DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

The role of autophagy and the lysosomal pathway in the clearance of Tau aggregates

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 Doutor Arjan Buist (Janssen Pharmaceutica) e do Professor Doutor Carlos Duarte (FCTUC)

Alberto José Carpinteiro Soares

2015

The work presented in this thesis resulted from a partnership between the University of Coimbra and Janssen Pharmaceutica NV, Beerse I. All experimental activities were performed at Janssen Pharmaceutica NV, Beerse I, a Johnson & Johnson pharmaceutical research and development facility in Beerse, Belgium.

Beerse, 2015

Acknowledgments

Antes de iniciar esta secção queria apenas deixar uma palavra de agradecimento a quem me apoiou mais neste projeto: a minha família. Não tenho maneira de agradecer aos meus pais por todo o apoio e esforço físico, emocional e financeiro que depositaram em mim ao longo dos últimos anos. Por me terem dado a liberdade de seguir os meus sonhos o meu mais profundo obrigado!

First I would like to acknowledge to Arjan for this amazing experience and work throughout this year. Thank you for your scientific knowledge and experience as well as all your advices, patience and trust on my work. Your enthusiasm in the lab was contagious and I'm sure will be a model for my future career.

I would also like to thank to Dieder to accept me in his group. Thank you for your interest, trust and all the critic input you gave to my work as well as all the positive discussions we had about science. It will be a pleasure to work with you in the next years!

Special thanks to Kathleen Callaerts for her help, experience and joy inside the lab. I would also like to thank to Luanda for amazing review to this work as well as critic opinion on my data. Thank you to all the Tau group for the help you offered me throughout this year and all the pleasant scientific discussions that made me a better scientist. I would like to specially thank to Sara Calafate for all the help and patience with me, as well as the friendship and crazy conversations both inside and outside the lab.

I also wish to thank Professor Carlos Duarte for supervising my work and all your knowledge and scientific enthusiasm. Also thanks to Professor Emilia Duarte for allowing me with this great opportunity. Thank you both for the amazing first year and all the help provided.

I would like to thank all my friends in Portugal for all the support they gave me through this year. For making home a little closer, thank you! Special thanks to my friends who came all the way to Belgium to bring us warm and joy. Thank you to all the intern students, especially to Harvey and Joe, and all new friends for the amazing weekends, nights and beers spend together. Thank you!

A special thanks to Ana Cascalho. Thank you for all the support, friendship, joy and happiness through this year. It was definitely a pleasure to share this adventure and experience with you.

Finally I would like thank all the Janssen staff. We will see each other in the future. Thank you for the opportunity to begin my scientific career in such a stimulating research environment.

Resumo

A proteína Tau é responsável pela ligação e estabilização dos microtúbulos (MT) no citoesqueleto, sendo fundamental na função neuronal. A atividade desta proteína pode ser regulada por modificações pós-translacionais, como a fosforilação, que promovem a separação dos microtúbulos. A alteração na conformação da Tau provocada por uma deficiente regulação, como a híper-fosforilação, causa destabilização dos MT e agregação da mesma em filamentos helicoidais emparelhados e tranças neurofibrilares. Estas estruturas são uma das principais características na doença de Alzheimer (AD), e o seu processo de formação pode representar um dos principais motivos que leva a morte celular nas Tauopatias, inclusivamente AD e outras patologias neurodegenerativas.

Nos últimos anos, recursos têm sido empregues na descoberta de novas estratégias que permitam diminuir a formação ou diminuam a quantidade de agregados da Tau dentro das células. Estudos recentes identificaram a indução da autofagia através da rapamicina como um dos potenciais alvos no aumento da remoção de agregados proteicos associados a doenças neurodegenerativas, melhorando também a esperança de vida em ratos e outros modelos.

Recentemente, o nosso laboratório desenvolveu um modelo celular baseado no trabalho de Guo e Lee (2011) que mimetiza a agregação intracelular da Tau depois de induzida a expressão de uma forma mutada desta proteína seguido do *seeding* com fibrilas K18:P301L pré-agregadas. Neste estudo, foi possível demonstrar como a utilização destes modelos permite identificar novos compostos com atividade nas vias de redução da Tau. Curiosamente estas moléculas foram responsáveis pelo desenvolvimento de um fenótipo vesicular que identificámos como sendo lisossomas, derivados de um possível estímulo na via endócitica. As alterações na morfologia sub-celular foram acompanhadas por modificações em marcadores de autofagia, sem aumento no fluxo autofágico. Estes dados sugerem que o aumento na degradação de proteínas e estruturas por autofagia poderão ter origem em efeitos colaterais de outras vias em detrimento do estímulo direto.

Para além disso, testamos uma série de moléculas com atividade reconhecida e validadas para induzir autofagia ou bloquear a degradação no lisossoma. Foi demonstrado que no nosso modelo, a ativação da autofagia não é responsável pela remoção de agregados. Por outro lado, provámos que os lisossomas são extremamente importantes da degradação de agregados da Tau.

Por fim, usámos o fator de transcrição EB (TFEB) para aumentar a biogénese de lisossomas e a autofagia. Células transfectadas com este fator apresentaram menos agregados de Tau e um aumento na viabilidade celular. Quando considerados em conjunto, estes resultados demonstram que a biogénese de lisossomas seguida por estímulos na autofagia podem ser mais importantes do que a ativação da autofagia por si só.

Concluindo, com este projeto foi não só possível identificar os mecanismos dos compostos responsáveis pela degradação dos agregados de Tau, como também foi possível validar o TFEB como um potencial novo alvo na descoberta de novos fármacos.

Palavras-chave: Doença de Alzheimer, Tau, Agregação da Tau, Degradação da Tau, Sistema autofagia-lisossoma.

Abstract

Tau protein is responsible for binding and stabilizing microtubules (MT) in the cytoskeleton, thus supporting neuronal function. This protein activity can be regulated by post-translation modifications, such as phosphorylation, which promotes MT detachment. Tau misfolding provoked by abnormal regulation, like hyperphosphorilation, causes MT destabilization and Tau aggregation into paired helical filaments (PHF) and neurofibrillary tangles (NFTs). These structures are one of the main hallmarks in Alzheimer's disease (AD), and its formation process may represent the principal motive for cell death in many Tauopathies, including AD and other neurodegenerative disorders.

Over the last years, great efforts have been placed to find new strategies to either diminish the build-up or decrease the amount of aggregated Tau inside cells. Recent studies have identified induction of autophagy through rapamycin as a potential target in increasing the clearance of aggregated proteins associated with neurodegenerative diseases, as well as ameliorating life expectancy in rats and other animal models.

Recently, our lab developed a cellular model based on the work by Guo and Lee (2011) that mimics the intracellular aggregation of Tau after overexpression of a mutated form of this protein and seeding with pre-aggregated K18:P301L fibrils. In this study, we have taken advantage of the developed model to discover new compounds active in Tau reduction pathways. Interestingly these molecules were responsible for the development of a vesicular phenotype that we identified as lysosomes due to a possible stimulation of the endocytic pathway. The change of the sub cellular morphology was followed by changes in autophagy markers, with no increase in the autophagic flux. This suggests an increment in the degradation of proteins and structures by autophagy as a collateral result from the activation of other pathways rather than a direct stimulus.

Furthermore, we have tested a series of molecules with known and validated activity to induce autophagy or disable degradation via the lysosome. We showed that at least in our model, autophagy activation is not responsible for the clearance of aggregates. On the other hand, we have proven that lysosomes play a critical role in Tau aggregates degradation.

Finally, we have used the transcription factor EB (TFEB) to intensify lysosomal biogenesis and autophagy. Cells transfected with this transcription factor had less Tau aggregates and cell viability was slightly increased. When considered together, these results show that lysosomal biogenesis followed by autophagy stimulation may be more important for clearance of Tau aggregates than autophagy by itself.

In conclusion, we have not only determined the mechanisms targeted by the compounds responsible for the degradation of Tau aggregates, but also validated TFEB as a potential new target for drug discovery.

Key-words: Alzheimer's disease, Tau, Tau aggregation, Tau clearance, Autophagy-lysosomal system

INDEX

Abbreviations

AAV – Adeno-associated virus

Aβ – β amyloid peptide

AD – Alzheimer's disease

ALS – Amyloid lateral sclerosis

APOE – Apolipoprotein E

APP – Amyloid precursor protein

ATG – Autophagy related genes

ATP – Adenosine triphosphate

BBB – Blood brain barrier

BSA – Bovine serum albumin

CDK5 – Cyclin dependent kinase 5

CHIP – Carboxy-terminus of Hsc70-interacting proteins

CLEAR – Coordinated lysosomal expression and regulation network

CMA – Chaperone mediated autophagy

CNS - Central Nervous System

DIV – Day *in vitro*

DMEM – Dulbecco's modified Eagle's medium

Dox - Doxycycline

EOAD – Early-onset AD

ER – Endoplasmic reticulum

FAP – Familiar form of AD

FTDP-17 – Frontotemporal dementia and Parkinsonism linked to chromosome 17

GFP – Green fluorescence protein

GOF – Gain of function

GSK3 – Glycogen synthase kinase

HBSS – Hank's balanced salt solution

HD – Huntington's disease

HTS – High-throughput screening

HSP – Heat Shock Protein

hTauP301L – human full-length Tau containing P301L mutation

KD – Knock Down

LAMP1 – Lysosome associated protein

LC3B – Light chain 3B

LMP – Lysosomal membrane proteins

LOAD – Late-onset AD

LOF – Loss of function

LSD – Lysosomal storage diseases

MAP – Microtubule associated protein

MAPT – Microtubule associated protein Tau

MARKs – Microtubule affinity-regulating kinase

MBD – Microtubule binding domain

MOI – Multiplicity of infection

MPR – Mannose-6-phosphate receptor

MT – Microtubule

mTOR – Mammalian target of rapamycin

MVB – Multi-vesicular bodies

NFDM – Non-fat dry milk

NFTs – Neurofibrillary tangles

NP – Neurotic plaques

PBS – Phosphate Buffered Solution

PD – Parkinson's disease

PenStrep – Penicillin-Streptomycin

PICALM – Phosphatidylinositol-binding clathrin assembly protein

PHF – Paired helical filaments

PI3K – Phosphoinositol 3-kinase

PKA – Protein kinase A

PTEN – Phosphate and tensin homolog

PSEN - Presenilin

PP2A – Protein phosphatase 2A

PrP – Prionic Protein

QBI – QBI-HEK 293A Cells

R – Microtubule Repeat domain

RF – Risk factors

RFP – Red fluorescence protein

ROS – Reactive oxygen species

RT – Room temperature

Ser – Serine residues

TFEB – Transcription factor EB

TGN – *trans*-Golgi network

Thr – Threonine residues

Tyr – Tyrosine residues

UPS – Ubiquitin-proteasome system

V-ATPase – Vacuolar-type H^+-APTase

WT – Wild-type

Chapter 1 - Introduction

Neurodegenerative disorders are a form of pathologies that cause a progressive loss of neuronal cells in particular regions of the nervous system, thus leading to a decrease in neuronal functions. The most common forms of these disorders are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) (Dupuis, 2013). These pathologies represent a personal and a social burden, as it affects not only the capability of one to be independent and perform its daily life activities, but also affects its family and health system who spend time and resources taking care of this patients. Latest studies indicate that in all Europe there are an estimated 7.5 million people suffering from dementias, costing around 105 billion € in direct and indirect healthcare costs in 2010 (Olesen, 2011) [Figure 1]. The risk to develop AD is considered to double every 5 years after 65 years of age, and it's estimated that in 2030 around 15-20 million people will suffer from this type of dementia in the US.

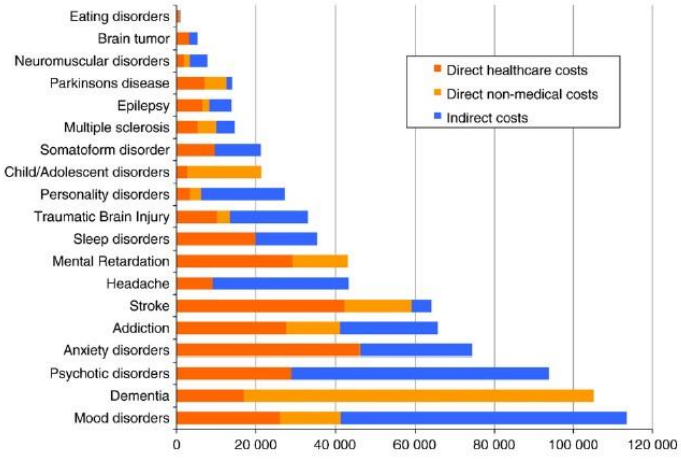

Figure 1 – Total cost of brain disorders and type of cost *(Olesen, 2011)*

Interestingly, all of these diseases share some common points: they're all age related and have a higher incidence in older people, a higher loss of specific neurons, mitochondrial dysfunction, axonal transport disrupted, higher reactive oxygen species (ROS) production, neuroinflamatory state, with consequent protein misfolding and aggregation. This last feature seems to be the main cause of neurodegenerative disorders since they are present in all of them, showing a direct relationship between protein aggregation and the disease (Shastry, 2013).

