DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Post-translational modifications of tau protein: implications for Alzheimer's disease

Rui Miguel Ferreira Gomes

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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 Professora Doutora Sandra Morais Cardoso (Universidade de Coimbra) e co-orientação da Professora Doutora Emília Duarte (Universidade de Coimbra)

Rui Miguel Ferreira Gomes

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Índice

Abstract 5
List of Abbreviation 9
Chapter 1-Introduction 11
1.1 General introduction 13
1.2. Mechanisms of Alzheimer disease 14
1.2.1 Aβ cascade hypothesis 14
1.2.2 Mitochondrial cascade hypothesis 15
1.3 Alzheimer disease is a tauopathy 18
1.3.1 Structure and function of tau protein 19
1.4 Autophagy in AD 23
1.5. Importance of MT-network in AD 26
Chapter 2-Aims 29
Chapter 3-Materials & Methods 33
3.1 Biological Materials 35
3.1.1 The cytoplasmic hybrid (cybrid) lines 35
3.1.2 Parental neuroblatoma cell line SH-SY5Y (SH) 37
3.1.3 Parental neuroblatoma cell line SH-SY5Y Tau P301L mutant cells (P301L) 37
3.2 Cell treatment 37
3.3 Chemicals 38
3.4 Cell Viability assay 39
3.5 Apoptotic cell death evaluation 39
3.5.1 Caspases activation 39
3.6 Immunoblotting 40
3.7 Statistical analysis 41
Chapter 4-Results 43
4.1 Effects of AK1, TubA and C646 on toxicity and apoptotic cell death in cybrid cells lines 45
4.2 Modulation of the levels of acetilation by specific inhibition of SIRT2, HDAC6 and p300 in cybrid cells 48
4.3 Alterations in autophagic activity in cybrid cells 51
4.4 Evaluation of the role of acetylation of tau and tubulin in SH and P301L cells 53
4.5 Role of HDAC6 and p300 on macroautophagy in SH and P301L cells. 56

Chapter 5-Discussion..... 59
Chapter 6-Conclusion..... 67
Chapter 7-References 71

Abstract

Alzheimer´s disease (AD) is the first most prevalent neurodegenerative disorder affecting, nearly 36 million peopleworldwide.While the mechanisms involved in the etiology of AD are unknown, it is believed that mitochondrial dysfunction is correlated with sporadic cases of the disease. The two primary pathological hallmarks are: amyloid plaques, composed of amyloid-β (Aβ), a cleavage peptide derived from amyloid precursor protein (APP), and neurofibrillary tangles (NFTs), primarily composed of hyperphosphorylated tau protein. This studywe aimed tounderstand the influence ofacetylation,a post-translational modification that affectsseveralproteins,oftauprotein andtubulinin thedevelopmentof AD. Given thatmitochondrial dysfunctionhas been widelyimplicated inADpathogenesis,we used cybrids cell lines, controland AD, which are used to studythe contributionof the mitochondrial DNA(mtDNA) in cellularphenotypic changes. In thiscellmodelwe ascertainedthe contribution ofp300acetyltransferase(p300), histone deacetylase6(HDAC6)andsirtuin2(SIRT2) tophenotypicchangesinAD, using the specific inhibitors C646, Tubastatin A and AK1, respectively. Under theseconditionswe observed that inhibition of SIRT2 is effective in protecting cells against apoptosis activation. It was foundthatp300hasinfluence ontauphosphorylation levelsand inautophagic flux in AD. In addition,we also found thatthis enzymemodulates theacetylationof tau andtubulin. In the case ofHDAC6 the results confirmed thatits inhibitiondecreasedphosphorylatedandacetylatedtau levelsand improvesautophagicflux inAD. With the inhibitionofSIRT2 we observed adecrease inthe phosphorylatedtau levelsand an improvement ofautophagicflux in AD.Subsequentlywe useda model oftauopathy, SH-SY5Y TauP301L (P301L),which is characterized byabnormal accumulation ofphosphorylated tau.The results in these cell linesconfirmed that tau acetylation correlates with its phosphorylation state.

Overall, our data suggest thattau acetylationcan contribute tothe formation ofNFT. We observed that reestablishment of microtubule (MT) network stability, through improvement of MT acetylation and decreased phosphorylation of tau, contributes to rescue autophagy and consequently cells from apoptotic cell death.

Keywords: Alzheimer Disease (AD), Tau, acetylation, neurofibrillary tangles (NFTs).

Resumo

Doença de Alzheimer (DA) é a doença neurodegenerativa mais prevalente afetando, em todo o mundo, cerca de 36 milhões de pessoas. Apesar da etiologia envolvida no desenvolvimento de DA ser desconhecido, acredita-se que a disfunção mitocondrial poderá estar relacionada com os casos esporádicos da doença. As duas características patológicas primárias são: placas amilóides, compostas de amilóide-β (Aβ), clivagem de um péptido derivado da proteína precursora da amilóide (APP), e emaranhados neurofibrilares (NFTs), compostos principalmente por proteína tau hiperfosforilada.

Com este estudo pretendemos compreender a influência da acetilação, uma modificação pós-traducional que afecta diversas proteinas, da proteina tau e tubulina para desenvolvimento desta patologia.Tendo em conta que a disfunção mitocondrial tem sido amplamente implicada na patogénese da DA, usámos linhas celulares cibridas, controlo e DA, que são utilizadas para estudar a contribuição do DNA mitocondrial (mtDNA) nas alteraçoes fenotipicas celulares. Neste modelo celular averiguamos o contributo da p300 acetiltransferase (p300), histona deacetilase 6 (HDAC6) e sirtuin 2 (SIRT2) para as alterações fenotipicas visiveis em DA. Para isso utilizamos inibidores especificos destas enzimas moduladoras da acetilação. Nestas condições observamos que estas enzimas desempenham um papel importante no processo apoptotico que culmina com morte celular. Constatou-se que a p300 tem influência nos niveis de fosforilação da proteina tau e na diminuição do fluxo autofágico em DA. Os resultados confirmaram que a inibição da SIRT2 é benéfica para as células de DA pois induz uma diminuição dos niveis de tau fosforilada e uma melhoria do fluxo autofágico em DA.

Posteriormente utilizamos um modelo de tauopatia, SH-SY5Y TauP301L (P301L), que se caracteriza pela acumulação anormal de tau fosforilada, utilizando a linha humana de neuroblastoma, SH-SY5Y (SH), como controlo. Os resultados mostraram que existe uma correlação entre a acetilação da tau e os seus níveis de fosforilação. A diminuiçao dos niveis de fosfo-tau em DA quando a p300 é inibida sugere que acetilação pode contribuir para a formação das NFT. Além disso parece haver uma relação entre a acetilação da tubulina e a melhoria do fluxo autofágico.

Palavras-chave: Doença de Alzheimer (DA), Tau, acetilação, tranças neurofibrilares (NFTs)

List of Abbreviation

Abbreviation	Description
AD	Alzheimer's disease
Aβ	Amyloid β Peptide
ALP	Autophagosome-lysosome pathway
APOE 4	Apolipoprotein E4
APP	Amyloid Precursor Proteins
ATRA	*All-trans* retinoic acid
AVs	Autophagic Vacuoles
Cdk-5	Kinase-5 cyclin-dependent
CMA	Chaperone-mediated autophagy
COX	Cytochrome C Oxidase
ETC	Electron transport chain
fAD	familial Alzheimer Disease
FTD	Frontotemporal dementia
GSK-3β	Glycogen synthase kinase 3 beta
HAT	Histone acetyltransferase
HDAC6	Histone Deacetylase 6
LC3	MT-associated light chain 3
Lys (K)	Lysine
MAP	MT-Associated Protein
MCI	Mild cognitive impairment
MT	Microtubule
mtDNA	Mitochondrial Deoxyribonucleic Acid
NAP	Davunetide
NFT	Neurofibrillary Tangles
NRB	Neighbor of Brac 1
p300	p300 acetyltransferase
P301L	SH-SY5Y TauP301L mutant cell line
PHF	Paired helical filaments
PP-2A	Phosphatase 2A
PP-1A	Phosphatase 1A
PSP	Progressive supranuclear palsy
ROS	Species reactive oxygen
sAD	sporadic Alzheimer Disease
Ser (S)	Serine
SH	SH-SY5Y cell line
SIRT2	Sirtuin 2
TubA	Tubatastin A
UPS	Ubiquitin proteasome system

Chapter 1

Introduction

1.1 General introduction

Alzheimer's disease (AD) is the most common form of dementia and was first described in 1906 by the psychiatrist Alois Alzheimer(Alzheimer, Stelzmann et al. 1995). Pathologically, it is characterized by the loss of neurons in the cortex and hippocampus (Gomez-Isla, Price et al. 1996)and the presence of two hallmarkers, extracellular amyloid plaquesand neurofibrillary tangles (NFTs). Clinically, AD patients manifest symptoms of loss of ability to remember the information recently acquired, difficulty to complete social and work activity and deteriorate movement coordination and pattern recognition.

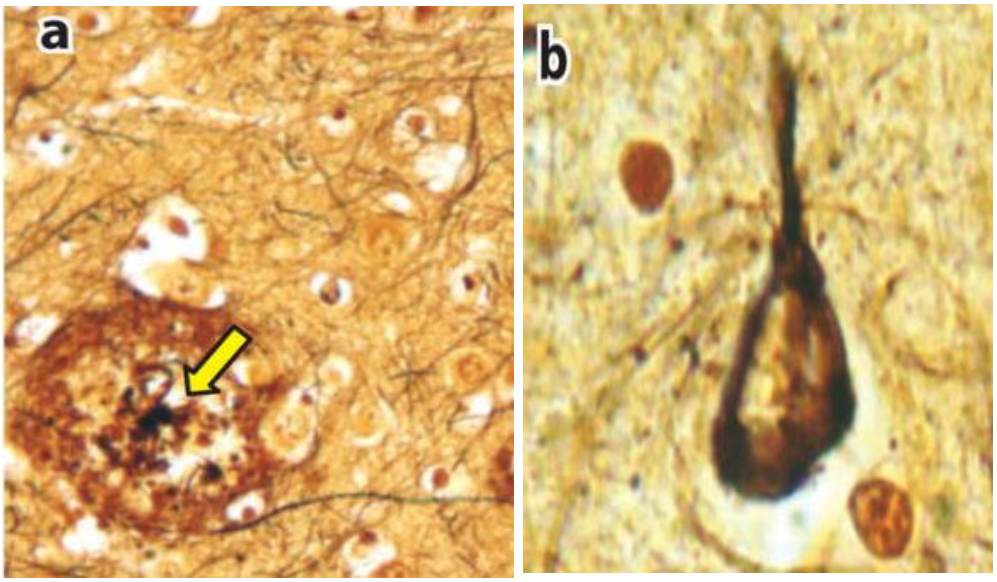


Figure 1.1-**Histopathological hallmarks of Alzheimer's disease**. A- Senile plaque of Aβ; B- Neurofibrillary tangles of hyperphosphorylated Tau. (adaptedfromO`Brien andWong 2011)

The etiological mechanisms underlying to genesis of AD remain unclear but aging is considered the main risk factor for the development of AD. Most cases are sporadic (sAD), late-onset forms of AD,corresponding to about 99% of patients with this disease. These forms arise among all genetic forms of the disease, manifesting in late stages on

the life however it is belived that genetic and environmental factors act synergistically in onset of the disease. The remaining percentage is the familial AD cases (fAD) correlated with genetic inheritance and appear at an unusually early age.AD is a progressive and chronic condition and currently there are no treatments to cure it but the emergence of the role of mitochondria, tau and tubulin proteins in AD etiopathogenesis open new windows for the identification of new therapeutical targets.

1.2.Mechanisms of Alzheimer disease

1.2.1 Aβ cascade hypothesis

Aβ cascade hypothesis emerged after the discovery that mutations in precursor protein of Aβ gene (APP),located on chromosome 21 caused AD. In 1992, Hardy developed this vision, which considers the involvement of amyloid plaque in the origin of the disease process(Hardy and Higgins 1992). The role of Aβondisease progression is supported by countless studies in animal and cellular models, spanning moleculargenetics and neuropathology where it was shown that problems in the metabolism of APP and therefore aggregation and deposition of Aβ would have a central role to the pathogenesis of AD(Hardy and Higgins 1992, Holtzman, Bales et al. 1999, Hardy and Selkoe 2002). The increased risk associated with having a family history of AD is not entirely explained by the fact that an individual has inherited the apolipoprotein E (APOE) ε4 risk gene(Holtzman, Bales et al. 1999, Laws, Hone et al. 2003, Raber, Huang et al. 2004, O'Brien and Wong 2011).

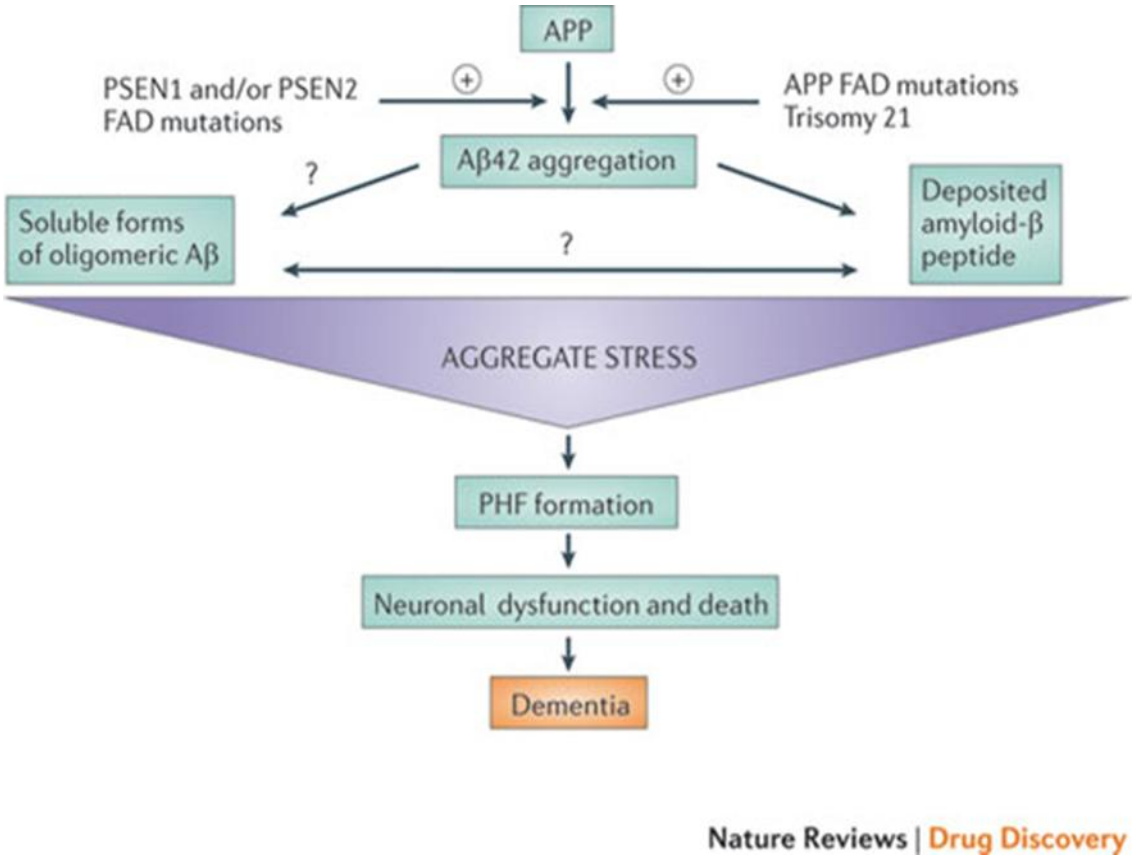


Figure 1.2- **The amyloid cascade hypothesis.**This theory relied on genetic studies that showed that mutations APP, PS1, or PS2 cause AD contributed to the formulation of this hypothesis.(adapted from Karren, Mercken et al. 2011)

However, several objections to this proposal are emerging namely, that the number of plates do not correlated to the severity of dementia. Thus, the hypothesis was directed to smaller forms of Aβ(Karran, Mercken et al. 2011). Over time this hypothesis has undergone changes, currently considering the effect of oligomeric form of Aβ, the main responsible for pathological alterations in AD(Karran, Mercken et al. 2011).

1.2.2 Mitochondrial cascade hypothesis

Emerged in 2004, by Swerdlow and Khan, an hypothesis that helped explain the sporadic cases of AD(Swerdlow and Khan 2004). Several studies have shown the key role of mitochondrial in several intracellular processes helping to provide important data for the emergence of this theory.Studies showed the relationship between mitochondrial

dysfunction and aging once it has been observed that electron transport chain (ETC) activity declines with age (Trounce, Byrne et al. 1989, Ojaimi, Masters et al. 1999) and oxidative stress increases with age(Cadenas and Davies 2000). It was seen that pre-translational mRNA oxidation may contribute to protein aggregation and in AD patients brains was observed excessive mRNA oxidation, but only in some transcribed RNAs(Nunomura, Perry et al. 1999, Nunomura, Hofer et al. 2009). Futhermore, results showed several polymorphisms in nuclear and mitochondrial genes of the ETC subunits indicating a possible influence on the development of aging, and neurodegeneration(De Benedictis, Rose et al. 1999, van der Walt, Nicodemus et al. 2003). This theory relies on the central idea that mitochondrial dysfunction occupies a upstream position in the pathogenesis of the disease and similar mechanisms underlie brain ageing and AD, fig.1.3(Swerdlow and Khan 2004, Swerdlow and Khan 2009). The parallelism between this theory and Aβ cascade hypothesis is mitochondrial dysfunction and consequently species reactive oxygen (ROS) overexpression primarily responsible for the development of this pathology.

