, c-AMP protein dependent

PMSF, phenylmethanesulfonylfluoride

PS-1, presenilin-1

PS-2, presenilin-2

ROS, reactive oxygen species

SAD, late onset sporadic Alzehiemer´s disease

SHSY-5Y ρ+, human neuroblastoma cell line

SHSY-5Y ρ0, mtDNA-depleted neuroblastoma cell line

Sir2, silent information regulator factor 2

SIRT2, suirtuin2

TBS, Tris Buffered Saline

TIM, translocase of the inner membrane

TOM, translocase of the outer membrane

UPR, unfolded protein response

VIP, vasoactive intestinal peptide

βAPP, amyloid-β precursor protein

Δψm, mitochondrial membrane potential

Mitocôndria: o elo comum que desencadeia a patologia da βA e Tau na doença de Alzheimer

Resumo

A disfunção mitocondrial tem sido largamente implicada na etiologia da doença de Alzheimer (DA). Tendo em conta que a mitocôndria está implicada num vasto número de processos celulares, tem-lhe sido atribuída uma posição central na cascata de eventos que conduzem à neurodegeneração observada na forma esporádica da DA. É consensual que o peptídeo β-amilóide (βA) adicionado extracelularmente é captado pelas células induzindo citotoxicidade. Tendo em conta este facto usámos uma linha humana clonal com origem em neuroblastoma, SHSY-5Y, a qual foi exposta ao peptídeo βA_{1-42}. Nestas condições observámos uma redução nos níveis de ATP bem como um decréscimo na actividade do complexo IV da cadeia respiratória mitocondrial (COX). Para além das alterações descritas, o rácio NAD^+/NADH estava aumentado após exposição das células ao petídeo βA_{1-42}. Estas alterações a nível mitocondrial, induzidas pelo petídeo βA_{1-42}, afectam a rede microtubular onde aumentos de tubulina livre e modificações pós-traducionais da Tau estão implicadas. Observámos que os níveis de acetilação da tubulina estavam diminuídos em células expostas aos petídeos βA. Está descrito que a sirtuina 2 (SIRT2), um membro da família de desacetilases, requer nicotinamida adenina dinucleotídeo (NAD^+) para a sua activação. A SIRT2 tem como substrato preferencial a tubulina o que nos levou a propor a hipótese de que o aumento de NAD^+ induzido pelos peptídeos βA poderá activar a SIRT2 culminando na ruptura da rede de microtúbulos. Como consequência desta disfunção microtubular o transporte axonal ficará comprometido. Constatamos, através da determinação dos níveis das isoformas

da proteína "microtubule-associated light chain 3" (LC3), LC3I e LC3II a activação da macroautofagia. Visto que aumentos nos níveis de LC3II são indicadores da activação da macroautofagia pudemos concluir que o peptídeo βA usado activa e altera o processo autofágico, uma vez que este mecanismo protector necessita de uma rede microtubular funcional para que as vesículas autofágiacs (VAs) sejam transportadas até aos lisossomas. Estas observações guiaram a investigação com base na hipótese de que o uso de agentes estabilizadores dos microtúbulos poderá constituir uma estratégia terapêutica para a DA. Verificámos uma redução nos níveis da hiperfosforilação da Tau em células pré-tratadas com uma concentração não tóxica de taxol. Este pré-tratamento com taxol impediu também o aumento dos níveis de LC3II. De acordo com estas observações, o taxol revelou eficácia na redução de oligómeros de βA nas fracções citosólicas, reduzindo também a activação de caspases 9 e 3. Os petídeos βA citosólicos são endereçados para a mitocôndria promovendo a sua disfunção. Taxol foi eficaz em reduzir os rácios anormais de NAD^+/NADH, melhorar os níveis de retenção mitocondrial de rodamina 123. O resultado mais interessante, que indícia a importância de uma nova terapêutica ao nível dos microtúbulos, mostrou que o taxol reduziu o conteúdo mitocondrial de oligómeros de βA. As observações descritas apontam para que uma terapêutica a nível microtubular é eficaz em restabelecer o transporte retrógrado de mitocôndrias e de VAs, permitindo que mitocôndrias disfuncionais e agregados proteicos possam ser degradados por macroautofagia. Em paralelo, usámos células SHSY-5Y ρ0, depletadas do seu DNA mitocondrial mas que conseguem manter o $\Delta\Psi_m$ e os níveis de ATP. Observámos que estas células ρ0 têm níveis reduzidos de tubulina acetilada o que aponta para uma disfunção ao nível da dinâmica dos microtúbulos. Esta alteração ao nível dos microtúbulos correlaciona-se com um aumento dos níveis de Tau hiperfosforilada o que foi revertido com um tratamento de

24h com taxol. No que respeita à autofagocitose verificamos que não está alterada nesta células. Embora as células sem DNA mitocondrial tenham alterações em proteínas associadas aos microtubulos (tubulina acetilada e Tau hiperfosforilada) como mantêm os níveis de ATP, conseguem assegurar o tráfego intracelular.

Palavras chave: Doença de Alzheimer, mitocôndria, βA, Tau, autofagia.

Mitochondria: the common up-stream driver of Aβ and Tau pathology in Alzheimer´s disease

Summary

Mitochondrial dysfunction has been widely implicated in Alzheimer´s disease (AD) etiology. Since mitochondria control a wide range of cellular processes the up-stream position of this organelle in the cascade of events leading to neurodegeneration in AD has been recognized. It is generally accepted that amyloid β-peptide (Aβ) added extracellulary is up-taken by cells and induces cytotoxicity. In this regard we used a clonal human neuroblastoma cell line, SHSY-5Y, which was exposed to $A\beta_{1-42}$. In this case we observed a reduction in ATP production and a reduction in the activity of mitochondrial respiratory chain complex IV (COX). Further, NAD^+/NADH ratio was increased in cells exposed to $A\beta_{1-42}$. These mitochondrial alterations induced by $A\beta_{1-42}$ affect the microtubular network by inducing an increase in free tubulin and Tau post-translational modifications. Moreover, we observed that tubulin acetylation levels where decreased in cells exposed to Aβ. It is documented that SIRT2, a member of the sirtuin family of deacetylases, requires nicotinamide adenine dinucleotide (NAD^+) for their activation. SIRT2 has as preferential substrate tubulin which made us hypothesize that Aβ-induced NAD^+ increase levels, may activate SIRT2 which could induce a microtubule network breakdown. In consequence of microtubule cytoskeleton collapse axonal transport becomes compromised. We accessed macroautophagy by evaluating microtubule-associated light chain 3 (LC3) isoforms, LC3I and LC3II. Since higher LC3II levels are indicator of activated macroautophagy, we could conclude that Aβ was able to simultaneously activate and impair autophagy. Such observations guided the

investigation based on the hypothesis that microtubule stabilizing agents can constitute a strategy for AD therapy. We verified a reduction in Tau hyperphosphorylation levels in cells pretreated with non toxic concentrations of taxol. Moreover, we could prevent LC3II levels from rising with taxol pretreatment. In accordance, taxol successfully reduced Aβ oligomers in cytosolic, and reduced caspase 9 and 3 activation. Cytosolic Aβ is reported to be imported by mitochondria where exerts further toxicity. Taxol reduced NAD^+/NADH abnormal ratios, ameliorated mitochondrial rhodamine 123 retention and, most interesting, mitochondrial Aβ oligomers content were significantly reduced. These observations point out that an intervention at a microtubule level may be effective in restoring mitochondrial and AVs retrograde transport, enabling damaged mitochondria and protein aggregates to be degraded by the lysosomes. In parallel, we used SHSY-5Y ρ0 cells which are mtDNA depleted, but maintain $\Delta\Psi_m$ and ATP levels. We found that these ρ0 cells have reduced levels of acetylated tubulin which entail microtubule dynamics impairment. Furthermore these cells showed high levels of hyperphosphorylated Tau which was ameliorated with 24h treatment with taxol. Taking into consideration autophagocytosis we found no significant alterations between mtDNA depleted cells and parental cells. So, although we found alterations in related microtubule proteins (acetylated tubulin and Tau hyperphosphorylation) we speculate that ATP levels maintenance allowed these cells to maintain their intracellular traffic.

Keywords: Alzheimer´s disease, mitochondria, Aβ, Tau, autophagy.

Chapter 1

Introdution

1.1 General introduction

Mitochondria are uniquely poised to play a key role in neuronal cell survival or death because they are regulators of both energy metabolism and apoptotic pathways (Duchen et al., 2004) (Fig. 1). In fact, this organelle plays an important role as ATP producers, as regulators of intracellular calcium homeostasis and producers of endogenous reactive oxygen species (ROS). Like no other cellular organelle, mitochondria possess their own DNA (mtDNA) that encodes the subunits of the oxidative phosphorylation (OXPHOS) system (Fukui & Moraes, 2008). Since respiratory chain is not completely efficient, ROS are produced during aerobic respiration, for instance, superoxide anion that can be converted into other ROS species such as hydrogen peroxide (Balaban et al., 2005; Fukui & Moraes, 2008). Since ROS production occurs during normal cellular metabolism in cells, the ageing process is associated with a marked decline in mitochondrial function, characterized by a decrease in OXPHOS and ATP synthesis, an increase in mtDNA mutations, an increase in abnormal mitochondrial cristae structures and a marked rise in free radical production (Lin & Beal 2006; Shi et al., 2008).

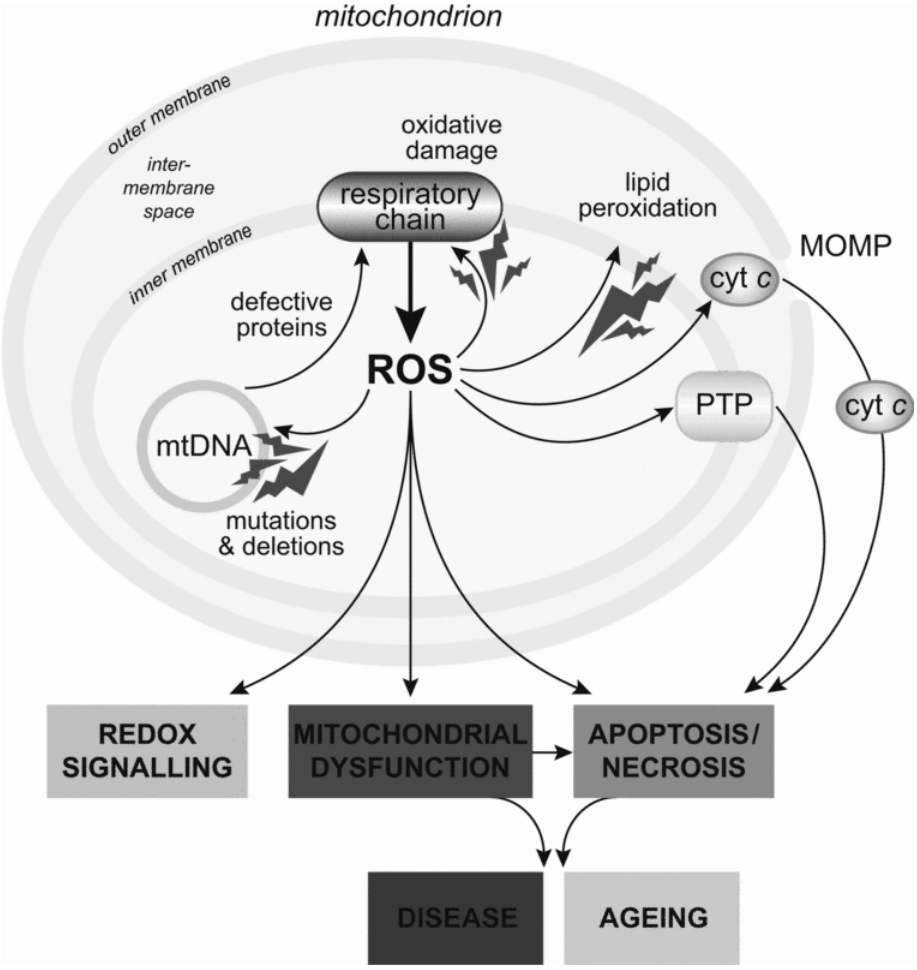


Fig.1. ROS production by mitochondria. ROS can damage mitochondrial proteins, membranes, DNA, impairing the ability to produce ATP and other metabolic functions. Mitochondrial oxidative damage can induce the release cytochrome c into the cytosol and activate apoptotic cell death cascades. Mitochondrial ROS may act as modulatable redox signal, reversibly affecting the activity of a range of functions in mitochondria, cytosol and nucleos. Overall, mitochondria is likely to contribute to a wide range of pathologies (adapted from Murphy et al., 2009).

In this point of view, ageing is considered the pivotal risk factor for the development of Alzheimer´s disease (AD) (Swerdlow & Khan 2004; Lin & Beal 2006; Swerdlow and Khan 2009). These late onset sporadic (SAD) forms of AD arise among all genetic forms of the disease, manifesting in late stages of the life although it is believed that genetic and environmental factors act synergistically in the onset of the disease (Anandatheerthavarada et al., 2003; Pereira et al., 2005). Since AD is a progressive and chronic condition and actually there are no available therapeutics that can alleviate, or even cure the disease, the involvement of mitochondria in a wide range of cellular processes opens new windows for the identification of new therapeutical targets.

1.2 Age-related Alzheimer´s disease

Mutations dominantly inherited in presenilin-1 (PS-1), presenilin-2 (PS-2) and amyloid precursor protein (APP) allowed to explain the early-onset familial forms of AD (FAD) although, only a small percentage of patients exhibit this familial trait (Laws et al., 2003). The vast majority of AD patients develop this condition later in life where mutations are rarely found (Shepherded et al., 2009). Characterized to be multi-factorial and heterogeneous, sporadic AD may be the result of stochastic driven ageing process. Ageing exerts a deleterious effect on brain activity and there have been some clues stating environmental and epigenetic variations that contribute to AD pathology (Cacabelos et al., 2005). Genetic analyses suggest that there are likely to be several genes exerting influence in the susceptibility of an individual to AD (Mattson 2004). The ε4 allele of the apolipoprotein E (apoE) gene (APOE) was identified as a major risk factor for late-onset AD across populations (Laws et al., 2003, Raber et al., 2004; Reddy & McWeeney 2006; Belinson & Michaelson 2009; Bu 2009). ApoE plays an important

role in the metabolism of lipoproteins and cholesterol and has been associated with increased deposition of Aβ, brain inflammation, impaired neuronal plasticity and repair (Belinson & Michaelson 2009). Even though, the correlation between the presence of ApoE alleles and the emergence of AD sporadic cases is well documented, their products, even present in homozygosity, are not determinant for AD development (Laws et al., 2003). Besides, in the vast majority of AD sporadic cases the referred alleles are absent. The etiology of AD remains elusive and, in the last years, the first steps have been taken in order to find therapeutic targets that allow a true beneficial outcome, since this disease represents a major health problem in developed societies, and is predicted to increase disproportionately as the global elderly population increases (Howel et al., 2005).

1.3 Mitochondrial dysfunction involvement in AD

Proposed by Swerdlow and Khan (Swerdlow & Khan 2004), the mitochondrial cascade hypothesis enabled to compass some handicaps of another proposed theory for AD etiology, the Aβ cascade hypothesis proposed by Hardy and Selkoe (2002). Hardy and Selkoe stated the primordial event in AD etiopathology was the production of Aβ peptides, from APP processing. The accumulation of these peptides in brain culminated in generalized neurodegeneration observed in AD (Fig. 2). Most recently, many authors affirmed that this theory should be applied in cases of early-onset AD (Selkoe 2001; Cummings & Cole 2002; Swerdlow & Khan 2004; Swedlow 2007).