1.1 Alzheimer disease

Alzheimer's disease (AD) is the most common form of dementia worldwide, accounting for 50 to 75% of the cases, making an estimated 6 million patients in Europe and 35,6 million worldwide (Duthey, 2013). Although it was first reported in 1904 by German neuropathologist Alois Alzheimer, the causes by which the condition progresses are still unclarified and there isn't a cure that stops our reverse the course of the pathology. The life expectancy after AD diagnosis is set around seven years, although the beginning of the disease is defined several years before the first symptoms. Being a degenerative disorder, AD patients have a gradual decline in cognitive function and social behaviour, thus affecting patient activity and leading to a situation of total dependence on caregivers to do basic tasks (Natalwala, 2013).

1.1.1 Diagnosis

AD is characterized by the gradual depletion of cognitive function, with a progressive loss in learning and memory formation capability in the early-years (Dupuis, 2013). As the disease progresses the symptoms evolve to aphasia (disturbance in language comprehension and ability to speak), apraxia (inability to execute voluntary movements), and agnosia (loss in the capability to recognise people, environments, objects and shapes) (Natalwala, 2013).

Diagnosing AD is still one of the most challenging tasks for clinicians. The lack of reliable biomarkers and the coexistence of the disease features with normal age related symptomatology rise as obstacles for a diagnosis. Today's Alzheimer classification is based on the criteria set by the Diagnostic and Statistical Manual of Mental Disorders (DMS-IV) and the National Institute of Neurological Disorders and Stroke-Alzheimer disease and Related Disorders (NINCDS-ADRDA). The DMS-IV based diagnosis states the presence of memory disorder and the impairment in at least one cognitive function that affects social interaction or activities of daily leaving. As for the NINCDS-ADRDA the impairment of cognitive function that interferes in social behaviour is not mandatory, but it stipulates that there must be more than one symptom that indicates memory loss or other cognitive deficits (Scheltens, Feldman, & Dubois, 2007).

Mild cognitive impairment
Variably defined but includes subjective memory or cognitive symptoms or both, objective memory or cognitive impairment or both, and generally unaffected activities of daily living; affected people do not meet currently accepted dementia or AD diagnostic criteria

Amnestic mild cognitive impairment
A more specified term describing a subtype of mild cognitive impairment, in which there are subjective memory symptoms and objective memory impairment; other cognitive domains and activities of daily living are generally unaffected; affected people do not meet currently accepted dementia or AD diagnostic criteria

Preclinical AD
The long asymptomatic period between the first brain lesions and the first appearance of symptoms and which concerns normal individuals that later fulfil AD diagnostic criteria

Prodromal AD
The symptomatic predementia phase of AD, generally included in the mild cognitive impairment category; this phase is characterised by symptoms not severe enough to meet currently accepted diagnostic criteria for AD

AD dementia
The phase of AD where symptoms are sufficiently severe to meet currently accepted dementia and AD diagnostic criteria

Panel 1- Different forms of dementia that present the same symptoms and could be mistaken *(Scheltens, Feldman, & Dubois, 2007).*

Nevertheless, the current criteria is outdated and inaccurate for distinguishing AD from other conditions like Mild Cognitive Impairment, or to assess the different stages of the disease (preclinical AD, prodromal AD, and AD dementia) [Panel 1]. The development and improvement of MRI and PET scans as allowed the evolution of new biomarkers that are now being tested and approved for diagnosis purposes (Mosconi & Leon, 2007).

1.1.1.1 Neuropathology

AD is a neurodegenerative disease with a diverse set of symptoms that are related with the decline of multiple parts of the cortex. Post-mortem studies performed in Alzheimer's patients showed

the presence of two pathological hallmarks: abnormal Tau proteins that aggregate forming the Neurofibrillary tangles (NFTs), and extracellular filaments known as β-amyloid plaques formed by the small insoluble peptide Aβ (Vickers, Dickson, Adlard, & Saunders, 2000).

Regarding the NFTs, they're found both inside cellular compartments and extracellular. This fibrils are partially-insoluble, hyperphosphorylated Tau aggregates. They can be found in particular brain regions of AD patients, people with other neurodegenerative disorders and dementias as well as in normal aging individuals. (Pennanen, Schild, & Gotz, 2004)

As for β amyloid plaques (AβPs), this are extracellular aggregates of Aβ, a small peptide formed by 40-42 amino acids that is produced when APP is cleaved by a different pathway, as it will be explained ahead. AβPs can be subdivided in 3 main types, being the neurotic plaques (NP) the most important in degeneration. The NP, or "senile plaques", are more likely to be involved in cognitive impairment than the other diffuse AβPs. Besides, they are usually found near degenerating axons and neurons that contain NFTs (Nelson, Neltner, & Scheff, 2014).

In the early stages of the disease, AβPs and NFTs seem to be more distributed in the hippocampus and the medial temporal lobes, where the neuronal loss is more significant. These areas have been proven to be affected long before the first traits of the disease appear (de Leon, Rusinek, & Mosconi, 2007). The fact that these regions suffer for higher neuronal death and synaptic loss could help to explain the first symptoms of memory, learning and cognitive impairment. Eventually the lesions spread to other parts of the brain during mild AD phase, namely in the adjacent inferior temporal and posterior cingulate cortex. In the later stages, lesions start to

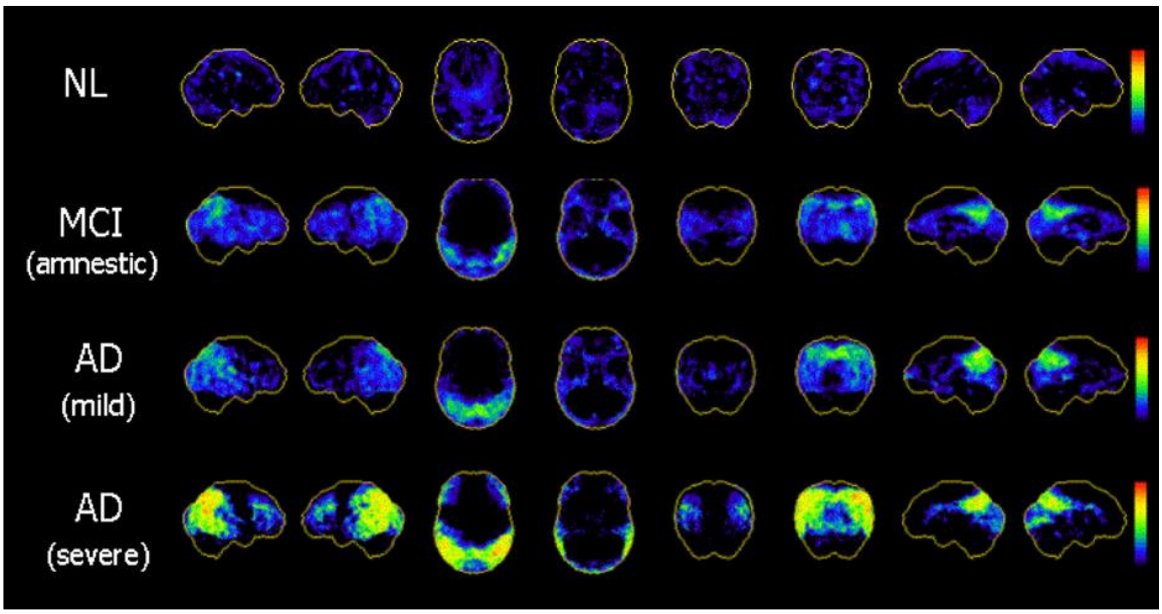

Figure 2 – PET scan showing the difference in cognitive impairment through the reduction in the metabolism. Results where compared with normal healthy individuals. Hypomethabolism was only found in MCI and AD in specific regions of the brain. NL – Normal, MCI – Mild cognitive impairment, AD- Alzheimer's disease. *(de Leon, Rusinek, & Mosconi, 2007)*

appear in parietotemporal and prefrontal association cortices, regions responsible for perception, attention and language (Braak & Braak, 1991).

1.1.2 Risk factors and genetics

The two main hallmarks of AD –amyloid-β deposits and Tau aggregates- start to appear long before the first clinical manifestations begin (Trojanowski, Toledo, & Shaw, 2012). During several years, risk factors (RF) could exert their effect in patient's brains, triggering the first steps for the onset of the disease and supporting its development. Although age cannot be seen as a RF itself, it is seen as the common denominator for the onset of the disorder. It is well stablished that with the aging process, the incidence of Alzheimer's cases increases with it. As for RF in the standard context, cardiovascular risk factors (CVRF) are found to be one of the most important elements. From this last, obesity (Young, Budge, Cherbuin, & Anstey, 2011), hypertension (more particularly higher level of diastolic blood pressure during midlife and lower at advanced age) (Peskind, Breitner, & Kukull, 2007), diabetes (Fang, et al., 2011), and high levels of cholesterol have been correlated with a higher predisposition for AD (Trojanowski, Shaw, Weiner, & Toledo, 2012). More recently high levels of cortisol and glucocorticoid from stress and post-traumatic stress disorder have been correlated with a greater incidence of this disorder (Chang, Wang, Lu, & Huang, 2009). Other RF identified are: alcohol consumption, thrombosis, homocysteine, smoking and existing cardiovascular disease (Breteler, 2000).

Although the environmental factors play an important role in the pathology, genetic risk factors have always been identified as a strong possible responsible in AD. Depending on the age of onset, we can identify two forms: early onset (<65 years-old) form of AD (EOFAD), and late onset (after 65 years-old) form of AD (LOFAD) (Nacmias, Bagnoli, & Sorbi, 2001). EOFAD is usually related with the familiar form of AD (FAD), mostly related to autosomal dominant mutations in proteins like: amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) (Van Broeckhoven, Sleegers, & Bettens, 2013). APP is the transmembrane neuronal protein that is sequentially cleaved first by β-secretase and then by γ-secretase, which is formed by a complex of PSEN1 and PSEN2, and that is responsible for the release of the Aβ peptide that forms the AβPs (Goate & Karch, 2014)

As for LOFAD, *ApolipoproteinE* (APOE) is the main identified risk factor. APOE is responsible for cholesterol transport, neuroplasticity, and inflammation. It also regulates the Aβ metabolism through the interaction with LRP1 receptor. Its gene is located on chromosome 19q13.2 and encodes three possible alleles: ε2, ε3, ε4 (Breteler, 2000). One allele, APOε4, is responsible for a 3 fold increased risk in AD, and two APOε4 for 15 fold risk when compared with APOε3ε3. On the

other hand, the expression of APOε2 gene reduces the probability to develop the condition (Haines, Cupples, & Farrer, 1997)

In spite of the mutation presented previously, there are an increasing number of polymorphisms identify that seem to be related with the disease. These don´t have the power to develop the disorder by itself, but when combined in the genome, they can increase the risk and probability associated with AD. Recent advances in microarrays have allowed to disclose several polymorphisms in AD patients: ABCA7, BIN1, CASS4, CD33, CD2AP, CELF1, CLU, CR1, DSG2, EPHA1, FERMT2, HLA-DRB5-DBR1, INPP5D, MS4A, MEF2C, NME8, PICALM, PTK2B, SLC24H4-RIN3, SORL1, ZCWPW1 (Goate & Karch, 2014). This polymorphisms were identified in genome-wide association studies (GWAS) using big groups of patients of EOFAD and LOFAD (Lambert, 2013).

1.1.3 Molecular hallmarks of AD

AD could be explained by the existence of two major macromolecules. Aβ plaques were the first to be discovered and associated with the condition, being considered through many years as the most important player. The second theory involve Tau aggregation resulting in NFTs, and although it was proposed more recently, it has gained many supporters. Currently, specialists assume both hypotheses are correct, contributing to the pathology through different mechanisms.

1.1.3.1 Amyloid hypothesis

Amyloid hypothesis is centred on the Aβ peptide which appears as β amyloid plaques near meningeal and cerebral vessels. Aβ is a small peptide (40-42 amino acids) that results from the cleavage of the Amyloid protein precursor (APP).

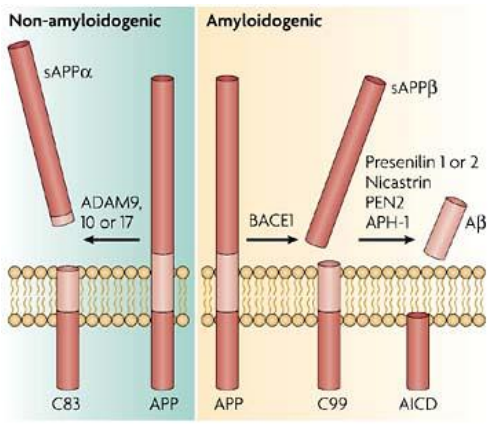

Figure 3 – Schematic representation of the two main pathways in APP metabolism *(Oddo, Green, & LaFerla, 2007)*.