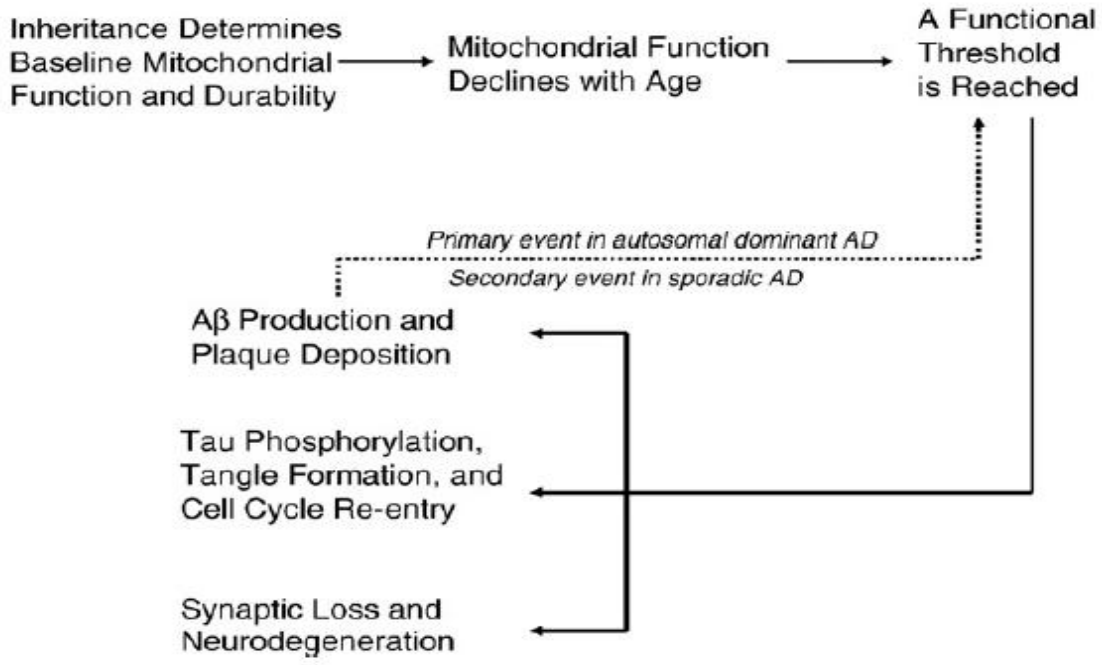


Figure 1.3- **Mitochondrial cascade hypothesis.** This hypothesis, which applies to sporadic AD, mitochondria are at the apex of the cascade. Age-related mitochondrial deficts drives a functional threshold that give rise to histopathological hallmarks of Alzheimer's disease.(adapted from Swerdlow and Khan 2009)

Supporting Swerdlow and Khan point of view, epidemiological studies have demonstrated the importance of maternal genetic inheritancefor the development of AD in descendants(Swerdlow and Khan 2004). Several studies on mitochondrial DNA (mtDNA) evidenced its importance on the development of neurodegenerative diseases(Swerdlow and Khan 2004). Observations have shown that mtDNA accumulats mutations over a person's lifespan influencing aging and could cause AD(Swerdlow and Khan 2004).Others data that refute this theory based on evidence that mitochondrial dysfunction are visible in sporadic AD and age-matched control in several tissues, such liver, muscle, fibroblasts, and brain(Swerdlow and Khan 2004, Beal 2005, Shi, Guo et al. 2008). Relevant to AD, it was observed observed in the brain and platelets from AD patients, a decrease in cytochrome c oxidase (COX) activity in early stages of the disease(Cardoso, Proenca et al. 2004). Fibroblasts from sporadic AD patients showed mitochondrial morphology and distribution abnormalities when compared to human fibroblasts from the normal subjects(Wang, Su et al. 2008). These results showed that AD can constitute a systemic disease affecting several tissues although it is most visible in the brain, since neurons are post-mitotic cells. Studies withmild cognitive impairment (MCI) subjects allowed to strengthen rational idea that the mitochondrial dysfunction is an early event in AD etiology. MCI is considered an intermediate stage between the expected cognitive decline of normal aging and AD(Petersen 2004). In fact, just as in AD, was observed in MCI subjects the early markers of metabolic dysfunction. It was observed high levels of mtDNA oxidative damage in both conditions, AD and MCI subjects compared to control(Swerdlow and Khan 2004, Migliore, Fontana et al. 2005). Further, studies with MCI subjects platelets showed deficit in ETC especially in COX as already observed in AD(Valla, Schneider et al. 2006). Other studies with AD and MCI subjects founded changes in oxidative stress markers, where there was an increased lipid peroxidation and a decrease in antioxidant defenses(Ansari and Scheff 2010, Padurariu, Ciobica et al. 2010). More recently, a study of AD and MCI was pursued in cybrid cell lines (cytoplasmic hybrid) that allowed to complete the evidence that had emerged so far about the mitochondriabeing in an upstream position in AD etiology.

The analysis of AD and MCI cybrid cell lines showed similar characteristics to AD and MCI subjects. The AD and MCI lines showed reduced COX activity, reduced

respiratory flux and decrease viability in comparison to control cybrids(Onyango, Tuttle et al. 2005, Silva, Santana et al. 2013, Silva, Selfridge et al. 2013).

1.3Alzheimer disease is a tauopathy

Tauopathies are a class of neurodegenerative diseases associated with the pathological aggregation of tau protein in the human brain, where AD is included (Crespo-Biel, Theunis et al. 2012). It is characterized by the presence of NFT containing hyperphosphorylated tau protein. The NFT founded in AD brains are composed of tau protein abnormally phosphorylated(Grundke-Iqbal, Iqbal et al. 1986, Goedert, Wischik et al. 1988, Wischik, Novak et al. 1988). Posteriors studies led to consider that tau tangles or tau protein could be at the beginning of AD. These studies founded that the number of NFT were closely related to the degree and duration of dementia(Berg, McKeel et al. 1998, Giannakopoulos, Herrmann et al. 2003). It is assumed that the agglomerated of phosphorylated tau protein plays a physiological role on the pathology of tauopathies. One of the strongest evidence of the involvement of tau protein in the origin of a disease is the cause of frontotemporal dementia (FTD). Genetic studies of families carrying this dementia showed numerous mutations in the protein gene that are linked to the accumulation of hyperphosphorylated tau protein in neurons and glial cells(Seelaar, Rohrer et al. 2011). FTD is the second commonest form of dementia that can be cause by a pathogenic mutation in tau (P301) gene (Lossos, Reches et al. 2003, Kar, Kuo et al. 2005).In other diseases such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) tau tangles are also present although not consider the cause of the disease(Pittman, Fung et al. 2006). In fAD, no mutations were detected in the gene for tau protein but studies have shown the presence of tau gene polymorphisms but no causal role in the pathogenesis of AD(Myers, Kaleem et al. 2005, Mukherjee, Kauwe et al. 2007). Although the relationship between tau protein and other AD brain phenomena is not yet intensely analyzed, its importance for the axonal transport give particular attention to this protein in this pathology. In addition, evidence has shown that the phosphorylation of tau has been considered a link between oxidative stress, mitochondrial dysfunction and failure synaptic pathology in AD(Adalbert, Gilley et al. 2007, Mondragon-Rodriguez, Perry et al. 2013).

1.3.1 Structure and function of tau protein

Tau was disclosedas a microtubules (MT)-associated protein (MAP). This protein is expressed in the brain, specifically in neurons, but was also detected tau mRNA in other cell types in several peripheral tissues(Lee 1990, Neumann, Farias et al. 2011, Lee and Leugers 2012). Human tau is encoded by a single gene, MAPT, located on chromosome 17(Kolarova, Garcia-Sierra et al. 2012). Alternative splicing of MAPT gene transcription produces predominantly six tau isoforms that are differentially expressed during development of the brain. Tau plays an important role in tubulin assembly into MTs that defines their normal morphology and provides structural support to the neurons but also in membrane interactions, facilitation of enzyme anchoring, organelle trafficking and cell differentiation and polarization(Ittner, Ke et al. 2011, Mandelkow and Mandelkow 2012).We can find it predominantly in axons but also residing in somatodendritic locations. Tau can be found in other brain cells, notably oligodendrocytes, astrocytes and endothelial cells. The six isoforms of tau protein vary in size from 352 to 441 amino acid residues and are classified by containing either 3 (3R) or 4 (4R) repeat domains involved in MT binding or for having one (1N), two (2N), or zero (0N) amino terminal inserts of 29 amino acids each(Kolarova, Garcia-Sierra et al. 2012, Lee and Leugers 2012). The flanking sequences upstream and downstream of these sites have been studied enough to realize the influence it has on MTs binding (Lee and Leugers 2012).

1.3.1.1 Phosphorylation

Phosphorylation of tau protein is the most well documented post-translational modification in the AD. There are over 85 Ser/Thr-Pro motifs in tau that can act as potential phosphorylation sites, most of which are located in the proline-rich region (P-region; residues 172–251) and the C-terminal tail region (C-region; residues 368–441)(Hanger, Seereeram et al. 2009). It has been described that this post-translational modification regulates tau binding to MTs (Liu, Li et al. 2007).Lindwall and Cole revealed that tau protein, in an unphosphorylated state, is more efficient at promoting

MTs assembly(Lindwall and Cole 1984).Normal levels of phosphorylation is required for the optimal function of tau, but the hyperphosphorylated state causes conformational changes thatcan make tau to lose its biological functions inneurons (Kolarova, Garcia-Sierra et al. 2012, Beharry, Cohen et al. 2014).Studies founded that somephosphorylated sites in paired helical filaments (PHF)-taucontribute to pathological processes in AD. Noteworthy, the AT8 (epitope pS199 / pS202 / pT205), PHF-1 (epitope pS396 / pS404) and pS262 phosphorylation sites havea greater immunoreactivity in human AD brain tissue showing to be efficient markers for AD(Mondragon-Rodriguez, Perry et al. 2013). Moreover, it has been proposed that under this pathological situation, phospho-status of tau is abnormally regulated by the action of several kinases and phosphatases including: glycogen synthase kinase 3 beta (GSK-3 β), kinase-5 cyclin-dependent (Cdk- 5), and protein phosphatase 1A (PP-1A) and 2A (PP-2A)(Gong and Iqbal 2008, Boutajangout, Sigurdsson et al. 2011). GSK-3β has received significant attention because it may play a major role in tau phosphorylation regulation in both physiological and pathological conditions(Gong and Iqbal 2008, Boutajangout, Sigurdsson et al. 2011). PP2A plays a key role sinceit is responsible for 70% of tau dephosphorylation in the brain(Boutajangout, Sigurdsson et al. 2011, Beharry, Cohen et al. 2014).

1.3.1.2Acetylation

Recently, itwas demonstrated that tau can be modified by lysine (lys) acetylation. This modification of tau has been classified as a new potential regulatory modification implicated in AD and other neurodegenerative disorders(Beharry, Cohen et al. 2014). Studies suggests that acetylated tau is not beneficial since it promotes pathological tau aggregation. Min and colaboradores reported in primary neurons, tauopathy mouse models and AD that acetylation of tau prevents degradation of phosphorylated tau(Min, Cho et al. 2010). Mass spectrometry analysis showed that acetylation of four lysine residues, in which the lys 280 (K280), located in the second MTs-binding repeat (^{275}VQIINKK^{280}), appeared to be the site most critical in modulating tau–MT binding (Cohen, Guo et al. 2011).

Another study showed, by immunohistochemistry, that the acetylated tau had greater relevance in cases of moderate to severe stage of AD and founded a similar distribution

of acetylated tau and hyperphosphorylated tau in AD(Irwin, Cohen et al. 2012).Futhermore, otherstudiesshowed significant reactivity to polyclonal antibody specific for lys residue 280 of the tau protein(Irwin, Cohen et al. 2013).On the opposite side, another theory believes that acetylation may be beneficial to prevent aggregation of tau.In vitro studies showed that acetylation of KXGS motifs of tau protein (located in the MT-binding domain, playing a crucial role in binding and stabilizing MTs) reduce the propensity to aggregation(Cook, Carlomagno et al. 2014). It was also shown that acetylation and phosphorylation compete to modulate tau on KXGS motifs(Cook, Carlomagno et al. 2014). In addition, it was observed that KXGS motifs are hypoacetylated in patients with AD, suggesting that the loss of acetylation of KXGS motifs in tau can drive to tau-induced neurodegeneration(Cook, Carlomagno et al. 2014). The authors concluded that increased acetylation of KXGS motifs in tau is crucial to a decrease the aggregation,which makes it a promising approach to delay or prevent disease progression in tauopathies(Cook, Carlomagno et al. 2014, Cook, Stankowski et al. 2014).

Some enzymes that regulate the acetylation have already been identifyed. Enzymes that add an acetyl group to tau are called acetyl transferases, such histone acetyltransferase (HAT) or lysine acetyltransferase (p300) and CREB-binding protein(Irwin, Cohen et al. 2012, Cook, Carlomagno et al. 2014). Deacetylases, such Sirtuin 1(SIRT1) and histone deacetylase 6 (HDAC6) remove an acetyl group(Min, Cho et al. 2010). It was observed that HDAC6 inhibition increases the acetylation of KXGS motifs, acetylation of KXGS motifs decreases tau polymerization(Cook, Carlomagno et al. 2014). HDAC6 expression levels have been shown to be elevated in AD patient's brains and in a transgenic mouse model of Aβ amyloidosis, moreover the activity of this enzyme was also high(Cook, Carlomagno et al. 2014). Studies have determinedthat SIRT1 levels are decreased in AD patient's brains(Min, Cho et al. 2010). Furthermore, in primary neurons was seen that the inhibition of SIRT1 blocks tau polyubiquitination and subsequent degradation via the ubiquitin proteasome system (UPS), resulting in p-tau accumulation(Min, Cho et al. 2010). Unlike the SIRT1, it was seen that the inhibition of p300 enabled the elimination of p-tau in mouse models of tauopathies(Min, Cho et al. 2010).

1.3.1.3 Other post-translational modifications

The mechanism leading to tau hyperphosphorylation still unknown but it was found that other post-translational modifications of tau interfere with tau function and aggregation.The impact of these changes in tau aggregation into NFTs are subdivided in two categories (fig. 1.4): pro-aggregation and anti-aggregation (Martin, Latypova et al. 2011).

In pro-aggregating category are included truncation(Garcia-Sierra, Mondragon-Rodriguez et al. 2008), glycation(Li, Liu et al. 2012), polyamination(Wang, Dickson et al. 2008), ubiquitination(Riederer, Leuba et al. 2013), nitration(Reyes, Fu et al. 2011), sumoylation(Takahashi, Ishida et al. 2008), and oxidation(Riederer, Leuba et al. 2013). On the opposite side, as anti-aggregating there are glycosylation(Wang, Grundke-Iqbal et al. 1996) and prolyl-isomerization(Bulbarelli, Lonati et al. 2009).

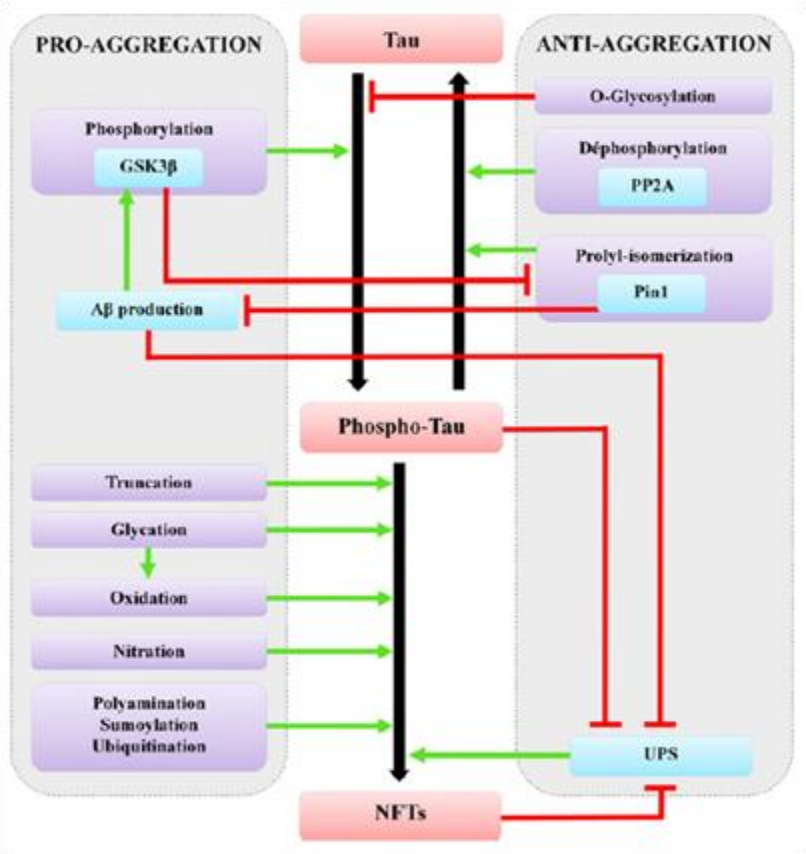


Figure 1.4-**Scheme of the influence of various post-translational modifications in the process of aggregation of tau**. Cellular mechanisms that impact tau aggregation into NFTs are subdivided in two categories: pro-aggregation and anti-aggregation mechanisms. This model integrates direct and indirect impacts of Ab, GSK3b, PP2A, Pin1 and UPS on the formation of NFTs. Phospho-Tau: phosphorylated tau, NFTs: neurofibrillary tangles, Ab: Amyloid b-peptide, GSK3b: glycogen synthase kinase-3b, PP2A: protein phophatase-2A, Pin1: peptidyl-prolyl cis/trans isomerase, UPS: ubiquitin–proteasome system. (adapted from Martin *et al.*, 2011)

1.4Autophagy in AD

It was De Duve, in 1963, who first came up with the word autophagy that appeared as a degradative process of double-membrane vesicle, the autophagosome, which contained inside intracytoplasmic material(De Duve 1963). Currently, autophagy amajor cell degradation system, is seen as an essencial quality control pathway for maintaining intracellular homeostasis(Glick, Barth et al. 2010). Autopagy is induced by different

adverse conditions such as limited nutrients, low oxygen levels, and decreased energy supply, and its providing essential elements for celular metabolism(Lynch-Day, Mao et al. 2012). There are several types of autophagy, via different pathways by which cargo is delivered to the lysosome: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA)(Glick, Barth et al. 2010). In diseases with protein aggregates as AD with accumulation of protein oligomers and aggregatesmacroautophagy may be overactivated(Cardoso, Pereira et al. 2010). Macroautophagy is a process that involves the sequestration of cellular contents by double-membraned structures called autophagosome(Glick, Barth et al. 2010).The latter acquires the enzymes required for the degradation of its content after fusion with the lysosome**.** Macroautophagy is activated in response to starvation(Mizushima, Yamamoto et al. 2004), physical and chemical stressors (Levine and Klionsky 2004). This process is crucial for cell homeostasis because plays an important role in the continuous turnover of organelles and clearance of misfolded and damaged proteins (Komatsu, Waguri et al. 2005, Komatsu, Kominami et al. 2006). Macroautophagy is a complex process controlled by a protein machinery that regulates several steps of this process. Autophagy is a selective process that is ensured mainly by specific adaptors which includes p62 / SQSMT1, Nix andneighbor of Brca1(NRB)(Lippai and Low 2014). Start of autophagy is controlled by the Atg1/ULK1 complex and leds to the formation of a double-membrane structure, called phagophore, that can sequestrate the cytoplasm region for degradation(Glick, Barth et al. 2010). Several studies have investigated the origin of this membrane and found out that mamalian cells may have a varied origin(Tooze and Yoshimori 2010). The next step, elongation, is largely governed by multiple proteins, namelyMT-associated light chain 3 (LC3), and allows the cargo accommodation resulting in the formation of autophagossome or autophagic vacuole (AV)(Glick, Barth et al. 2010). After fusion with lysosome, a new entity is formed, autolysosome, where the degradation process takes place by proteolytic enzymes(Nixon 2007, Glick, Barth et al. 2010).