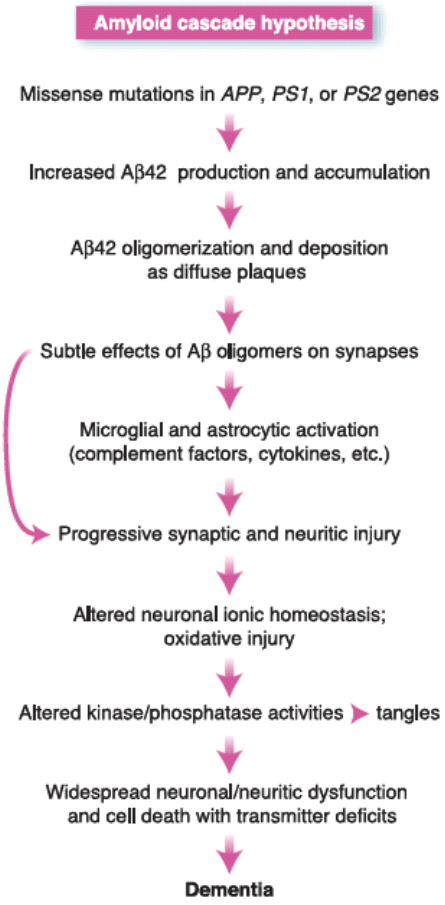


Fig.2. The amyloid cascade hypothesis. Mutations in APP, PS1 and PS2 genes provide a genetic framework for the emerging of AD. (adapted from Hardy and Selkoe, 2002).

On the other hand, mitochondrial cascade hypothesis relies on the central idea that similar mechanisms underlie brain ageing and AD (Swerdlow 2007). Mitochondrial dysfunction is believed to occupy an upstream position in the pathogenesis of the disease, giving rise to the histopathological and pathophysiological features of AD (Swerdlow & Khan 2004). Supporting Swerdlow and Khan point of view is the systemic mitochondrial dysfunction in AD, which cannot simply represent a consequence of neurodegeneration (Swerdlow 2007). In addition, inherited polymorphic

variations of mtDNA together with the age-related mitochondrial changes determine a functional threshold that induces AD-characteristic histopathology (Swerdlow & Khan 2009). The bridge between mitochondrial cascade hypothesis and Aβ cascade hypothesis is mitochondrial dysfunction and consequent ROS overproduction (Fig. 3).

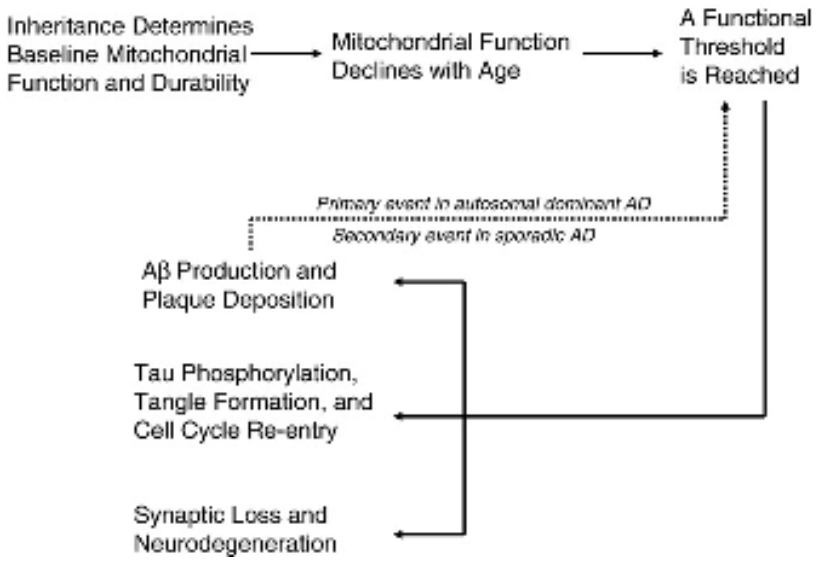


Fig.3. Mitochondrial cascade hypothesis. Age-related mitochondrial deficits drives a functional threshold that give rise to AD-characteristic histophatologies, including processing of APP to Aβ (adapted from Swerdlow and Khan, 2009).

Over the years many evidence have been presented in order to unify the cellular processes responsible for ageing, mitochondrial dysfunction and, consequently, neurodegenerative disease such as late onset AD. A number of studies have demonstrated that mitochondrial integrity declines with age (Shigenaga et al., 1994),

affecting multiple systems in the brain such as memory, learning and sensory processes (Aliev et al., 2009; Boumezbeur et al., 2010). Age-dependent mitochondrial abnormalities consist in decline in mitochondrial function, namely decrease in oxidative phosphorylation and ATP synthesis, such as an increasing in ROS production and accumulation of mtDNA mutations (Cortopassi & Wong 1999; Beal 2005; Shi et al., 2008). Experiments performed with platelets from AD subjects, age-matched controls and young control subjects showed that mitochondrial membrane potential was higher in young controls than both AD patients and aged control subjects (Shi et al., 2008). Furthermore, AD subjects and age-matched controls showed tolerance to the addiction of Aβ in opposition to young controls suggesting a response to a chronic mild stimulation. Recently, Boumezbeur and colleagues (2010) used magnetic resonance spectroscopy to access neuronal mitochondrial metabolism of healthy elderly and young volunteers. It could be concluded that during healthy ageing occurs a reduction in neuronal mitochondrial metabolism and altered glial mitochondrial metabolism, in comparison with young subjects, which may be responsible for declines in brain function. Taken together, these observations point to mitochondrial dysfunction occurring during ageing what can predispose elderly individuals to age-related disorders. To further support the rational of mitochondrial dysfunction as an early event in AD etiology, Mild Cognitive Impairment (MCI) subjects have been studied. MCI is considered a nosological entity or a translational state between normal ageing and AD (Morris et al., 2008; Padurariu et al., 2010). In fact, the early markers of metabolic dysfunction observed in AD have also been found in MCI subjects, such as mitochondrial DNA (mtDNA) oxidative damage, reported as a major risk for AD development and progression (Hirai et al., 2001; Swerdlow & Khan 2004; Manczak et al., 2004; Migliore et al., 2005). Studies made in lymphocytes showed significantly

higher levels of primary oxidative mtDNA damage in lymphocytes from AD and MCI subjects compared with control subjects (Migliore et al., 2005). Further, deficits in mitochondrial electric transport chain (ETC) have been verified, particularly in cytochrome c oxidase (COX) platelets from MCI subjects in agreement with observations made in platelets from AD patients (Valla et al., 2006). More recently, changes in oxidative stress markers were detected in serum from MCI subjects. A positive correlation was detected between the decreased antioxidant defense and increased lipid peroxidation in AD and MCI patients when compared with age-matched controls (Padurariu et al., 2010). Altogether, these data gathered from healthy elderly people and MCI subjects leave open the possibility that mitochondrial dysfunction occur *a priori* of symptom onset and it is possible that other pathological changes also take place before the disease manifestation (Valla et al., 2006). Furthermore, observations from AD patients report a defective activity of COX in early stages of the disease. COX activity was found to be decreased in platelets from AD subjects whereas the protein subunits are normally present (Cardoso et al., 2004) and this fact is not a consequence of tissue degeneration, ROS-induced membrane damage, deficient antioxidant defenses neither decreased synthesis rates. This data supports the idea that mitochondrial dysfunction is a primary or at least a non-amyloid dependent process (Cardoso et al., 2004). Fibroblasts from sAD patients revealed a greater amount of abnormal mitochondrial morphology and distribution when compared to age-matched normal human fibroblasts (Wang et al., 2008). These studies performed in peripheral blood allowed to suggest that AD can constitute a systemic disease affecting several tissues. Observation from *post mortem* AD brain samples revealed an increased oxidative damage and decreased COX activity (Bosseti et al., 2002), lower percentage of normal mitochondria and a higher percentage of the mitochondria with broken cristae

compared with age-matched controls (Hirai et al., 2001; Wang et al., 2009). Important was the fact that the described abnormalities were detected in neurons lacking of neurofibrillary tangles (NFT) and before Aβ deposition (Hirai et al., 2001). In order to further complete this amount of evidence that place mitochondria in an upstream position in AD etiology, AD cybrid cell lines (cytoplasmic hybrid) have shown to correlate in many ways to AD pathophysiology, for instance, decreased COX activity (Swerdlow et al., 1997; Cardoso et al., 2004), decline in bioenergetics over time in culture and decrease viability in comparison to control cybrids (Onyango et al., 2005). It is well accepted that changes observed in AD cybrids recapitulate the early events of the disease (Trimmer et al., 2004). Cybrid technique was first described by King and Attardi (1989) and is based on the central idea that cybrid cells lines result from the repopulation of a host mtDNA depleted cell (ρ0) with different mtDNA, having the same nuclear background.

1.4 AD: a disorder of protein misfolding

Independent of its etiology, AD is characterized clinically by chronic and progressive dementia, including severe memory loss, profound changes in behavior and personality, incapacitating patients even in their daily tasks (Pereira et al., 2005). Histopathological hallmarks are intracellular NFT of abnormally phosphorylated Tau; neuropil threads, dystrophic neurites and extracellular senile plaques composed of Aβ (Iqbal & Grundle-Iqbal 2008) (Fig. 4).

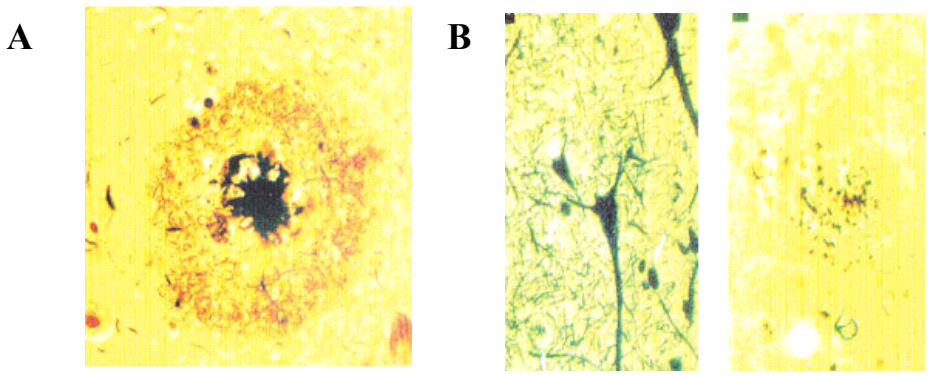


Fig.4. AD Histopathological hallmarks. (A) Senile plaque of Aβ. (B) Neurofibrillary tangles of hyperphosphorylated Tau. (adapted from Maccioni et al., 2001).

The presence of the referred hallmarks in AD brains is a necessary prerequisite for the definitive *postmortem* diagnosis of AD. Anatomically, AD brains are characterized by a severe atrophy which correlates with a reduction of cell density in brain regions involved in learning and memory, as a consequence of generalized degeneration of synapses and death of neurons (Mattson 2004). The course of AD neurodegeneration is initiated in the entorhinal cortex and spreads to the hippocampus, temporal cortex, frontoparietal cortex and, at last, to subcortical nuclei causing severe dementia (Reddy & McWeeney 2006). Since the first description of an AD patient by Alois Alzheimer, some decades were required to finally isolate and identify AD related brain areas and histopathological hallmarks (Small & Duff 2008). This isolation allowed inferring that senile or neuritic plaques are mainly composed of deposits of amyloid fibrils surrounded by dystrophic neuritis, activated microglia and reactive astrocytes (Sorrentino & Bonavita 2007). More specific, in AD brains Aβ is concentrated around meningeal and cerebral vessels and in the gray matter as Aβ plaques (Götz et al., 2004).

Aβ is a 4kDa proteolytic fragment that presents more often two carboxyl-terminal variants of Aβ: $A\beta_{1-40}$ mainly secreted from cultured cells and found in cerebrospinal fluid and $A\beta_{1-42}$ which is the main component of amyloid deposits in the brain (Neve et al., 2000), resulting from cleavage of amyloid-β precursor protein (βAPP) (Selkoe 2001; Reddy & McWeeney 2006). APP is a type I integral membrane glycoprotein through a single transmembrane domain (Evin et al., 2003; Mattson 2004) which possesses a large extracellular glycosylated N terminus and a shorter cytoplasmic C terminus (Marlow et al., 2003). Aβ region is located at the cell surface or in the luminal side of endoplasmic reticulum (ER) and Golgi membranes where part of the peptide is anchored. Along the secretory pathway APP is cleaved sequentially by β-secretase (BACE) at the N-terminal end (Marlow et al., 2003) originating sβAPP fragment and a membrane-associated C-terminal fragment, βCTF (Sambamurti et al., 2002; Mattson 2004; Yu et al., 2004). Then suffers cleavage by γ-secretase, a proteolytic complex composed of presenilin, nicastrin, APH-1 and PEN-2 (Götz et al., 2008) at the C-terminal of βCTF, in the amyloidogenic pathway (Anandatheerthavarada et al., 2003; Mattson 2004) originating a series of beta-sheet containing peptides, the Aβ peptides (Pereira et al 2005). The second classical neuropathological lesion present in AD brains is NFT which consist in intracellular bundles of abnormal fibers aggregated into paired helical filaments (PHF) (Selkoe 2001; Sorrentino & Bonavita 2007). NFT were found to be mainly composed of an abnormally hyperphosphorylated form of the microtubule-associated protein, Tau. Tau physiological functions inside the cell include the assembly and stabilization of microtubules (Götz et al., 2004; Zhang et al., 2009). Other roles have been attributed to Tau, such as signal transduction, organization of neuronal cytoskeleton, intracellular axonal transport, generation of cell polarity and shape and anchoring of phosphatases and kinases (Maas et al., 2000; Götz et al., 2004; Sorrentino & Bonavita 2007). These

structures are found in cell bodies, apical and distal dendrites and in dystrophic neurites associated with amyloid plaques (Götz et al., 2008). In an AD context it was shown that Tau was phosphorylated 3 to 4 folds higher due to the activity of some groups of kinases like, glycogen-synthase kinase-3β (GSK-3β), cyclin-dependent protein kinase 5 (cdk5), c-AMP protein dependent (PKA) and stress activated protein kinases (Gong & Iqbal 2008). The result of an augmented phosphorylation is a change in its conformation, the dissociation of Tau from microtubules, and a potential increase in toxicity (Götz et al., 2008; Zhang et al., 2009). Ultimately, these changes in microtubule conformation affect morphology and biological functions of neurons (Zhang et al., 2009).