APP is a type I transmembrane glycoprotein involved in several mechanisms inside the cell, particularly: lipid metabolism, cell adhesion, synaptogenesis, neuroprotection and neurotrophic effects (Inestrosa, Nunez, Colombres, & Morgan, 2004). APP is encoded by one gene that contains 19 exons, and that can generate three isoforms through alternative splicing. These proteins are classified according to the number of amino acids present: 770, 751 and 695 (Chasseigneaux & Allinquant, 2012).

APP can be metabolized by two pathways with different outcomes [figure 3]. In physiological conditions, amyloid protein precursor is cleaved by α-secretase enzymes (ADAM 9, 10 or 17), thus originating the soluble peptide sAPPα in what is called the non-amyologenic pathway. This molecule was shown to be involved in neuroprotection, neurogenesis, cell proliferation, among others (Chasseigneaux & Allinquant, 2012). Since the breakdown is performed within the Aβ region no harmful component is released. The resulting domain, the C83, is then cleaved by γ-secretase, releasing a short fragment called p3 that doesn't show any physiological activity (Oddo, Green, & LaFerla, 2007).

The amyologenic pathway begins with the cleavage of APP by β-secretase, or BACE, that releases the peptide sAPPβ from the transmembrane element C99. After this step, γ-secretase (PEN2, APH-1, presenilin, nicastrin) cleaves C99 releasing the Aβ monomer to the extracellular environment. The resulting Aβ molecule can have 40 amino acids – the more common, designated $A\beta_{40}$ – and the 42 amino acids, which accounts for only 10% but is more prone to aggregate and presents higher neurotoxicity (Oddo, Green, & LaFerla, 2007).

In the familiar form of AD, mutations were found in APP and in the enzymes responsible for the metabolism of Aβ like PEN2, by the PSEN1 gene (Takahashi, Almeida, & Gouras, 2005). On the other hand, in the sporadic form there's not only a decrease in this enzymes, but also a deficiency in miRNA. This is responsible for higher levels of β-secretase, and APOE, mainly by the isoform $APOE_4$, which tend to decrease the rate of its clearance (De Strooper, Delacourte, Papadopoulou, Horré, & Hébert, 2009).

The formation and release of the Aβ peptide seems to play an important physiological role inside the central nervous system. Studies suggest that at low concentrations that do not allow the formation of oligomers, the monomers function as: trophic factors to undifferentiated hippocampal cells, protecting against excitotoxicity, stimulus to differentiation and proliferation, and is released during brain development (Chasseigneaux & Allinquant, 2012).

In contrast, in pathological conditions, the higher cleavage and release of Aβ is responsible for the aggregation of the same in oligomers (from 3-4 monomers up to 24) in structures that are sometimes called Aβ-derived diffusible ligands (ADDLs). Evidence suggest that is this form that is responsible for the primary neuronal circuit loss mainly through disruption of synaptic plasticity and interaction with dendritic spines by changing is function, maintenance and morphology (Klein, Viola, & Lacor, 2007). Furthermore, ADDLs can also interact with mGluR5, reducing its movement and promoting its clustering. This event is responsible for an increase in intracellular calcium that can cause excitotoxicity (Triller, Klein, & Marianne Renner, 2006). In later stages oligomers will

aggregate even more leading to the formation of fibrils and amyloid plaques which present less neurotoxicity than the oligomers (Glockshuber & Finder, 2007).

Aβ and its aggregates tend to accumulate in the extracellular space since its precursor, APP, is a transmembrane protein that is cleaved in the outer side. Nevertheless, recent studies have proven that Aβ can accumulate inside the cell and more important, that the oligomer can spread from one neuron to another through neuron-to-neuron transmission. Although not fully understood, this mechanism represents a step forward in the comprehension and understanding of the spreading in AD through anatomical connections and symptoms (Marcusson, Granseth, Agholme, & Nath, 2012).

1.1.3.2 Tau hypothesis

The second theory to be proposed was the Tau hypothesis. This one states that the neuronal death is based on the hyper phosphorylation and consequent aggregation of the microtubule-associated protein Tau (MAPT), a small protein responsible for the stabilization of microtubules. MAPT, or simply Tau as it is more commonly denominated, tends to aggregate in structures called neurofibrillary tangles (NFTs) which are commonly present in a set of diseases called "tauopathies" that could comprehend: Alzheimer's disease, Down's syndrome, progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementias like Parkinsonism linked to chromosome 17 (FTDP-17). (Yao & Hall, 2005)

A correlation in AD was found between the presence of NFTs and neuronal death in the structures already focused above. Nowadays the pathological diagnosis made on post-mortem must include the presence of these structures, proving therefore its relevance (Smith, Nunomura, Moreira, & Lee, 2005).

Today's debate in Tau hypothesis is focused on which is the real cause for neuronal death: from one side it can be the loss of stabilizing microtubules function by Tau, but on the other hand it can be explained by a gain of toxic function of the oligomers. Since this is a wide and vast area, we will discuss the Tau hypothesis in the next chapters.

1.1.3.2 Link between Aß and Tau

Recent studies showed that there is in fact a co-existence of both mechanisms of pathology, rather than just one. It has been observed that the Aβ accumulation precedes and promotes the Tau hyperphosphorylation (Ribé, Perez, & Gómez-Islaa, 2005). Even more, in post-mortem analysis to the brain structures of AD and Down's syndrome patients, $A\beta_{42}$ oligomers were found in CA1

pyramidal neurons with early signs of Tau hyperphosphorilation. This data highlight the idea of a temporal relation between the two elements (Takahashi, Capetillo-Zarate, & Gouras, 2010).

Evidence also suggests that both molecules can have a synergetic effect neuronal toxicity. Firstly Aβ oligomers are responsible for altering some physiological processes, affecting not only synaptic transmission and receptors proper function, but also dendrites and axons morphology and integrity. Secondly, NFTs lose the ability to stabilize the microtubules thus interfering with axonal transport of vesicles and mitochondria. Finally the two factors are directly toxic to neurons, meaning that there will be an accentuated loss of cells (Coleman & Yao, 2003).

In the past years several efforts have been made to develop animal models to mimic AD in order to study and comprehend better the mechanism behind it. Transgenic mice were breed to express human mutant forms of APP and Tau, and results showed that not only Aβ precedes Tau hyperphosphorilation, but also that Tau is an important collaborator in amyloid toxicity. In this, Tau knock-out and knock-down has proven to decrease Aβ harmful effect, therefor demonstrating that this dementia is caused by the combined action of the two hypothesis (Ittner, Ke, Delerue, & Wölfing, 2010).

1.2 Tau protein

Tau is a small protein found in high concentration at the central nervous system (CNS). Known for its ability to control microtubule polymerization and stabilization, Tau plays a critical role in cytoskeleton organization, axonal transport and neurotransmission machinery (Clavaguera, Tolnay, & Grueninger, 2014). It belongs to the group of microtubule-associated proteins (MAPs) and it's encoded by the MAPT gene localized on the chromosome 17q.21.31 (Andreadis, William, & Kosik, 1992).

From the chromosome processing, exons 2, 3 and 10 undergo a process of alternative splicing, yielding a total of 6 isoforms in the CNS. The exons 4A, 6 and 8 are not expressed. The protein is composed by 4 regions [figure 4]:

1. An acidic region on the N-terminus (exons 1-5);
2. A region rich in proline (exon 7 and beginning of 9);
3. The microtubule-binding domain (MBD), responsible for the interaction with microtubules (exons 9 to 12).It has a 18 amino acid repeat denominated R1, R2, R3 and R4;
4. The C-terminus encoded by exon 13 (Andreadis, William, & Kosik, 1992).

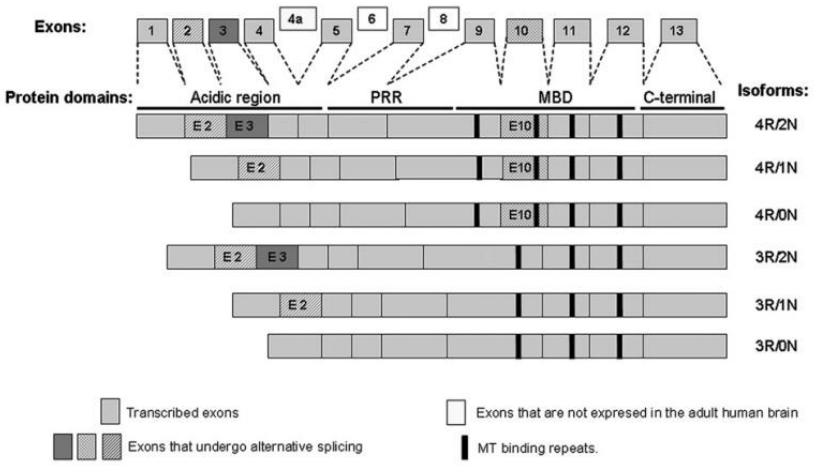

Figure 4 – Schematic representation of Tau mRNA and its alternative splicing, as well as the different regions. The black bars that encode the MT binding repeats represent the 18-amino acids repeats that differentiate the 3R and 4R isoforms. *(Cardenas, Ardiles, Barraza, & Caviedes, 2012)*

The six different isoforms can be divided using two criteria. From one side the existence of none, one or two insertions of 29 amino acids repeats in the acidic region is responsible for the 0N, 1N and 2N forms. On the other side, two groups come from the MBD constitution: the 3R where exon 10 is spliced; and the 4R where exon 10 is encoded. This way, we can classify the 6 different isoforms in: 0N3R, 1N3R, 2N3R, 0N4R, 1N4R and the longest 2N4R (Bullmann, Holzer, Mori, & Arendt, 2009).

The difference in the presence of the exon 10 can help to explain the differences in the affinity for the microtubules, to which 4R isoforms present stronger connections and higher stabilization properties than 3R (Panda, Samuel, Massie, & Wilson, 2003). The ratio between the 3R and 4R isoforms change during life-time as a response to different development stages, e.g.: during embryonic development, the brain needs a more dynamic structure to allow a higher rate of mitosis and motility, whereas the adult brain demands a more stable cytoskeleton. This means that during periods of neuronal plasticity there is an increase in Tau 3R form as a mechanism to change microtubule dynamics (Sennvik, Boekhoorn, & Van Leuven, 2007). Recent evidence shows an imbalance of the ratio of 3R and 4R Tau in some tauopathies like Down's syndrome and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Cardenas, Ardiles, Barraza, & Caviedes, 2012).

1.2.1 Post-transcriptional modifications

Tau protein can undergo many post-transcriptional modifications that are responsible for regulating its activity and the conformational state. Not all of these modifications are responsible for inducing aggregation in NFTs. For instance, glycation can be responsible for a higher oxidation and less degradation of Tau, but is unable by itself to induce aggregation. Among the changes

responsible for this process we can point phosphorylation, truncation, nitration, polyamination, ubiquitination, and oxidation. On the other hand, changes like glycosylation and prolyl-isomerization are responsible for the opposite effects (Martin, Latypova, & Terro, 2011)

1.2.1.1 Tau kinases

Phosphorylation is the most important post-transcriptional modification in Tau since it has a direct role in changes on the cytoskeleton organization and is also responsible for the aggregation in NFTs when occurring in pathological levels. In physiological conditions, this protein is phosphorylated at about 31 serine, tyrosine and threonine residues. Studies suggest that the phosphorylation of Ser262 and 356 by MARK (microtubule affinity-regulating kinase), Ser214 by protein kinase A (PKA) or Ser416 by Ca^{2+}calmodulin-dependent protein kinase (CAMK), could have moderate influence on tau-microtubule interactions (Mandelkow & Maldelvow, 1998).

The MBD (regions R1-R4) is known to possess a consensus motif KXGS that can be phosphorylated on Ser262 and 214 promoting Tau detachment, thus destabilizing microtubule organization. This process doesn't necessarily mean pathology since microtubule rearrangement is necessary for neuronal development, mainly for cell migration and division at early stages. In fact, phosphorylation at Ser214 and several other Ser-Pro motifs represents a critical process for mitotic cells. For instance MARK activity appears to be important for the establishment of cell polarity (Illenberger, 1998). This could be achieved by an increase in MBD phosphorylation and a decrease in the flanking domains. However, its deregulation can be one more mechanism by which aggregation can occur. This supports the idea that tauopathies result from a change in the phosphorylation pattern (Stoothoff & Johnson, 2005).

The correct functioning of Tau is therefore tightly related with its state of phosphorylation, which is in its turn the result of kinase and phosphatase activities and the equilibrium they promote. Changes in this ratio promotes an abnormal phosphorylation of Tau that eventually may cause aggregation of the protein in NFTs. In the next sections we will study the action of the three main kinases: GSK3, CDK5 and MAPK.