Studies related to the role of macroautophagy in the pathogenesis of AD have shown that this process is impaired and symultaneously induced in brain samples from patients with AD and peripheral models of AD(Nixon 2007). In neocortical biopsies from AD brains, abundant AVs including autophagosomes, amphisomes, multilamellar bodies, and autolysosomes, were seen suggesting that the normally efficient autophagic process is altered(Cataldo, Paskevich et al. 1991, Nixon 2007) (Fig.1.5).

Furthermore, it was reported that AVs showed to be enriched in APP and components needed to generate Aβ peptide(Cras, Kawai et al. 1991). This impairment of autophagosome-lysosome pathway (ALP) will lead to an increase of Aβ peptide production, which in turn exert cytotoxic effects as described in the literature like mitochondrial dysfunction (Yu, Cuervo et al. 2005, Boland, Kumar et al. 2008).

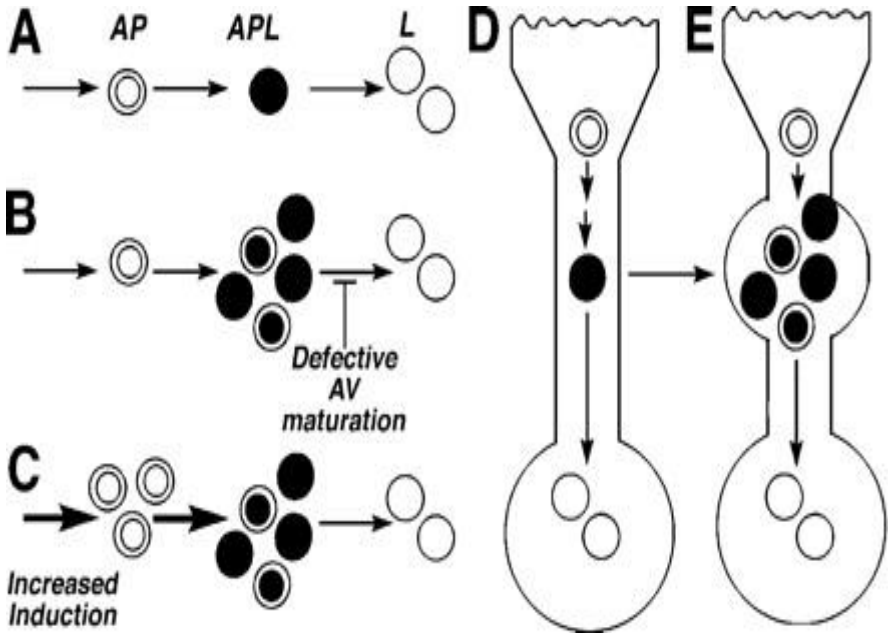


Figure1.5- **Models of AV accumulation leading to elevated Aß levels.** In the schemes we can see (A) the usual progression from macroautophagy; (B) impaired or delayed maturation of autophagosomes to lysosomes; (C) induction of acute maximum macroautophagy. These conditions (B and C) lead to AV accumulation and consequently Aβ generation. In D we see the normal progress of AVs to lysosomes within neurons;Disrupted retrograde transport is one of the mechanisms that lead to AV accumulation which is visible in AD (E) This accumulation leads an exacerbation of Aβ peptide which in turn exert cytotoxic effects in neurons.(adapted from Yu, Cuervo et al. 2005)

This impairment in ALP can induce neuronal cell death directly or by activating apoptosis. Also, it was observed a decrease in beclin mRNA levels, an important protein for induction of autophagy in MCI subjects and AD patients(Lee and Gao 2008, Pickford, Masliah et al. 2008).

One hypothesis to explain the AV accumulation that occurs in AD is based on the failure of the last step of ALP, the degradation of autophagic cargo by lysosomal

enzymes. It has been described several mutations in a range of lysosomal proteins which could induce minor degradative capacity (Nixon and Cataldo 2006). The defective lysosomal acidification and lysosomal proteolytic disruption are major contributors to autophagy failure and its pathological consequences in AD(Wolfe, Lee et al. 2013).

In opposition, other evidence suggest that the failure of the autophagic process is due to a MT cytoskeleton abnormalities, which can be responsible for the impairment of AV retrograde transport towards the cell body where lysosomes are located. In support of this hypothesis, it has been shown that MT depolymerizing agents disrupt vesicular transport inducing rapid accumulation of AV as was seen in AD(Silva, Esteves et al. 2011).

1.5. Importance of MT-network in AD

The first connection between autophagy and MTs was proposed for more than a decade ago. Indeed, pioneering studies demonstrated that use of depolarizing agents of MTs interfered with autophagic process (Amenta, Sargus et al. 1977, Aplin, Jasionowski et al. 1992, Seglen, Berg et al. 1996). In addition, studies related to the role of MTs in autophagic process demonstrated that the disruption of the MT affects several steps in this process(Fass, Shvets et al. 2006, Kochl, Hu et al. 2006). Relevant to AD pathology, is the impairment of intracellular trafficking mediated by MT disassembly leads to the accumulation of autophagosomes as mentioned above. In 1989, Matsuyama conceived the hypothesis that impairment of MT is an explanation for the pathogenesis of AD(Matsuyama and Jarvik 1989). Impairments of MTs system and its disruption through the use of pharmacologic agents have reproduced certain abnormalities observed in AD(Matsuyama and Jarvik 1989). A study revealed a reduction in the number and length of MTs in pyramidal neurons from AD(Cash, Aliev et al. 2003). More, this decrease is not related to the formation of NFTs(Cash, Aliev et al. 2003). However, others have shown that the phosphorylation of tau protein and consequently its aggregation causes a decrease in MTs assembly(Iqbal, Liu et al. 2009).

Another important aspect in the modulation of MT dynamics is acetylation. This post-translational modification occurs in C-terminal tail of α-tubulin which affects the

function ofMTs in most cells(L'Hernault and Rosenbaum 1985, LeDizet and Piperno 1987, Cueva, Hsin et al. 2012, Topalidou, Keller et al. 2012).

Fan Zhang et al, 2015, reported that in AD brain's an increase in the levelsof acetylated tubulin in comparison with controls (Zhang, Su et al. 2015). In addition, studies point to the importance of MTacetylation for axonal transport. The first enzyme associated with MTs wasHDAC6 that deacetylates α-tubulin at lys 40 was reported both in vitro and in cells(Hubbert, Guardiola et al. 2002, Matsuyama, Shimazu et al. 2002, Bertos, Gilquin et al. 2004). HDAC6 levels are increased and a reduction of the α-tubulin acetylation (K40) in NFTs were observed in tissue samples postmortem AD patients (Hempen and Brion 1996, Ding, Dolan et al. 2008). Studies also show an improvement in cognitive and deficits in animalmodels of AD with inhibition of HDAC6(Kilgore, Miller et al. 2010, Govindarajan, Rao et al. 2013).This enzyme has recently been linked to intracellular trafficking in AD(Deribe, Wild et al. 2009, Chen, Owens et al. 2010).In hippocampal neurons in vivo and in vitro, HDAC6 knockout wasable to recovermitochondrial traffic against Aβ-induced impairment.This view is supported by data indicating that Aβ peptides lead to cytoskeletal abnormalities, specifically the impairment of intracellular transport(Govindarajan, Rao et al. 2013). Moreover, it was demonstrated that excess tau leads to an inhibition of HDAC6 that prevents the completionof autophagy(Perez, Santa-Maria et al. 2009). Other studies have pointed to the MT-stabilizing agents prevented neuronal dysfunction. Taxol, a proven drug for breast cancer that stabilizes MT, has proved beneficial in several models of AD. It was seen that the use of taxol or analogues protects against Aβ25–35 toxicity(Li, Faibushevich et al. 2003, Silva, Esteves et al. 2011), prevented tau hyperphosphorylation(Michaelis, Dobrowsky et al. 2002)and restored the levels of synaptic proteins(Butler, Bendiske et al. 2007). Another drug with the same characteristics, NAP (davunetide), reduce tau hyperphosphorylation, improving MTreorganization and increased neuritenumber in a variety of models(Gozes and Divinski 2004, Matsuoka, Gray et al. 2007)

Chapter 2

Aims

Aim

The development of new and efficient disease-modifying therapies is one of the major challenges in the AD field. Although several hypotheses have been proposed to explain the molecular mechanisms underlying the pathogenesis of sAD, growing evidence has highlighted the role of mitochondrial dysfunction and the disruption of mechanisms which rely on mitochondrial bioenergetics in sAD. Acetylation of several proteins, such as tubuline and tau protein has also been associated with AD. Recently acetylation of tau protein has gained a significant importance for understanding the formation of NFTs. In this work, we aim to address the role of acetylation (tubuline and/or tau) on the phosphorylation of tau protein and in the regulation of the autophagy-lysosomal pathway.Therefore, the specific aims of this work were:

1- Uncover the role of SIRT2, HDAC6 and p300 in the acetylation of tau and tubulin.
2- To evaluate the effects of acetylation modulators on AD pathologic features.
3- To elucidate the involvement of HDAC6, p300 and SIRT2 in autophagy completion.

To achive these purpose we used four cell models: control and AD cybrids, parentaland mutant Tau P301L SH-SY5Y cells lines.

Chapter 3

Materials & Methods

3.1 Biological Materials

3.1.1 The cytoplasmic hybrid (cybrid) lines

Cybrid cells were maintained in 75cm^2 tissue culture flasks under a humidified atmosphere of 95% air, 5% CO2, at 37ºC. Cybrid cells were grown in DMEM, supplemented with 10% non-dialysed bovine serum, 1% penicillin–streptomycin solution and 44mM sodium bicarbonate.

3.1.1.1 Generation of transmitochondrial hybrid (cybrids) cell lines

Cybrid tecnique was first described by King Attardi (1989) and consists of the transfer of mtDNA exogenous to mtDNA-depleted recipient cells (Rho0 line cells), generating hybrid cell lines (cybrids)(King and Attardi 1989).
In figure 2.1 we see the various steps of the technique as described by (Swerdlow, Parks et al. 1997)
The SH-SY5Y cell line was depleted from mitochondrial DNA by long-term ethidium bromide exposure. These mtDNA-depleted cells were mixed with platelets isolated from different individual sAD and disease-free control subjects. Thus, the resulting control, AD cybrids expressed the nuclear genes of the recipiente Rho0 cell line and the mitochondrial genes of the platelet donor.

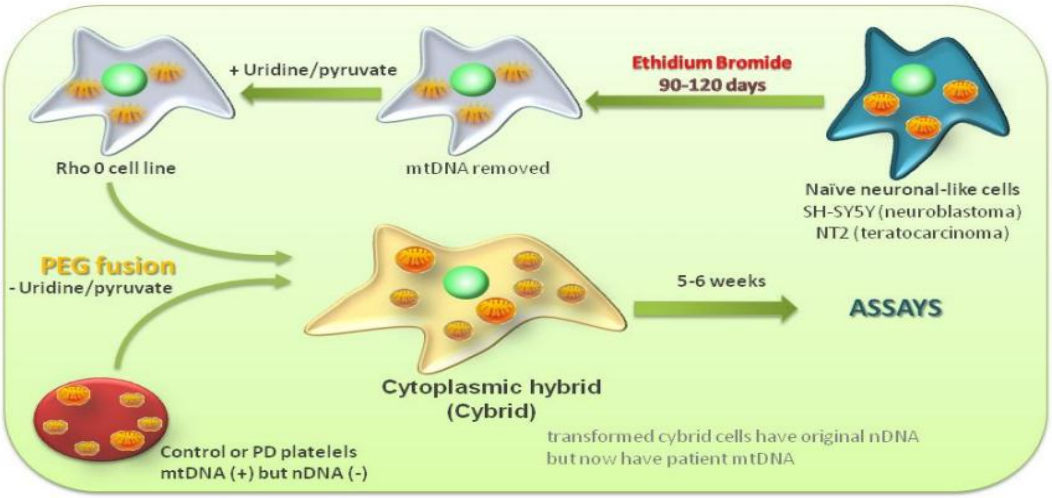


Figure 3.1- **Cybrid results from the repopulation of the Rho0 cell line with mtDNA exogenous.** Rho0 cell line results from the elimination of mtDNA of immortalized cell lines by the presence of ethidium bromide.Rho0 cells are then fused with patient platelets, which contain mitochondria but not nuclei.In this case, mtDNA exogenous is derived from platelets isolated from different individual sAD subjects and disease-free control subjects. So, differences in function between control and AD cybrids cell lines likely arise through differences in their mtDNA.(adapted from Arduíno 2012)

3.1.1.2 Cybrid cells diferentiation

Cybrid cell lines with an SH-SY5Y background present a commited neuronal precursor stage of differentiation. SH-SY5Yprecursor cells can be induced by *all-trans* retinoic acid (ATRA) to differentiate *in vitro* allowing the expression of neuronal markers, including neurofilament, neuron-specific enzymes and growth-associated protein(Encinas, Iglesias et al. 2000).

Cybrid cell lines were diferentiated to form process-bearing neuronal cells according to the procedure of Paquet-Durand *et al.(*Paquet-Durand, Tan et al. 2003*)*. Control and AD cybrids cell line where plated at a confluence of about 70%. After 24h medium was renewed to DMEM containing 1% of FBS, 200 µg/ml sodium pyruvate, 150 µg/ml uridine, 1% penicillin–streptomycin solution and freshly supplemented with 10 µM ATRA, renewed every 2 days. Cells were harvested and experiments were performed 12-14 days after the beginning of the differentiation.

3.1.2 Parental neuroblatoma cell line SH-SY5Y (SH)

SH-SY5Y human neuroblastoma cells were purchased from ATCC. Cells were maintained in 75cm^2 tissue culture flasks under a humidified atmosphere of 95% air, 5% CO2, at 37ºC. Cells were grown in DMEM, supplemented with 10% non-dialysed bovine serum, 1% penicillin–streptomycin solution and 44mM Sodium bicarbonate.

3.1.3 Parental neuroblatoma cell line SH-SY5Y TauP301L mutant cells (P301L)

SH-SY5Y cells were stably transfected as described by(Hoerndli, Pelech et al. 2007). Briefly, SHSY5Y cells were transfected with constructs encoding P301L mutant (P) human tau (using the longest human tau isoform, htau40), under the control of the CMV promoter. SH-SY5Y untransfected cells were used as control. Cells were maintained in 75cm^2 tissue culture flasks under a humidified atmosphere of 95% air, 5% CO2, at 37ºC. Cells were grown in DMEM, supplemented with 10% non-dialysed bovine serum, , 1% penicillin–streptomycin solution and 44mM Sodium bicarbonate.To select the cells with P301L construct was used blasticidin antibiotic at a concentration of 3ug/ml.

3.2 Cell treatment

Differentiated cybrids, SH and P301L cells were exposed to 10 μM AK1 (specific inhibitor of SIRT2) , 5 μM Tubastatin A (tubA) (specific inhibitor of HDAC6) and 2.5 μM C646 (specific inhibitor of p300) during 24h.

For the analysis of LC3 and p62, were added 20 mM ammonium chloride and 20 μM leupeptin in the culture medium in the last 4 h of treatment.

3.3 Chemicals

The most common chemicals used were as categorized in the Table II.1.

Table 3.1-**Chemicals with the corresponding company.**

CHEMICAL	COMPANY
Antibodies	
Goat alkaline phosphatase-conjugated antimouse	GE Healthcare (Buckinghamshire, UK)
Goat alkaline phosphatase-conjugated antirabbit	GE Healthcare (Buckinghamshire, UK)
Mouse mAb Anti-acetylated tubulin (Acetyl K40)	Sigma-Aldrich Co. (St. Louis, MO, USA)
Mouse mAb Anti-actin	Sigma-Aldrich Co. (St. Louis, MO, USA)
Mouse mAb anti-Cytochrome C	BD Biosciences (San Diego, CA, USA)
Rabbit pAb anti-LC3B	Cell Signaling (Danvers, MA, USA)
Rabbit pAb anti-SQSTM1/p62	Sigma-Aldrich Co. (St. Louis, MO, USA)
Rabbit pAb anti-TOM20	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Rabbit pAb anti-tau (Acetyl K280)	AnaSpec (Fremont, CA, USA)
Rabbit pAb anti-tau (phospho S396)	Santa Cruz Biotechnology (Santa Cruz, CA, USA
Enzyme inhibitors	
Tubastatin A (tubA)	BioVision Inc. (Milpitas, CA, USA)
C646	Calbiochem(Billerica, MA, USA)
Ak1	ChemBridge Co. (San Diego, CA,USA)
Leupeptin	Sigma-Aldrich Co. (St. Louis, MO, USA)
Western blotting purpose	
Enhanced chemifluorescence (ECF) reagente	GE Healthcare (Buckinghamshire, UK)
HybondTM-P PVDF membrane	GE Healthcare (Buckinghamshire, UK)
ImmobilonTM-P PVDF membrane	Millipore (Billerica, MA, USA)

3.4 Cell Viability assay

3.4.1 MTT reduction test

Cell viability was determined by the colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. In viable cells, dehydrogenases metabolize MTT into a formazan that absorbs light at 570 nm. Cybrid cells were seeded in 24-well plates.After treatments, cells were washed with PBS and incubated and replaced 500µl MTT (0.5mg/mL). After 2h incubation at 37°C, the formazan precipitates were solubilized with 500µl of acidic isopropanol (0.04M HCl/isopropanol). The absorbance was measured at 570 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices). Cell reduction ability was expressed as a percentage of control.

3.5 Apoptotic cell death evaluation

3.5.1 Caspases activation

Caspase activation was measured using a colorimetric method described by(Cregan, MacLaurin et al. 1999), in which the substract clivage is monitored at 405 nm. Cybrid cells were seeded in petri dishes (10cm). After 24h treatment, cells were washed and placed in hypotonic lysis buffer (25 mM HEPES,1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$) and supplemented with (0.1 mM phenylmethanesulfonylfluoride (PMSF), 2 mM dithiothreitol (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 14000 rmp 4ºC. The resulting supernatant was subjected to protein quantification. Protein content was determined by the bicinchoninic (BCA) protein assay using the BCA kit (Pierce Thermo Fisher Scientific, Rockford, IL). Lysates (50 µg of protein) were incubated at 37ºC for 2 h in 25mM HEPES, pH 7.5 containning 0.1 % CHAPS, 10% sucrose, 2mM DTT and 40µM Ac-DEVD-pNA (Calbiochem) to determine caspase 3 activation.