1.5 Mitochondrial metabolic control of Sirtuin 2 regulation

Mammalian sirtuins are homologues of the silent information regulator factor 2 (Sir2), first described in *Saccharomyces cerevisiae* (Denu 2005; Michan & Sinclair 2007). These proteins have been found in organisms from all domains of life, ranging from prokaryotes to humans (Outeiro et al., 2008; Julien et al., 2009). The sirtuin (SIRT) family is composed of seven members (SIRT1-SIRT7) who are characterized by the presence of an approximately 200-amino-acid core (Vaquero et al., 2006) that require nicotinamide adenine dinucleotide (NAD^+) for their enzymatic activity. These proteins catalyze mainly deacetylation reactions in a variety of substrates including histones, transcription factors and apoptotic modulators (Michan & Sinclair 2007; Outeiro et al., 2008; Yu et al., 2009). Although the molecular mechanisms underlying the ageing process are not fully understood, in the last years a body of evidence emerged pointing to sirtuins as mediators of life extension, described in a wide range of organisms

including yeast, insects and rodents (Outeiro et al., 2008; Han 2009). Since it is recognized that ageing and neurodegenerative diseases such as AD are intimately connected, the understanding of sirtuin-mediated pathways can, in the future, provide feasible therapeutic targets in the treatment and prevention of such conditions (Han 2009). It is consensual that sirtuins differ in their sub-cellular localization, where SIRT1, SIRT6 and SIRT7 are predominantly in the nucleus, SIRT3, SIRT4 and SITR5 in the mitochondria, whether SIRT2 resides mostly in the cytoplasm (Michan & Sinclair 2007). Various cellular functions have been attributed to SIRT2 for instance its translocation to the nucleus during the G_2/M phase where deacetylation of histone H_4 takes place (Yu & Auwerx 2009). SIRT2 can be found associated with the microtubule network showing affinity to acetylated tubulin as a substrate, in comparison to acetylated histone (North et al., 2003). North and colleagues (2003) demonstrated using GFP-SIRT2 that SIRT2 deacetylated Lys40 of α-tubulin which co-localize with the microtubule network. In the same experiments, using SIRT2 knockdown mice it was shown that tubulin was hyperacetylated. Even though sirtuins have been related with some potential protective effects, not everything is perfect in the sirtuin world. In fact, SIRT2 activation has been reported to promote cell death (Han 2009). In acute or chronic neurodegenerative diseases, it has been described alterations in mitochondrial metabolic function and oxidative stress. Indeed, it is evident an impairment of the main mitochondrial enzymes, resulting in ATP depletion (Mattson 2000) which can be attributed to an increase in ROS production (Pereira et al., 1999; Butterfiled & Lauderback 2002). Among these mitochondrial enzymes, pyruvate dehydrogenase (PDH) and α-ketoglutarate are vulnerable to oxidative modification due to their sulfhydryl groups (Pocernich & Butterfield 2003). Since these enzymes are responsible for the conversion of oxidized NAD^+ into NADH, which is vital to mitochondrial

respiration and oxidative phosphorylation, their inhibition by acrolein result in higher levels of NAD^+. Acrolein was shown to be augmented in AD brains comparing with age-matched controls and it was proved that it could inhibit state 3 respiration without reducing mitochondrial complexes I-IV activity (Porcernich & Butterfield 2003). In parallel, age-related reductions in mitochondrial respiratory chain and increase in ROS production have also been described (Prihar et al., 2008; Brewer 2000). Parihar and collaborators (2008) demonstrated that in neurons from aged rat there was a significant decline in resting NADH concentration, when compared with embryonic and middle-aged neurons, which have a larger pool of NADH for metabolism. Furthermore, Chong and collaborators (Chong et al., 2005) demonstrated that nicotinamide (NIC), a known inhibitor of sirtuin activity could reduce anoxia-induced neuronal injury in primary hippocampal rat neuronal cultures and increase cell viability, preventing caspase activity, reducing DNA fragmentation and increased phosphorylation of proteins like Akt and Bad, preventing apoptosis. Of course, only indirectly we can infer that, at least part of these results is due to SIRT2 inhibition. More recently, studies made in 3xTg-AD mice found that NIC was able to prevent cognitive deficits by the inhibition of brain sirtuins associated with increased levels of deacetylated tubulin (Green et al., 2008). Further, 3xTg-AD mice at 8 months of age have high levels of Tau phosphorylation in serine and threonine residues. Remarkably, treatment with NIC could significantly reduce Thr231-phosphoTau . Since acetylation of tubulin, a primary substrate of SIRT2 was implicated in NIC-dependent results, we can infer that SIRT2 inhibition by NIC results in neuroprotection in this AD model.

1.6 Mitochondrial metabolic control of microtubule network

Neurons are highly differentiated cells with extensive synapses, branched dendritic arbors, and axons that can achieve a considerable size. Communication between the synapses and cell body is crucial for neuronal functioning and survival in a way that neurons are susceptible to disruption of microtubule-dependent transport (Trimmer & Borland 2005). It has been widely documented that AD neurons have changes at cytoskeleton level and a reduction of assembled microtubules is evident when compared with control cases (Cash et al., 2003; Santa-María et al., 2005). Disruption in microtubule dynamics results in incapacity of many subcellular processes to go on which conducts to retrograde degeneration accompanied by synaptic loss and neuronal cell death (Li et al., 2007). Studies performed in AD retinoic acid-differentiated cybrids showed that the velocity of both mitochondrial and lysosomal transport along neurites was significantly reduced when compared to differentiated control cybrids (Trimmer & Borland 2005). This result suggests a disruption in the transport machinery of both organelles in consequence of dysfunctional mitochondria contained in AD cybrids. Further evidence point to mitochondrial dysfunction and associated ROS production induced changes in the redox equilibrium, having a straight influence on microtubule disassembly (Santa-María et al., 2005). Cash and co-workers (2003) found, in brain biopsies of AD patients, alterations in the microtubules of pyramidal neurons, mitochondrial abnormalities and increase oxidative damage, before detectable changes in Tau. ROS overproduction can affect microtubules in such way that quinones are likely to be involved. It was found in cell culture experiments that the interaction between tubulin-quinone resulted in a decrease of microtubule polymerization rather than in microfilaments (Santa-María et al., 2005). In non pathological situations there is

a finely regulated balance between kinase and phosphatase activation that seems to be broken in AD (Ebneth et al., 1998). Indeed, it was shown that Aβ could bind to Tau in solution which potentiates GSK3β activation and Tau phosphorylation impairing its physiological function (Guo et al., 1998). When Tau is not available to play its normal role cells can suffer serious damage due to microtubule deficient organization. Various studies showed that Tau over-expression induced changes in cellular shape, loss of polarization and delayed cell growth, where mitochondrial distribution is dramatically altered and acquiring a clustered aspect (Ebneth et al., 1998). Further studies, using Tau over-expression models, concluded that cells presented shorter neurites with impaired microtubule transport which promoted an increased sensitive to oxidative stress due to lower mitochondrial content (Stamer et al., 2002; Mandelkow et al., 2003).

1.7 Mitochondrial metabolic control of the autophagic-lysosomal pathway

In age related diseases like AD, protein oligomerization and aggregation are prominent features that trigger neuronal disturbances including over-activation of macroautophagy (Cardoso et al., 2009) (Fig.5). Macroautophagy, herein referred as autophagy, is a degradative pathway of the lysossomal system that allows the removal of cellular organelles and long-lived proteins (Boland et al., 2008).

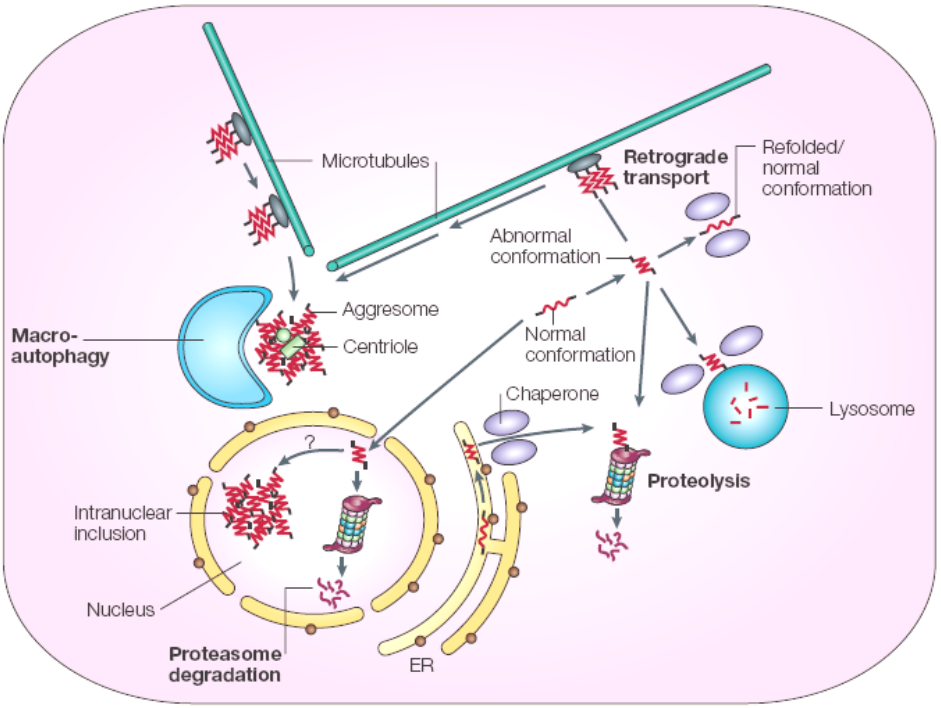


Fig.5. Abnormal aggregated proteins clearance. If a protein cannot be refolded by molecular chaperones it is targeted to the endosome/lysosome system or proteasome degradation. Alteratively, protein aggregates may suffer retrograde transport along microtubule network towards the centriole where they can form an aggresome which can be ultimatly degraded by macroautophagy. (adapted from Ross and Poirier, 2005).

Autophagy is a tightly regulated process that begins with the formation of a cytosolic membrane under the control of multiple proteins, including microtubule-associated light chain 3 (LC3). This complex controls the membrane elongation that sequestrates a region of the cytoplasm into a double membrane autophagic vacuole (AV) or autophagosome (Levine & Kroemer 2008). The degradation of sequestered materials is completed when AVs fuse with lysosomes (autophagolysosomes), process that allows the acidification and acquisition of proteolytic enzymes (Yu et al., 2005; Nixon 2007;

Klionsky 2008). First described as a mainly starvation inducible process, it is now recognized the constitutive role of autophagy in the brain (Nixon 2007). This "self-eating" process is crucial for cellular maintenance providing essential elements for cellular metabolism (Cuervo 2004). Further, it is well documented that the autophagosome-lysosome pathway (ALP) is simultaneously impaired and induced in AD brains (Nixon 2007). AVs accumulate within dystrophic neurites in AD brains and in AD animal models, suggesting a progressive dysfunction of ALP process (Yu 2005; Nixon et al., 2005) and consequently protein aggregates turnover is blocked. Low levels of mRNA of beclin 1, a protein required for induction of autophagy *in vivo*, have been documented in MCI subjects and AD patients (Lee & Gao 2008; Pickford et al., 2008). In APP mice model this low level of beclin 1 is also verified and is associated with enhancement in Aβ pathology, where impaired autophagy may be involved (Pickford et al., 2008). Likewise, mitochondrial dynamics impairment has been widely implicated in neurodegenerative disorders such as AD (Chan 2006). Mitochondria are degraded by the autophagic process, herein referred as mitophagy (Chen and Yan 2007). It was verified an increase in mitochondrial degradation products in vulnerable AD neurons, suggesting an augmented mitochondrial turn-over by mitophagy (Moreira et al., 2007). Electron microscopic analyses of AD brain samples, lipoic acid (LA) and COX-1 revealed that LA become associated with AVs in opposition to control cases. These observations demonstrated that mitochondria are key targets of autophagy in AD (Moreira et al., 2007). A close interaction between AVs accumulation and Aβ deposition has been demonstrated. Endosomes and AVs, isolated from a variety of tissues, showed to be enriched in APP and APP secretases, namely γ-secretase components most precisely PS1 and nicastrin, needed to generate Aβ peptides (Yu et al., 2004; Mizushima 2005). This impairment in ALP leads to an exacerbation of Aβ

production known to exert cytotoxicity (Boland et al., 2008). Yang and co-workers (2008) reported that activated caspase-3 was found within dystrophic neurites, most exactly in AVs associated with amyloid plaques in PS/APP mice. Pro-apoptotic proteins will accumulate and apoptotic cell death can take place culminating in the neurodegeneration process observed in AD brains. Valid proofs have been presented pointing to the link between Aβ-mediated toxicity and mitochondrial dysfunction. Aβ species have been found associated with mitochondria in human AD brains (Hirai et al., 2001; Caspersen et al., 2005; Manczak et al., 2006; Devi et al., 2006). Many studies showed that Aβ added extracellularly can act intracellularly promoting mitochondrial dysfunction (Anandatheerthavarada et al., 2003; Hansson Petersen et al., 2008) by lowering enzymatic activities. Aβ treatments in isolated rat brain mitochondria induced deficits in energy metabolism (Chen & Yan 2007; Casley et al., 2002). Data obtained using PC12 and NT2 cells showed that Aβ peptides decrease the activity of MRC complexes (Pereira et al., 1999; Cardoso et al., 2001). PC12 cell line bearing APP Swedish mutation (APPsw PC12) showed a decreased in COX activity and failure in other MRC enzymes, when compared with wild type APP bearing cells or empty vector transfected cells (Keil et al., 2004). Furthermore, it was demonstrated that Aβ is targeted to mitochondria, and arrested by TOM-40, one of the mitochondrial translocase proteins being the transport towards the inner membrane assured by translocase of the inner membrane (TIM) (Anandatheerthavarada et al., 2003; Hansson Petersen et al., 2008). Along with the described effects of Aβ in mitochondrial metabolism, mitochondrial fusion and fission is also compromised. Abnormal mitochondrial fission results in fragmented and bioenergetically impaired mitochondria (Barsoum et al., 2006). Studies using cerebrocortical neurons in culture exposed to Aβ, showed an increase in S-nitrosylation of Drp1 (Westermann 2009) culminating in SNO-Drp1 dimmer formation,

similar to those found in AD brain patients (Cho et al., 2009). The formation of such dimmers results in augmented GTPase activity favoring mitochondrial fragmentation process and neuronal synaptic activity failure (Cho et al., 2009). To further address the role of APP and Aβ causing mitochondrial dysfunction and consequent neuronal cell death through modulation of mitochondrial fusion and fission, M17 cells were transfected in order to over-express APPwt or APPsw. APP over-expression induced fragmentation and abnormal mitochondrial distribution in both constructions of transfected M17 cells (Wang et al., 2008). After all, oxidative damage is one major trigger of mitochondrial dynamics impairment. In primary cortical neuronal cultures submitted to NO donor, SNOC, uncontrolled fission revealed to be an upstream and early event in SNOC-mediated neuronal cell death. The augmented rate of fission could be followed by 3D time-lapse fluorescence imaging, where it was evident the fragmentation of mitochondrial into several isolated organelles (Barsoum et al., 2006). Normal human fibroblasts treated with H_2O_2 increased cellular ROS, induced dynamin-like protein 1 (DLP1) reduction, which regulates mitochondrial fission and distribution. The alteration in distribution of mitochondria was comparable to what was observed in sporadic AD fibroblasts (Wang et al., 2008). As discussed before, mitochondrial dysfunction triggers abnormalities along microtubule cytoskeleton that can be responsible for AVs retrograde transport impairment, towards the cell body where lysosomes are located. In support, it has been shown that microtubule depolymerizing agents disrupt vesicular transport inducing rapid accumulation of AVs (Kolchl et al., 2006). In opposition, microtubule polymerizing agents prevent cognitive deficits in AD mice model (Matsuoka et al., 2008). Directly affecting mitochondria some uncouplers were shown to disturb mitochondrial dynamics (Cappelleti et al., 2005). In post-mitotic cells the continuous accumulation of oxidative damage, triggers the accumulation of

non-functional mitochondria, process that induces autophagy. Besides, damaged oxidative-modified proteins potentiate lipofuscin accumulation into the lysosome (Zheng et al., 2006). Lipofuscin is a polymeric material composed of oxidatively modified protein and lipid residues, which decreases lysosomal capacity and sensitizes cells to oxidative stress (Terman et al., 2006). Such evidence point to an interplay between mitochondrial dysfunction, impair microtubule network, and activation of the autophagic process, which creates a positive feedback loop where AVs are being continuously produced and accumulated due to lack of degradation within lysosomes (Cataldo et al., 1996; Yu et al., 2005; Cardoso et al., 2009).