GSK3

Glycogen synthase kinase 3 (GSK3) is the most important and studied kinase in Tau phosphorylation process, therefore it was previously known as Tau kinase I. This enzyme is constituted by two very similar forms: GSK3α and GSK3β. Although expressed by two different genes, they share 85% homology (Schaffer, Wiedau-Pazos, & Geschwind, 2003). GSK-3 is

constitutively active and can be inactivated by phosphorylation of Ser^{21} in GSK3α and Ser^{9} in GSK3β (Hernández, de Barreda, & Avila, 2010).

GSK3 is responsible for the phosphorylation of 17 serine and 5 threonine residues in the surroundings and inside of the MBD in physiological conditions. Because of the close interaction between this enzyme and the MBD, the structure responsible for the interaction with microtubules, GSK3 is considered a critical player preventing the interaction between the two structures. (Hanger, Anderton, & Noble, 2009).

Recently it was found that in AD cases, GSK3 can phosphorylate Tau in 29 sites besides the ones usually involved. (Hanger, Anderton, & Noble, 2009). GSK3 overexpression in mice is also able to induce hyperphosphorylation as well as neuronal death (Lucas, Hernandez, & Avila, 2001) (Bhat, Leonov, & Lee, 2002). In addition, Aβ oligomers were demonstrated to induce GSK3 expression, and in rat hippocampal neurons Aβ exposure is capable of inducing tau phosphorylation and apoptosis (Amadoro, Corsetti, & Calissano, 2011). This data strengthens not only the role of GSK3 enzyme in AD, but also the existing relation between Tau and amyloid hypothesis.

CDK5

CDK5 belongs to the group of cyclin-dependent kinases family. Even though it doesn't have activity as a cell cycle regulator. When enzymatically active, CDK5 is responsible for a phosphorylation profile similar to GSK3, but for this to happen it needs to be associated with its co-activator p39/35 and p29/25. Since p35 is abundant in neurons, this kinase is highly active in these cells, making the complex CDK5/p35 a major player in brain development and functioning. In addition, p25 (an alternative truncated form of p35) can also interact with this enzyme and is present in all cells with lesser expression. This form is thought to be the responsible for the dynamics in microtubules associated with mitosis (Stoothoff & Johnson, 2005).

Higher activation of CDK5 is found in some neurodegenerative disorders and tauopathies, mainly because of elevated levels of p25 in brains of Alzheimer's patients (Rademakers, Sleegers, Theuns, & Van den Broeck, 2005). This could be due to longer half-life and expression of p25 in pathogenic conditions, which will eventually deregulate the cells ability to control phosphorylation when compared to the CDK5/p35 complex. Moreover, post-mortem studies in Alzheimer's patient's brains showed a higher ratio of p25/35, reinforcing the hyperphosphorylated stage (Cruz, Tseng, & Goldman, 2003).

The CDK5/p53 complex share a pattern of phosphorylation in Tau similar to the one found in GSK3, and in the case of Tau at least 11 residues that are found in AD can serve as substrates for CDK5.

Studies showed that the presence of this enzyme could be the responsible for some of the changes seen in AD (Baumann, Mandelkow, & Biernat, 1993). In addition, it's thought that CDK5 is responsible for a first phosphorylation of Tau in specific sites that will make it a better substrate for GSK3. Finally, CDK5 can also phosphorylate neurofilaments, APP and presenilin-1 (Lau, Howlett, & Kesavapany, 2002).

MARK

Microtubule affinity-regulating kinase (MARK) is an enzyme that regulates a different set of cellular functions like: cell division, organelle trafficking, cellular growth and microtubule stability, and other undisclosed activities. Its activity can be regulated by many post-transcriptional modification, mainly through phosphorylation, being the position of the amino acid modified critical for the final outcome. Activation of MARK1 can be achieved by the MARK2 and STK11 kinases due to phosphorylation at Thr208. On the other hand, MARK2 can be inhibited by GSK3β also by phosphorylation at Ser219. This could be seen as negative feedback signalling and a strategy from the cell to reduce the amount of kinases and phosphorylation (Matenia & Mandelkow, 2009).

Generally Tau is phosphorylated by MARK in the serine residues of the KXGS domains in the R domains, more precisely at Ser262, 293, 324 and 356. Besides these regions, MARK can also act in Ser305 and 320. These post-transcriptional modifications are responsible for the detachment of Tau from microtubules, inducing its self-aggregation (Drewes, Ebneth, & Preuss, 1997).

In conclusion, this enzymes can be responsible for some of the changes seen in AD pathology, making Tau kinases inhibitors a great starting point for research and the development of new drugs.

1.2.1.2 Phosphatases

To counteract the actions of the kinases, cells have developed a mechanism that quickly dephosphorylates Tau protein by the involvement of phosphatases. Several of these proteins have already been identified, like: PP1, PP2B and the most important and studied PP2A.

Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that accounts for almost 1% of all proteins present in cellular tissues. Because of is function and abundance, this enzyme plays a critical role in development, cell growth and specially in regulating signalling cascades (Janssens & GORIS, 2001). PP2A activity allows the cell to control its functions by maintaining a state of dephosphorylation. In our case, isoform ABαC is responsible for Tau binding and it is likely the main Tau phosphatase (Sontag, Nunbhakdi-Craig, & Lee, 1996). Phosphatases are a major

regulator in cellular activity and its activity allows neurons to maintain axonal stability as well as preventing hyperphosphorylation of Tau.

1.2.2 Tau pathogenicity

As it was already stated, one of the main hallmarks of AD is the existence of abnormal quantities of Tau protein free in the cytoplasm that tend to form aggregates. In fact, this pathological process is not exclusive of Alzheimer's but instead is characteristic of many dementias generally known as tauopathies. Other conditions in which neurofibrillary tangles are commonly observed include frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), corticobasal dementia (CBD) and Pick's disease. In the next sections we will go deeper in the cellular and molecular mechanisms involved in these pathogenesis.

1.2.2.1 Tau Hyperphosphorylation

Early studies in brains of AD and other tauopathies patients, have identified Tau tangles in higher concentrations and in a hyperphosphorylated state (Ihara, Nukina, Miura , & Ogawara, 1986). Further reports indicated that this process occurs independently of the pathology or the underlying cause for it. Even more important it was shown that the deposition of Tau aggregates happened even in the absence of a gene mutation leading to amino acid changes or alterations in the 4R/3R ration, only needing to be hyperphosphorylated to assemble (Hanger, Anderton, & Noble, 2009).

As it was already covered in previous chapters, Tau phosphorylation can occur in 3 types of residues: serine, tyrosine and threonine. In normal conditions, Tau can be phosphorylated in 31 residues of its 85 possible sites (45 serines, 35 threonines and 5 tyrosines). However, in pathological situations this number can increase to 45 (maybe even more), most of them in the MBD, causing the decrease in the binding of Tau to microtubules (Hanger, Anderton, & Noble, 2009). After the dissociation from the microtubules, this protein continues to be modified through phosphorylation and other alterations that increase the chance of aggregation. Whether phosphorylation is the main catalyser for Tau aggregation is still a matter of debate, mainly because *in vitro* assembly of aggregates is inefficient. The same process can be however bypassed by the use of polyanions like heparin and oxidizing molecules like DTT (Paudel & Li, 1999).

In addition, some Tau mutations could be responsible for a higher predisposition to phosphorylation and therefor to aggregation. One of these mutations is hTauP301L, where the substitution of the proline for the leucine amino acid in the MBD is responsible for a higher affinity to the kinases, increasing its phosphorylation and consequent detachment from microtubules.

Therefore, this mutation is used in transgenic mice and cellular models to induce Tau aggregation to study the implications of tau hyperphosphorylation and formation of NFTs (Dutschmann, Menuet, & Stettner, 2010). Other mutations found in some tauopathies on Tau with impact on its activity are: G272V, ΔK280, P301S, V337M, R406W (Lee, Jung, & Hall, 2009).

One of the theories for the abnormal rate of Tau phosphorylation involves one of its main kinases, the GSK3. Recent experiments showed that the Aβ peptide is responsible for the GSK3 overexpression and activation through the insulin pathway, thus contributing to an increase in its kinase activity (Avila, de Barreda, & Engel, 2010) (Hernández, 2010). This hypothesis could help to explain not only the coexistence of Aβ peptides and hyperphosphorylated Tau in AD patients, but also the first appearance of the amyloid oligomers followed by the NFTs.

Besides GSK3, other kinases and phosphatases seem to be involved in the abnormal rate of phosphorylation, explaining the different patterns seen in Tau. Among the kinases, CDK5 and DYRK1A by direct action, and CK1 and c-Abl by priming action, are proven to be related with the pathological effects in the protein (Hanger, Anderton, & Noble, 2009). Since so many major proteins are involved in different pathways, enzyme inhibitors discovery for therapeutic uses has been delayed. Interestingly, it was found that it is the free form of phosphorylated Tau that produces direct toxicity for the cells rather than its tangles. This suggests that the aggregation process could be a self-defence mechanism of the cell in order to increase its survival (Crespo-Biel, Theunis, & Borghgraef, 2014) (Morsch, Simon, & Coleman, 1999). In the next chapters we will discuss the main characteristics and process of Tau aggregation.

1.2.2.2 NFTs

Although there's still a great discussion about the mechanisms by which aggregation occurs, it is commonly accepted that the imbalance in phosphatases and kinases activity is responsible for the phosphorylation of residues in the MBD and that the dysfunction in the clearance pathways leads to an accumulation of free phosphorylated Tau (Hanger, Anderton, & Noble, 2009). [Figure 6]

The increase in soluble hyperphosphorylated Tau in the cytosol makes the protein more prone to interaction with other elements responsible for post-translational modifications. This process is responsible for the loss of Tau native form by reducing its natural folding and increasing the β-sheet conformation. In fact, the β-sheet conformation seems to be a critical feature of Tau in order for the beginning of the nucleation process (Chirita, Congdon, Yin, & Kuret, 2005). Thereby the protein becomes more stable leading to its dimerization in an anti-parallel manner. Since the dimers are highly stable there is a rapid formation that eventually evolves to the development of oligomers. In the last years, additional attention has been given to Tau oligomers and its possible harmful effects. Last reports indicate that oligomers not only appear in the early phase of AD and FTDP, but also demonstrated higher toxic effects for the cell when compared with other Tau elements,

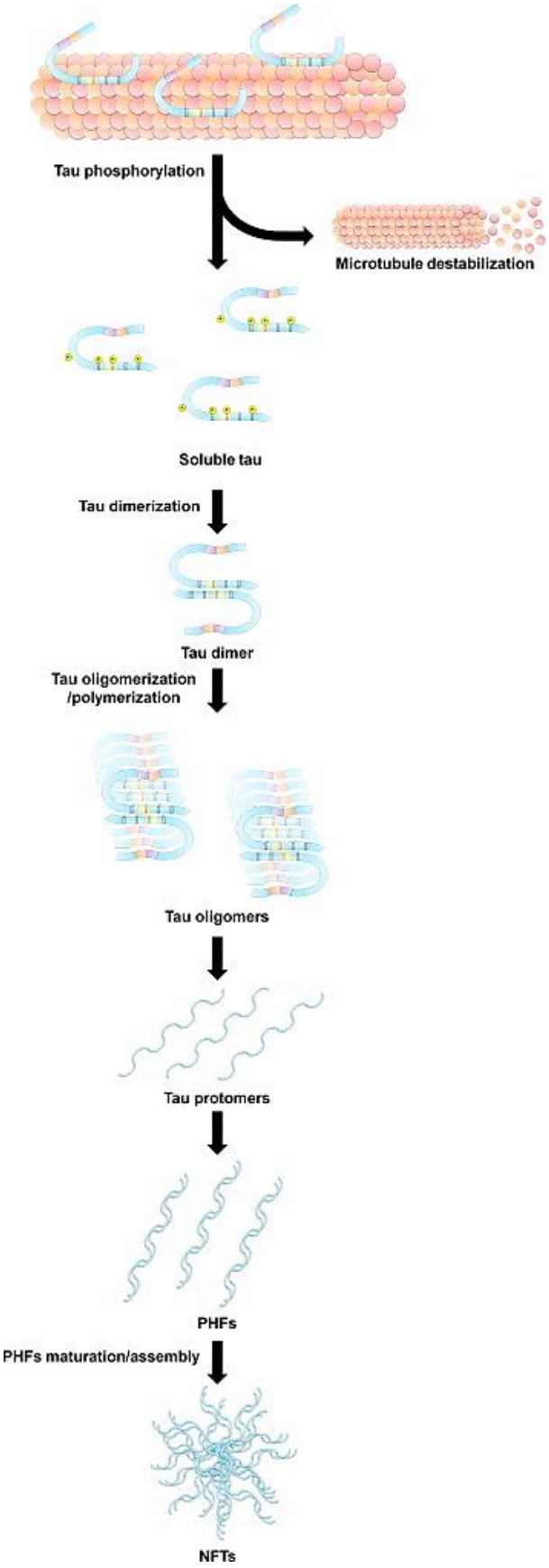

Figure 6 – Process of NFTs formation. In physiological conditions Tau protein stabilizes microtubules. However, when it's highly phosphorylated tends to aggregate in oligomers and NFTs *(Martin, Latypova, & Terro, 2011)*

inducing synaptic and mitochondrial dysfunction as well as memory impairment (Maeda, Sahara, & Saito, 2006) (Lasagna-Reeves, Castillo-Carranza, & Sengupta, 2011).