3.6 Immunoblotting

Cybrid cells were plated in petri dishes (10cm) for determination of phospho-tau, acetylated-tau, acetylated-tubulin, LC3 and p62. In order to determine the citochrome C, cells were plated in 75 cm^2 flask. SHSY-5Y and P301L cells were plated in 6-well plates (10cm) at a density of 2.5×10^5 cells/mL for determination of phospho-tau levels, acetylated-tau, acetylated-tubulin, LC3 and p62.

After washing, cells were scraped and resuspended in ice-cold hypotonic lysis buffer supplemented with 0.1 M PMSF, 0.2 M DTT and 1:1000 diluition of a protease inhibitors cocktails. To detect phospho-Tau, cell lysates were prepared using the lysis buffer supplemented with more 2mM ortovanadate and 50mM sodium fluoride. For analysis of acetylated-tau and acetylated-tubulin, cell lysates were prepared using the lysis buffer supplemented. For analysis of LC3II and p62, cell lysates were prepared using the lysis buffer supplemented with more 1% Triton X-100. The cellular extracts were frozen and defrozen three times to favor cells disruption, centrifuged at 13600 rpm for 10 min, at 4 °C, and the resulting supernatant collected. To prepare the mitochondrial fractions for detect cytochrome C (cyt C), cells were scraped and homogenized in lysis buffer with sucrose (250mM Sacarose, 20mM Hepes, 1mM EGTA, 1mM EDTA) supplemented with 0.1 M phenylmethanesulfonylfluoride (PMSF), 0.2 M dithiothreitol (DTT) and 1:1000 diluition of a protease inhibitors cocktails. Homogenization was done in Potter-Elvejhem homogenizer with teflon pestle. The homogenates were centrifuged at 2200 rpm for 20 min, 4ºC. The supernatants were then removed and were centrifuged again at 10600 rpm for 20min ,4ºC. The pellet (mitochondrial fraction) was resuspended in 20µL of lysis buffer with sucrose supplemented.

Protein content was determined by the bicinchoninic (BCA) protein assay using the BCA kit (Pierce Thermo Fisher Scientific, Rockford, IL) For the SDS-PAGE the samples were denaturated with six times concentrated denaturating buffer at 100 °C, for 5 min.

Equivalent amounts of protein were resolved by electrophoresis in 10-15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked for 1h at room temperature in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween with gently agitation. The membranes were subsequently incubated overnight at 4 °C with gently

agitation with the specific primary antibodies. The rabbit polyclonal anti-tau (phospho S396) (1:750), the rabbit polyclonal anti-tau (Acetyl K280) (1:750), the rabbit polyclonal anti-SQSTM1/p62 (1:1000), the rabbit polyclonal anti-TOM20 (1:1000), the mouse monoclonal anti-acetylated tubulin (1:1000), and the rabbit polyclonal anti-LC3B (1:1000), the mouse monoclonal anti-actin (1:5000) the mouse monoclonal anti-cytochrome C (1:350)Then, membranes were washed three times (5 min), with TBS containing 0.05% Tween (TBS-T) and incubated with anti-rabbit and anti-mouse IgG secondary antibodies (1:20000)for 2h at room temperature with gentle shaking. After three washes with TBS-T (5 min), specific bands of immunoreactive proteins were visualized after membrane incubation with enhanced chemifluorescence reagent (ECF) for 5 min in a VersaDoc Imaging System (Bio-Rad), and the density of protein bands was calculated using the Quantity One Program (Bio-Rad).

3.7 Statistical analysis

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the one-way ANOVA followed by Bonferroni Multiple-Comparisons Procedure as post-hoc test or paired t-test with the program prism 6 (GraphPad Software, San Diego, CA). A P value <0.05 was considered statistically significant.

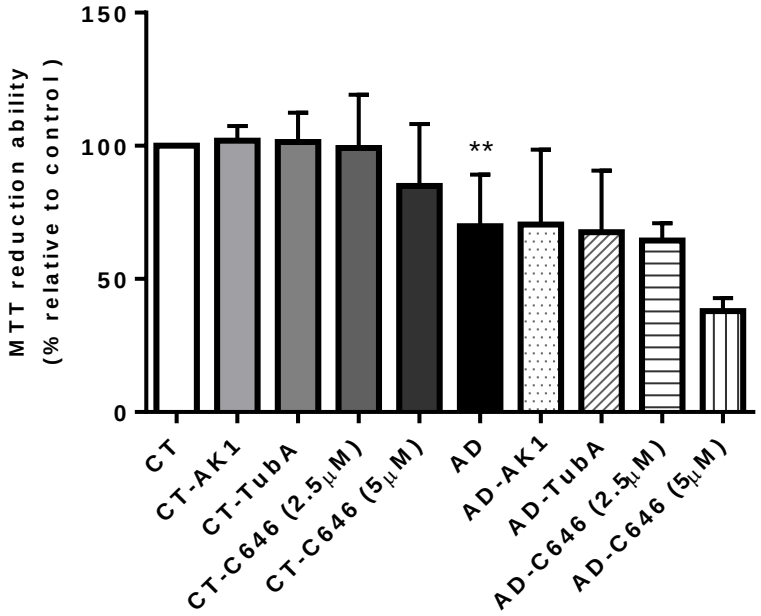
Chapter 4

Results

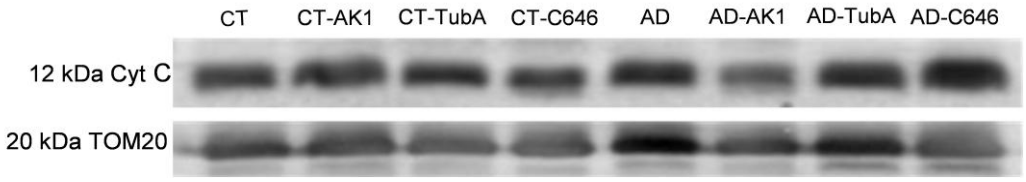
4.1 Effects of AK1, TubA and C646 on toxicity and apoptotic cell death in cybrid cells lines.

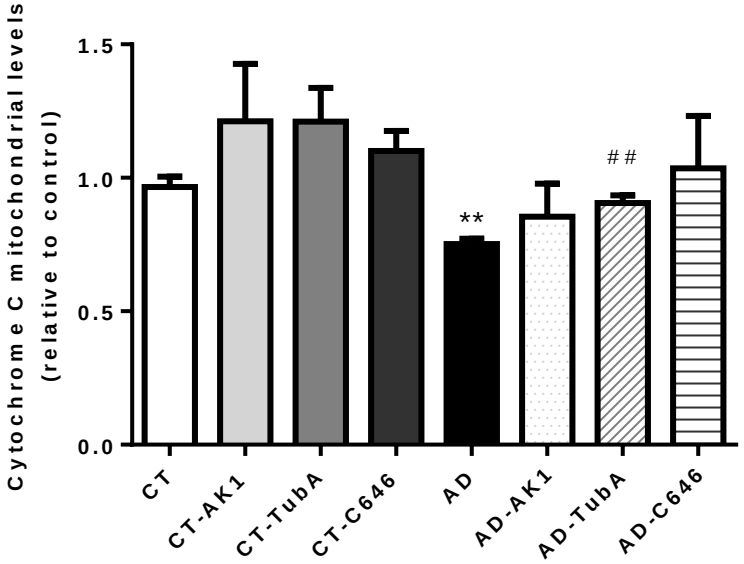
MTT was used in order to estabelish a concentration of AK1, TubA and C646 that did notinduced cell toxicity (fig 4.1 A). Concentrations10 μM AK1, 5 μM TubA and 2.5 μM C646 did not significantly affect cell viability in both lines. The concentration 5 μM C646 presents a significant reduction in cell viability. We next decided to examine the effects of these compounds in activation of the intrinsic apoptotic pathway in cybrid cellular model. Aditionally, we also evaluated cytochrome C levels in mitochondrial fraction by Western blot (fig 4.1 B).The translocation of cytochrome C from mitochondria (whereby it relies under physiological conditions) to citosol can be used as an additional apoptosis marker (Scorrano and Korsmeyer 2003). These results relate only to the mitochondrial fraction since in the cytosolic fraction was not possible to detect the cytochrome C.We analyzed the caspase-3 activation, an effector caspase in the apoptotic process, to better characterize the cell death mechanisms mediated by these compounds (fig 4.1 C). Caspase 3 activity and mitochondrila cytochrome C content revealed that AD cybridshad a significant increase in the apoptotic pathway when compared with CTcybrids. Furthermore, AK1 decreased apoptosis activation in AD cybrids.

A


MTT reduction ability
(% relative to control)
150
100
50
0
**
CT
CT-AK1
CT-TubA
CT-C646 (2.5μM)
CT-C646 (5μM)
AD
AD-AK1
AD-TubA
AD-C646 (2.5μM)
AD-C646 (5μM)

B


CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646
12 kDa Cyt C
20 kDa TOM20


Cytochrome C mitochondrial levels
(relative to control)
1.5
1.0
0.5
0.0
**
##
CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646

C

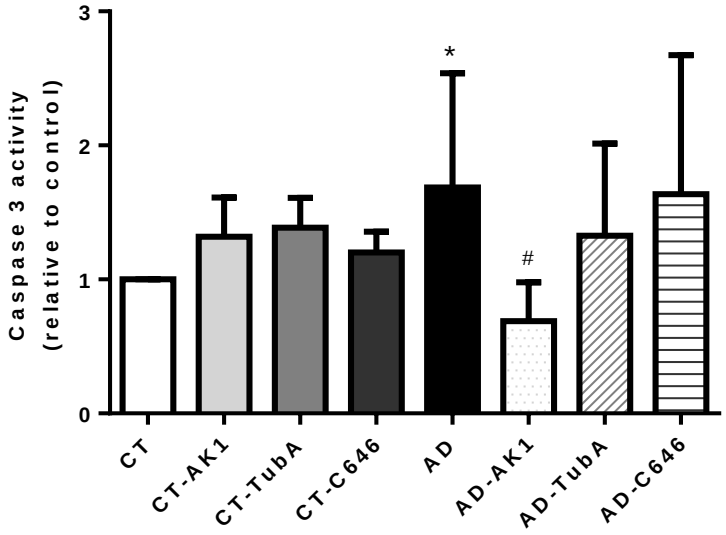


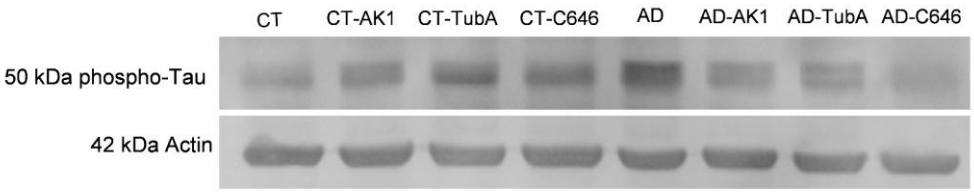
Figure 4.1-**Effects of Ak1, TubA and C646 on MTT reduction, caspase 3-like activation and cytochrome C levels (mitochondrial fraction) in cybrid cells**.**(A)** Control, AD cybrids were incubated with 10 μM AK1; 5 μM Tub A; 2.5 μM and 5 μM C646.Cell viability was determined by following changes in cell reduction capacity by the MTT as described in Material and Methods section. **(B)** Control, AD cybrids were incubated with 10 μM AK1; 5 μM TubA; 2.5 μM C646. Cytochrome C levels were determined by Western blot as described in Materials and Methods section and it was corrected with TOM 20 levels. Representative immunoblot for cytochrome c levels.**(C)** Control, AD cybrids were incubated with 10 μM AK1; 5 μM Tub A; 2.5 μM C646. Caspase activation was evaluated spectrophotometrically at 405 nm as described in Chapter II.

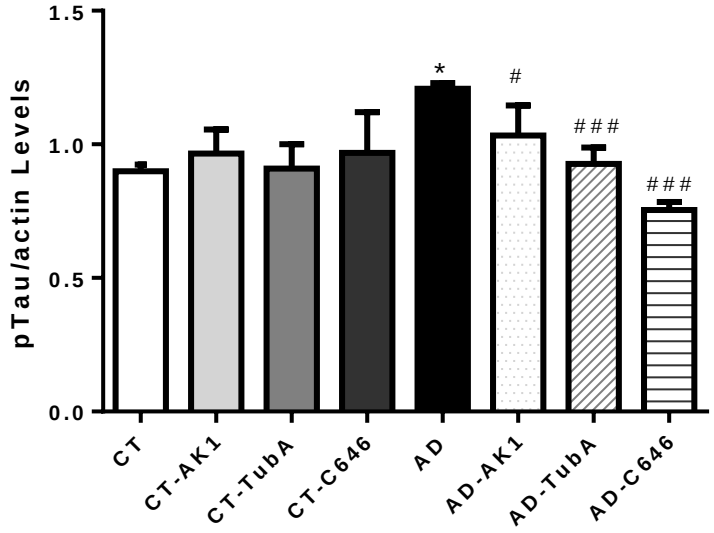
Data are expressed with the mean ±SEM values derived from 4 to 6 independent experiments. * $p < 0.05$, ** $p < 0.01$ significantly different as compared CT . # $p < 0.05$, ## $p < 0.01$ significantly different as compared AD.

CT-control of control cybrids cell lines; AD- control of AD cybrids cell lines.

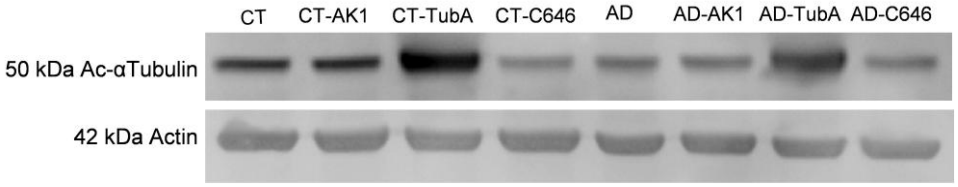
4.2Modulation of the levels of acetilation by specific inhibition of SIRT2, HDAC6 and p300 in cybrid cells

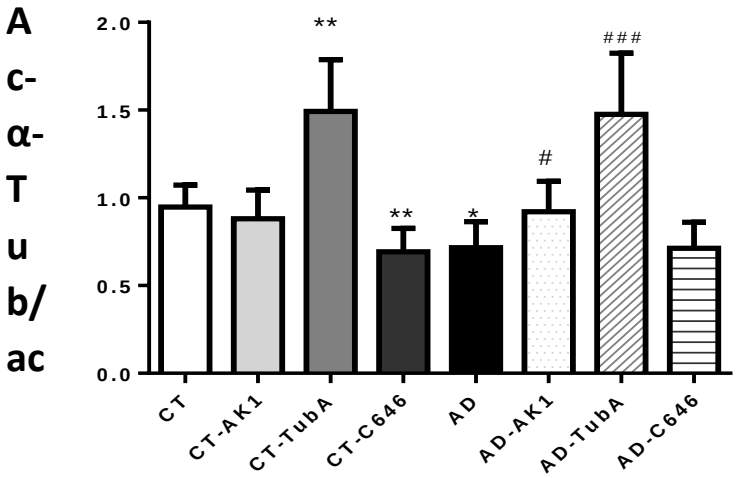
Studies indicate the importance of tau post-translational modifications in the phosphorylationof tau protein (Martin, Latypova et al. 2011). Recent studies indicated that acetylation plays an important role in the phosphorylation state of tau protein (Cook, Stankowski et al. 2014). The importance of acetylation has also been widely studied in the MTnetwork since it improves its stability, although this relationship is not clearcut (Hubbert, Guardiola et al. 2002).Firstly, we evaluate the levels of phosphorylated tau protein at ser396 residue in cybrid cells incubated with inhibitors under study. We observed that AD cybrids has a significant increase of phospho-tau when compared with CT (fig. 4.2 A). The AK1, TubA and C646 promoted a significant decrease in the levels of phosphorylated tau protein at ser396 residue in AD cybrids (fig. 4.2 A). Importantly,we saw a significant reductionin tau phosphorylation with the inhibition of p300.Then we decided to directly explore the role ofp300 acetytransferase, SIRT2 and HDAC 6 deacetylases on acetilation ofα-tubulin in cybrid cells. Thus, we determined the acetylation state of α-tubulin in cybrid models and analyzed the effects ofspecific inhibitors(fig.4.2 B). Under basal conditions, AD cybrids showed decreased acetylation levels at lys40 of α-tubulin relative to CT cybrids. Treatment with TubA significantly restored the acetylation of α-tubulin. The p300 inhibition decreases significantly acetylation of α-tubulin in CT cybrids.Furthermore, AK1 increased α-tubulinacetylationin AD cybrids.To evaluate the acetylation state of tau in these models, we analyzed the effect of the already mentioned inhibitors in the levels of acetylated tau protein at lys280 residue (fig. 4.2 C). We observed that the inhibition of p300significantly decrease the levels of acetylated tau in AD cybrids.


CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646
50 kDa phospho-Tau
42 kDa Actin


pTau/actin Levels
1.5
1.0
0.5
0.0
*
#
###
###
CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646

B


CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646
50 kDa Ac-αTubulin
42 kDa Actin


Ac-α-Tub/ac
2.0
1.5
1.0
0.5
0.0
**
**
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###
CT
CT-AK1
CT-TubA
CT-C646
AD
AD-AK1
AD-TubA
AD-C646

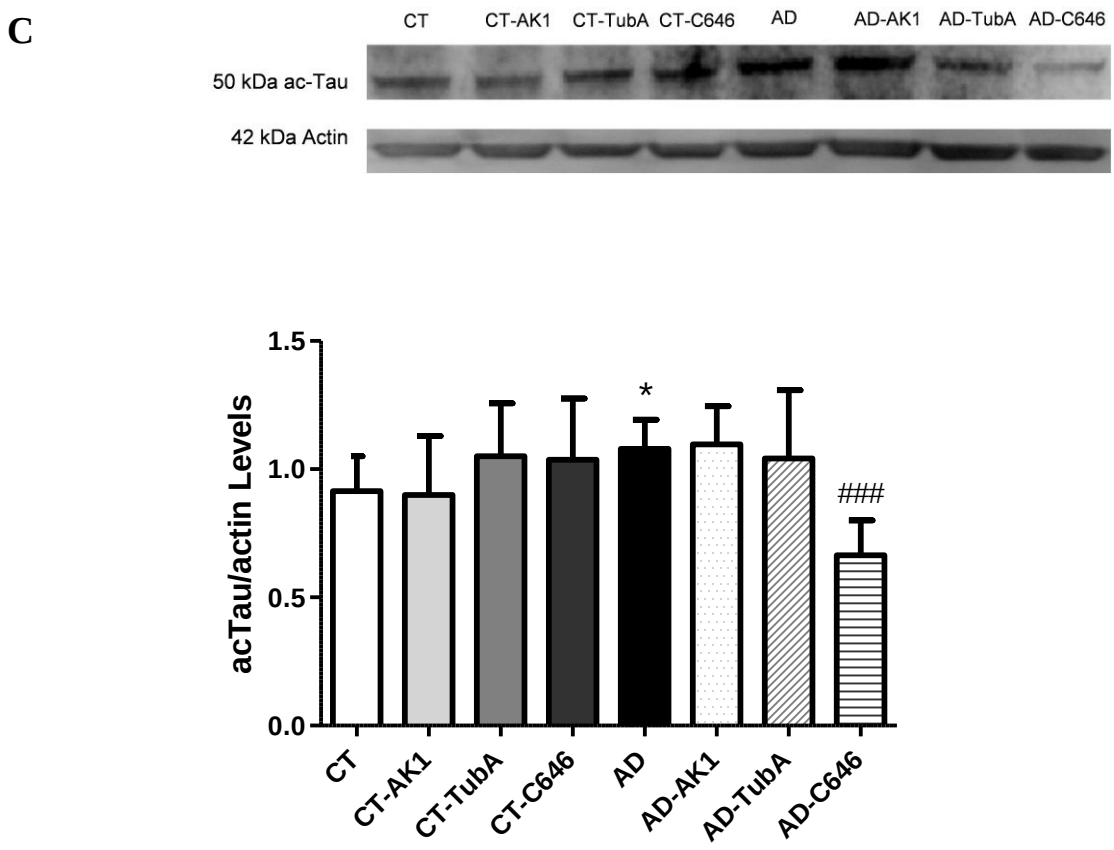
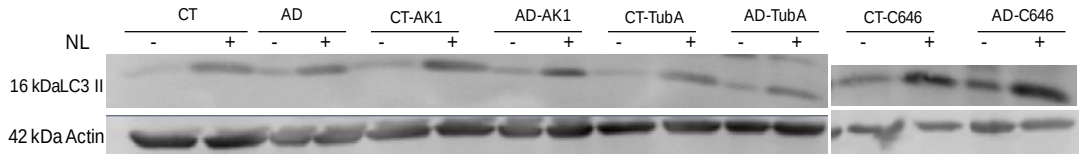


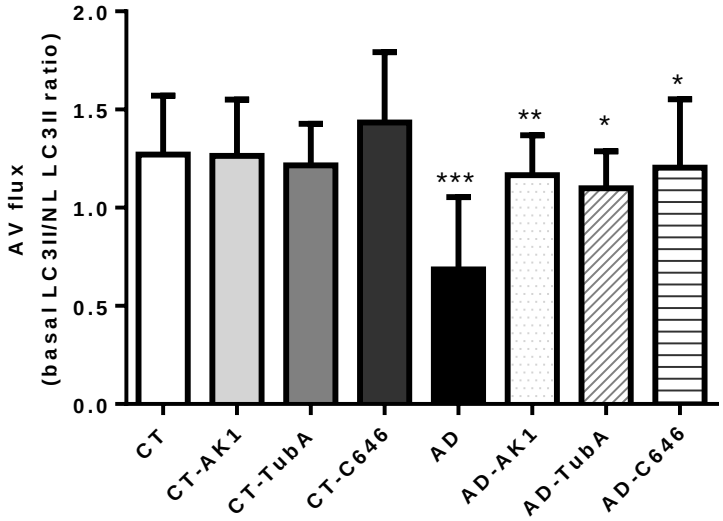
Figure 4.2- **Effects of Ak1, TubA and C646 on phospho-tau , acetylated α-tubulinand acetylated tau in cybrid cells**.Cells from CT and AD cybrids were treated 10 μM AK1, 5 μM TubA,2.5 μM C646. **(A)**Western blot analysis of phospho-tau levels. **(B)** Western blot analysis of acetylated α-tubulin levels. **(C)** Western blot analysis of acetylated tau levels. Representative immunoblot for phospho-tau , acetylated α-tubulin and acetylated tau levels. Data represent the mean ±SEM values derived from 5 to 7 independent experiments. * $p < 0.05$, ** $p < 0.01$ significantly different as compared CT cybrid. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ significantly different as compared AD cybrid.

4.3 Alterations in autophagic activity in cybrid cells

Autophagic failure found in our AD cybrids model can be due to an alteration in autophagosomes traffic along the MTssystem. Studies related the role of MT in the formation of autophagosomes (Fass, Shvets et al. 2006) and motility of these (Kochl, Hu et al. 2006). Specifically, acetylation of tubulin results in higher affinity of motorproteins to MTs(Reed, Cai et al. 2006)and improvestheir stability.In order to understand the role of acetylation (either tubulin or tau) on autophagy, we evaluated the levels of LC3II and p62. When compared with CT cybrids, AD cybrids has reduced autophagic flux, as mesured by the ratio between basaland NH4CL/leupeptine treated LC3 II and p62 levels (fig.4.3 A and B). The treatment with the inhibitors used in this study led to an improvement of the autophagic flux in AD cybrid (fig. 4.3 A).

A





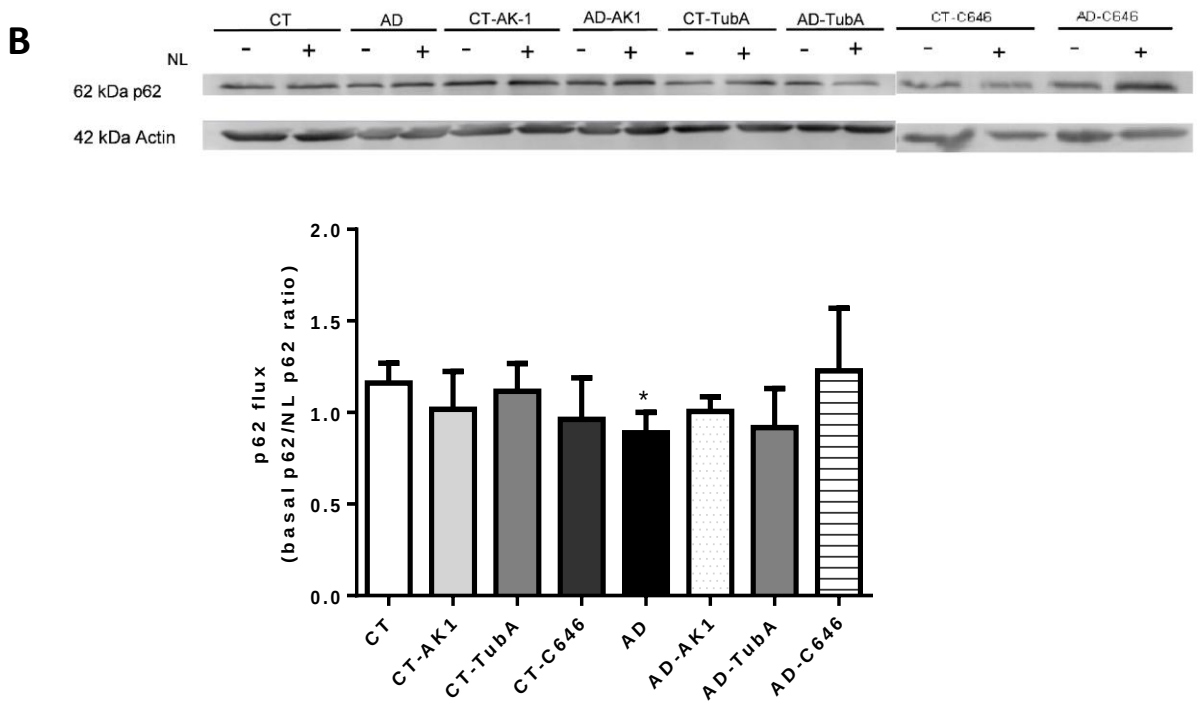
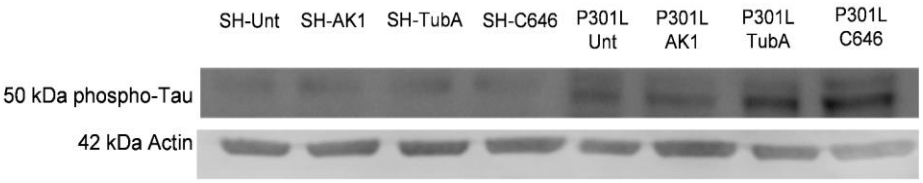


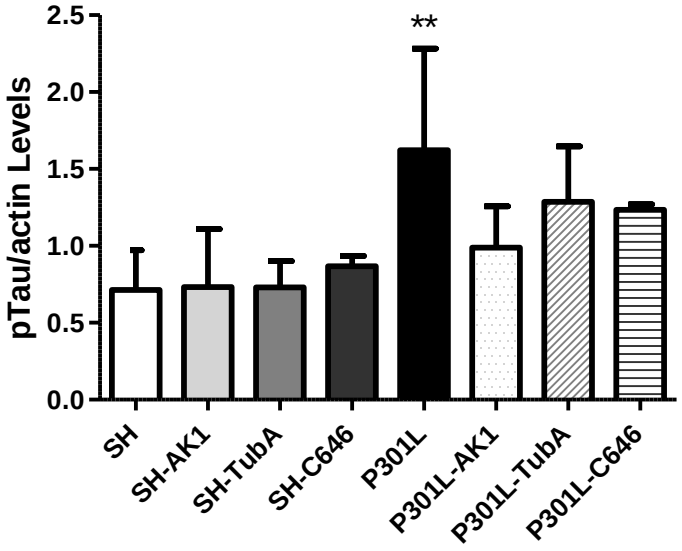
Figure 4.3- **Effect of AK1, TubA and C646 on cybrid cells autophagic markers.** Cells from CT and AD cybrids were treated 20 μM AK1, 5 μM TubA,2.5 μM C646 and were examined by immunoblotting using the anti-LC3-II (**A**) and anti-p62 (**B**) antibodies. Representative immunoblot for LC3 II and p62 levels. Data represent the mean ±SEM values derived from five independent experiments. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ significantly different as compared CT cybrid.

4.4Evaluation of the role of acetylation oftau and tubulinin SH and P301L cells

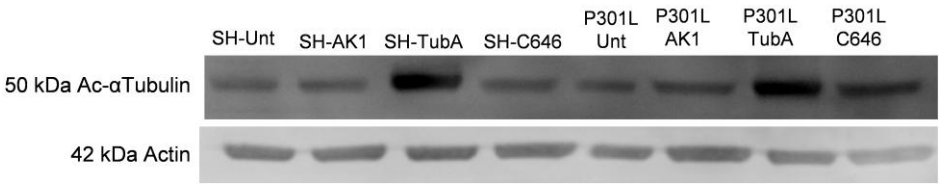
To confirm the importance of tau phosphorylation and consequently the formation of NFTs in AD we used a cellular model featuring the progressive tauopathy.The SH-SY5Y tauP301L mutant cells are characterized by the accumulation of hyperphosphorilated tau and developement of NFTs that serves as a model for FTD and AD. We evaluate the effects of AK1, TubA and C646 in this cell line and in control SH-SY5Y. From the analysis of the figure 4.4 A we found that the inhibition of p300, SIRT2 and HDAC6leads to a non-significant decrease of phosphorylated tau levels in P301L. We also found a significant increase of tubulin acetylation levels, afterAK1 and TubA treatments (figure 4.4 B. In figure 4.4 C, we see that under basal conditions, P301L showed increase acetylation levels at K280 of tau relative to SH. In addition, we observed that the inhibition of p300 significantly decrease the levels of acetylated tau in SH.

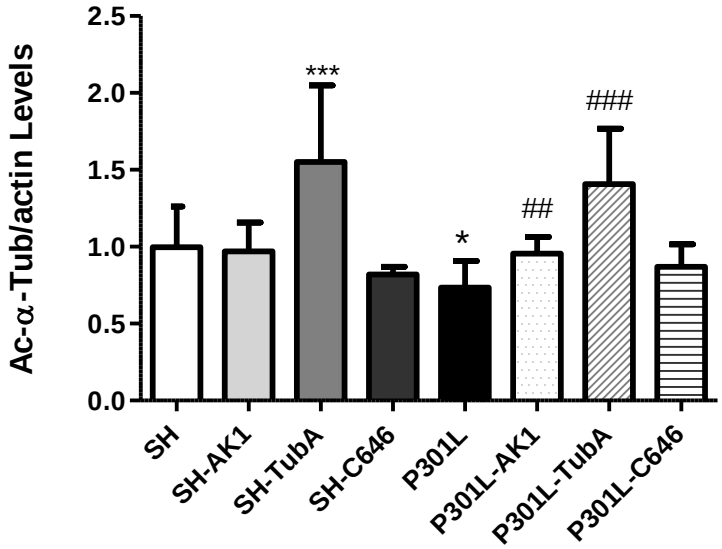
A


SH-Unt
SH-AK1
SH-TubA
SH-C646
P301L Unt
P301L AK1
P301L TubA
P301L C646
50 kDa phospho-Tau
42 kDa Actin


pTau/actin Levels
2.5
2.0
1.5
1.0
0.5
0.0
**
SH
SH-AK1
SH-TubA
SH-C646
P301L
P301L-AK1
P301L-TubA
P301L-C646

B


SH-Unt
SH-AK1
SH-TubA
SH-C646
P301L Unt
P301L AK1
P301L TubA
P301L C646
50 kDa Ac-αTubulin
42 kDa Actin


Ac-α-Tub/actin Levels
2.5
2.0
1.5
1.0
0.5
0.0
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SH
SH-AK1
SH-TubA
SH-C646
P301L
P301L-AK1
P301L-TubA
P301L-C646

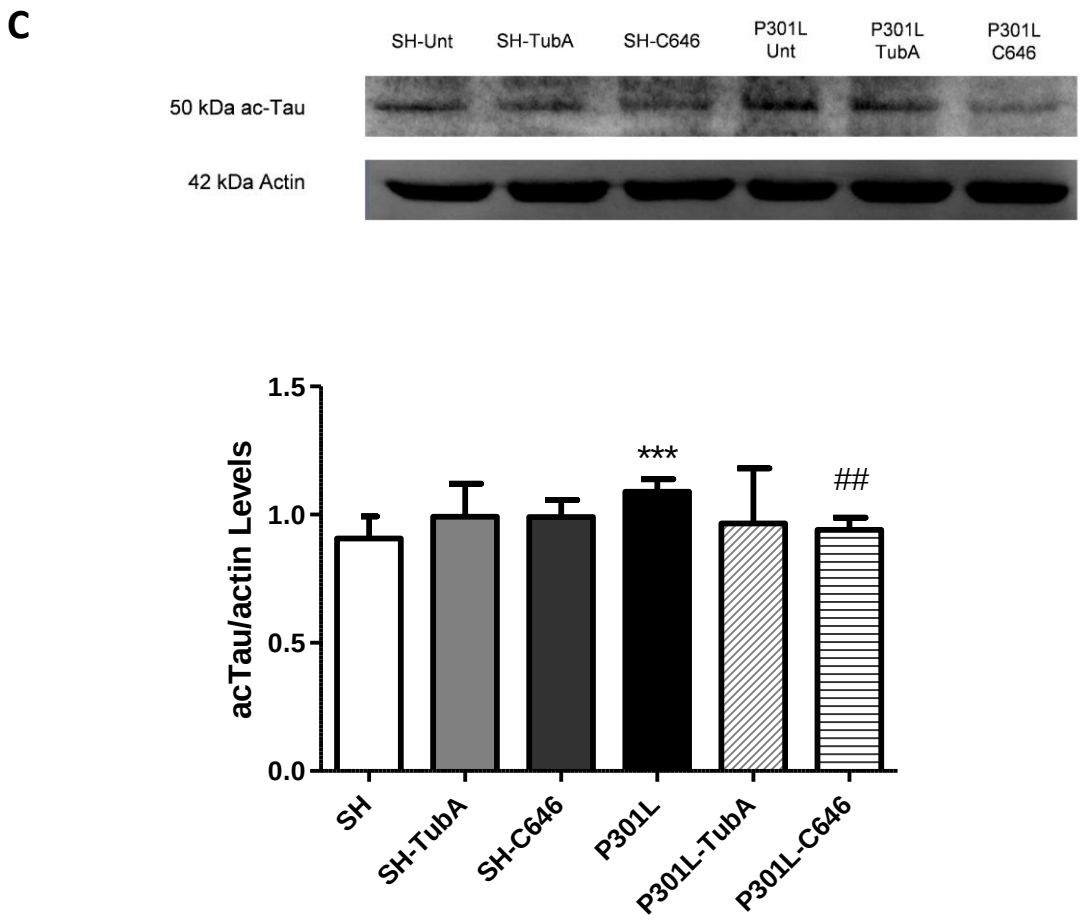
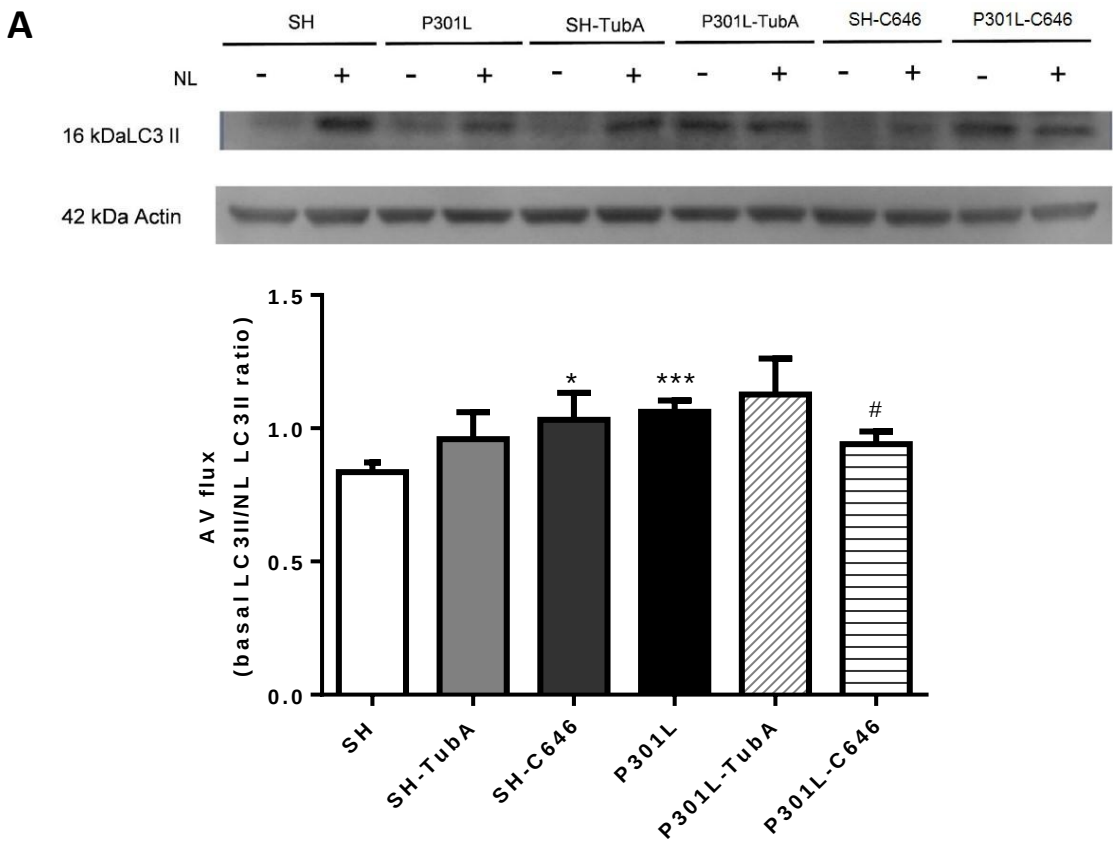


Figure 4.4- **Effects of modelation of acetylation on phospho-tau , acetylated α-tubulinand acetylated tau in SH and P301L cells. (A)** SH and P301L cells were incubated with 20 μM AK1; 5 μM Tub A; 2.5 μM C646 and were examined by immunoblotting using the anti-phospho tau antibody. **(B)** SH and P301L cells were incubated with 20μM AK1, 5 μM Tub A, 2.5 μM C646 and were examined by immunoblotting using the anti-phospho tau antibody. **(C)**Cells from SH and P301L cells were treated 5 μM Tub A,2.5 μM C646 and were examined by immunoblotting using the anti-acetyl-Tau (K280) antibody. Representative immunoblot for phospho-tau , acetylated α-tubulin and acetylated tau levels. Data represent the mean ±SEM values derived from 4 to 7 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different as compared SH. ## $p < 0.01$, ### $p < 0.001$ significantly different as compared P301L.