1.8 Therapeutic intervention at microtubule network level

Along with new insights in the molecular and genetic mechanisms that underlying AD pathology, over the years there has been intense search for drugs that could slow the neurodegeneration process. The efficacy of therapeutic agents tested under clinical trials was not good, so it seems important to adjust pharmacological intervention to the elucidation of the still unclear molecular pathogenic events in AD and to disclosure new ways to achieve earlier diagnosis (Michaelis et al., 2003). Since one major hallmark of AD is the deposition of plaques of Aβ and hyperphosphorylation of Tau, many efforts have been taken regarding the process of misfolded protein accumulation. In this regard, the disruption of microtubule network induced Tau hyperphosphorylation led to propose that microtubule stabilizing agents may prevent neuronal dystrophy (Lee et al., 1994). In a rat model it was demonstrated that taxol protects cortical neurons from toxicity by $A\beta_{25-35}$, decreasing calpain activation and cdk5/p25 complexes formation (Li et al., 2003). Pretreatment with taxol showed to be effective in preventing Tau

hyperphosphorylation (Michaelis et al., 2002) and in reducing Aβ-induced apoptosis (Chen et al., 2001). Further, Michaelis and colleagues (2005) tested various microtubule-stabilizing agents which demonstrated protective effects against the $A\beta_{25-35}$ and $A\beta_{1-42}$ peptide found in AD brains plaques. Endoplasmic reticulum (ER) stress has been widely implicated in Aβ-mediated toxicity (Nakagawa et al., 2000; Ferreiro et al., 2006). Microtubule stabilizers were able to reduce early events in ER stress response to Aβ and thapsigargin treated cells (Seyb et al., 2006). These results suggest that microtubule integrity avoid unfolded protein response (UPR) activation and consequently ER stress responses. Besides protein oligomer and fibril accumulation, synaptic failure with loss of essential synaptic components is intimately associated with dementia of AD (Butler et al., 2007). The disruption of microtubule cytoskeleton by accumulation of proteins leads to axonopathy and synaptic pathology (Butler et al., 2007). In hippocampal slice model of lysosomal dysfunction it was found that pretreatment with TX67, an analogue of taxol, restored acetylation levels of tubulin, which is considered a good indicator of microtubule stability. TX67 was also able to restore pre and post synaptic proteins levels, rescuing hippocampal synapses in such model (Butler et al., 2007). Although taxol and analogues showed potential therapeutic application for AD, it is known to have little effectiveness in the access to the central nervous system (CNS) (Liu et al., 2002). Moreover, a systemic taxol delivery is associated to the activation of apoptotic cell death in dividing cells (Divinski et al., 2006). Studies using a vasoactive intestinal peptide (VIP) showed that this compound induce neuroprotective effects, stimulating glial cells to produce neurotrofic factors that mediate neuroprotection (Gozes et al., 2005). NAP is an eight amino acid peptide derived from activity-dependent neuroprotective protein (ADNP), one of the neurotrofic factors that mediate VIP protective effects (Gozes et al., 2005; Vulih-Shultzman et al.,

2007). Remarkably, NAP was shown to be able to cross blood brain barrier after systemic or intranasal administration (Gozes et al., 2005), protecting neurons even in the absence of glial cells (Zemlyak et al., 2000). Tackling the target of NAP-induced neuroprotection, this peptide was shown to interact with microtubule cytoskeleton after cellular internalization mimicking taxol neuroprotection effects (Divinski et al., 2004), improving microtubule reorganization and increasing neuritis number (Divinski et al., 2006). *In vitro* and *in vivo* studies demonstrated that NAP is efficient in reducing Tau hyperphosphorilation in a variety of models (Gozes et al., 2004; Vulih-Shultzman et al., 2007; Shiryaev et al., 2009). Using $ADNP^{+/-}$ mice which exhibit Tau pathology and correlated cognitive deficits, NAP was able to partially ameliorate degenerative features (Vulih-Shultzman et al., 2007). Moreover, in 3xTg-AD mice treatments with NAP induced a reduced Aβ accumulation and Tau hyperphosphorylation (Matsouka et al., 2007; 2008). Further studies point to NAP being effective in neuroprotection against diverse insults. In fact this octapeptide protected newborn rat cortical neurons from $A\beta_{1-42}$ toxicity (Gozes et al., 2008). One indicator of mitochondrial dysfunction is the release of cytochrome c from mitochondria to cytosol that activates downstream caspases triggering cell death. NAP treatment in cortical neurons from rats submitted to oxygen-glucose deprivation lowered the levels of cytochrome c release (Zemlyak et al 2009). This result lead to hypothesize that NAP uptaked by neurons stabilizes microtubules and offers protection against cytocrome c release related mitochondrial dysfunction and against apoptosis activation due to cytoskeleton damage (Zemlyak et al., 2009). This neuroprotective ability of NAP demonstrated in a wide range of experimental settings paved the path for clinical experiments. NAP is currently in phase II of clinical trials, by Allon Therapeutics Inc. in MCI subjects. Clinical tests results indicate an improvement in memory skills when compared with placebo treated subjects (Shiryaev et al., 2009).

1.9 Objectives of this study

While the etiology of AD remains largely unclear, there is accumulating evidence from a variety of models that suggests mitochondrial dysfunction as prominent feature in disease etiology. Further, recent data suggests that Aβ deposition is potentiated by autophagic-liposomal pathway (ALP) impairment. In this regard the ultimate goal of this work consisted in address how mitochondrial dysfunction triggers ALP impairment. In this situation Aβ production and deposition becomes increased which potentiate Tau post-translational modifications and promotes cell death. The bridge linking these two events seems to be microtubule network breakdown. To address the importance of microtubule cytoskeleton in Aβ-mediated toxicity we tested the potential protective role of taxol, a microtubule stabilizing agent. To achieve these purposes we used two cell models: SHSY-5Y parental and SHSY-5Y ρ0 cell lines.

Chapter 2

Materials & Methods

2.1 Biological Material

2.1.1 Cell lines

2.1.1 Parental neuroblastoma cell line SHSY-5Y ρ+

SHSY-5Y human neuroblastoma cells containing mitochondrial DNA (ρ+) were purchased from ATCC. Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37IC and 5% CO2.

2.1.2 mtDNA depleted SHSY-5Y ρ0 cells

The SHSY-5Y ρ0 cell line was depleted from mitochondrial DNA by long-term ethidium bromide exposure as described by Swerdlow and co-workers (1997). Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37ºC and 5% CO2.

2.2 Chemicals and Cell media

Paclitaxel (taxol) and Nocodazole were obtained from Sigma (St. Louis, MO, USA). The synthetic $A\beta_{1-42}$ peptide was obtained from Bachem (Bubendorf, Switzerland). DMEM medium was obtained from Gibco-Invitrogen. Non-dialyzed fetal bovine serum was obtained from Gibco-Invitrogen. SHSY-5Y ρ+ cell growth medium consisted in DMEM and Ham´s F12 medium with 10% supplemental non-dialyzed fetal bovine serum and 100 IU/ml penicillin and 50 μg/ml streptomycin. SHSY-5Y ρ0 cell growth

medium consisted of SHSY-5Y growth medium supplemented with 200 μg/ ml sodium pyruvate, 150 μg/ml uridine.

2.3 Toxicity studies

2.3.1 In SHSY-5Y ρ+ cells

For the MTT assay, cells were plated in 24-well plates at a density of 0.1 $\times 10^6$ cells/well. For rhodamine 123 measurements, cells were plated in 24-well plates at a density of 0.05 $\times 10^6$ cells/well. For ATP determinations, cells were plated in 12-well plates at a density of 0.2 $\times 10^6$ cells/well. Immunocytochemistry was performed in cells grown on coverslips in 12-well plates at density of 0.05 $\times 10^6$ cells/well. For measurements of caspase enzyme activities, Western blot analysis, determinations of electron transport chain (ETC) enzyme activities and NAD^+/NADH levels cells were plated in petri dishes (10 cm) at a density of 2.5 $\times 10^6$ cells/dish. For western blot, immunocytochemestry, rhodamine 123 and NAD^+/NADH levels twenty-four hours after seeding of cells the medium was refreshed and taxol was added from a DMSO-taxol stock solution with a final concentration of 2 nM. Four hours later $A\beta_{1-42}$ was added in the same well/petri dish of the previous described taxol incubation with a final concentration of 1μM. Other wells/petri dishes were incubated with 5nM of nocodazole, 1μM of $A\beta_{1-42}$ and 2nM of taxol, respectively. The final concentration of DMSO in culture media did not exceed 0.05% (v/v) and under these conditions, no alterations in cell's viability were observed. In the case of ETC activities and ATP determinations

cells were exposed to 1μM of $A\beta_{1-42}$ for 24 hours. For all experimental procedures, controls were performed in the absence of the stress agents.

2.3.2 In SHSY-5Y ρ0 cells

To minimize cell death after plating, SHSY-5Y ρ0 cells were scrapped directly from the growing flask. However, the cell medium was refreshed before incubation with 2nM of taxol, during 24 hours. Controls were performed in the absence of taxol.

2.4 Cell viability assay

2.4.1 MTT reduction test

Cell viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). In viable cells, the enzyme succinate dehydrogenase metabolizes MTT into a formazan that absorbs light at 570 nm. Following the cell treatment protocol the medium was aspirated and 0.5 ml MTT (0.5 mg/ml) was added to each well. The plate was then incubated at 37 ºC for 3 h. At the end of the incubation period the formazan precipitates were solubilized with 0.5 ml of acidic isopropanol (0.04 M HCl/Isopropanol). The absorbance was measured at 570 nm. Cell reduction ability was expressed as a percentage of the control.

2.5 Mitochondrial function evaluation

2.5.1 Cytochrome c Oxidase (Complex IV) assay

Complex IV activity was determined upon Aβ exposure using the method of Wharton and Tzagotoff (1967), which measures the oxidation of reduced cytochrome *c* by cytochrome *c* oxidase at 550 nm. To prepare reduced cytochrome *c*, cytochrome *c* was mixed with a few crystals of ascorbate and partitioned by a dialysis membrane overnight against 0.01 M phosphate buffer, pH 7.0 at 4 ºC. The reduced cytochrome *c* concentration was then determined using 0.1 M potassium ferricyanide. The reaction mixture contained 0.01 M potassium phosphate, pH 7.0, and 50 μM reduced cytochrome *c*. The reaction was initiated by addition of the sample at 30 ºC. The pseudo first order rate constant (K) was calculated, because the reaction is of first order with respect to cytochrome *c*. Results are expressed both as K per minute per milligram of protein.

2.5.2 Analysis of adenine nucleotides

Cells were extracted, on ice, with 0.4 M perchloric acid. Cells were next centrifuged at 14,000 rpm for 5 min at 4 ºC. The resulting pellets were solubilized with 1 M NaOH to further analyse protein content by Bradford protein assay (Bio-Rad, Hercules, CA, USA). The resulting supernatants were neutralized with 5 M KOH and 2.5 M Tris, pH 7–8, and then centrifuged at 14,000 rpm for 5 min at 4 ºC. These supernatants were assayed for adenine nucleotides (ATP, ADP and AMP) by reversed-phase HPLC as described by Stocchi et al. (1985). The chromatographic apparatus used was a Beckam Gold System, consisting of a 126 Binary Pump Model and a 166 Variable UV detector

that was controlled by computer. The column was a Lichrospher 100 RP-18 (5 lm) from Merk (Germany). An isocratic elution with 100 mM KH2PO4 buffer at pH 7.4 and 1% methanol was performed at a flow rate of 1.2 ml/min. The adenine nucleotides were detected at 254 nm for 5 min.

2.5.3 Quantification of NAD^+/NADH levels

Cells were harvested and assayed for NAD^+/NADH levels using the MBL International Corporation Kit. Briefly, cells were resuspended in an extraction buffer. The cell lysates were incubated at 37 °C for 60 min with NAD cycling mix and NADH developer. Total NAD and NADH were detected by absorbance change at 450 nm.

2.6 Apoptotic cell death evaluation

2.6.1 Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

Changes in mitochondrial membrane potential were estimated using the fluorescent cationic dye rhodamine 123 (Rh123) (Sigma). After 24h treatment, cells were loaded with 0.5 μM Rh123 (in the dark, at 37 ºC) and the fluorescence (λexc = 505 nm and λem = 525 nm) was recorded during 45 min before, and also for 10 min after mitochondrial depolarization, using a Spectramax Plus 384 spectrofluoremeter (Molecular Devices). Maximal mitochondrial depolarization (Δψm collapse) was performed in every individual experiment by adding 1 μM FCCP (protoionophore), which was always preceded by oligomycin (2 μg/ml) to prevent ATP synthase reversal. Rh123 retention was determined by the difference between total fluorescence (after depolarization) and

the initial value of fluorescence. Because positively charged Rh123 is retained by functional mitochondria with a high Δψm, a decrease of cellular retention of Rh123 has been associated with a decrease in Δψm.

2.6.2 Caspases activation

Caspase activation was measured using a colorimetric method described by Cregan et al. (1999), in which the substrate cleavage is monitored at 405 nm. After 24h treatment, cells were washed and placed in buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl2, and protease inhibitors (0.1 M PMSF, 2 mM DTT, and a 1:1000 dilution of a protease inhibitor cocktail). Cells were harvested by scraping and frozen three times on liquid nitrogen. The lysate was centrifuged for 10 min at 14,000rpm 4 ºC. The resulting supernatant was stored at -80 ºC. Protein concentrations were measured by the Bradford method. Lysates (50 μg of protein) were incubated at 37 ºC for 2 h in 25 mM HEPES, pH 7.5 containing 0.1% CHAPS, 10% sucrose, 2 mM DTT, and 40 μM Ac-LEHD-pNA or 40 μM Ac-DEVD-pNA (Calbiochem) to determine caspase 9 or caspase 3 activation, respectively.

2.7 Immunoblotting

Individual cell lines were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 2% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, pH 7.5, 2 mM MgCl2, 1 mM EDTA and 1 mM EGTA supplemented with 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail). Cell suspensions were frozen three times in liquid nitrogen and centrifuged at 20,000×g for 10 min. To detect Tau

PHF, cell lysates were prepared using the lysis buffer, in the absence of Triton supplemented with 2 mM ortovanadate and 50 mM sodium fluoride.

For the analysis of Aβ aggregates, the supernatants (Triton soluble fractions) were collected and saved and the pellets (Triton insoluble fractions) were ressuspended in 1× specific sample buffer (0.2 M Tris–HCl, pH 6.8, 40% glycerol, 2% SDS, 0.005% Coomassie). For the analysis of mitochondrial Aβ aggregates cells were washed with PBS, scraped in a buffer containing 250 mM sucrose, 20 mM Hepes, 1 mM EDTA, 1 mM EGTA, and protease inhibitors (0.1 M PMSF, 0.2 M DTT, and 1:1000 dilution of a protease inhibitor cocktail) and homogenized. Cells were frozen three times on liquid nitrogen and the lysate was centrifuged at 2200 rpm for 12 min at 4 ºC. The resulting supernatant was centrifuged at 10600 rpm for 10 min at 4 ºC. Pellets resulting from this step constitute a crude mitochondrial fraction. Triton soluble fractions and mitochondrial fractions containing same amounts of protein were diluted (1:2) with sample buffer and were separated by electrophoresis on a 4–16% Tris–Tricine SDS gel (Klafki et al., 1996).

To prepare soluble and polymeric tubulin fractions, cells were very gently washed twice with a microtubule stabilizing buffer (0.1 M N-morpholinoethanesulfonic acid, pH 6.75; 1 mM MgSO4; 2 mM EGTA; 0.1 mM EDTA; 4 M glycerol). Soluble proteins were extracted for 4–6 min at 37°C in 100 µl of microtubule stabilizing buffer containing 1% Triton X-100. The remaining contents of the culture dish were scraped in 100 µl of 25 mM Tris (pH 6.8) and 0.1% SDS. This suspension was frozen three times in liquid nitrogen (Joshi and Cleveland, 1989).

The total amount of resulting cell lysates obtained were removed and stored at −80 °C. Protein content was determined using Bradford protein assay (Bio- Rad, Hercules, CA, USA).

For the SDS-PAGE experiments samples were resolved by electrophoresis in SDS polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). For the PAGE experiments samples were not boiled to minimize disaggregation prior to electrophoresis. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk or 5% BSA for phosphorilated proteins and 0.1% Tween in TBS for 1h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4°C with gentle agitation (1:10,000 mouse monoclonal anti-alpha-tubulin antibody from SIGMA, 1: 1000 6E10 mouse monoclonal anti-Aβ, mouse anti-PHF-Tau (clone AT8; 1:250) and anti-Tau (1:400) from Pierce Endogen (Rockford, IL), 1:20000 mouse monoclonal anti-acetylated tubulin from SIGMA, 1:1000 rabbit polyclonal anti-SirT2 from Cell Signaling, 1:1000 rabbit polyclonal anti LC3B from Cell Signaling, 1:500 mouse monoclonal anti Beclin 1 from BD Biosciences, 1:300 mouse monoclonal anti-OXPHOS complex IV subunit I from Molecular Probes, 1:500 mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase from Millipore (Billerica, MA, USA). Membranes were washed with TBS containing 0.1% nonfat milk and 0.1% Tween three times (each time for 15 min), and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature with gentle agitation. After three washes specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare). Fluorescence signals were detected using a Biorad Versa-Doc Imager, and band densities were determined using Quantity One Software.