The oligomers continue to aggregate between them until they reach the length of 20nm. At this time the fibrillization begins with the formation of filaments called protomers that eventually forms dimers in a highly stable structure called paired helical filaments (PHF). Over time this structure assemble together forming one of the most characteristic elements of AD, the neurofibrillary tangles (NFTs) (Maeda, Sahara, & Saito, 2006).

In the first years of research in AD at molecular level, it was believed that NFTs and PHFs where the main responsible for neuronal toxicity. This assumption was based not only on the post mortem studies, where the coexistence of these fibrils with neuronal lesions point a possible relation, but also by the fact that these aggregates are involved in some cellular dynamics alteration. Among other modifications, the NFT and PHF where associated with disruption in vesicular, mitochondrial and endoplasmic reticulum transport through the axons and dendrites (Ebneth, Godemann, Stamer, & Illenberger, 1998).

However, this paradigm was changed in the recent years, supported by the appearance of new models of the disease. Nowadays it's accepted that the existence of NFTs inclusions in neurons could not be seen as a specific biomarker for tauopathies, since it was found that cells can survive several years with the tangles (Morsch, Simon, & Coleman, 1999). Furthermore, NFTs deposition may play a critical role as a response factor to increase neuronal survival and act as a neuroprotector. One of the positive actions associated with NFTs is the action as anti-oxidant factor. By preventing oxidative stress it can prevent neuronal loss by apoptosis and also is responsible for the non-activation of kinases by reactive oxygen species (ROS) (Smith, Casadesus, Joseph, & Perry, 2002). Since oxidative damage appears in the initial stages of AD, it correlates well with the early manifestation of NFTs (Nunomura , Perry, Aliev, & Hirai , 2001).

Taking into account the last considerations, questions arise whether the neuronal loss is a reflection of toxic effects provoked by modified Tau, or by dysregulation in microtubules dynamics. These possibilities will be discussed in the next sections.

1.2.2.3 Tau gain and loss of function

Although important breakthroughs have been made during the last years in understanding AD, many questions still stand unsolved. One of the mysteries is how Tau correlates with neuronal death. To explain this event there are two theories: the loss or gain of function [Figure 7].

Loss of function (LOF)

One of the most well-known functions of Tau protein is the role in microtubule stabilization. Microtubules are special cellular elements made from dimers of α and β-tubulin that polymerize in hollow cylinder structures from the microtubule organizing centres (MTOCs). These are responsible for many cellular functions like: formation of the cytoskeleton, that gives structural support and organization; providing tracks for vesicular and organelles transportation; involved in mitoses and meiosis, particularly in the formation of the mitotic spindles and chromosome division. Because of the normal functions the cell has to perform, microtubules constantly need to assemble and disassemble, making this structure a highly dynamic one. Nevertheless, cytoskeleton must preserve some stability, especially in neurons, in order to allow the normal traffic of cellular components like mitochondria's and vesicles. To achieve this, Tau and MAP2 among other interacting proteins, are responsible for the binding and stabilization (McNally , 1996).

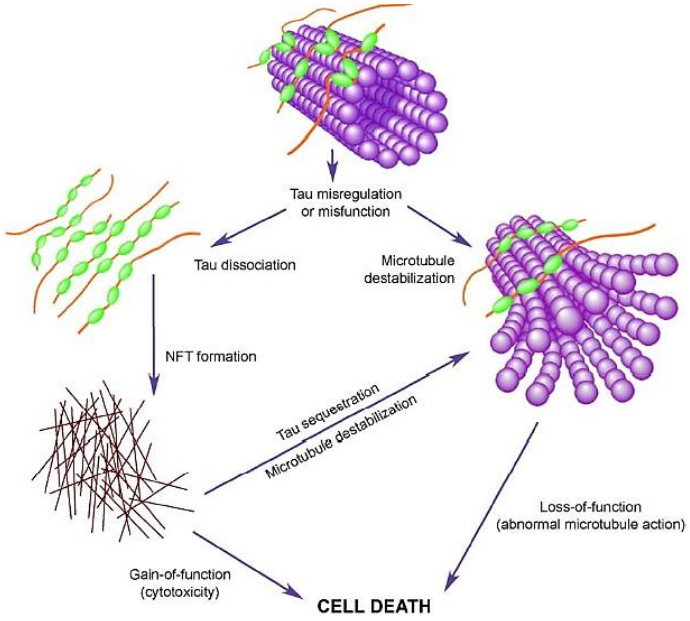

Figure 7 – The 3 different hypothesis for the neurotoxicity caused by Tau dysfunction: the gain-of-function, the loss-of-function, and the incorporation of elements from the two pathways. Adapted from: *(Feinstein & Wilson, 2005)*

In Tauopathies and particularly in AD, the hyperphosphorylation of Tau and subsequent detachment from microtubules creates an impairment of its normal function. This creates a disruption in the normal organelles transportation that has a critical impact on synapses, mainly because of the higher demand of energy, synthesis and vesicles. When Tau hyperphosphorylates, it compromises the regular transportation, leaving the cells with less capability to deal with stress and prone to cellular death (Mandelkow, Stamer, & Vogel, 2003). In addition, it was shown that neuronal death provoked by microtubule destabilization correlates well with the structural impairment and cognition deficits present in dementias associated with tauopathies (DeKosky, Schef, & Styren, 1996).

Moreover, it was found that Tau can also interact with actin filaments. This element is also part of the cytoskeleton being responsible for the mediation of the transport and the structural features

of the cell. Although it is still unclear what the specific role of Tau is in this structure it was reported that the increase in its hyperphosphorylation can destabilize the actin organization (Ko, DeTure, Sahara, & Chihab, 2005). In addition, it was also found that Tau can bind to cellular membranes and that as in the other situations already presented, the over-phosphorylation is responsible for a decrease in the binding (Pooler, Usardi, Evans, & Philpott, 2012).

All combined, the loss of function hypothesis is based on the decreased physiological activity of Tau provoked by a pathological phosphorylation. Without this mechanism the cell loses the ability to function properly, thus becoming more susceptible to environmental aggressions that finally culminates in neuronal death.

Gain-of-function (GOF)

In contrast to the last proposed hypothesis, the gain-of-function pathways is based on the novel properties of Tau after hyperphosphorylation. As it was already stated during this work, Tau hyperphosphorylation and aggregation was proven to present a higher toxicity for the cell, mainly by the presence of the oligomers. This was proven not only by an increase in neuronal death, but also by the loss of synaptic markers, the induction of mitochondrial dysfunction and the impairment in memory encoding and consolidation (Lasagna-Reeves, Castillo-Carranza, & Sengupta, 2011).

Interestingly the formation of NFTs has been correlated with a reduction in the overall neuronal death, leading to the idea that the formation of these structures could act as survival strategy (Yoshiyama, Higuchi, & Zhang, 2007). The idea behind this hypothesis is that by aggregating all the harmful Tau, i.e.: Tau oligomers, the cell lowers the impact of its toxic effect.

One of the proposed mechanisms that leads to Tau induced toxicity is *via* the activation of the tyrosine protein kinase FYN. In this model, the accumulated hyperphosphorylated Tau in dendrites interacts with FYN that is targeted to the postsynaptic region where it phosphorylates the NMDAR. This event promotes the formation of complexes between the receptor and the post-synaptic density (PSD95), thus creating a cluster of glutamatergic receptors that can be responsible for excitotoxicity (Spires-Jones, Stoothoff, & de Calignon, 2009) (Ittner & Götz, 2011).

Another interesting model for the gain of toxicity is the Tau axis hypothesis for AD. This combines the action of β-amyloid and Tau and is divided in two phases. In the first stage, the hyperphosphorylated Tau targets FYN to interact with NMDARs that sensitizes the receptor for the amyloid-β toxicity. On a second period, the NMDAR is continually stimulated by β-amyloid plaques present in the AD patient brain. Since β-amyloid increases Tau phosphorylation, and Tau

acts on the NMDAR, there is a constant aggression to the neurons. This model helps to explain the progression features present in AD (Ittner & Götz, 2011).

In conclusion, none of the hypothesis described above occurs in an independent manner, rather it is a mixture of both. In this theory, genetic and environmental factors are combined to promote Tau hyperphosphorylation and detachment from microtubules. The loss of stability is responsible for the impairment of cellular transportation, thus disrupting the normal neuronal function and leaving the cell more susceptible to environmental changes. In addition, fibrils formation is responsible for the abduction of free Tau that is responsible for the regulation of microtubules dynamics. Furthermore, the harmful effects of Tau and β-amyloid on post-synaptic receptors and other organelles like mitochondria, are responsible for direct aggression to neurons, thus inducing their death (Feinstein & Wilson, 2005).

1.3 Prion-like propagation of Tau

One of the main challenges in Tauopathies like AD, is to comprehend and understand the mechanisms by which the disease progresses. As it was already stated, the presence of NFTs and the neuronal death in specific regions of the brain correlates well with dementia progression, particularly with clinical symptoms. Nevertheless, the reason by which there is specificity in the regions affected and why there are similarities between different neurodegenerative disorders, cannot be explained by the theories already covered. One of the possible mechanisms that has been more explored recently is the Prions hypothesis.

1.3.1 Prions Hypothesis

The prions hypothesis is based on a set of common diseases, known as prion diseases, which share some features with neurodegenerative disorders. This include time progression that can be adapted to explain some of the features. This concept was first introduced in 1986 by Stanley B. Prusiner, which stated that a misfolded protein, commonly known as prion, was the main cause of several diseases known as transmissible spongiform encephalopathies (TSEs, also called Prions disease) that include among others: Kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker (GSS) disease, and others identified in animals (Prusiner, Prions: Novel Infectious Pathogens, 1986) (Liberski, 2014) (Heppner, Prinz, & Aguzzi, 2001).

Prions were first described as proteins lacking nuclear acids that were able to induce protein aggregation and had infectious properties similar to the ones observed in bacteria and virus. Nowadays this concept has evolved to assume that prions are made of prion protein (PrP) aggregates. The normal prion protein (PrP^{C}) is a membrane protein expressed in all cells, with especially localization in neurons and glia. It is constituted by 209 amino acids (in humans) with

unknown activity that presents a three α-helix structure (Viles, Cohen, & Prusiner, 1999). However, the infectious element is made by a protease-resistance isoform, the PrP^{Sc}, which is only present in patients with prions disease. This has a different constitution that changes the conformation from the normal α-helix structure to a β-sheet, thus increasing its ability to aggregate and forming the prion (Hope, 2000).

The reason by which PrP is changed in patients with prions disease is still unclear. It was found that the mutation in amino acid 101 (from P to L) is responsible for two conformations of the protein: the non β-form and the β-form. From these two, only the last one has shown the ability to induce prions disease (Kaneko, Ball, & Wille, 2000). This is due to the fact that PrP^{Sc} is able to bind to PrP^{C} thereby changing its natural structure and catalysing the polymerization of new protease-resistance molecules to its surface. This process is known as heterogeneous nucleation, and is the responsible for the exponential growth rate of prions (Orgel , 1996).

Another factor for the rapid increase in its concentration is the fact that at some point de polymer breaks and originates two similar structures that will continue to aggregate more PrP. This process is known as seeding, and only a few of these structures per cell are needed for an exponential growth, only depending on the existence of monomers. The oligomeric forms act as seeds by stabilizing the transformation of PrP monomeric forms (Masel, Jansen, & Nowak, 1999). [Figure 8]

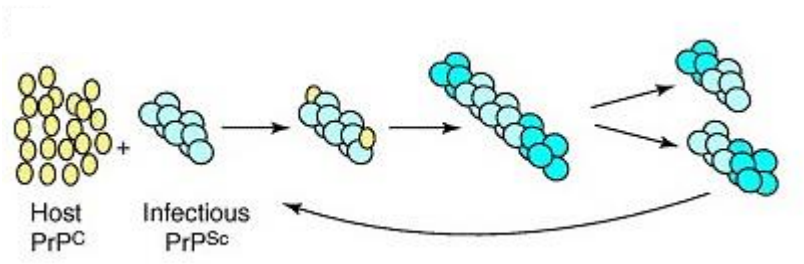

Figure 8 – Proposed model for prions formation. After the induction of aggregation by PrP^{Sc} the strains start to form and grow until a time where it breaks. The new structure will then act as a seed. Adapted from *(Soto, Estrada, & Castilla, Amyloids, prions and the inherent infectious nature of misfolded protein aggregates, 2006)*

Another important aspect of the prions disease characteristics is the infection-like behaviour that correlates with the seeding effect. This feature is responsible for the progression of the disorders from one region of the brain to the rest of the neuronal circuits and to adjacent localisations. This is only possible due to the fact that one oligomer of PrP^{Sc} is responsible for the conversion of the PrP normal proteins, therefore only a single seed is needed in one cell to induce polymerization (Sigurdsson, Wisniewski, & Frangione, 2002) (Soto & Saborío, 2001). Since it was proven that prions are able to move intra and extra-cellular, it is possible to explain the sequential impact on neuronal circuits. In fact, the ability to transfer between cells is a central feature of prions disease,

and in some cases, it´s even possible to cross between species (Kuku and BSE). This principal is also important in the development of models, as it will be discussed furthermore.