SH-control of SH-SY5Y cell lines; P301L- control of SHSY5Y TauP301L mutant cells.

4.5 Role of HDAC6 and p300 on macroautophagy in SH and P301L cells.

Additionally, we evaluate the role of HDAC 6 and p300 enzymes in autophagic turnover in SH and P301L cells. In order to understand the effects of these enzymes we studied the levels of p62 and LC3II (fig. 4.5 A and B). Under basal conditions, P301L showed increased of autophagic flux relative to SH (fig. 4.5 A).When compared with SH, P301L has increased autophagic flux (fig.4.5 A). The treatment with C646 leds to an improvement of the autophagic flux in SH (fig. 4.5 A).With the same inhibitor,the results showed a significantreduced in AV flux (fig. 4.5 A) anda increase in p62 flux (fig. 4.5 B)in P301L line.



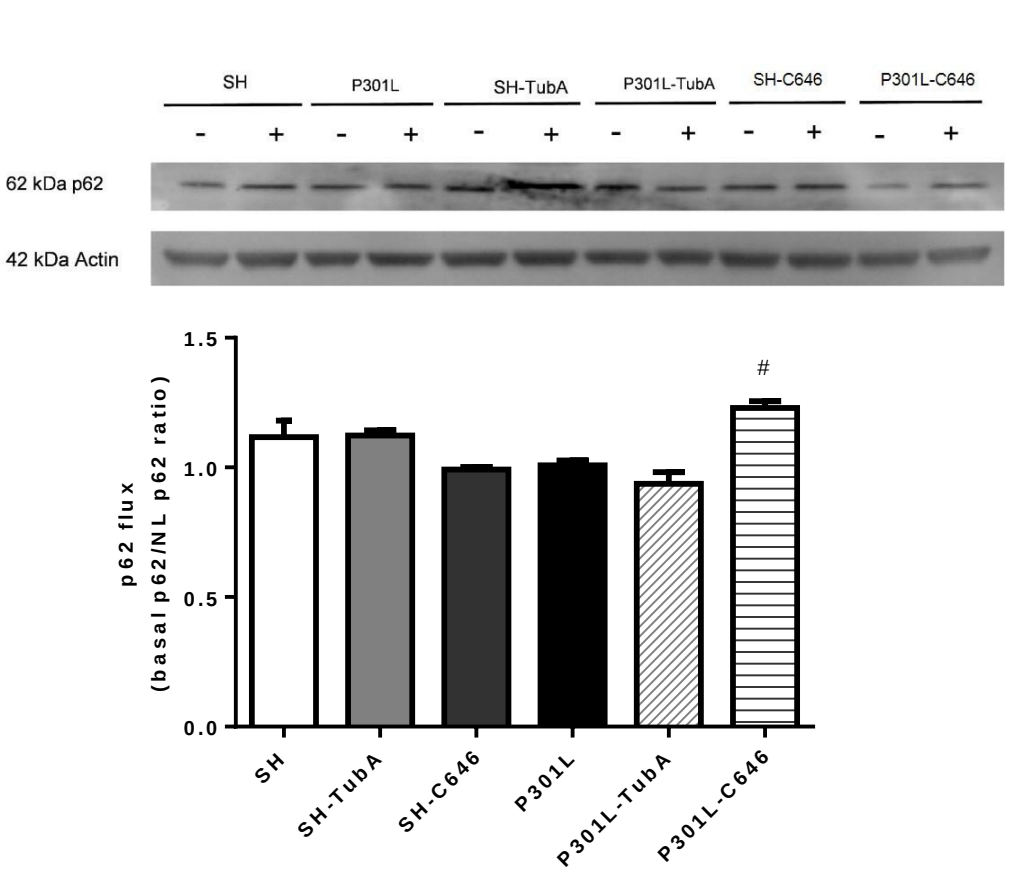


Figure 4.5- **Effect of TubA and C646 on SH and P301L cells autophagic markers.** Cells from SH and P301L were treated 5 μM Tubastatin A,2.5 μM C646 and were examined by immunoblotting using the anti-LC3-II (**A**) and anti p62 (**B**). Representative immunoblot for LC3 II and p62 levels. Data represent the mean ±SEM values derived from 2 to 4 independent experiments. * $p < 0.05$, *** $p < 0.001$significantly different as compared SH. # $p < 0.05$significantly different as compared P301L

Chapter 5

Discussion

Mitochondrial dysfunction has been widely implicated in AD etiology, and many authors claim it is a primordial event in the pathogenesis of the disease(Moreira, Cardoso et al. 2006, Swerdlow and Khan 2009, Silva, Esteves et al. 2011).The present study shows significant evidence of the involvement of mitochondrial dysfunction in AD that sets off changes in cellular metabolism that culminate with tau post-translational modifications.In this study, we provide evidence forthe role of α-tubulin acetylation at lys40 andtau acetylation at lys280 in autophagic flux impairment in AD.

Our work usingcybrid cell lines, a model used in the study of AD, gives particular relevanceto mitochondrial function believed to have a key role in the origin of AD pathology. Cybrid results from the repopulation of the Rho0 cellline with exogenous mtDNA. In this case, mtDNA is derived from platelets isolated from different individual sAD patientsand disease-free control subjects. So, differences in function between control and AD cybrids cell lines likely arise through differences in their mtDNA (Wilkins, Carl et al. 2014). Numerous studies have clearly associated neuronal loss in AD with activation of apoptosis (Smale, Nichols et al. 1995, Eckert, Keil et al. 2003).Apoptosis is executed by a family of proteasesthat have a cysteine at their active site and cleave their target proteins at specific aspartic acids, called caspases. In mammalian cells, a major caspase activation pathway resultsfroma variety of apoptotic stimuli that causes cytochrome c release from mitochondria, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death(Jiang and Wang 2004). Previous studies using cybrids have shown an increase in apoptotic markers (Cardoso, Proenca et al. 2004, Zhang, Liu et al. 2011). Our results corroborate previous observations demonstrating a reduction in cell viability in AD cybrids**(fig. 4.1 A)**. Moreover, the analysis of two apoptotic markers, namelymitochondrial cytochromec levels **(fig. 4.1 B)** and caspase-3 activation**(fig. 4.1 C)**, confirm that AD cybrids have increased commitment to apoptosis in basal conditions.In this study, we analyzed the role of three enzymes (SIRT2, HDAC6 and p300) that play an important role in the modulation of acetylation of several proteins (Hubbert, Guardiola et al. 2002, North, Marshall et al. 2003, de Oliveira, Sarkander et al. 2012). Our results revealed a decrease in the activation of apoptosis after treatment with AK1, that inhibit SIRT2 **(fig. 4.1 B and C)**.It was seen that SIRT2 inhibition significantly decreased oxidative stress-induced cell death in differentiated PC12 cells(Nie, Hong et al. 2014). The results obtained show that specific SIRT2 inhibition

with AK1 leds to slightly increase in mitochondrial cytochromeclevels but it must be pointed out the significant decrease in the activity of caspase 3 **(fig. 4.1 C)** indicating the protective role of SIRT2 inhibition. In accordance, SIRT2 specific inhibitor, AK1, was proven to prevent neuronal loss in rTg4510 mice when injected directly in the hippocampus while having no toxic effects (Spires-Jones, Fox et al. 2012).p300 and HDAC enzymes have also been implicated in the apoptotic process (Rouaux, Jokic et al. 2003, Chuang, Leng et al. 2009). Our results show a significant roleof p300 and HDAC6 in apoptosis since its inhibition leads to anincrease in the levels of mitochondrial cytochrome c in AD cybrid**(fig. 4.1 B)**.

Abnormal phosphorylation of tau protein has been proposed to play an important role in neurodegeneration in AD.In AD brains it was found that tau phosphorylation at Ser396 is significantly increased when compared with other phosphorylation sites and constitutes an early site of tau phosphorylation, occurring prior to the appearance of NFTs(Mondragon-Rodriguez, Perry et al. 2014).Our data in AD cybrid showed a significant increase in phosphorylated tau levels at Ser396 providing evidence that this cell line has similar characteristics to AD brain pathology, emphasizing that AD is not brain limited and can be observed in peripheral tissues.**(fig. 4.2 A)**. Recently, studies have shown that tau is post-translationallymodified by lysine acetylation, which impairs normal tau function and is associated with the NFTs formation process(Min, Cho et al. 2010, Irwin, Cohen et al. 2012, Cook, Stankowski et al. 2014). HDAC6 and p300 enzymes are recognized as modulators of tau acetylation (Min, Cho et al. 2010, Cook, Carlomagno et al. 2014). Tau acetylation by p300 was seen in the early stages of AD and hyperacetylation impairs tau degradation, promoting the accumulation of abnormally phosphorylated tau(Min, Cho et al. 2010, Irwin, Cohen et al. 2012). Furthermore, inhibition p300 reduces acetylated tau and leads to degradation of pathogenic phosphorylated tau (Min, Cho et al. 2010).In AD cybrids, the results obtained show that inhibition of p300decreasedacetylated tau levels **(fig. 4.2C)**andcontributed to the reduction of phosphorylatedtau levels **(fig. 4.2 A)**.In addition, we found that the inhibition of HDAC6 promotes a reduction in acetylated tau **(fig. 4.2 C)**and inhibition of tau phosphorylation(**fig. 4.2 A)**. Ding and co-workers observed interaction between tau protein and HDAC6 in HEK cells stably expressing tauand found that itsinhibition or knockdown attenuate the phosphorylation of various tau residues (Ding, Dolan et al. 2008). AK1 treatment in AD cybrid showed a decrease

of phosphorylated tau **(fig. 4.2 A)** but apparently there is no relationship between SIRT 2 and deacetylation of tau protein **(fig. 4.2 C)**. Although we found no evidence that tau is a SIRT2 target, reduction of p-tau levels may be due to increased levels of acetylated tubulin**(fig. 4.2 B)**. Indeed, it was observed in NFT-bearing neurons the reduction in acetylated α-tubulin and consequently reduction in MT stability(Hempen and Brion 1996). The increase of acetylated tubulin levels by SIRT2inhibition stabilizes MTs and this stabilization allows tau binding. Thus, tau protein ceases to be a target for kinases, which leads to a decrease of phospho-tau levels.Besides the analysis in cybrids we also found changes in phosphorylated and acetylated tau levels in neuroblastoma SH-SY5Y parental cells and SH-SY5Y cells transfected with tau constructs that carry P301L mutation. P301L is a cell line that is characterized by the accumulation of phosphorylated tau and is widely used to study tauopathies. As expected, levels of phosphorylated tau protein in P301L are increased relatively to the parental SH cells**(fig.4.4 A),**which is accompanied by an increase of acetylated tau in this cell line**(fig.4.4 C)**. In P301L cells we found a slight decrease in tau acetylation levelsafterp300 inhibitionwhich correlates with the decrease in phosphorylated-tau levels. Previous *in vitro* studies revealed that the pharmacological inhibition of HDAC6 activity by TubA resulted the increased acetylated tau levels and an increase in pathological hyperphosphorylated tau in oligodendroglial cell lines (Noack, Leyk et al. 2014).

Tubulin is one of the substrates of HDAC6 and SIRT2. Tubulin deacetylation by HDAC6 is well documented in several models(Hubbert, Guardiola et al. 2002, Zhang, Li et al. 2003). North et al 2003 reported that a SIRT2 functions as a tubulin deacetylase both *in vitro* and *in vivo*(North, Marshall et al. 2003). One study has shown that inhibition of this enzyme increases the acetylation of tubulin in 3xTg-AD mice model.(Green, Steffan et al. 2008). In our cybrid model we observed a significant increase in acetylated tubulin with the inhibition of HDAC6 **(fig. 4.2 B)**. These results corroborate studies in AD models that observed increase in acetylated tubulin by inhibiting HDAC6. (Govindarajan, Rao et al. 2013, Zhang, Su et al. 2015). This result was expected since this enzyme is the major MT deacetyase (Zhang, Li et al. 2003). The data obtainedwith the inhibition of SIRT2 by AK1 also indicate that the decrease in acetylated tubulin levels in AD cells can be that can bealtered**(fig. 4.2 B)**. This result confirmed the role of SIRT2 as a tubulin deacetylase.Although there are no described

evidence in the literature in respect of tubulin as a p300substrate, the results show a significant decrease in acetylated tubulin in the CTgroup**(fig.4.2 B)**. Although the exact mechanisms by which decreases p300 acetylated tubulin is not clarified, it was noted that this enzyme modulates the function of the enzymes HDAC6 and SIRT2. It was reported that p300 interacts with and acetylates HDAC6 resulting down-regulation of HDAC6 deacetylase activity(Han, Jeong et al. 2009). They found that this acetylation led to the inhibition of tubulin deacetylation and suppression of Sp1 transcriptional activity (Han, Jeong et al. 2009).Similar results were observed forSIRT2. It was found that acetylation of SIRT2 mediated by p300 led to attenuation of deacetylation activity, which increased the transcriptional activity of p53 (Han, Jeong et al. 2009). These results show that p300 can regulate the acetylation status of tubulin indirectly through the action of SIRT2 and HDAC6. Our data in CT cellscorroborated this hypothesis despite we do not see a further decrease in acetylated tubulin levels in AD cells (**fig. 4.2 B)**.Consistent with the results obtained in cybrids, we confirm an increase in acetylated tubulin levels by inhibition of HDAC6 in parental SH and P301L **(fig. 4.4 B)**. We also found that inhibition of SIRT2 with a higher concentration of AK1 (20 µM) can increase the acetylation of tubulin in both lines **(fig. 4.4 B)**. With regard to p300 inhibition we did not observed any change in the levels of tubulin acetylation, indicating that p300 not play any role at this level these cell lines **(fig. 4.4 B)**.

The stability of MTs have an important role in intracellular trafficking, with greater relevance on autophagic process in AD(Silva, Esteves et al. 2011).It is believed that acetylation of tubulin have an important role in the stability of MTs(Cueva, Hsin et al. 2012, Kull and Sloboda 2014). It was reported in AD brains an decrease in the levels of acetylated tubulin in comparison with controls (Zhang, Su et al. 2015).AD is characterized by accumulation of autophagic vacuoles and this accumulation has been associated with the impairment of MTs(Nixon, Wegiel et al. 2005, Nixon 2007, Silva, Esteves et al. 2011). In accordance with the above, we show a clear reduction of autophagic flux in AD **(fig. 4.3 A)** as well as anaccumulationof p62 levels **(fig. 4.3 B)**.The role of HDAC6 in autophagy is controversial. Studies point to the importance of this enzyme in autophagic responses in conditions of cell stress, such as the inhibition of ubiquitin-proteasome or in cells with containing misfolded proteins(Iwata, Riley et al. 2005). Others have demonstrated that inhibition of this enzyme increases the acetylation of MTs and contributes to an improvement of autophagy. (Xiong, Zhao et al. 2013,

McLendon, Ferguson et al. 2014). This study shows that inhibition of SIRT2,HDAC6 and p300 contributes to an improvement of autophagic flux in the AD **(fig. 4.3 A)**.SIRT2 over-activation has been associated with reduction of autophagy efficiency (Zhao, Yang et al. 2010, Gal, Bang et al. 2012). The inhibition of this enzyme rescued autophagic flux, restoring the fusion of autophagosomes with lysosomes.Despite the fact that all three inhibitors rescued autophagy in AD cells, it should be noted that only AK1 was able to reduce the activation of caspase 3, and we attribute this result to the fact that inhibiting SIRT2 improves MTs assembly and intracellular transport, enabling autophagic vacuoles to fuse with lysosomes. Concerning HDAC6 and p300, although we observe some protection in autophagic flux, the fact that these enzymes are implicated in many other cellular processes may result in lack efficiency in rescuing cells from apoptosis. It is well documented phosphorylation state of tau affects its capacity to bind and stabilize MTs, favouring its aggregation, which culminates with a decrease in MTs assembly.We analysed the two markers of autophagy in the parental SH and P301L cell lines. It was found that there is an increase inautophagic flowin P301L cells, indicating that the cells are mounting a compensatory response in an attempt to eliminate the increase of phosphorylated tau and other protein aggregates that may accumulate **(fig 4.5 A)**.C646 has improved the autophagic flux in SH but in P301L decreases the autophagic flux **(fig 4.5 A)**with an accumulation of p62 levels **(fig 4.5 B)**.

We conclude that p300 may have influence the levels of phosphorylated tau in AD. In the case of SIRT2, we have found that when it is inhibited, the phosphorylated tau levels decrease only in AD cybrids. This may be due to the increase in acetylated tubulin. Regarding the HDAC6, we observed that its inhibition leads to a decrease of phosphorylayedtau in AD cybrid, which may be due to an improvement of autophagic process.Nevertheless,this decrease can be related with a decrease of tau acetylation that we know contributes to a better clearence of the phosphorylated tau levels.