2.8 Immunocytochemistry

2.8.1 Confocal microscopy visualization of LC3B and tubulin

After 24h treatment parental SHSY-5Y parental and SHSY-5Y ρ0 cells were washed twice with PBS and fixed for 30 min at room temperature using 4% paraformaldehyde. The fixed cells were washed again with PBS, permeabilized with 0.2% Triton X-100, and blocked with 3% BSA. The permeabilized cells were incubated with primary antibody (1:2,000 monoclonal anti-alpha-tubulin from Sigma; and 1: 300 polyclonal anti- LC3B from Cell Signaling). Afterward cells were incubated 1h with appropriate secondary antibody (1:250 alexa fluor 594 and 488 from Molecular probes, Eugene, OR, USA). Finally cells were washed in PBS, incubated for 5 minutes with Hoechst 33342 (15mg/L in PBS, pH 7.4) in the dark. Cells were visualized by confocal microscopy.

2.9 Data analysis

All data were expressed as mean ± SEM of at least three independent experiments performed in triplicate. Statistical analyses performed using one-way ANOVA followed by Bonferroni Multiple-Comparisons Procedure as post-hoc test or paired t-test. A P value < 0.005 was considered statistically significant.

Chapter 3

Results

3.1 Aβ, taxol and nocodazole toxicity in SHSY-5Y ρ+. Aβ and taxol toxicity in SHSY-5Y ρ0.

We used the ability of cells to reduce MTT as a parameter of cell viability, in order to establish a concentration of taxol and nocodazole that did not interfere with SHSY-5Y cells proliferation. In parallel, we established a concentration of Aβ that, despite being toxic, do not induce necrosis (Cardoso SM, PhD thesis). SHSY-5Y ρ0 were used to determine non toxic taxol concentrations. As previously described in NT2 ρ0 cells, Aβ did not induce alterations in SHSY-5Y ρ0 cells viability (Cardoso et al., 2001).

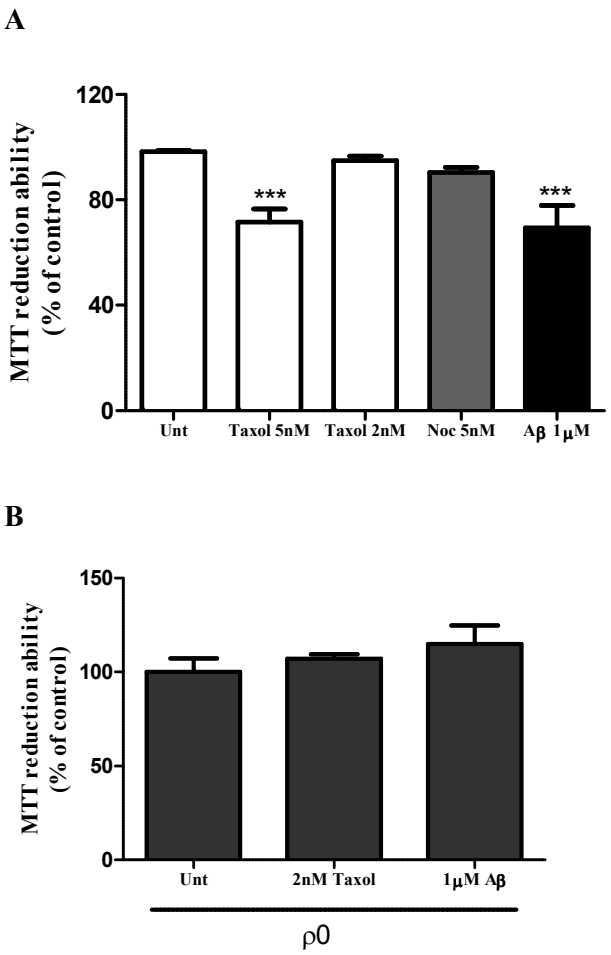


Fig.6. Effects of taxol, nocodazole and Aβ on MTT reduction. (A) SHSY-5Y ρ+ cells where incubated with 5nM, 2nM taxol; 5 nM nocodazole or 1μM of

$A\beta_{1-42}$ mainly constituted of oligomeric forms. (B) SHSY-5Y ρ0 cells where incubated with 2 nM taxol or 1μM of $A\beta_{1-42}$. Data are expressed as a percentage of the untreated SHSY-5Y cells, with the mean ± SEM from three independent experiments. ***p < 0.001, significantly different from the untreated cells.

3.2 Mitochondrial function in SHSY-5Y ρ+ exposed to Aβ and in SHSY-5Y ρ0

As previously described Aβ treated cells have lower COX activity than untreated SHSY-5Y ρ+ cells (Cardoso et al., 2001) (Table I). Moreover, SHSY-5Y ρ0 cells had no detectable COX activity (data not shown). ATP levels, in SHSY-5Y ρ+ cells exposed to 1μM of $A\beta_{1-42}$, decreased approximately 35 % as compared to untreated cells. In ρ0 cells, ATP levels were similar to those measured in ρ+ cells (Fig. 7A).

Table I

	Complex IV activity(K/min/mg)
Untreated	0,000562 ± 0,000101
Aβ	0,000384±0,000118*

Activity of mitochondrial respiratory complex IV (COX) in SHSY5Y untreated cells and exposed to 1μM Aβ. * P < 0.05, significantly different as compared to untreated SHSY5Y cells.

We further analyzed NAD^+/NADH ratio in ρ+ and ρ0 cells. We observed that Aβ exposure significantly increased the levels of NAD^+ in ρ+ cells which was prevented by

pre-treatment with taxol. Nocodazole also induced an increase in NADH to NAD^+ turnover (Fig. 7B). These observations are in accordance with the hypothesis that Aβ peptides added extracellulary are up-taken by cells and are targeted to the mitochondria, where exerts further toxicity (Anandatheerthavarada et al., 2003; Hansson Petersen et al., 2008). Interestingly, NAD^+/NADH ratio was significantly increased in untreated ρ0 cells as compared to untreated ρ+ cells. Similarly to what we observed in Aβ treated ρ+ cells, taxol partially prevented the increase in NAD^+ levels in untreated ρ0 cells (Fig. 7C).

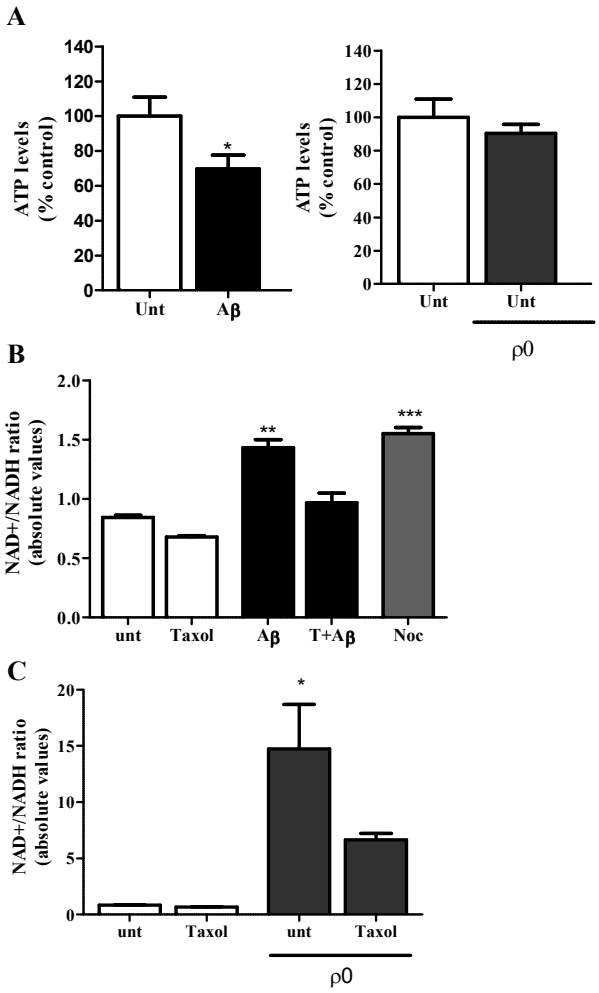


Fig.7. (A) ATP levels in SHSY-5Y ρ+ and ρ0 cells untreated and ρ+ cells exposed to 1μM Aβ. ATP levels where measured by HPLC as described in

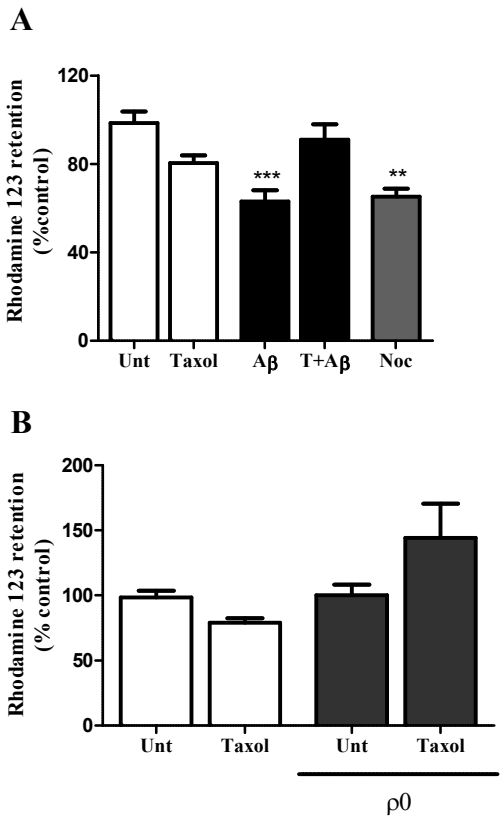
Materials and Methods. Data are expressed as a percentage of untreated ρ+ cells values, with the mean ± SEM derived from triplicate determinations from four independent experiments. *p < 0.05 significantly different compared with ρ+ untreated cells. (B) NAD^+/NADH ratio in SHSY5Y ρ+ cells incubated with taxol, nocodazole and Aβ as described in Materials and Methods. (C) NAD^+/NADH ratio in SHSY5Y ρ0 cells incubated with taxol, as described in Materials and Methods. Data are expressed as absolute values, with the mean ± SEM derived from three independent experiments. *p < 0.05 ; **p < 0.01; ***p < 0.001, significantly different compared with ρ+ untreated cells.

3.3 Activation of the intrinsic apoptotic pathway

Rhodamine 123 retention reflects the status of the mitochondrial membrane potential, and declines as the mitochondrial membrane potential is dissipated. As shown in Fig. 8A, Aβ treatment decreased rhodamine 123 retention in ρ+ cells. Taxol was able to ameliorate this Aβ-induced decline in mitochondrial membrane potential in ρ+ cells. Similar to Aβ induced alteration in rhodamine 123 retention, nocodazole treatment led to a decrease in mitochondrial membrane potential. ρ0 cells were able to maintain mitochondrial membrane potential, although not statistically significant. Taxol treatment increased rhodamine 123 mitochondrial retention in ρ0 cells (Fig. 8C).

A decrease in mitochondrial membrane potential induced by Aβ peptides can activate the mitochondrial apoptotic pathway (Cardoso et al., 2002). In order to clarify if Aβ-mediated insult activated the intrinsic apoptotic pathway in our cellular model, we showed that Aβ and nocodazole exposure significantly activated caspases 9 and 3. Pre-

treatment with taxol was able to reduce Aβ–mediated caspases 9 and 3 activation (Fig. 8C and D).



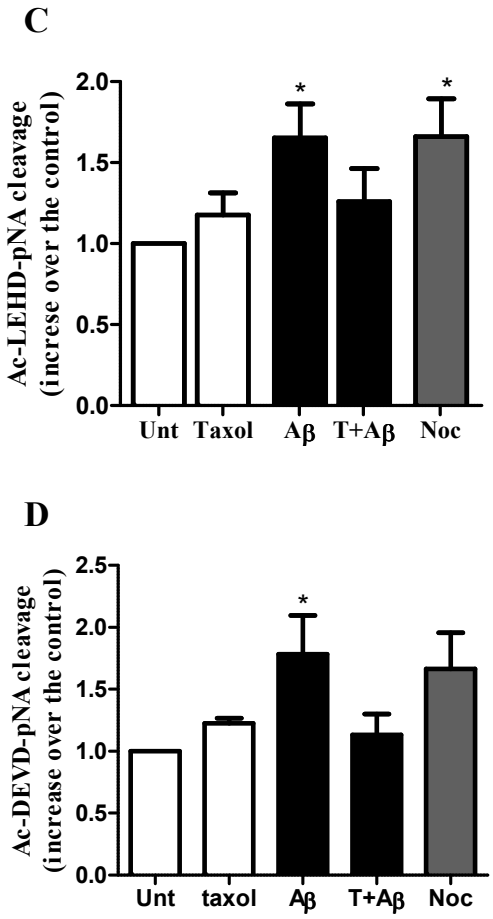


Fig.8. Mitochondrial membrane potential and caspase 9-like and caspase 3-like activation in SHSY-5Y cells, incubated with taxol, nocodazole and Aβ. (A) Mitochondrial membrane potential was expressed as the percentage of rhodamine 123 retention in ρ+ untreated cells, with the mean ± SEM values derived from four independent experiments. **p < 0.01, ***p < 0.001 significantly different as compared to untreated cells. (B) Mitochondrial membrane potential in SHSY-5Y ρ0 was expressed as the percentage of rhodamine 123 retention in ρ+ untreated cells, with the mean±SEM derived from three independent experiments. Caspase activity was measured spectrophotometrically at 405 nm, as described in Materials and Methods. (C) Caspase 9 activation was determined by Ac-LEHD-pNA cleavage. (D) Caspase 3 activation was determined by Ac-DEVD-pNA cleavage. Data

represent the mean ± SEM values derived from five independent determinations. * $P < 0.05$, significantly different as compared to untreated SHSY-5Y ρ+ cells.

3.4 Mitochondrial metabolism alteration drives microtubule network disruption.

Aβ-mediated mitochondrial dysfunction led to an increase in NAD^+ levels, a well known sirtuin cofactor. Moreover, SIRT2, a tubulin deacetylase, have been associated to alpha-synuclein induced toxicity (Outeiro et al., 2007). We accessed SIRT2 activation indirectly by determining tubulin acetylation levels, since tubulin is a substrate of this enzyme. SIRT2 expression levels were similar in our cellular models (Fig. 9).

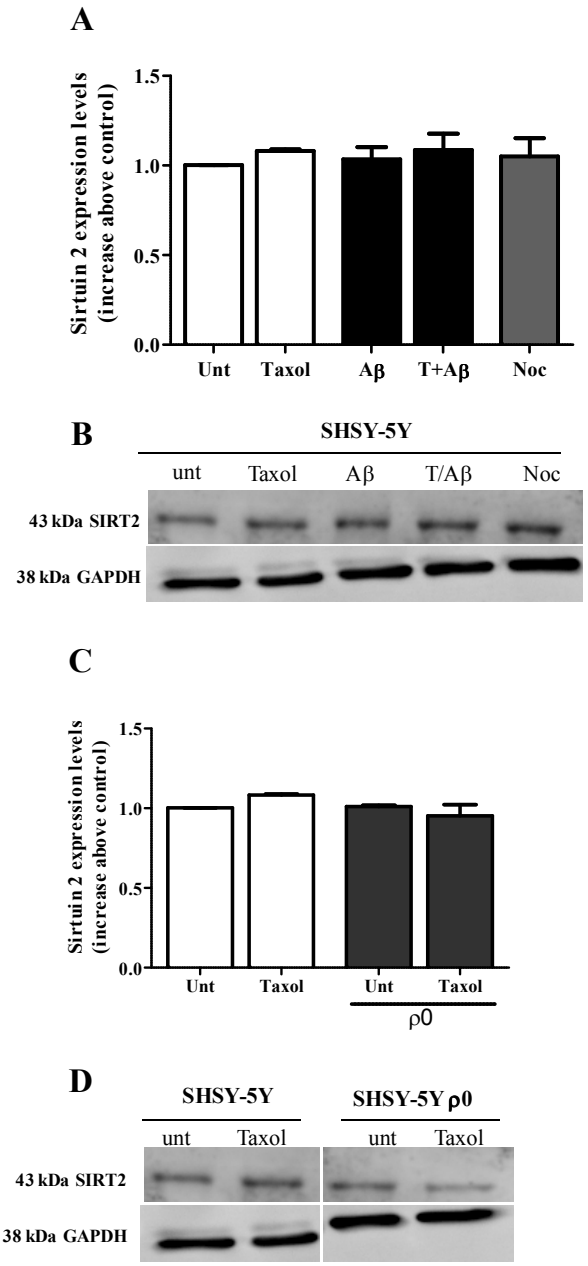


Fig.9. SIRT 2 expression in SHSY-5Y ρ+ and SHSY-5Y ρ0 cells. (A) and (B) Densitometry and Western blot analysis of Sirtuin 2 levels in SHSY-5Y ρ+ cells untreated or incubated with taxol, nocodazole and Aβ after correcting with GAPDH. (C) and (D) Densitometry and Western blot analysis of Sirtuin 2 levels in SHSY-5Y ρ0 cells untreated or incubated incubated with taxol, after correcting with GAPDH.