1.3.2 Prion-like behaviour of Tau

Three decades ago a model based on the prions disease mechanism was proposed as a possible explanation for AD progression among other neurodegenerative disorders (Prusiner, 1984). However, only a few years ago this hypothesis started to be explored and tested for the different pathologies based on the new research in protein aggregation and spreading between cells (Goedert, Clavaguera, & Tolnay, 2010). Since the prions disorder involves the formation and spreading of misfolded proteins with β-sheet structure, the same principle can be applied to β-amyloid, Tau and α-synuclein proteins [Figure 5] (Frost & Diamond, 2010). These proteins that share the prion-like characteristics are named "prionoids" (Aguzzi & Rajendran, 2009).

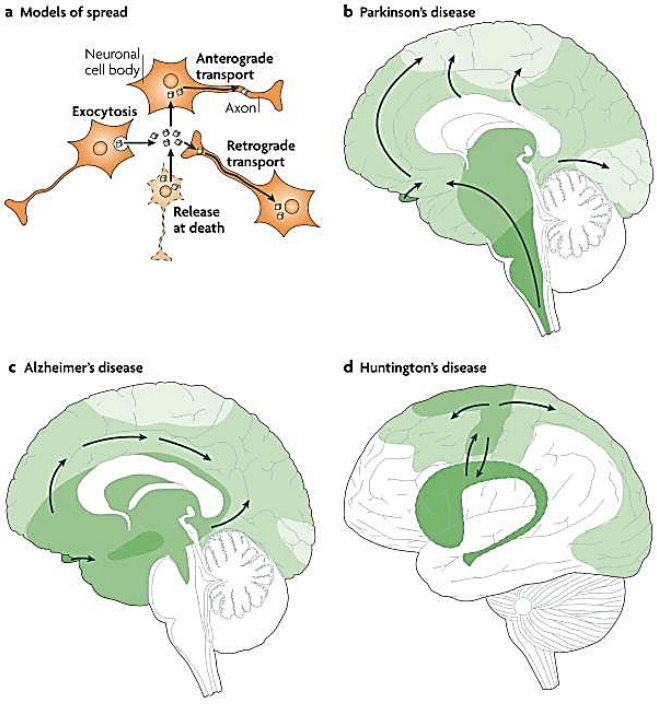

Figure 5 – Prion behaviour in neurodegenerative disorders. **A** Protein aggregates can be released at neuronal death or exocytosis and uptake by endocytosis by adjacent neurons. **B, C, D** Spatiotemporally spreading of protein aggregates in different pathologies *(Kopito & Brundin, 2010)*.

In AD cases, both β-amyloid and Tau where shown to be able to form strains after contact with mutated proteins. Exposure to different prionoids conformation could lead to different strain structures and phenotypes, thus explaining the various clinical symptoms in patients (Tanaka, Collins, Toyama, & Weissman, 2006). The fact that Tau has six isoforms and all of them can be expressed in NFTs support the last theory.

A critical feature of this model that helps to explain the behaviour of Tau in patient's brains is the ability to spread from one neuron to another and to propagate from one cell to another (Fukutani, Kobayashi, & Nakamura, 1995). Tau aggregates were shown to be able to enter the cell by endocytosis via engulfment of the cellular membrane, and not by penetrating the membrane as it was first proposed. Afterwards the aggregates are able to induce the aggregation of other Tau proteins that don't have a mutation. Like in the prions disease, they change its conformation and

begins to polymerise (Frost, Jacks, & Diamond, 2009). The same experiment with β-amyloid demonstrated some of these properties, although extracellular amyloid β sheet molecules lack some of the seeding capabilities present in neurofibrillary tangles (Petkova, Leapman, & Guo, 2005).

Experiments in other forms of tauopathies, more precisely argyrophilic grains (AGs), showed a spatial and temporal spreading Tau inclusions that correlates well with the observation in AD. In these cases the abnormal tangles started in a specific region of CNS and with time spread to the other associated zones (Saito, Ruberu, & Sawabe, 2004). In addition, it was also proven recently that different Tau strains are able to propagate through mouse neurons and induce different tauopathies (Sanders, Kaufman, DeVos, & Diamond, 2014).

As described above, Tau has de ability to behave as a prion until a certain point. Not only this helps to explain some of the features observed in tauopathies, but also constitutes an opportunity to develop new models for aggregation. Shortly, it's possible to have cell lines expressing either wild-type or mutated forms of Tau and induce aggregation inside cells by simply adding pre-aggregated pathological Tau to the medium (Lee & Guo, 2011). This model is highly useful for HTS, as it allows to induce pathological hallmarks, like Tau aggregation, in a short period of time.

1.4 Tau degradation

As it was covered before, phosphatases play a critical role controlling the amounts of free Tau in the cytoplasm, therefor controlling its physiological function. Besides this enzymes, there are two systems responsible for maintaining a physiological amount of free Tau in the cytoplasm of the cell that regulate its degradation: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system.

1.4.1 Ubiquitin-protease system

The ubiquitin-protease system is responsible for the regulation of protein levels and their quality control by elimination of the ones that are misfolded, damaged or mutated (Glickman & Ciechanover, 2002). The process begins with the conjugation of the UPS substrate with a 76 residues protein, designated Ub, in a series of steps mediated through an enzymatic cascade (E1, E2 and E3 enzymes). The polyubiquitinated protein is then targeted to a structure composed by at least 33 subunits, called proteasome, to be degraded in smaller peptides of 3 to 24 amino acids (Pickart & Cohen, 2004).

Studies showed that UPS is a major player regulating Tau concentration in cells, therefor being directly involved in its aggregation process. The PHF tangles exhibit preferential ubiquitination on

Lys254, Lys311, and Lys353 residues (MBD region), showing relation between the amino acids affected and the decision whether Tau is degraded or aggregates (Cripps, Thomas, Jeng, & et al, 2006).The evidences also suggested that dysregulation of proteasome proper function is responsible for accumulation of ubiquitinated Tau proteins in PHF and NFTs.

CHIP/Hsp70

The most important enzymes mediating the ubiquitin process are the CHIP, Hsp70 and Hsp90 proteins. Concerning carboxyl terminus of the Hsc70–interacting protein (CHIP), this is an E3 ligase of Tau responsible for the last step of its ubuquitnation.

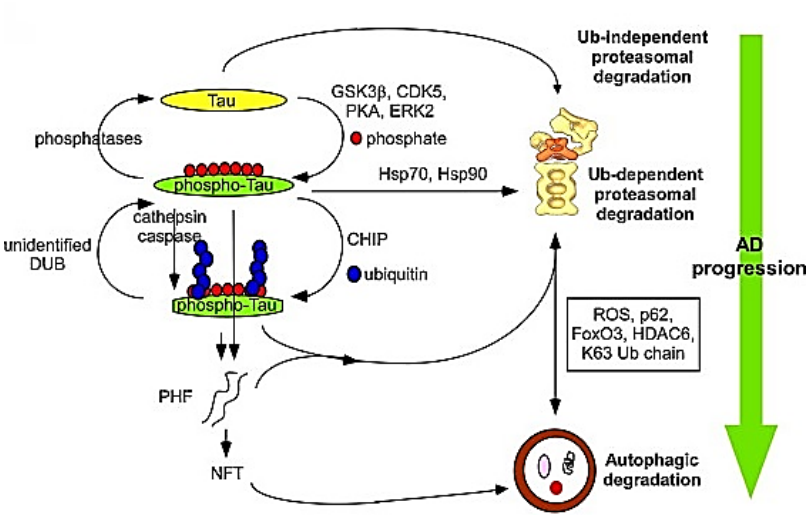

Figure 6 – Different pathways for Tau degradation. In this picture we can relate the different step in the degradation of Tau as AD progresses, involving the UPS via CHIP and Hsp70/90 and the Autophagic mechanism. *(Petrucelli, Dickson, & Kehoe, 2004)*

Additionally, CHIP interacts with Hsp70 as a co-chaperone, mediating the direct link between the chaperone quality control system and the UPS, thus allowing a degradation path for the proteins who cannot be refolded (Petrucelli, Dickson, & Kehoe, 2004).

Hsp70 and Hsp90 are heat shock proteins (Hsp) or chaperone molecules and their main function is to help in the correct folding of newly synthesised proteins and refolding of the tertiary structure, thus preventing their aggregation. Hsp70 is one of the most important chaperons in the cell. Not only does Hsp70 interact with polypeptides produced in the ribosome, thus preventing early misfolding, but it can also interact with already synthesized proteins in a *de novo* posttranslational folding process for the ones that need the help of intermediaries to reach the functional structure. Approximately 15-20% of all the proteins in the cell interact directly or indirectly with this chaperone during their biogenesis, mainly because of its capability to interact with other chaperones like Hsp40 and Hsp90 (Feldman & Frydman, 1995).

In relation with Tau, Hsp70 and Hsp90 where proven to promote Tau solubilisation and prevent its aggregation. Moreover these chaperones maybe responsible for targeting the hyperphosphorylated and Tau to the proteasome machinery, via CHIP, for its degradation (Dou, Netzer, Takashima, & Xu, 2003). Therefore, after taking into account all these data, we can propose the chaperone and CHIP pathways as possible new targets for pharmacological drugs.

1.4.2 Autophagy-lysosome system

Autophagy is a general name given to the process of targeting long-lived proteins and damaged cellular organelles in the lysosome. Not only this process is important regulating the clearance of damaged protein and structures, it also represents a survival mechanism that can be activated in starvation situations to increase the recycling of nutrients and macromolecules important for cell maintenance. Induction of autophagy is responsible for the uptake of obsolete molecules that in the end will be degraded in the lysosome, therefor generating energy and building blocks that will be released in the cytoplasm to be used (Tee & Dunlop, 2014).

Autophagy was first observed in 1956 by Johannes Rhodin through the study of subcellular organelles in kidney epithelium cells using electron microscopy. At this time it was only possible to observe mitochondria's surrounded by a bilayer membrane. However, with the development of microscopy techniques it was possible to see organelles being degraded in 1959, and finally the term *autophagy* was introduced by Christian de Duve from the Greek word "self-eating" (de Duve, 1963). The development of new genetic and biochemical technics in the past decades as allowed scientists to understand better how autophagy is processed and what genes play a role in it (Selgen, 2011). Nevertheless, many questions are still unsolved and the study of its process is still complex and full of potential bias while analysing data. In the next pages we will address how autophagy proceeds from the initial stimulus to the final degradation of a product and how it can be studied.

1.4.2.1 Autophagy and autophagosome formation

Nowadays autophagy can be divided in three different subtypes: a) **macroautophagy**, a process by which a *de novo* formed isolation membrane expands and engulfs a portion of the cytoplasm – known as '*in bulk'* - or a specific structure – usually mitochondria, although it can comprehend almost very sub cellular component – to create a double-membrane vesicle denominated as autophagosome. The autophagosome is responsible for cargo delivery to the lysosome where it´s degraded; b) **microautophagy**, when invaginations of the lysosome or late autophagosome membrane allow the incorporation of proteins or small structures by a selective or *in bulk* targeting to the lumen of the same; c) and **chaperone-mediated autophagy** (CMA), a procedure where the targeting motif of a protein is recognized by heat shock proteins (e.g., HSC 70) that are responsible for the delivery of the same to transmembrane proteins in the lysosome, thus enabling the substrate translocation (Mizushima, Yoshimori, & Levine, 2010). [Figure 7]

Even though they have different denominations, the mechanisms have the same goal of degrading outdated and useless components of the cell in the lysosome, thus promoting its reusing and recycling. Macroautophagy, herein referred simply as autophagy, represents the most important and broadly studied process of degradation, therefor we will lay emphasis in this mechanism, taking into consideration the importance of the other two.