Chapter 6

Conclusion

Conclusion

AD is a disabling neurodegenerative disorder that is strongly associated with ageing, increasing exponentially its incidence above the age of 65.The therapies tested until now to slow the disease development has focused on reducing Aβ levels in AD brains but have repeatedly failed in clinical trials. The search for new targets will be preponderant to improve the lives of patients with AD. The work presented in this thesis provides evidence that modulation of tau acetylation may become a potential target to counteract AD pathology. Furthermore, we showed possible therapeutic targets that may contribute to improving the autophagic flux in AD since it was previously described that this process is impaired.

The results presented in this thesis showed that SIRT2 does not deacetylatetau protein. The p300 appears to work directly by decreasing tau acetylation levels and indirectly as a modulator of tubulin acetylation. In addition, we found that acetylation is involved in tau phosphorylation. Furthermore we also saw that the inhibition of p300 and HDAC6 contributed to the reduction of phosphorylated tau in AD probably due to decrease in acetylated tau in AD cybrids.

It was also shown that acetylation of tubulin stabilizes MTs and favors tau binding to MTs, decreasing phosphorylated tau levels. The inhibition of SIRT2, p300 and HDAC6, rescued autophagy in AD group but only SIRT2 inhibition increased acetylated tubulin levels and decreased apoptosis activation.

Although the present findings strongly suggest that SIRT2, p300 and HDAC6 could holdpotential therapeutic effectsin AD, further studies are required to better understand the function of each enzyme on AD pathologic features and autophagic process.

Besides, *in vivo* studies performed in animal models of AD will reinforce the potential of these enzymes as therapeutic targets to “fight” this devastating neurodegenerative disease.

Moreover, a better understanding of the effects of tau acetylation is crucial since it have been documented contradictory roles for this tau post translational modifications in terms of AD progression. Studies with new modulators of tau acetylation well as the use of different inhibitors or the knockdown of enzymes that target tau are essential to understand the effects of post-translational modification.

Chapter 7

References

Adalbert, R., J. Gilley and M. P. Coleman (2007). "Abeta, tau and ApoE4 in Alzheimer's disease: the axonal connection." Trends Mol Med**13**(4): 135-142.

Alzheimer, A., R. A. Stelzmann, H. N. Schnitzlein and F. R. Murtagh (1995). "An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde"." Clin Anat**8**(6): 429-431.

Amenta, J. S., M. J. Sargus and F. M. Baccino (1977). "Effect of microtubular or translational inhibitors on general cell protein degradation. Evidence for a dual catabolic pathway." Biochem J**168**(2): 223-227.

Ansari, M. A. and S. W. Scheff (2010). "Oxidative stress in the progression of Alzheimer disease in the frontal cortex." J Neuropathol Exp Neurol**69**(2): 155-167.

Aplin, A., T. Jasionowski, D. L. Tuttle, S. E. Lenk and W. A. Dunn, Jr. (1992). "Cytoskeletal elements are required for the formation and maturation of autophagic vacuoles." J Cell Physiol**152**(3): 458-466.

Arduíno, DM (2012). "Mitochondria and Protein Homeostasis in Parkinson`s Disease." (Tese de Doutoramento), Universidade de Coimbra,Portugal.

Beal, M. F. (2005). "Mitochondria take center stage in aging and neurodegeneration." Ann Neurol**58**(4): 495-505.

Beharry, C., L. S. Cohen, J. Di, K. Ibrahim, S. Briffa-Mirabella and C. Alonso Adel (2014). "Tau-induced neurodegeneration: mechanisms and targets." Neurosci Bull**30**(2): 346-358.

Berg, L., D. W. McKeel, Jr., J. P. Miller, M. Storandt, E. H. Rubin, J. C. Morris, J. Baty, M. Coats, J. Norton, A. M. Goate, J. L. Price, M. Gearing, S. S. Mirra and A. M. Saunders (1998). "Clinicopathologic studies in cognitively healthy aging and Alzheimer's disease: relation of histologic markers to dementia severity, age, sex, and apolipoprotein E genotype." Arch Neurol**55**(3): 326-335.

Bertos, N. R., B. Gilquin, G. K. Chan, T. J. Yen, S. Khochbin and X. J. Yang (2004). "Role of the tetradecapeptide repeat domain of human histone deacetylase 6 in cytoplasmic retention." J Biol Chem**279**(46): 48246-48254.

Boland, B., A. Kumar, S. Lee, F. M. Platt, J. Wegiel, W. H. Yu and R. A. Nixon (2008). "Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease." J Neurosci**28**(27): 6926-6937.

Boutajangout, A., E. M. Sigurdsson and P. K. Krishnamurthy (2011). "Tau as a therapeutic target for Alzheimer's disease." Curr Alzheimer Res**8**(6): 666-677.

Bulbarelli, A., E. Lonati, E. Cazzaniga, M. Gregori and M. Masserini (2009). "Pin1 affects Tau phosphorylation in response to Abeta oligomers." Mol Cell Neurosci**42**(1): 75-80.

Butler, D., J. Bendiske, M. L. Michaelis, D. A. Karanian and B. A. Bahr (2007). "Microtubule-stabilizing agent prevents protein accumulation-induced loss of synaptic markers." Eur J Pharmacol**562**(1-2): 20-27.

Cadenas, E. and K. J. Davies (2000). "Mitochondrial free radical generation, oxidative stress, and aging." Free Radic Biol Med**29**(3-4): 222-230.

Cardoso, S. M., C. F. Pereira, P. I. Moreira, D. M. Arduino, A. R. Esteves and C. R. Oliveira (2010). "Mitochondrial control of autophagic lysosomal pathway in Alzheimer's disease." Exp Neurol**223**(2): 294-298.

Cardoso, S. M., M. T. Proenca, S. Santos, I. Santana and C. R. Oliveira (2004). "Cytochrome c oxidase is decreased in Alzheimer's disease platelets." Neurobiol Aging**25**(1): 105-110.

Cash, A. D., G. Aliev, S. L. Siedlak, A. Nunomura, H. Fujioka, X. Zhu, A. K. Raina, H. V. Vinters, M. Tabaton, A. B. Johnson, M. Paula-Barbosa, J. Avila, P. K. Jones, R. J. Castellani, M. A. Smith and G. Perry (2003). "Microtubule reduction in Alzheimer's disease and aging is independent of tau filament formation." Am J Pathol**162**(5): 1623-1627.

Cataldo, A. M., P. A. Paskevich, E. Kominami and R. A. Nixon (1991). "Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease." Proc Natl Acad Sci U S A**88**(24): 10998-11002.

Chen, S., G. C. Owens, H. Makarenkova and D. B. Edelman (2010). "HDAC6 regulates mitochondrial transport in hippocampal neurons." PLoS One**5**(5): e10848.
Chuang, D. M., Y. Leng, Z. Marinova, H. J. Kim and C. T. Chiu (2009). "Multiple roles of HDAC inhibition in neurodegenerative conditions." Trends Neurosci**32**(11): 591-601.
Cohen, T. J., J. L. Guo, D. E. Hurtado, L. K. Kwong, I. P. Mills, J. Q. Trojanowski and V. M. Lee (2011). "The acetylation of tau inhibits its function and promotes pathological tau aggregation." Nat Commun**2**: 252.
Cook, C., Y. Carlomagno, T. F. Gendron, J. Dunmore, K. Scheffel, C. Stetler, M. Davis, D. Dickson, M. Jarpe, M. DeTure and L. Petrucelli (2014). "Acetylation of the KXGS motifs in tau is a critical determinant in modulation of tau aggregation and clearance." Hum Mol Genet**23**(1): 104-116.
Cook, C., J. N. Stankowski, Y. Carlomagno, C. Stetler and L. Petrucelli (2014). "Acetylation: a new key to unlock tau's role in neurodegeneration." Alzheimers Res Ther**6**(3): 29.
Cras, P., M. Kawai, D. Lowery, P. Gonzalez-DeWhitt, B. Greenberg and G. Perry (1991). "Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein." Proc Natl Acad Sci U S A**88**(17): 7552-7556.
Cregan, S. P., J. G. MacLaurin, C. G. Craig, G. S. Robertson, D. W. Nicholson, D. S. Park and R. S. Slack (1999). "Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons." J Neurosci**19**(18): 7860-7869.
Crespo-Biel, N., C. Theunis and F. Van Leuven (2012). "Protein tau: prime cause of synaptic and neuronal degeneration in Alzheimer's disease." Int J Alzheimers Dis**2012**: 251426.
Cueva, J. G., J. Hsin, K. C. Huang and M. B. Goodman (2012). "Posttranslational acetylation of alpha-tubulin constrains protofilament number in native microtubules." Curr Biol**22**(12): 1066-1074.
De Benedictis, G., G. Rose, G. Carrieri, M. De Luca, E. Falcone, G. Passarino, M. Bonafe, D. Monti, G. Baggio, S. Bertolini, D. Mari, R. Mattace and C. Franceschi (1999). "Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans." FASEB J**13**(12): 1532-1536.
De Duve, C. (1963). "The lysosome." Sci Am**208**: 64-72.
de Oliveira, R. M., J. Sarkander, A. G. Kazantsev and T. F. Outeiro (2012). "SIRT2 as a Therapeutic Target for Age-Related Disorders." Front Pharmacol**3**: 82.
Deribe, Y. L., P. Wild, A. Chandrashaker, J. Curak, M. H. Schmidt, Y. Kalaidzidis, N. Milutinovic, I. Kratchmarova, L. Buerkle, M. J. Fetchko, P. Schmidt, S. Kittanakom, K. R. Brown, I. Jurisica, B. Blagoev, M. Zerial, I. Stagljar and I. Dikic (2009). "Regulation of epidermal growth factor receptor trafficking by lysine deacetylase HDAC6." Sci Signal**2**(102): ra84.
Ding, H., P. J. Dolan and G. V. Johnson (2008). "Histone deacetylase 6 interacts with the microtubule-associated protein tau." J Neurochem**106**(5): 2119-2130.
Eckert, A., U. Keil, C. A. Marques, A. Bonert, C. Frey, K. Schussel and W. E. Muller (2003). "Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease." Biochem Pharmacol**66**(8): 1627-1634.
Encinas, M., M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Cena, C. Gallego and J. X. Comella (2000). "Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells." J Neurochem**75**(3): 991-1003.
Fass, E., E. Shvets, I. Degani, K. Hirschberg and Z. Elazar (2006). "Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes." J Biol Chem**281**(47): 36303-36316.
Gal, J., Y. Bang and H. J. Choi (2012). "SIRT2 interferes with autophagy-mediated degradation of protein aggregates in neuronal cells under proteasome inhibition." Neurochem Int**61**(7): 992-1000.
Garcia-Sierra, F., S. Mondragon-Rodriguez and G. Basurto-Islas (2008). "Truncation of tau protein and its pathological significance in Alzheimer's disease." J Alzheimers Dis**14**(4): 401-409.

Giannakopoulos, P., F. R. Herrmann, T. Bussiere, C. Bouras, E. Kovari, D. P. Perl, J. H. Morrison, G. Gold and P. R. Hof (2003). "Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease." Neurology**60**(9): 1495-1500.
Glick, D., S. Barth and K. F. Macleod (2010). "Autophagy: cellular and molecular mechanisms." J Pathol**221**(1): 3-12.
Goedert, M., C. M. Wischik, R. A. Crowther, J. E. Walker and A. Klug (1988). "Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau." Proc Natl Acad Sci U S A**85**(11): 4051-4055.
Gomez-Isla, T., J. L. Price, D. W. McKeel, Jr., J. C. Morris, J. H. Growdon and B. T. Hyman (1996). "Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease." J Neurosci**16**(14): 4491-4500.
Gong, C. X. and K. Iqbal (2008). "Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease." Curr Med Chem**15**(23): 2321-2328.
Govindarajan, N., P. Rao, S. Burkhardt, F. Sananbenesi, O. M. Schluter, F. Bradke, J. Lu and A. Fischer (2013). "Reducing HDAC6 ameliorates cognitive deficits in a mouse model for Alzheimer's disease." EMBO Mol Med**5**(1): 52-63.
Gozes, I. and I. Divinski (2004). "The femtomolar-acting NAP interacts with microtubules: Novel aspects of astrocyte protection." J Alzheimers Dis**6**(6 Suppl): S37-41.
Green, K. N., J. S. Steffan, H. Martinez-Coria, X. Sun, S. S. Schreiber, L. M. Thompson and F. M. LaFerla (2008). "Nicotinamide restores cognition in Alzheimer's disease transgenic mice via a mechanism involving sirtuin inhibition and selective reduction of Thr231-phosphotau." J Neurosci**28**(45): 11500-11510.
Grundke-Iqbal, I., K. Iqbal, Y. C. Tung, M. Quinlan, H. M. Wisniewski and L. I. Binder (1986). "Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology." Proc Natl Acad Sci U S A**83**(13): 4913-4917.
Han, Y., H. M. Jeong, Y. H. Jin, Y. J. Kim, H. G. Jeong, C. Y. Yeo and K. Y. Lee (2009). "Acetylation of histone deacetylase 6 by p300 attenuates its deacetylase activity." Biochem Biophys Res Commun**383**(1): 88-92.
Hanger, D. P., A. Seereeram and W. Noble (2009). "Mediators of tau phosphorylation in the pathogenesis of Alzheimer's disease." Expert Rev Neurother**9**(11): 1647-1666.
Hardy, J. and D. J. Selkoe (2002). "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics." Science**297**(5580): 353-356.
Hardy, J. A. and G. A. Higgins (1992). "Alzheimer's disease: the amyloid cascade hypothesis." Science**256**(5054): 184-185.
Hempen, B. and J. P. Brion (1996). "Reduction of acetylated alpha-tubulin immunoreactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease." J Neuropathol Exp Neurol**55**(9): 964-972.
Hoerndli, F. J., S. Pelech, A. Papassotiropoulos and J. Gotz (2007). "Abeta treatment and P301L tau expression in an Alzheimer's disease tissue culture model act synergistically to promote aberrant cell cycle re-entry." Eur J Neurosci**26**(1): 60-72.
Holtzman, D. M., K. R. Bales, S. Wu, P. Bhat, M. Parsadanian, A. M. Fagan, L. K. Chang, Y. Sun and S. M. Paul (1999). "Expression of human apolipoprotein E reduces amyloid-beta deposition in a mouse model of Alzheimer's disease." J Clin Invest**103**(6): R15-R21.
Hubbert, C., A. Guardiola, R. Shao, Y. Kawaguchi, A. Ito, A. Nixon, M. Yoshida, X. F. Wang and T. P. Yao (2002). "HDAC6 is a microtubule-associated deacetylase." Nature**417**(6887): 455-458.
Iqbal, K., F. Liu, C. X. Gong, C. Alonso Adel and I. Grundke-Iqbal (2009). "Mechanisms of tau-induced neurodegeneration." Acta Neuropathol**118**(1): 53-69.
Irwin, D. J., T. J. Cohen, M. Grossman, S. E. Arnold, E. McCarty-Wood, V. M. Van Deerlin, V. M. Lee and J. Q. Trojanowski (2013). "Acetylated tau neuropathology in sporadic and hereditary tauopathies." Am J Pathol**183**(2): 344-351.

Irwin, D. J., T. J. Cohen, M. Grossman, S. E. Arnold, S. X. Xie, V. M. Lee and J. Q. Trojanowski (2012). "Acetylated tau, a novel pathological signature in Alzheimer's disease and other tauopathies." Brain**135**(Pt 3): 807-818.
Ittner, A., Y. D. Ke, J. van Eersel, A. Gladbach, J. Gotz and L. M. Ittner (2011). "Brief update on different roles of tau in neurodegeneration." IUBMB Life**63**(7): 495-502.
Iwata, A., B. E. Riley, J. A. Johnston and R. R. Kopito (2005). "HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin." J Biol Chem**280**(48): 40282-40292.
Jiang, X. and X. Wang (2004). "Cytochrome C-mediated apoptosis." Annu Rev Biochem**73**: 87-106.
Kar, A., D. Kuo, R. He, J. Zhou and J. Y. Wu (2005). "Tau alternative splicing and frontotemporal dementia." Alzheimer Dis Assoc Disord**19 Suppl 1**: S29-36.
Karran, E., M. Mercken and B. De Strooper (2011). "The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics." Nat Rev Drug Discov**10**(9): 698-712.
Kilgore, M., C. A. Miller, D. M. Fass, K. M. Hennig, S. J. Haggarty, J. D. Sweatt and G. Rumbaugh (2010). "Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease." Neuropsychopharmacology**35**(4): 870-880.
King, M. P. and G. Attardi (1989). "Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation." Science**246**(4929): 500-503.
Kochl, R., X. W. Hu, E. Y. Chan and S. A. Tooze (2006). "Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes." Traffic**7**(2): 129-145.
Kolarova, M., F. Garcia-Sierra, A. Bartos, J. Ricny and D. Ripova (2012). "Structure and pathology of tau protein in Alzheimer disease." Int J Alzheimers Dis**2012**: 731526.
Komatsu, M., E. Kominami and K. Tanaka (2006). "Autophagy and neurodegeneration." Autophagy**2**(4): 315-317.
Komatsu, M., S. Waguri, T. Ueno, J. Iwata, S. Murata, I. Tanida, J. Ezaki, N. Mizushima, Y. Ohsumi, Y. Uchiyama, E. Kominami, K. Tanaka and T. Chiba (2005). "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice." J Cell Biol**169**(3): 425-434.
Kull, F. J. and R. D. Sloboda (2014). "A slow dance for microtubule acetylation." Cell**157**(6): 1255-1256.
L'Hernault, S. W. and J. L. Rosenbaum (1985). "Reversal of the posttranslational modification on Chlamydomonas flagellar alpha-tubulin occurs during flagellar resorption." J Cell Biol**100**(2): 457-462.
Laws, S. M., E. Hone, S. Gandy and R. N. Martins (2003). "Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription." J Neurochem**84**(6): 1215-1236.
LeDizet, M. and G. Piperno (1987). "Identification of an acetylation site of Chlamydomonas alpha-tubulin." Proc Natl Acad Sci U S A**84**(16): 5720-5724.
Lee, G. (1990). "Tau protein: an update on structure and function." Cell Motil Cytoskeleton**15**(4): 199-203.
Lee, G. and C. J. Leugers (2012). "Tau and tauopathies." Prog Mol Biol Transl Sci**107**: 263-293.
Lee, J. A. and F. B. Gao (2008). "Regulation of Abeta pathology by beclin 1: a protective role for autophagy?" J Clin Invest**118**(6): 2015-2018.
Levine, B. and D. J. Klionsky (2004). "Development by self-digestion: molecular mechanisms and biological functions of autophagy." Dev Cell**6**(4): 463-477.
Li, G., A. Faibushevich, B. J. Turunen, S. O. Yoon, G. Georg, M. L. Michaelis and R. T. Dobrowsky (2003). "Stabilization of the cyclin-dependent kinase 5 activator, p35, by paclitaxel decreases beta-amyloid toxicity in cortical neurons." J Neurochem**84**(2): 347-362.
Li, J., D. Liu, L. Sun, Y. Lu and Z. Zhang (2012). "Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective." J Neurol Sci**317**(1-2): 1-5.