Since tubulin acetylation levels are a good indicator of microtubule stability (Butler et al., 2007), we evaluated SIRT2-induced alterations in acetylated tubulin levels in our models. Aβ treatment decreased tubulin acetylation in SHSY-5Y ρ+ cells, similarly to what we observed by nocodazole treatment. Accordingly to our previous results, taxol pretreatment in ρ+ and ρ0 cells reversed the decrease in acetylated tubulin levels (Fig. 10).

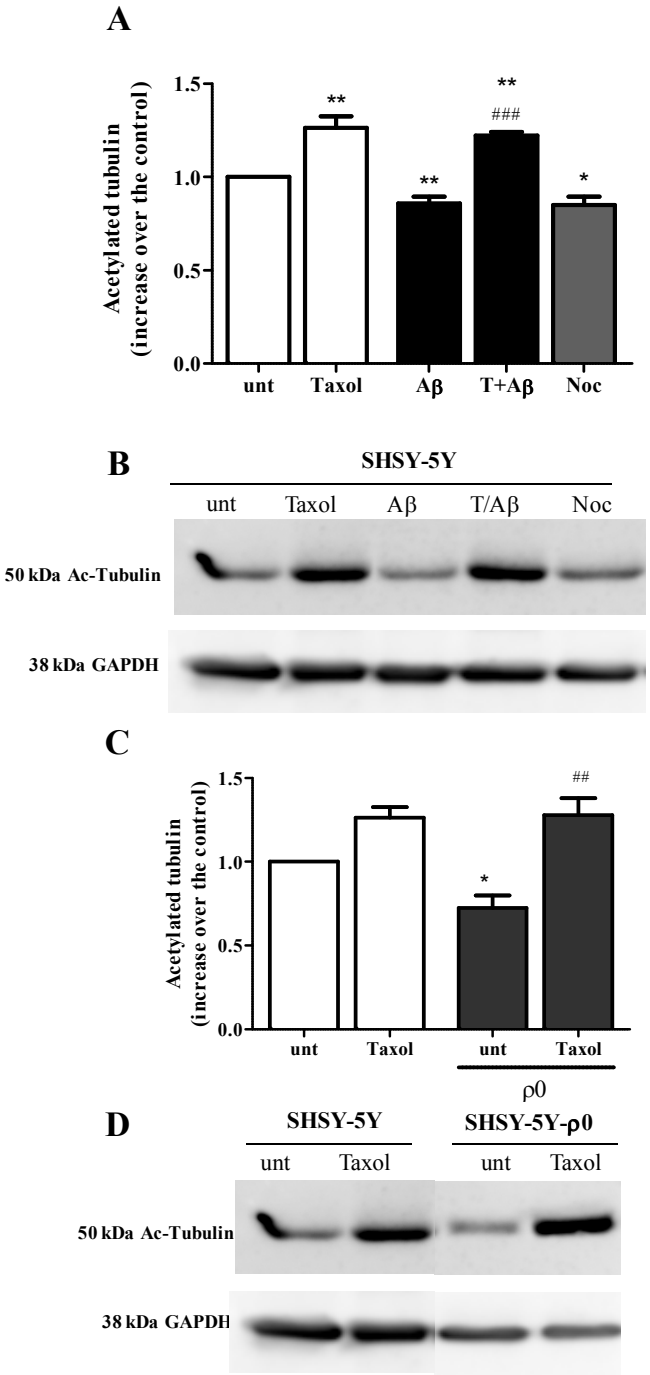


Fig. 10. Acetylated tubulin in SHSY-5Y ρ+ and SHSY-5Y ρ0 cells. (A) and (B) Densitometry and Western blot analysis of acetylated tubulin levels in SHSY-5Y ρ+ incubated with taxol, Nocodazole and Aβ, after correcting with GAPDH (C) and (D) Densitometry and Western blot analysis of tubulin acetylated levels in SHSY-5Y ρ0 cells untreated and exposed taxol, after

correcting with GAPDH. Data are expressed as the increase over untreated SHSY-5Y cells and represent mean ± SEM values derived from at least three independent determinations. * $p < 0.05$; **$p < 0.01$ significantly different as compared to untreated SHSY-5Y ρ+ cells. ## $p < 0.01$ significantly different as compared to untretaed SHSY-5Y ρ0 cells, ### $p< 0.001$ significantly different as compared to SHSY-5Y ρ+ cells exposed to Aβ.

Confocal microscopy analysis of tubulin showed that Aβ is able to induce microtubule network disruption being this structure less defined, similar to what we observed in nocodazole treated cells (Fig. 11A). ρ0 cells showed similar tubulin patterns as compared to untreated ρ+ cells (Fig. 11B).

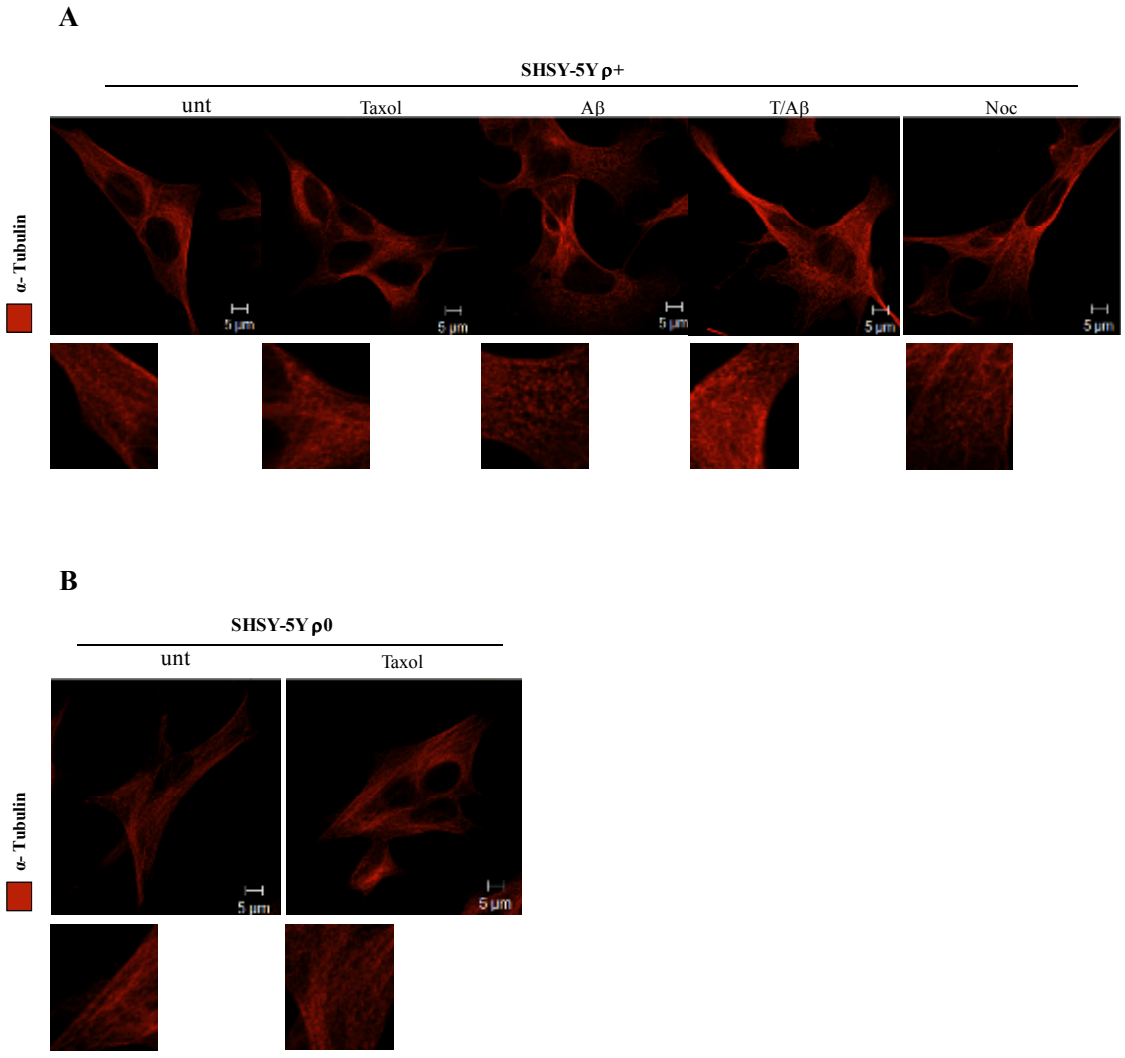


Fig. 11. α-tubulin staining in SHSY-5Y ρ+ and SHSY-5Y ρ0 cells. (A) α-tubulin staining in SHSY-5Y ρ+ untreated or incubated with taxol, Nocodazole and Aβ. (B) α-tubulin staining in SHSY-5Y ρ0 cells untreated or incubated with taxol.

Since microtubule dynamics is dependent on the availability of ATP, Aβ-induced ATP levels reduction and tubulin acetylation decrease compromise proper microtubule dynamics. These effects were confirmed by determining free tubulin levels by western blot analysis (Fig. 12A and B).

Taxol as microtubule targeted drug that stabilizes assemblies was effective in reducing free tubulin in the presence of Aβ, which can be visualized by immunocytochemistry (Fig. 11A) and western blot analysis (Fig. 12A and B).

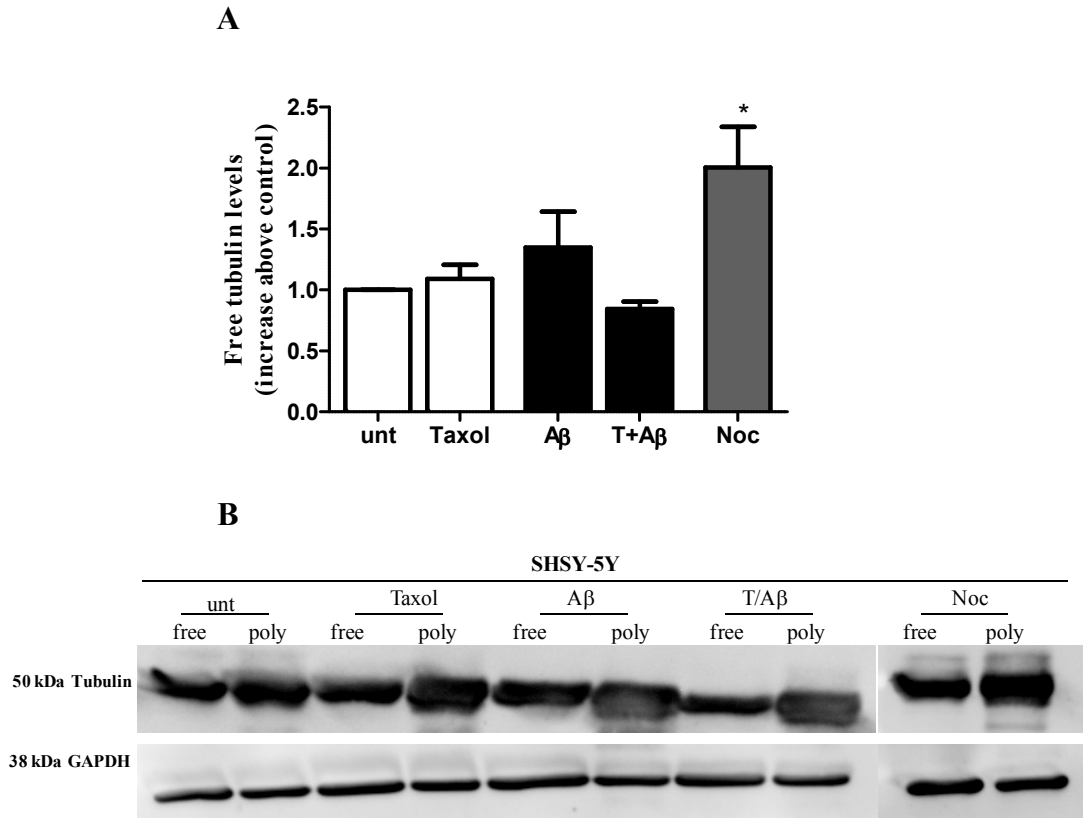


Fig. 12. Free tubulin in SHSY-5Y ρ+. (A) and (B) Densitometry and Western blot analysis of free tubulin in after correcting with GAPDH. Data are expressed as the increase over untreated SHSY-5Y cells and represent mean ± SEM values derived from at least three independent determinations. * $p < 0.05$ significantly different as compared to untreated SHSY-5Y ρ+ cells.

Evidences from our group, shown that $A\beta_{1-42}$ induces Tau hyperphosphorylation in cortical neurons (Resende et al., 2008). We further supported this evidence in our

cellular model (Fig. 13A and B). Taxol pre-treatment showed efficacy in reducing Tau phosphorylation at Serine 202 residue. Moreover, we showed that nocodazole was also able to induce slightly Tau hyperphosphorylation. Data obtained in ρ0 cells point to a non statistical increase of Tau hyperphosphorylation (Fig. 13C and D). Our results indicate that microtubule depolymerization potentiates Tau post-translational modifications.

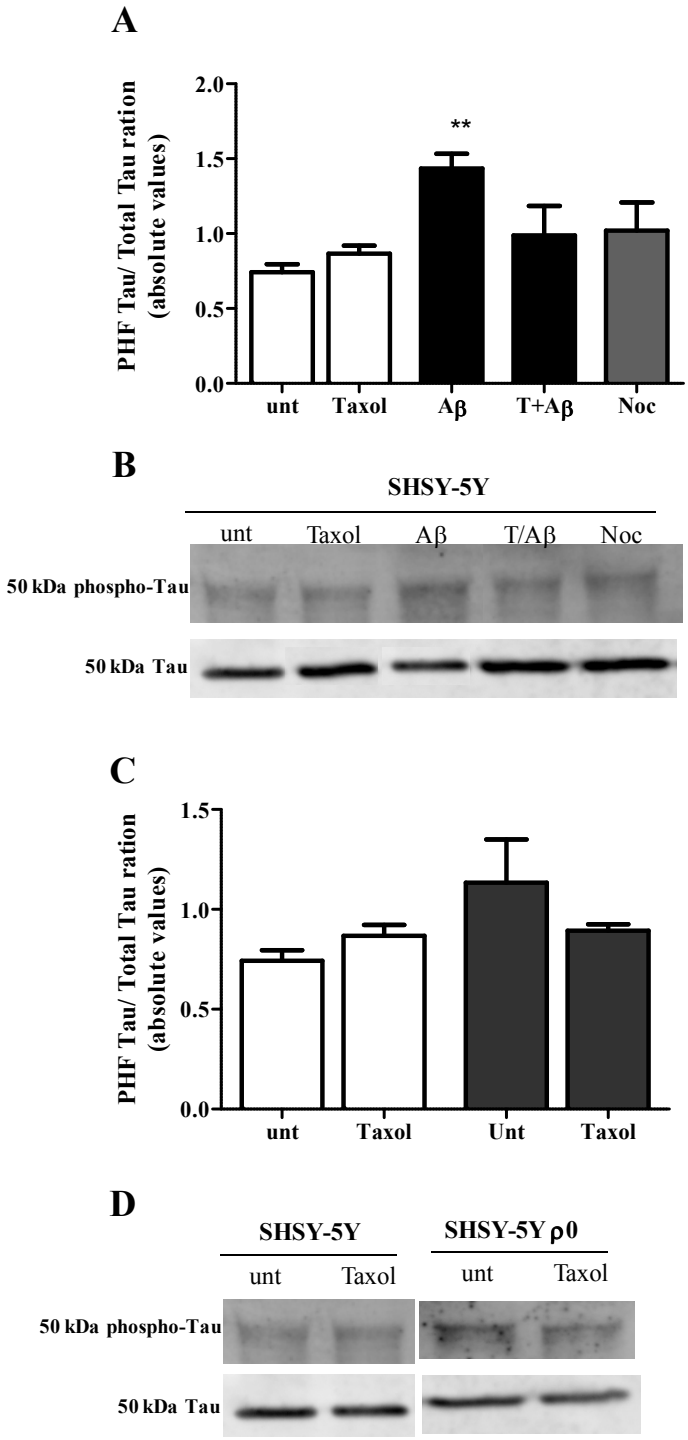


Fig. 13. Alterations in Tau hyperphosphorylation upon taxol, nocodazole and Aβ exposure. (A) and (B) Densitometry and Western blot analysis of PHF Tau/Tau ratio in SHSY-5Y ρ+ cells. (C) and (D) Densitometry and Western blot analysis of PHF Tau/Tau ratio in SHSY-5Y ρ0 cells. Data are

expressed as the increase over untreated SHSY-5Y cells and represent mean ± SEM values derived from at least three independent determinations. **p < 0.01 significantly different as compared to untreated SHSY-5Y ρ+ cells.