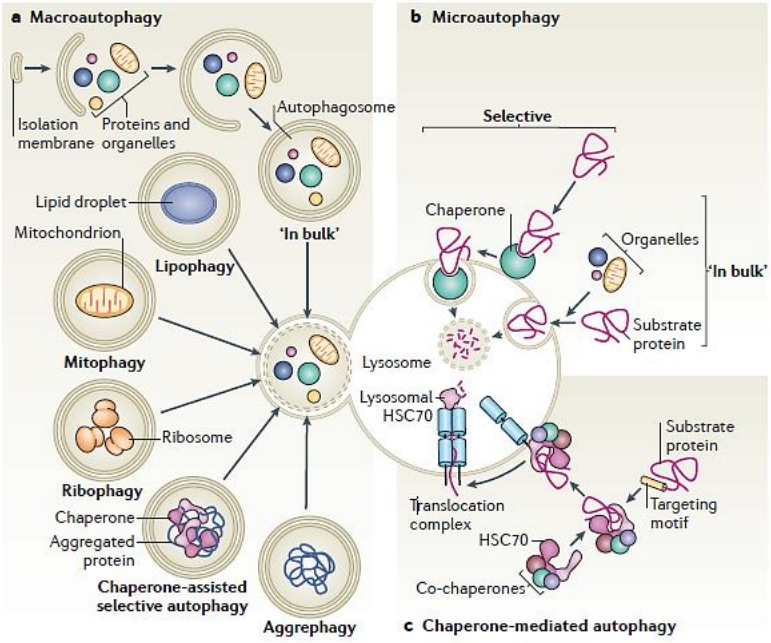

Figure 7 – The three types of autophagy. a) Macroautophagy is responsible for the formation of a double-membrane that isolates a substrate in a vesicle known as autophagosome that is delivered to the lysosome. b) In Microautophagy the invagination of lysosomal and autophagosomal membranes allows is responsible for substrate uptake. c) CMA uses chaperones and membrane proteins to deliver proteins into the lysosome. *(Cuervo, 2011)*

Autophagy is a well conserved process in all organisms, from simple eukaryotic organisms to mammals. Studies in yeasts have identified around 37 autophagy-related genes (ATGs), most of them codifying proteins with a human homologous. Briefly, this proteins are responsible for the recruitment and maturation of the membrane in the early stages and consequent maturation of the autophagosome. They work together in different complexes, like the ULK and PI3K complex, or in conjugation with LC3B. Besides the ATGs, there are also another group of proteins, commonly known as non-ATG, responsible for the initiation and control of the autophagy pathway. Among others, the role of this proteins can be subdivided in: 1) initiation of the autophagy process by kinases (mainly through mTOR, but also by RAPTOR and AMPK), 2) initiation and formation of the vesicles through coating proteins like Clathrin, 3) fusion of the autophagosome with the lysosome by SNAREs, 4) and regulators of the hole process by lipid-binding proteins and PI3K complex interactors (Ohsumi Y. , 2011).

On the overall, autophagy begins with the autophagosome formation and ends with the degradation of the substrate and release of the generated components by the lysosome. Autophagosome formation can be divided in three main steps: initiation, nucleation and expansion. This processes will be overviewed next.

Initiation. The initiation process begins with the ULK complex, which is composed by a series of proteins like: ULK1, ULK2, ATG13, FIP200 and ATG101 (Stork & Alers, 2012). This complex receives input from two main regulators: the mTORC1 and AMPK which integrate signals from other pathways. In normal fed conditions the mTORC1 (mammalian target of rapamycin complex 1) is bound to ULK and inactivates its action. In starvation stages however, the mTORC1 is inhibited and ULK becomes free, which leads to an increase in the kinase activity of ULK1 and ULK2 and to a consequent mediation of membrane recruitment (Tooze & Chan, 2009).

Also important for the beginning of the membrane formation is the class III PI3K complex, or Beclin 1 complex. This is composed by the vacuolar protein sorting 34 (VSP34), p150, Beclin 1 and ATG14 and interacts as well with ULK. When activated, the PI3K complex is also responsible for interactions with membranes that will target the nucleation process (Guan, 2013).

Nucleation. After initiation takes place, the ULK and PI3K complexes are responsible for the activation of pathways still not fully understood, that culminate in the increase of the autophagosome specific secondary messenger phosphatidyl-Inositol-3-phosphate (PtdIns(3)P or PIP3P) (Tooze & Simonsen, 2009).

Nevertheless, it seems likely that ULK phosphorylates VSP34 in the PI3K complex, initiating a set of events that begin with the recruitment of ATG proteins like DFCP1 and WIPI. This process allows the enrolment of proteins that are essential for the promotion and regulation of the membrane formation. Interestingly, VSP34 is also responsible for the formation of the early endosomal

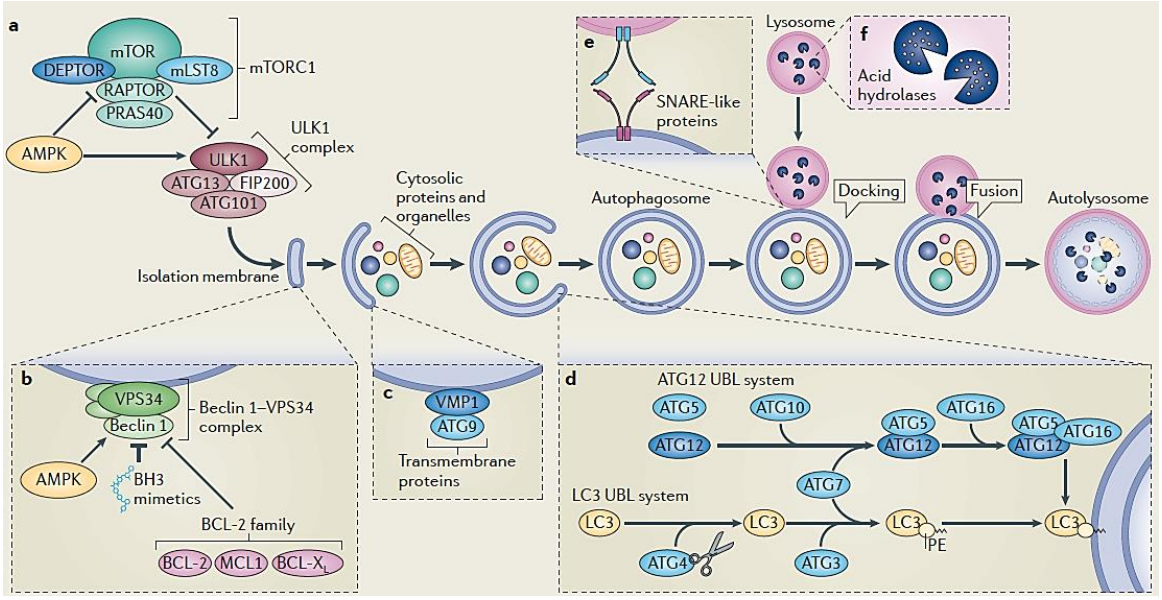

Figure 8 –Overview on the autophagosome formation. a) Protein complexes like mTORC1 and ULK1 are responsible for integrating signals from different pathways regulating the autophagy mechanism. b) Initiation of the process begins in the Beclin 1 –VSP34 complex with the PtnIns(3)P formation that triggers the recruitment of the membrane. c) Transmembrane proteins are critical for the stabilization and regulation of the membrane expansion. d) LC3 conjugation plays a critical role in the formation of the autophagosome, being one of the most widely used markers in autophagy *(Kroemer & Marino, 2014)*.

vesicle through a complex similar to PI3K that contains the UVRAG protein instead of ATG14. The endosomal pathway ends up being a primary source of vesicles that can be recycled or mature into lysosomes (Tooze & Simonsen, 2009).

Expansion. With the nucleation event a subset of ATG proteins, more precisely ATG12, ATG5 and ATG16L1, become active and available to interact with each other to form the ATG16L1 complex. After being formed, the complex is driven to the membrane where it has E3-like ligase activity, especially important in LC3B lipidation (Ohsumi Y. , 2011).

Microtubule-associated protein 1B light chain 3A (MAP1LC3B), or simply LC3B, is a soluble protein with a molecular weight between 16 and 18 kDa that is distributed ubiquitously in all mammalian tissues and was first discovered as the yeast homologue Atg8. After autophagy activation the cytosolic form, LC3-I (18 kDa), is conjugated with phosphatidylethanolamine (PE) through a reaction involving the ATG16L1 complex, thus originating what is commonly designated as the lapidated form, LC3-II (16 kDa). Besides the reaction promoted by ATG16L1 already covered, this modification can be also promoted through reactions similar to ubiquitination by Atg7 and Atg3 which have E1 and E2-like activity respectively (Kominami & Tanida, 2000) (Kominami & Sou, 2005). LC3-II is highly important on the recruitment and stability of the membrane while this is expanding. Even more it plays an important role in selective autophagy, as it can interact with the complex p62-SQSTM1 that binds to ubiquitin, allowing the targeting of structures and proteins flagged to autophagy (Ichimura & Komatsu, 2012).

1.4.2.2 Membrane origin and connection to other mechanism

The site where the process begins and the source of the membrane has been debated over the past years without a conclusive clear answer. It is commonly accepted that not only the origin of the membrane could be the result from synthesized *de novo* mechanisms, but also from cellular membranes of other organelles like the ER, Golgi, endosomes, mitochondria and plasma membrane.

Recent studies have identified the ER as the main provider of a platform that allows the initiation of autophagy. Many autophagic proteins and regulators seem to localize in the ER membrane or as protein complexes near this structure. The ULK1 and ATG16L1 systems have been shown to be present and active in the ER surface where they interact with other players like DFCP1, VMP1 and Beclin 1 leading to the synthesis of PIP3P and consequent autophagosome formation (Ktistakis & Walker, 2008).

It should be noted however that other organelles can still act as quick suppliers in the demanding stages, like the expansion process. In fact, different membrane providers and intervenient structures can modify the type of autophagy. For instance, the mitochondrial outer membrane marker NBD phosphotidyl-serine can be found in the autophagosomal membrane, suggesting a direct role in its formation as a membrane supplier (Lippincott-Schwartz & Hailey, 2010). Even more, mitochondrial proteins like BCL-2 has the ability to interact with the BH3 domain of Beclin 1 and can bind to pro-autophagic protein AMBRA1, stablishing a relation between the two organelles that could explain the selective autophagic process of mitochondrial degradation, mitophagy (Voeltz & Rowland, 2012).

Another important source of membrane, especially during the expansion and maturation of autophagosomes are the vesicles derived from the Golgi, plasma membrane and endosomes. They allow the delivery of different types of lipids and proteins produced *de novo* or recycled to the autophagosome, contributing this way to its final composition. Three proteins regulate the connection between this structures and autophagosome: ATG9, Rab proteins and class III PI3K complex. Their role and mechanism will be discussed in the next paragraphs.

ATG9 is a protein that under fed conditions is localized mainly in the *trans*-Golgi network (TGN) and late endosomes. However in starvation or low-nutrient conditions the distribution of this protein seems to favour the late endosome, increasing its dispersion into vesicles that interact with the expanding isolation membrane (Tooze & Orsi A., 2012) (Mari & Reggiori, 2010). Even though some of the functions and pathways of this protein are still unclear it is well stablished that ATG9 vesicles are an important source for membrane in autophagosome biogenesis (Ohsumi & Yamamoto, 2012).

VSP34 on its hand, forms a complex with other proteins creating the only class III PI3K known to date. It has a general distribution in all the vesicles inside the cell and is responsible for the formation of PtdIns(3)P, which plays a critical role in the formation of the autophagosome. This complex was recently detected on the Golgi, where along with Beclin 1 can interact with other resident proteins, like GAPR1, responsible for autophagy inhibition (Shoji-Kawata & Levine, 2013).

As for the RAB family, this is constituted by a group of small GTPases that regulate the events of formation and fusion between different vesicles in the endocytic pathway, even though some of them can be found in the Golgi and ER. They are particularly important for sorting the vesicles that will either a) continue to mature until they fuse with the lysosome, b) the ones that will be recycled and c) the ones that will fuse with the autophagosome to work as a source of membranes (Rink & Zerial, 2005). Other Rabs are responsible for different vesicle movements, for instance: Rab1 is

responsible for the promotion of ER to Golgi traffic and autophagosome formation, while Rab11 regulates the recycling of endosomes into autophagosomes (Colombo & Zoppino , 2010) (Fukuda & Itoh, 2008). Finally, a subset of the Rab family serves as helpers in complexes related with autophagy, like RAGA and RHEB that are essential for mTOR activity (Duran & Hall, 2012).

The endocytic pathway plays a critical role on autophagy, not only by supporting the membrane expansion, but also because it contributes to the development of structures like the lysosomes [Figure 9]. The pathway begins with the invagination of the plasma membrane promoted by the clathrin-coated pit formation mechanism. Once the structure is fully developed and independent from the membrane, clathrin detaches to be reused and the vesicles fuse between each other to form the early endosome. Early endosomes can then mature into late endosomes and multivesicular bodies (MVB) or be recycled to the plasma membrane or ER (Ravikumar & Rubinsztein, 2010). This process is regulated by Rab proteins, namely: Rab5 in early endosomes, Rab7 in late endosomes and Rab11 in recycling endosomes. In fact, it has been observed that Rab5 gets replaced by Rab7 through the endocytic pathway, and that Rab7 is a critical protein in the fusion process between the late endosome and the lysosome (Rink & Zerial, 2005). The specificity of the different Rab proteins regarding the structure they are associated with, helps the identification of the structure.