Lindwall, G. and R. D. Cole (1984). "Phosphorylation affects the ability of tau protein to promote microtubule assembly." J Biol Chem**259**(8): 5301-5305.
Lippai, M. and P. Low (2014). "The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy." Biomed Res Int**2014**: 832704.
Liu, F., B. Li, E. J. Tung, I. Grundke-Iqbal, K. Iqbal and C. X. Gong (2007). "Site-specific effects of tau phosphorylation on its microtubule assembly activity and self-aggregation." Eur J Neurosci**26**(12): 3429-3436.
Lossos, A., A. Reches, A. Gal, J. P. Newman, D. Soffer, J. M. Gomori, M. Boher, D. Ekstein, I. Biran, Z. Meiner, O. Abramsky and H. Rosenmann (2003). "Frontotemporal dementia and parkinsonism with the P301S tau gene mutation in a Jewish family." J Neurol**250**(6): 733-740.
Lynch-Day, M. A., K. Mao, K. Wang, M. Zhao and D. J. Klionsky (2012). "The role of autophagy in Parkinson's disease." Cold Spring Harb Perspect Med**2**(4): a009357.
Mandelkow, E. M. and E. Mandelkow (2012). "Biochemistry and cell biology of tau protein in neurofibrillary degeneration." Cold Spring Harb Perspect Med**2**(7): a006247.
Martin, L., X. Latypova and F. Terro (2011). "Post-translational modifications of tau protein: implications for Alzheimer's disease." Neurochem Int**58**(4): 458-471.
Matsuoka, Y., A. J. Gray, C. Hirata-Fukae, S. S. Minami, E. G. Waterhouse, M. P. Mattson, F. M. LaFerla, I. Gozes and P. S. Aisen (2007). "Intranasal NAP administration reduces accumulation of amyloid peptide and tau hyperphosphorylation in a transgenic mouse model of Alzheimer's disease at early pathological stage." J Mol Neurosci**31**(2): 165-170.
Matsuyama, A., T. Shimazu, Y. Sumida, A. Saito, Y. Yoshimatsu, D. Seigneurin-Berny, H. Osada, Y. Komatsu, N. Nishino, S. Khochbin, S. Horinouchi and M. Yoshida (2002). "In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation." EMBO J**21**(24): 6820-6831.
Matsuyama, S. S. and L. F. Jarvik (1989). "Hypothesis: microtubules, a key to Alzheimer disease." Proc Natl Acad Sci U S A**86**(20): 8152-8156.
McLendon, P. M., B. S. Ferguson, H. Osinska, M. S. Bhuiyan, J. James, T. A. McKinsey and J. Robbins (2014). "Tubulin hyperacetylation is adaptive in cardiac proteotoxicity by promoting autophagy." Proc Natl Acad Sci U S A**111**(48): E5178-5186.
Michaelis, M. L., R. T. Dobrowsky and G. Li (2002). "Tau neurofibrillary pathology and microtubule stability." J Mol Neurosci**19**(3): 289-293.
Migliore, L., I. Fontana, F. Trippi, R. Colognato, F. Coppede, G. Tognoni, B. Nucciarone and G. Siciliano (2005). "Oxidative DNA damage in peripheral leukocytes of mild cognitive impairment and AD patients." Neurobiol Aging**26**(5): 567-573.
Min, S. W., S. H. Cho, Y. Zhou, S. Schroeder, V. Haroutunian, W. W. Seeley, E. J. Huang, Y. Shen, E. Masliah, C. Mukherjee, D. Meyers, P. A. Cole, M. Ott and L. Gan (2010). "Acetylation of tau inhibits its degradation and contributes to tauopathy." Neuron**67**(6): 953-966.
Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori and Y. Ohsumi (2004). "In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker." Mol Biol Cell**15**(3): 1101-1111.
Mondragon-Rodriguez, S., G. Perry, J. Luna-Munoz, M. C. Acevedo-Aquino and S. Williams (2014). "Phosphorylation of tau protein at sites Ser(396-404) is one of the earliest events in Alzheimer's disease and Down syndrome." Neuropathol Appl Neurobiol**40**(2): 121-135.
Mondragon-Rodriguez, S., G. Perry, X. Zhu, P. I. Moreira, M. C. Acevedo-Aquino and S. Williams (2013). "Phosphorylation of tau protein as the link between oxidative stress, mitochondrial dysfunction, and connectivity failure: implications for Alzheimer's disease." Oxid Med Cell Longev**2013**: 940603.
Moreira, P. I., S. M. Cardoso, M. S. Santos and C. R. Oliveira (2006). "The key role of mitochondria in Alzheimer's disease." J Alzheimers Dis**9**(2): 101-110.
Mukherjee, O., J. S. Kauwe, K. Mayo, J. C. Morris and A. M. Goate (2007). "Haplotype-based association analysis of the MAPT locus in late onset Alzheimer's disease." BMC Genet**8**: 3.

Myers, A. J., M. Kaleem, L. Marlowe, A. M. Pittman, A. J. Lees, H. C. Fung, J. Duckworth, D. Leung, A. Gibson, C. M. Morris, R. de Silva and J. Hardy (2005). "The H1c haplotype at the MAPT locus is associated with Alzheimer's disease." Hum Mol Genet**14**(16): 2399-2404.
Neumann, K., G. Farias, A. Slachevsky, P. Perez and R. B. Maccioni (2011). "Human platelets tau: a potential peripheral marker for Alzheimer's disease." J Alzheimers Dis**25**(1): 103-109.
Nie, H., Y. Hong, X. Lu, J. Zhang, H. Chen, Y. Li, Y. Ma and W. Ying (2014). "SIRT2 mediates oxidative stress-induced apoptosis of differentiated PC12 cells." Neuroreport.
Nixon, R. A. (2007). "Autophagy, amyloidogenesis and Alzheimer disease." J Cell Sci**120**(Pt 23): 4081-4091.
Nixon, R. A. and A. M. Cataldo (2006). "Lysosomal system pathways: genes to neurodegeneration in Alzheimer's disease." J Alzheimers Dis**9**(3 Suppl): 277-289.
Nixon, R. A., J. Wegiel, A. Kumar, W. H. Yu, C. Peterhoff, A. Cataldo and A. M. Cuervo (2005). "Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study." J Neuropathol Exp Neurol**64**(2): 113-122.
Noack, M., J. Leyk and C. Richter-Landsberg (2014). "HDAC6 inhibition results in tau acetylation and modulates tau phosphorylation and degradation in oligodendrocytes." Glia**62**(4): 535-547.
North, B. J., B. L. Marshall, M. T. Borra, J. M. Denu and E. Verdin (2003). "The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase." Mol Cell**11**(2): 437-444.
Nunomura, A., T. Hofer, P. I. Moreira, R. J. Castellani, M. A. Smith and G. Perry (2009). "RNA oxidation in Alzheimer disease and related neurodegenerative disorders." Acta Neuropathol**118**(1): 151-166.
Nunomura, A., G. Perry, M. A. Pappolla, R. Wade, K. Hirai, S. Chiba and M. A. Smith (1999). "RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease." J Neurosci**19**(6): 1959-1964.
O'Brien, R. J. and P. C. Wong (2011). "Amyloid precursor protein processing and Alzheimer's disease." Annu Rev Neurosci**34**: 185-204.
Ojaimi, J., C. L. Masters, K. Opeskin, P. McKelvie and E. Byrne (1999). "Mitochondrial respiratory chain activity in the human brain as a function of age." Mech Ageing Dev**111**(1): 39-47.
Onyango, I. G., J. B. Tuttle and J. P. Bennett, Jr. (2005). "Altered intracellular signaling and reduced viability of Alzheimer's disease neuronal cybrids is reproduced by beta-amyloid peptide acting through receptor for advanced glycation end products (RAGE)." Mol Cell Neurosci**29**(2): 333-343.
Padurariu, M., A. Ciobica, L. Hritcu, B. Stoica, W. Bild and C. Stefanescu (2010). "Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease." Neurosci Lett**469**(1): 6-10.
Paquet-Durand, F., S. Tan and G. Bicker (2003). "Turning teratocarcinoma cells into neurons: rapid differentiation of NT-2 cells in floating spheres." Brain Res Dev Brain Res**142**(2): 161-167.
Perez, M., I. Santa-Maria, E. Gomez de Barreda, X. Zhu, R. Cuadros, J. R. Cabrero, F. Sanchez-Madrid, H. N. Dawson, M. P. Vitek, G. Perry, M. A. Smith and J. Avila (2009). "Tau--an inhibitor of deacetylase HDAC6 function." J Neurochem**109**(6): 1756-1766.
Petersen, R. C. (2004). "Mild cognitive impairment as a diagnostic entity." J Intern Med**256**(3): 183-194.
Pickford, F., E. Masliah, M. Britschgi, K. Lucin, R. Narasimhan, P. A. Jaeger, S. Small, B. Spencer, E. Rockenstein, B. Levine and T. Wyss-Coray (2008). "The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice." J Clin Invest**118**(6): 2190-2199.
Pittman, A. M., H. C. Fung and R. de Silva (2006). "Untangling the tau gene association with neurodegenerative disorders." Hum Mol Genet**15 Spec No 2**: R188-195.
Raber, J., Y. Huang and J. W. Ashford (2004). "ApoE genotype accounts for the vast majority of AD risk and AD pathology." Neurobiol Aging**25**(5): 641-650.

Reed, N. A., D. Cai, T. L. Blasius, G. T. Jih, E. Meyhofer, J. Gaertig and K. J. Verhey (2006). "Microtubule acetylation promotes kinesin-1 binding and transport." Curr Biol**16**(21): 2166-2172.

Reyes, J. F., Y. Fu, L. Vana, N. M. Kanaan and L. I. Binder (2011). "Tyrosine nitration within the proline-rich region of Tau in Alzheimer's disease." Am J Pathol**178**(5): 2275-2285.

Riederer, B. M., G. Leuba and Z. Elhajj (2013). "Oxidation and ubiquitination in neurodegeneration." Exp Biol Med (Maywood)**238**(5): 519-524.

Rouaux, C., N. Jokic, C. Mbebi, S. Boutillier, J. P. Loeffler and A. L. Boutillier (2003). "Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration." EMBO J**22**(24): 6537-6549.

Scorrano, L. and S. J. Korsmeyer (2003). "Mechanisms of cytochrome c release by proapoptotic BCL-2 family members." Biochem Biophys Res Commun**304**(3): 437-444.

Seelaar, H., J. D. Rohrer, Y. A. Pijnenburg, N. C. Fox and J. C. van Swieten (2011). "Clinical, genetic and pathological heterogeneity of frontotemporal dementia: a review." J Neurol Neurosurg Psychiatry**82**(5): 476-486.

Seglen, P. O., T. O. Berg, H. Blankson, M. Fengsrud, I. Holen and P. E. Stromhaug (1996). "Structural aspects of autophagy." Adv Exp Med Biol**389**: 103-111.

Shi, C., K. Guo, D. T. Yew, Z. Yao, E. L. Forster, H. Wang and J. Xu (2008). "Effects of ageing and Alzheimer's disease on mitochondrial function of human platelets." Exp Gerontol**43**(6): 589-594.

Silva, D. F., A. R. Esteves, D. M. Arduino, C. R. Oliveira and S. M. Cardoso (2011). "Amyloid-beta-induced mitochondrial dysfunction impairs the autophagic lysosomal pathway in a tubulin dependent pathway." J Alzheimers Dis**26**(3): 565-581.

Silva, D. F., A. R. Esteves, C. R. Oliveira and S. M. Cardoso (2011). "Mitochondria: the common upstream driver of amyloid-beta and tau pathology in Alzheimer's disease." Curr Alzheimer Res**8**(5): 563-572.

Silva, D. F., I. Santana, A. R. Esteves, I. Baldeiras, D. M. Arduino, C. R. Oliveira and S. M. Cardoso (2013). "Prodromal metabolic phenotype in MCI cybrids: implications for Alzheimer's disease." Curr Alzheimer Res**10**(2): 180-190.

Silva, D. F., J. E. Selfridge, J. Lu, L. E, N. Roy, L. Hutfles, J. M. Burns, E. K. Michaelis, S. Yan, S. M. Cardoso and R. H. Swerdlow (2013). "Bioenergetic flux, mitochondrial mass and mitochondrial morphology dynamics in AD and MCI cybrid cell lines." Hum Mol Genet**22**(19): 3931-3946.

Smale, G., N. R. Nichols, D. R. Brady, C. E. Finch and W. E. Horton, Jr. (1995). "Evidence for apoptotic cell death in Alzheimer's disease." Exp Neurol**133**(2): 225-230.

Spires-Jones, T. L., L. M. Fox, A. Rozkalne, R. Pitstick, G. A. Carlson and A. G. Kazantsev (2012). "Inhibition of Sirtuin 2 with Sulfobenzoic Acid Derivative AK1 is Non-Toxic and Potentially Neuroprotective in a Mouse Model of Frontotemporal Dementia." Front Pharmacol**3**: 42.

Swerdlow, R. H. and S. M. Khan (2004). "A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease." Med Hypotheses**63**(1): 8-20.

Swerdlow, R. H. and S. M. Khan (2009). "The Alzheimer's disease mitochondrial cascade hypothesis: an update." Exp Neurol**218**(2): 308-315.

Swerdlow, R. H., J. K. Parks, D. S. Cassarino, D. J. Maguire, R. S. Maguire, J. P. Bennett, Jr., R. E. Davis and W. D. Parker, Jr. (1997). "Cybrids in Alzheimer's disease: a cellular model of the disease?" Neurology**49**(4): 918-925.

Takahashi, K., M. Ishida, H. Komano and H. Takahashi (2008). "SUMO-1 immunoreactivity co-localizes with phospho-Tau in APP transgenic mice but not in mutant Tau transgenic mice." Neurosci Lett**441**(1): 90-93.

Tooze, S. A. and T. Yoshimori (2010). "The origin of the autophagosomal membrane." Nat Cell Biol**12**(9): 831-835.

Topalidou, I., C. Keller, N. Kalebic, K. C. Nguyen, H. Somhegyi, K. A. Politi, P. Heppenstall, D. H. Hall and M. Chalfie (2012). "Genetically separable functions of the MEC-17 tubulin acetyltransferase affect microtubule organization." Curr Biol**22**(12): 1057-1065.

Trounce, I., E. Byrne and S. Marzuki (1989). "Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing." Lancet**1**(8639): 637-639.
Valla, J., L. Schneider, T. Niedzielko, K. D. Coon, R. Caselli, M. N. Sabbagh, G. L. Ahern, L. Baxter, G. Alexander, D. G. Walker and E. M. Reiman (2006). "Impaired platelet mitochondrial activity in Alzheimer's disease and mild cognitive impairment." Mitochondrion**6**(6): 323-330.
van der Walt, J. M., K. K. Nicodemus, E. R. Martin, W. K. Scott, M. A. Nance, R. L. Watts, J. P. Hubble, J. L. Haines, W. C. Koller, K. Lyons, R. Pahwa, M. B. Stern, A. Colcher, B. C. Hiner, J. Jankovic, W. G. Ondo, F. H. Allen, Jr., C. G. Goetz, G. W. Small, F. Mastaglia, J. M. Stajich, A. C. McLaurin, L. T. Middleton, B. L. Scott, D. E. Schmechel, M. A. Pericak-Vance and J. M. Vance (2003). "Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease." Am J Hum Genet**72**(4): 804-811.
Wang, D. S., D. W. Dickson and J. S. Malter (2008). "Tissue transglutaminase, protein cross-linking and Alzheimer's disease: review and views." Int J Clin Exp Pathol**1**(1): 5-18.
Wang, J. Z., I. Grundke-Iqbal and K. Iqbal (1996). "Glycosylation of microtubule-associated protein tau: an abnormal posttranslational modification in Alzheimer's disease." Nat Med**2**(8): 871-875.
Wang, X., B. Su, H. Fujioka and X. Zhu (2008). "Dynamin-like protein 1 reduction underlies mitochondrial morphology and distribution abnormalities in fibroblasts from sporadic Alzheimer's disease patients." Am J Pathol**173**(2): 470-482.
Wilkins, H. M., S. M. Carl and R. H. Swerdlow (2014). "Cytoplasmic hybrid (cybrid) cell lines as a practical model for mitochondriopathies." Redox Biol**2C**: 619-631.
Wischik, C. M., M. Novak, P. C. Edwards, A. Klug, W. Tichelaar and R. A. Crowther (1988). "Structural characterization of the core of the paired helical filament of Alzheimer disease." Proc Natl Acad Sci U S A**85**(13): 4884-4888.
Wolfe, D. M., J. H. Lee, A. Kumar, S. Lee, S. J. Orenstein and R. A. Nixon (2013). "Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification." Eur J Neurosci**37**(12): 1949-1961.
Xiong, Y., K. Zhao, J. Wu, Z. Xu, S. Jin and Y. Q. Zhang (2013). "HDAC6 mutations rescue human tau-induced microtubule defects in Drosophila." Proc Natl Acad Sci U S A**110**(12): 4604-4609.
Yu, W. H., A. M. Cuervo, A. Kumar, C. M. Peterhoff, S. D. Schmidt, J. H. Lee, P. S. Mohan, M. Mercken, M. R. Farmery, L. O. Tjernberg, Y. Jiang, K. Duff, Y. Uchiyama, J. Naslund, P. M. Mathews, A. M. Cataldo and R. A. Nixon (2005). "Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease." J Cell Biol**171**(1): 87-98.
Zhang, F., B. Su, C. Wang, S. L. Siedlak, S. Mondragon-Rodriguez, H. G. Lee, X. Wang, G. Perry and X. Zhu (2015). "Posttranslational modifications of alpha-tubulin in alzheimer disease." Transl Neurodegener**4**: 9.
Zhang, H., Y. Liu, M. Lao, Z. Ma and X. Yi (2011). "Puerarin protects Alzheimer's disease neuronal cybrids from oxidant-stress induced apoptosis by inhibiting pro-death signaling pathways." Exp Gerontol**46**(1): 30-37.
Zhang, Y., N. Li, C. Caron, G. Matthias, D. Hess, S. Khochbin and P. Matthias (2003). "HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo." EMBO J**22**(5): 1168-1179.
Zhao, Y., J. Yang, W. Liao, X. Liu, H. Zhang, S. Wang, D. Wang, J. Feng, L. Yu and W. G. Zhu (2010). "Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity." Nat Cell Biol**12**(7): 665-675.