3.5 Impairment of macroautophagy triggered by microtubule alterations.

Growing evidence indicate that basal macroautophagy is essential for maintenance of cellular homeostasis through the degradation of mal-functioning organelles and protein aggregates. Since beclin-1 is required for macroautophagy induction we evaluated its expression in our cell models. We found no alterations in ρ+ cells with or without treatments (Fig. 14).

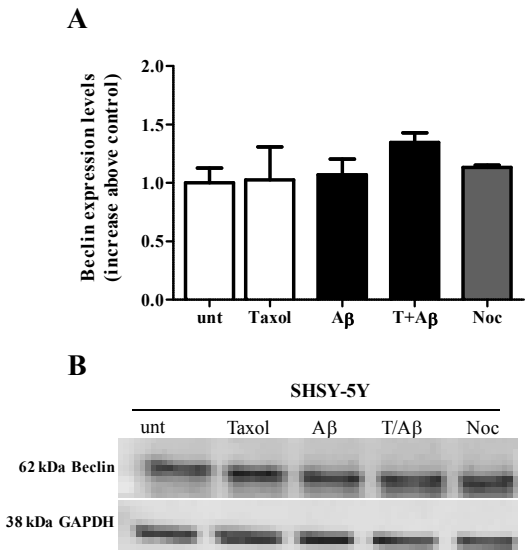


Fig. 14. Beclin levels in SHSY-5Y ρ+ cells. (A) and (B) Densitometry and Western blot analysis of Beclin levels after correcting with GAPDH.

In view of the fact that Aβ-driven microtubule dynamics impairment compromises AVs transport we evaluated the levels of LC3II, which are indicators of a stimulation of

macroautophagy. We observed an up-regulation of macroautophagy in Aβ treated cells that were partially prevented by taxol pretreatment (Fig. 15A and B). Data obtained in ρ0 cells indicated no significative alterations in autophagocytosis (Fig. 15C and D)

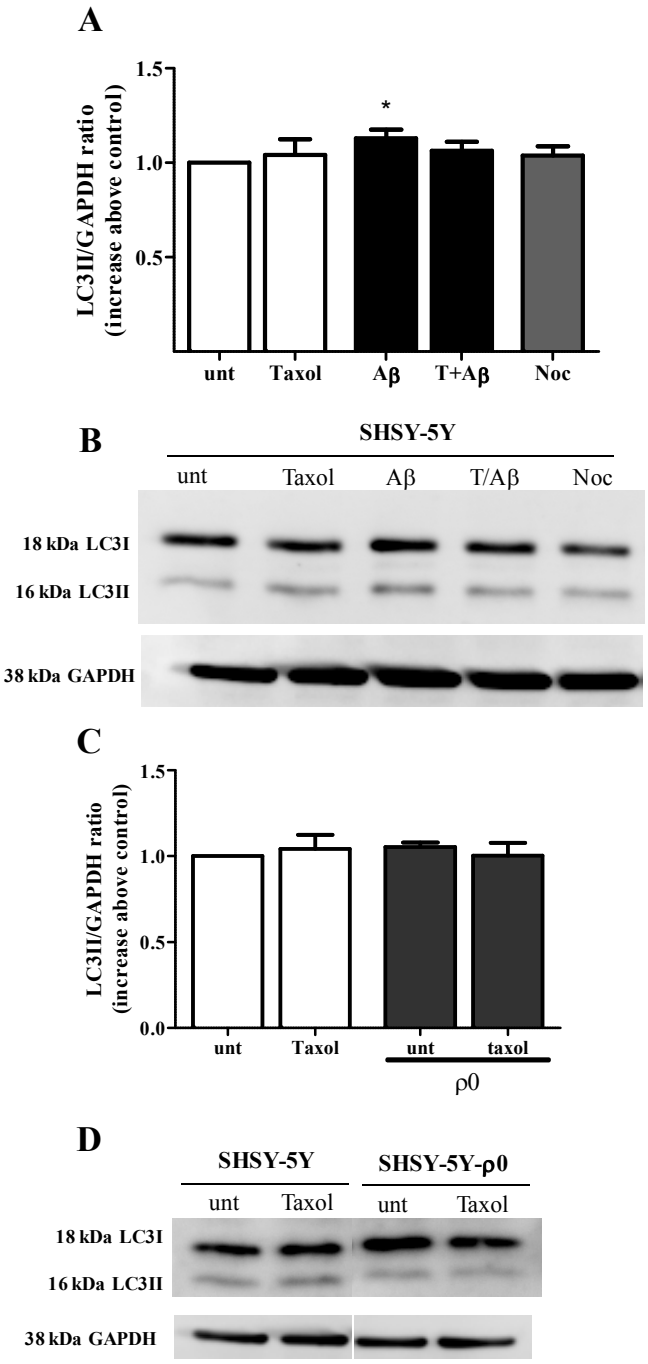


Fig.15. LC3II alterations in SHSY-5Y ρ+ and SHSY-5Y ρ0 cells. (A) and (B) Densitometry and western blot analysis of LC3II levels in SHSY-5Y ρ+

cells incubated with taxol, Nocodazole and Aβ after correcting with GAPDH. (C) and (D) Densitometry and western blot analysis of LC3II levels in SHSY-5Y ρ0 cells untretated or exposed to taxol after correcting with GAPDH. Data are expressed as the increase over untreated SHSY-5Y cells and represent mean ± SEM values derived from at least three independent determinations.* $p < 0.05$ significantly different as compared to untreated SHSY-5Y ρ+ cells.

Confocal images merging α-tubulin and LC3B showed that in Aβ treated cells LC3B fluorescence (green) is most spread along cells and has increased fluorescence indicating a possible activation of ALP pathway (Fig. 16).

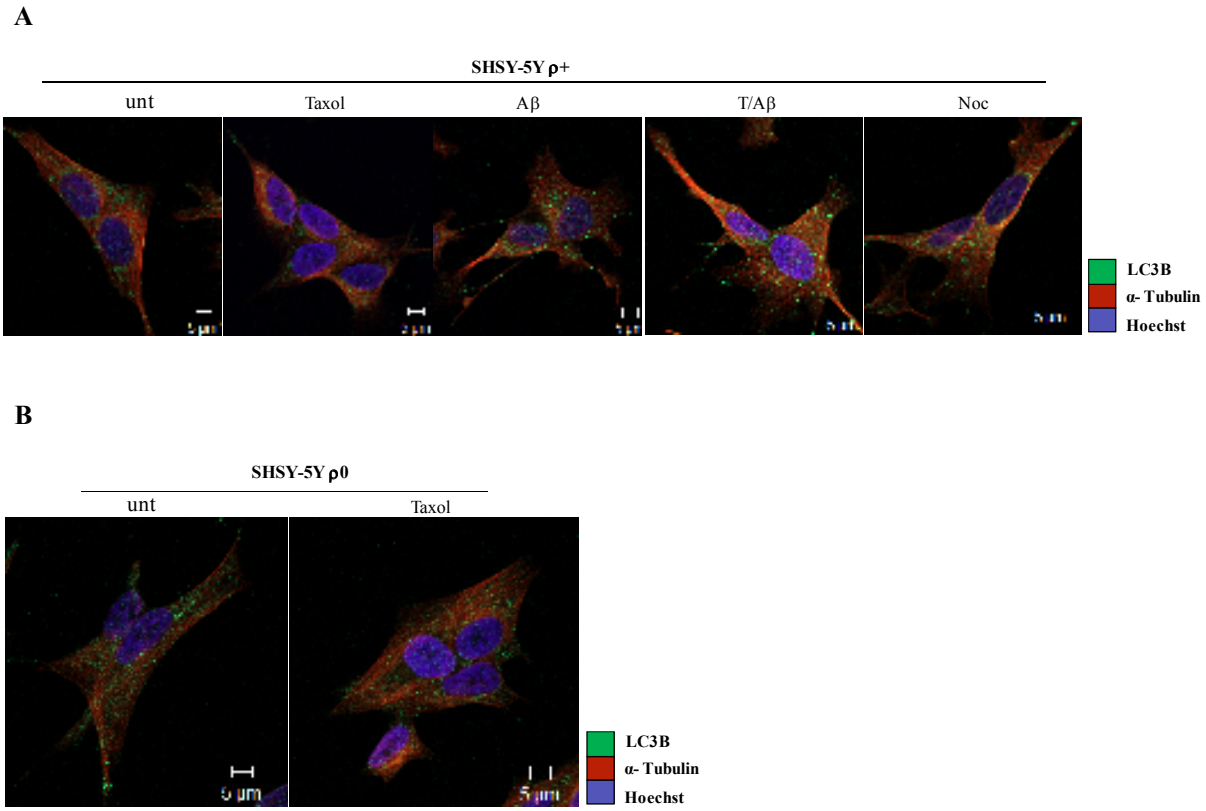


Fig. 16. LC3B staining alterations in SHSY-5Y ρ+ and SHSY-5Y ρ0 cells. (A) LC3B staining alterations in SHSY-5Y ρ+ cells untreated or incubated with taxol, Nocodazole and Aβ. (B) LC3B staining alterations in SHSY-5Y ρ0 cells untreated or exposed to taxol.

3.6 Microtubule stabilizer reduced Aβ oligomerization and mitochondrial Aβ content.

After 24 h incubation Taxol decreased Aβ triton insoluble oligomers in SHSY-5Y ρ+ cells, which indicated that microtubule breakdown inhibited Aβ clearance (Fig. 17).

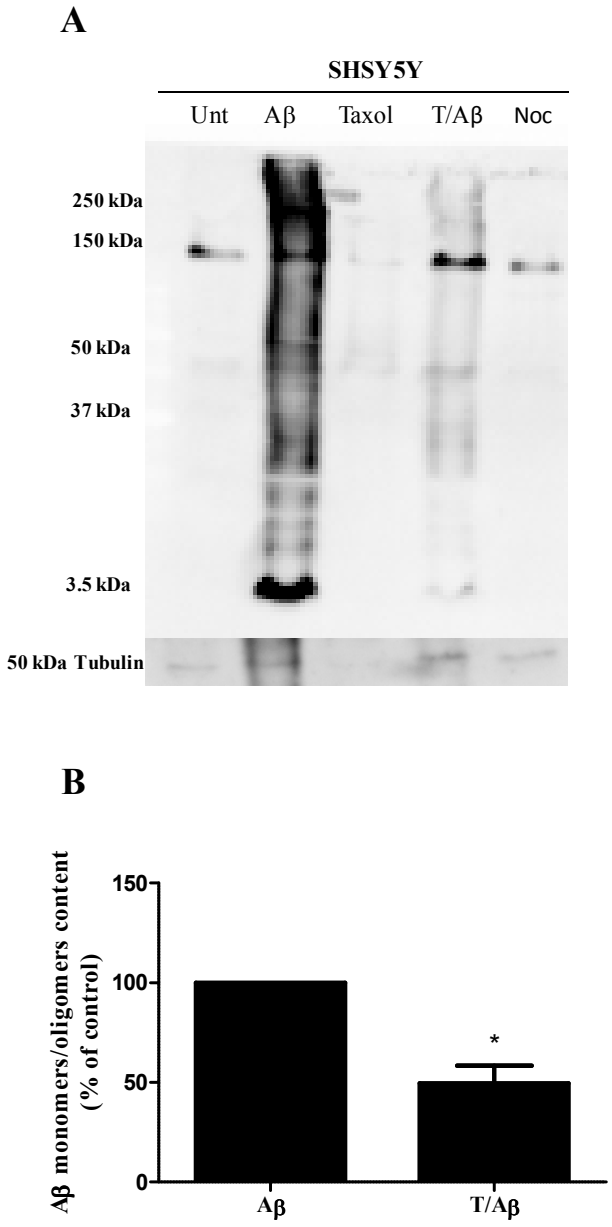


Fig.17. Cytosolic Aβ monomers/oligomers content in SHSY-5Y ρ+. (A) and (B) Densitometry and westernblot analysis of cytosolic Aβ monomers/oligomers content in cells exposed to taxol, nocodazole and Aβ after correcting with GAPDH. Data are expressed as the decrease over SHSY-5Y cells exposed to Aβ and represent mean ± SEM values derived from at least three independent determinations. * $p < 0.05$ significantly different as compared to Aβ treated SHSY-5Y ρ+ cells.

We have also showed that taxol could reduce mitochondrial Aβ (Fig. 18). This evidence point out to the crucial role of microtubule network for the degradation by ALP of damaged mitochondria loaded with Aβ peptides.

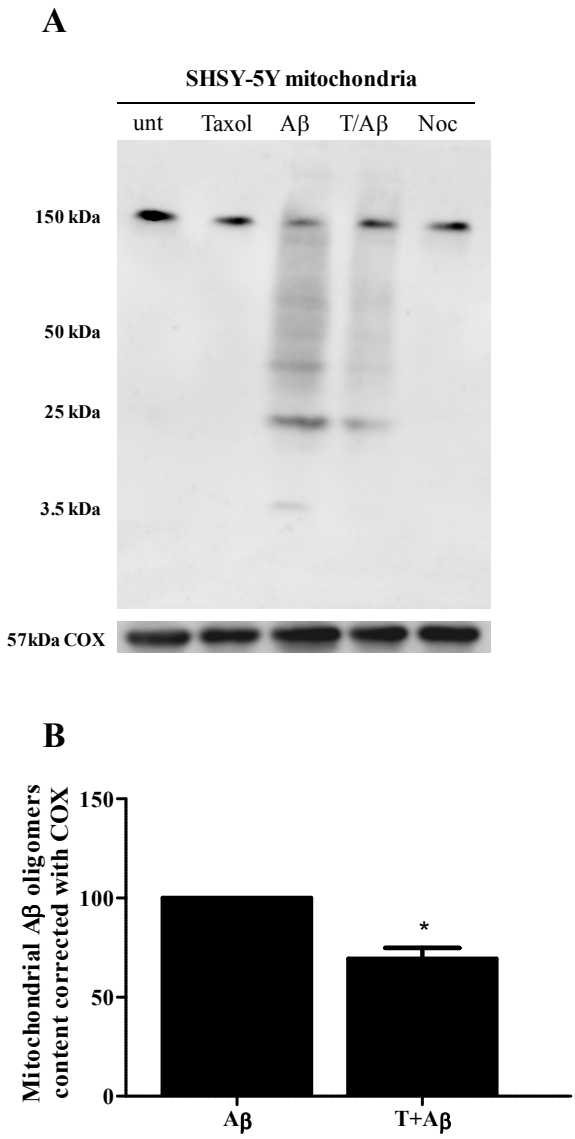


Fig.18. Mitochondrial Aβ monomers/oligomers content in SHSY-5Y ρ+. (A) and (B) Densitometry and westernblot analysis of mitochondrial Aβ monomers/oligomers content in cells exposed to taxol, nocodazole and Aβ after correcting with COX. Data are expressed as the decrease over SHSY-5Y cells exposed to Aβ and represent mean ± SEM values derived from at

least three independent determinations.* $p < 0.05$ significantly different as compared to Aβ treated SHSY-5Y ρ+ cells.

Chapter 4

Discussion

In this study we investigated the role of mitochondrial dysfunction in molecular events leading to protein missfolding and deposition in an AD cellular context. We provide evidence that mitochondrial dysfunction is up-stream in the cascade events that lead to cell demise, namely trigger microtubule network breakdown and macroautophagy impairment.

We started our work showing that $A\beta_{1-42}$ could induce a bioenergetics failure and alterations in NAD^+/NADH ratio levels. Lower ATP and higher NAD^+ levels could induce microtubule disassembly, where SIRT2 is likely to play a major role. Furthermore, we showed that taxol could prevent tubulin cytoskeleton damage induced by $A\beta_{1-42}$ rescuing cells from apoptotic cell death. Remarkably, Aβ oligomerization and mitochondrial Aβ content where reduced in the presence of taxol.

From AD patients brains and platelets to AD cellular models COX deficiency is a prominent pathological feature (Mancuso et al., 2003; Bosseti et al., 2003; Cardoso et al., 2004). Moreover, mitochondrial abnormalities have been found early in the disease progression, before amyloid deposition or NFT formation (Hirai et al., 2001). In our model which consisted in SHSY-5Y ρ+ cells exposed to $A\beta_{1-42}$ we observed a decrease in COX activity and lower ATP content. Aβ-induced metabolic dysfunction has been largely demonstrated. PC12 cells treated with increasing concentrations of Aβ peptides had mitochondrial alterations where complex I, II, III and IV deficits were evident (Pereira et al., 1999). Indeed, it was verified an impairment of glycolysis resulting in ATP depletion (Pereira et al., 1999). Although the exact mechanism by which Aβ induces cell death is not yet fully clarified, there is evidence that this peptide, added extracelullary, can act intracelullary, increasing mitochondrial dysfunction (Hansson Petersen et al., 2008), lowering antioxidant enzymatic activity, and increasing oxidative stress. Aβ treatments in mtDNA depleted NT2 cells that do not contain functional ETC,

could not exert cytotoxicity. This study was the ultimate evidence of the link between Aβ-mediated toxicity and mitochondrial dysfunction (Cardoso et al., 2001; Cardoso et al., 2009). Accordingly it was observed that Aβ species were associated with human AD brain mitochondria (Hirai et al., 2001; Caspersen et al., 2005; Devi et al., 2006; Manczak et al., 2006). Further, mitochondria isolated from human AD frontal cortex show inefficient MRC within dystrophic neurites, associated with Aβ peptides deposition in these regions (Perez-Gracia et al., 2008). The described effects of Aβ peptides targeted to mitochondria include failure in ETC enzymes namely COX and inhibition of ATP production (reviewed in Reddy and Beal), as our results also indicate. Our results and others point to the possibility that mitochondrial abnormalities start a sequence of cellular events that give rise to AD pathogenesis. Since we had established that $A\beta_{1-42}$ induced mitochondrial dysfunction we moved forward in order to tackle the connection between this event and the major AD pathological hallmarks: Aβ oligomerization and deposition ,and Tau hyperphosphorylation.

Given that microtubule dynamics is dependent on the availability of ATP we hypothesize about the importance of microtubule disassembly in AD. Evidence from pyramidal neurons from AD patients brain showed microtubule alterations concomitant with mitochondrial abnormalities before detectable NFT (Cash et al., 2003).

Tau protein as a neuronal microtubule associated protein (MAP) is important for microtubules proper function, neurite growth and maintenance (Stamer et al., 2002). It was demonstrated that overexpression of Tau results in a change in cellular shape, loss of polarization and delayed cell growth. In addition, mitochondrial distribution was profoundly altered becoming preferentially localized near the microtubule organization centre (MTOC) with a clustering aspect (Ebneth et al., 1998). Evidence from Tau overexpressing cells supports the referred idea, where transport along microtubules was

inhibited and this effect was prominent in the plus-end direction (Stamer et al., 2002; Mandelkow et al., 2003). Moreover, these cells have shorter neurites and showed to be more sensitive to oxidative stress, since the lower amount of mitochondria in cellular processes implies less bioenergetics leading to the retrograde degeneration or "dyeing back" phenomenon (Mandelkow et al., 2003). More recently, Guo and colleagues (2006) demonstrated that Aβ binds to Tau in solution, suggesting that this may be the precursor event to latter aggregation of both molecules. This binding enhances Tau phosphorylation which can form aggregates that may block the intracellular traffick, including AVs to lysosomes movement (Guo et al., 2006). In our model Aβ exposure triggered an increase in Tau hyperphosphorilation which was reverted by taxol. Nocodazole exposure could slightly enhance Tau hyperphosphorylation which made us hypothesize that, even in the presence of Aβ, if microtubules are stabilized Tau may not be dissociated from microtubules avoiding its availability to kinases that promote hyperphosphorylation.

Moreover, results obtained in SHSY-5Y cells, where Aβ-driven bioenergetics failure resulted in increased free tubulin levels. Besides free tubulin increase we have also found reduced levels of acetylated tubulin when cells were treated with $A\beta_{1-42}$. Tubulin acetylation is a reliable indicator of microtubule stability and has been found reduced in AD (Hempen and Brion, 1996). We associated lower levels of acetylated tubulin to higher NAD^+/NADH ratio observed under the same conditions. The bridge between these two events may be the activation of SIRT2, a cytoplasmic deacetylase known to have tubulin as a preferential substrate (North et al., 2003) which is dependent on the availability of NAD^+ for enzymatic activity. Because SIRT2 expression is not an indicator of its activation we access this enzyme activity indirectly by determining tubulin acetylation. Recent data from Henriques and colleagues (2010) showed in

primary neuronal cultures exposed to Aβ, tubulin cytoskeleton abnormalities, including reduced levels of acetylated tubulin, which impaired vesicular anterograde transport. Our microtubule alterations were reversed by taxol treatments indicating that microtubule network disruption are implicated in Aβ-induced citotoxicity. Microtubule disturbance may lead to an impairment of the autophagic pathway, since AVs are transport through microtubules (Cuervo, 2004). Evidence from AD brains showing massive AVs accumulation within dystrophic neurites (Nixon et al., 2005; Yu et al., 2005) suggested that autophagy is simultaneously impaired and induced in AD. Given that AVs in the brain are the major source of intracellular Aβ (Yu et al., 2005) an impairment of autophagic pathway compromise its normal clearance favoring Aβ oligomers production. Hung and co-workers (2009) found that Aβ peptides could activate autophagy increasing autophagosome formation and LC3II levels in SH-SY5Y/pEGFP-LC3 cell line. In accordance, we showed that Aβ is able to augment LC3II levels and that this effect could be partially reversed by taxol pretreatment. Moreover, experiences performed with vinblastine, an inhibitor of microtubule assembly during a period of few hours, caused the accumulation of AVs in neurites (Boland et al., 2008). Furthermore, Beclin-1 is required for autophagy induction *in vivo*, and it was reported that the levels of mRNA of this protein are reduced in subjects with middle cognitive impairment (MCI) and severe AD (Lee & Gao, 2008). Indeed, this reduction in Beclin-1 is associated with enhancement in Aβ pathology in APP mice model and the possible explanation is reduced autophagy. Interestingly, we do not see alterations induced by Aβ in Beclin-1 expression.

Yang and colleagues has reported that activated caspase 3 was found within dystrophic neurites, most exactly in AVs associated with amyloid plaques in PS/APP mice (Yang et al., 2008). Pro-apoptotic proteins will accumulate and apoptotic cell death can take

place culminating in neurodegeneration observed in AD brains. This imbalance between AVs formation and degradation verified in AD, drives cells into a so called "autophagic stress" (Chu, 2006). Clearance of AVs requires intact microtubules, so that they can go towards the cell body where the lysosomes are located (Nixon, 2007). In fact, taxol revealed efficacy in reducing Aβ insoluble oligomers which we attribute to microtubule dependent autophagy improvement. Autophagy acquires a protective role since Aβ oligomers are cleared and apoptotic cell death is attenuated in our cellular model. Our results point to a decrease in caspase 9 and 3 activation with taxol.

Indeed, it was observed an increase in mitochondrial degradation products in vulnerable AD neurons, suggesting an augmented mitochondrial turn-over by mitophagy (Moreira et al., 2007). The results obtained from our experiments show that taxol treatment can revert the loss of mitochondrial membrane potential and normalize NAD^+/NADH ratios. We hypothesize that Aβ are being targeted to mitochondria and arrested by TOM 40, one of the mitochondrial translocase proteins (Anandatheerthavarada et al., 2003). Then, the transport towards the inner membrane is assured by the translocase of the inner membrane (TIM) (Hansson Petersen et al., 2008).

Accordingly with our results, Aβ was also found within mitochondrial membrane, inducing COX dysfunction and lowering of ATP levels in transfected PC12 cells with APPwt and APPsw (Keil et al., 2004). Interestingly, Aβ oligomers in mitochondrial fractions where also lowered upon taxol exposure. These data prove that by restoring microtubule network, Aβ-damaged mitochondria are degraded by macroautophagy, giving further support to the idea that mitophagy is a prominent feature in AD.

Data from SHSY-5Y ρ0 cells show that these cells can maintain $\Delta\Psi_m$ and ATP levels similar to SHSY-5Y ρ+ cells despite the depletion of their mtDNA. This fact arise from

a “reversal” of ATP synthase activity, where nuclear encoded ATP synthase subunits assemble and maintain proton gradient across mitochondrial membrane (Nijlmans et al., 1995, Cardoso et al., 2001). Since these cells can maintain ATP levels microtubule breakdown could be prevented. Nevertheless, we found low levels of acetylated tubulin followed by huge increase in NAD^+ which indicated SIRT2 activation. As discussed before acetylation alteration induces microtubule instability, which can correlate with Tau hyperphosphorilation augmented levels. Taxol could prevent these alterations. Such data further support our idea that mitochondrial dysfunction is correlated with ALP alteration through microtubule network impairment.

Chapter 5

Final remarks

AD and other neurodegenerative disorders are expected to constitute a major health problem as life expectancy boost. Efforts have been taken in order to tackle innovate therapies namely, addressing mitochondrial dysfunction, a striking feature in AD, that has great influence in the etiology of the disease. The outcomes of mitochondrial abnormal metabolism: lower ATP levels, ROS overproduction; may deregulate many cellular processes, for instance, SIRT2 activation and consequent tubulin deacetylation may be implicated in microtubule network integrity. Upon microtubule network disruption axonal transport becomes compromised and the accumulation of autophagic vesicles in dystrophic neurites is prominent. The γ-secretase complex, described to be present in AVs membranes, cleaves APP leading to a major production of Aβ peptides. The increased accumulation of Aβ peptides results from the lack of fusion between AVs and lysosomes. Cytosolic Aβ may be then imported into the mitochondria where exerts further toxicity. A feedback mechanism becomes established where the impaired autophagic pathway results in a burden of Aβ peptides production which worsen mitochondrial dysfunction (Figure19).

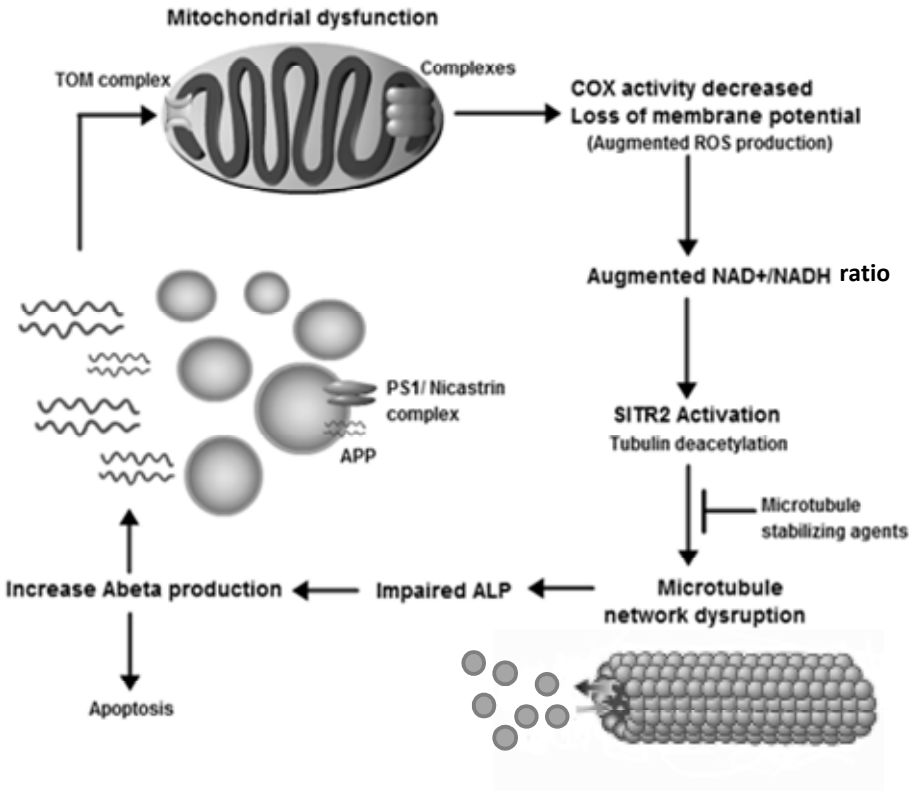


Fig.19. Positive feedback mechanism in AD etiogenesis. Mitochondrial dysfunction is the leading step in the molecular mechanisms underlying aged-related AD. The consequent bioenergetics failure leads to microtubule network breakdown impeding AVs retrograde transport to occur and lysosomal fusion. The impairment in autophagic pathway favors the accumulation of toxic protein oligomers (Aβ) and dysfunctional mitochondria which culminates in the neurodegeneration pathway observed in AD brains.

Data presented in this thesis show that $A\beta_{1\text{-}42}$ added extracellulary induce mitochondrial dysfunction by lowering ATP levels, decreasing in COX activity and increasing

NAD^+/NADH ratios. These events drive microtubule destabilization interfering with proper dynamic of this structure. Microtubule dynamics is compromised in two different ways: (1) ATP levels decrease the capacity of polymerization that culminated in augmented free tubulin ratios; (2) SIRT2 is activated in consequence to NAD^+ increased availability, deacetylates tubulin and promotes further microtubule instability. Given that tubulin cytoskeleton is no longer efficient, AVs retrograde transport is impeded and accumulates within cell. The absence of proper clearance of degradative products favors Aβ oligomerization and deposition which activates mitochondrial dependent apoptotic cell death.

These observations indicated us that microtubule stabilizers could ameliorate Aβ-dependent microtubule damage. In fact we proved that pretreatment with taxol was efficient recuing cells from augmented free tubulin and tubulin deacetylation. Moreover Tau hyperphosphorylation caused by Aβ–mediated mitochondrial damage was decreased upon taxol exposure. Since microtubule integrity is restored upon taxol treatment Aβ oligomerization and deposition was partially reverted, in accordance with a decrease in caspase activation. Autophagy is likely to be implied in this process because LC3II levels partially decreased with taxol treatment, indicating that a functional microtubule network allows AVs to fuse with lysosomes. Interestingly, the presence of taxol decreased mitochondrial Aβ oligomers content, protected against mitochondrial rhodamine retention loss and decreased NAD^+ augmented levels. These results show that restored microtubule integrity enabled dysfunctional mitochondria to be degraded by mitophagy.

Although, taxol cannot cross the blood brain barrier, our data point to a microtubule targeted therapy in AD. Currently, in our laboratory we are testing the effects of NAP, a targeted microtubule peptide that act at a microtubule level. NAP passed the toxicity

tests and is now being tested in AD patients in phase II clinical trials. It will be interesting to address in our models whether this peptide improves cell viability.

Overall, we propose a new therapeutic target: microtubules, as a reliable future restorative strategy in AD, since they are likely to be perturbed early in the disease progression. This approach allows for an intervention up-stream in the events leading to AD pathogenesis.

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