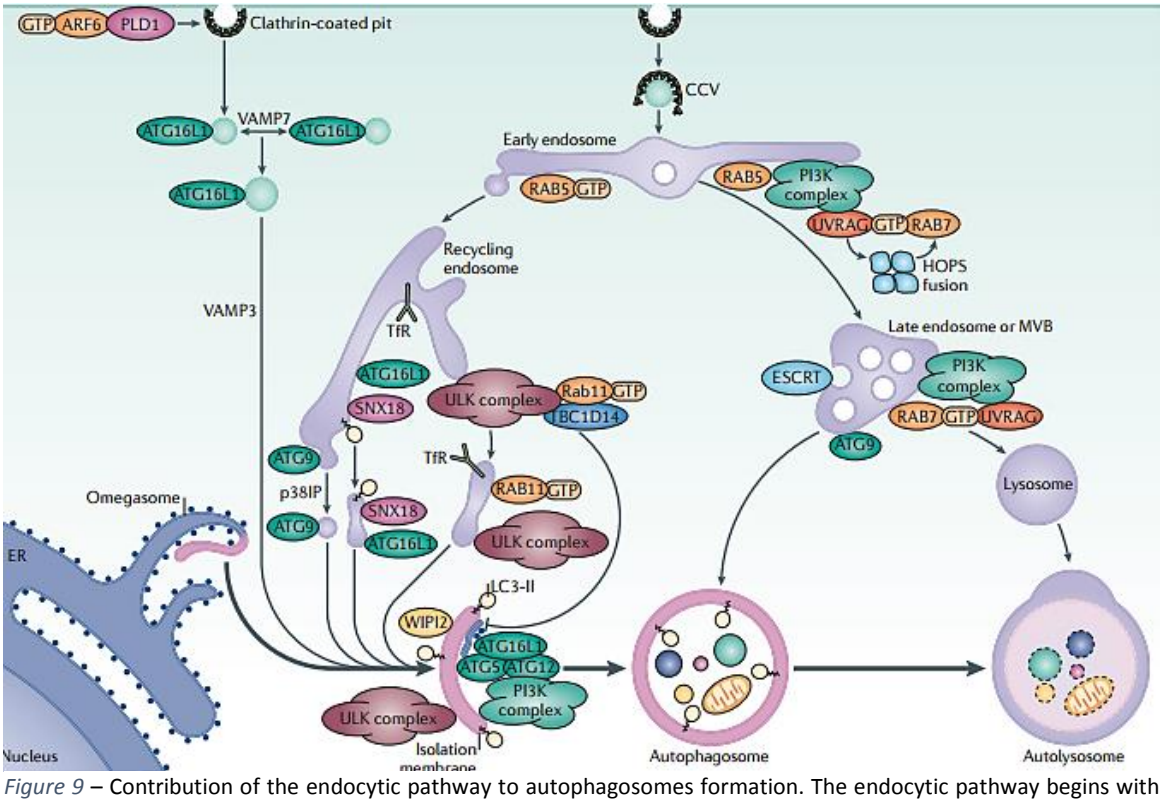

Figure 9 – Contribution of the endocytic pathway to autophagosomes formation. The endocytic pathway begins with the invagination of the cellular membrane by clathrin-coated vesicles. After the fusion with the early endosome, other vesicles can be formed and its fate is regulated mainly through the action of proteins belonging to the Rab family. Shortly vesicles from the endosome can maturate into late endosomes or multivesicular bodies, being recycled back to the plasma membrane or help in the expansion of the autophagosome *(Tooze & Lamb, 2013)*.

1.4.2.3 *Lysosomes and lysosomal biogenesis*

Lysosomes are subcellular organelles inside animal cells that constitute the primary degradative compartment. They receive their substrate through fusion with other vesicles that carry cellular debris, organelles and pathological microorganisms. The delivery can be achieved by fusion with autophagosomes, phagocytosis and late endosomes. There are around 50 soluble acid hydrolases responsible for the degradation of cargo and another 120 lysosomal membrane proteins (LMP) that regulate lysosomal fusion events and help to maintain the organelle integrity and pH (Sleat & Lübke, 2009).

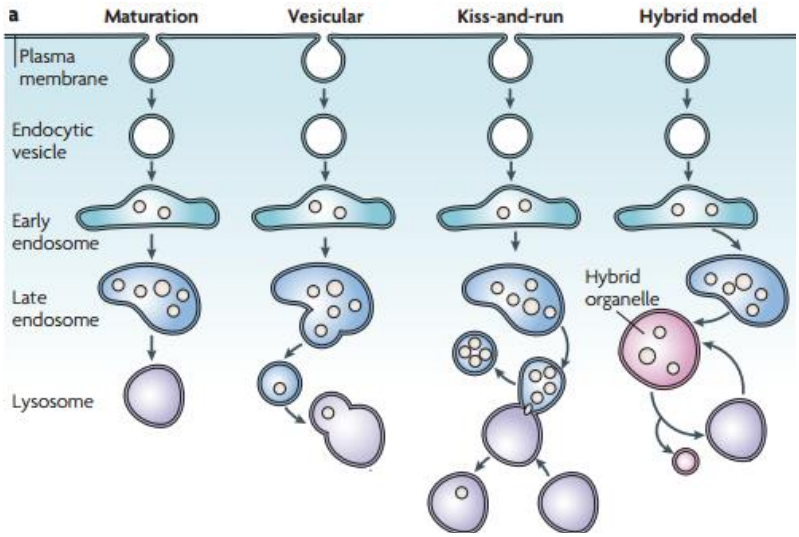

Figure 10 - Lysosomal delivery by the endosomal pathway. The maturation process involves the sequential addition of lysosomal components and loosing of late endosomal ones. In the second, vesicles detach from the late endosome to fuse with mature lysosomes. In the "kiss-and-run", small vesicles fuse temporarily with the lysosome to deliver cargo but are rapidly recycled. Finally, the hybrid model a mixed organelle that operates as a bridge, contains both endosomal and lysosomal components and latter maturation is required *(Bright & Paul Luzio, 2007)*.

Lysosomes formation and biogenesis is a critical step for cargo degradation since it represents the final stop in the process, and relies mainly on vesicle flux of the endocytic pathway. A summary of the process can be observed in Figure 10. Not only this process provides a good source of membrane to the new lysosomes, but also the interaction between the endocytic vesicles with the ER and Golgi provides the shipping of LMP and hydrolases to the final structure. The transcription and processing of new acid hydrolases starts in the cis-Golgi complex where they are tagged with mannose-6-phosphate and bind to mannose-6-phosphate receptors (MPRs). The delivery of this lysosomal proteins is taken care by the trans-Golgi Network (TGN) that communicates directly with the early endosomes, thus helping in lysosomal biogenesis (Bright & Paul Luzio, 2007).

One important feature of lysosomes is their acidic nature, which is achieved by the presence of proton-pumping vacuolar ATPases (Mellman & Helenius, 1986). This characteristic is critical for the function of the acidic hydrolases that require low pH to function properly. Also, the constant decrease of pH during endosomes maturation is responsible for the detachment of lysosomal proteins from MPRs and substrates engulfed by endocytosis from their receptors, thus allowing the delivery of cargo and the recycling of membrane receptors (Seamen, 2014). As the early

endosome matures into late endosome, the pH starts do decrease from 6 to 5, reaching 4,5 when it gets to the lysosomes. Also during this process there is the sorting of cargo that will continue. This allows the cell to manipulate and select which substrate will be degraded (Bright & Paul Luzio, 2007).

Lysosomal biogenesis is regulated by the transcription factor EB (TFEB) that is responsible for the expression of the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network. Together they are responsible for the transcription almost 450 genes, codifying proteins for most of the acid hydrolases, LMP, structural and functional related proteins and V-ATPases (Yang & Yamamoto, 2014). TFEB activity is manipulated by pathways related with nutrient availability and cell survival, such as the mTOR and AMPK ones (Ballabio & Settembre, 2011). Regarding mTOR, its suppression seems to be an inducer of lysosomes and autophagosome proteins expression, compliant with its autophagy inducer properties. Besides this, lysosome fusion blockage through Atg5 and Atg7 knockdown has the opposite effect, showing a decrease in lysosomal activity (Shen & Zhou, 2013).

1.4.2.4 Autolysosomes

Autolysosomes formation is the result of the fusion between lysosomes and autophagosome or amphisomes (autophagosomes that bind previously with endosomes), and is the last step of autophagy that promotes the delivery of the cargo to the place where the degradation will occur. Because the process is crucial for cell survival and cell homeostasis, many proteins act as regulators of the mechanism, mainly: lysosomal membrane proteins, such as V-ATPases and LAMP1; GTPases like Rab7; ATP binding proteins (UVRAG - UV resistance associated gene protein); cytoskeleton proteins; homotypic fusion and protein sorting (HOPS) complex; and SNARE proteins (Mizushima & Shen, 2014).

The SNARE complex is the most studied group of fusion proteins on the cell, being known to mediate the merging of most of the vesicles. Despite the general knowledge on this proteins, only a few set of SNAREs have been identified as mediators of autophagosome-lysosome fusion in mammalian cells. The vesicle-associated membrane protein 7 (VAMP7) is recognized as a major regulator of the process, and its sequestration in cholesterol enriched region of the endolysosomal membrane is thought to be responsible for lysosomal storage diseases (LSDs) (Ballabio & Fraldi, 2010). More recently the synthaxin (Stx) 17 (a Q-SNARE protein) has been identified as a central protein in the fusion regulation of lysosomes with autophagosomes, as well as in endosomal vesicles (Itakura & Mizushima, 2012).

1.4.2.5 Methods for the study of autophagy

The study of autophagy and its mechanisms has witnessed a great increase in interest over the past years, supported by recent works that connect this process with areas as important as cancer, neurodegenerative disorders, immunology and diabetes. Despite the attention it has received, many problems still stand when approaching autophagy. If from one side it is almost impossible to quantify a dynamic process with "stationary" methodologies, from the other one has to take into account that "form" or structures doesn't mean necessarily "function" or degradation. This being said the next paragraphs summarize the existing technics in autophagy study.

After the expansion process is finished and the membrane enclosures on itself, most of the ATG proteins dissociate to be recycled, while LC3 continues bound to the inner part of the autophagosome along with some other proteins. Due to this fact, LC3-II is widely used as a marker for autophagy and autophagosomes formation, since not only it's possible to determine the amount that is converted in the lipidated form, but also it can be tagged with GFP or monomeric tandem probes (mRFP-GFP) to study its localization and morphology by immunofluorescence (Kominami & Tanida, 2008). Moreover, biochemical assay like western blot can be used with or without autophagy inhibitors (e.g.: Bafilomycin A1) to study how a certain treatment influences the autophagic flux (Rubinsztein, Cuervo, & Klionsky, 2009).

However, special concern has to be taken into account while analysing data regarding LC3 expression. One should play attention to the fact that LC3 itself ends up being degraded by proteases after a fusion between autophagosomes and lysosomes. Also important is the fact that the process between activation of autophagy and autophagosome formation and degradation is rather quick. As an example, an observable increase in the amount of LC3 can imply an increase in autophagy or a decrease in the degradation rate. On the other hand, but in the same line, the inexistence of a meaningful difference doesn't transduce directly to no changes in the autophagic flux, as it can implicate the simultaneous increase in formation and degradation as well (Rubinsztein, Cuervo, & Klionsky, 2009).

In addition to LC3, other assays can be used to study autophagy. Electron microscopy, the first to be used in the identification of the mechanism in 1956, is still considered the best approach to show differences in autophagosome formation and lysosomal activity. The analysis has however to be confirmed by someone with experience in the field and results should always be validated with other methodologies in order to reduce false positive. Another approached developed in the 1970's tries to evaluate cargo degradation through long-lived proteins labelled with isotopes to measure lysosomal function (Mizushima , 2010).

In more recent years, the progress of genetic technics, like gene knock-down and more importantly, transgenic models such as yeasts, have allowed the identification of new genes and proteins with activity on autophagy (Delorme-Axford & Klionsky, 2015) (Sun & Du, 2013). This evolution, used in combination with the already mentioned techniques have expand our knowledge in the autophagy flux modifiers that are currently used as control compounds. Just as a small example, bafilomycin A1 is known to inhibit autophagy and all the endosomal pathway through acidic pH dispersion via vesicular-proton pump inactivation, while chloroquine has the same effect but through direct changes in the acidic lysosomal environment. On the other hand, Rapamycin induces autophagy *via* mTOR inhibition and Trehalose has the same action but through an mTOR independent pathway (Norberg, 2015).

It should be enlightened that there is no perfect technique or methodology to study autophagy in cells. Instead, one as to evaluate the best suited models for each case, taking into account the strong points and flaws that all have and use different assays before drawing any conclusions.

1.4.2.6 Autophagy in AD and Tauopathies

Many studies using cells, animals and even humans have found a correlation between the autophagy deregulation and neurodegenerative disorders. Autophagy is a highly important mechanism for the quality control of some organelles and proteins especially in neuronal cells. Potentially toxic components need to be identified, targeted, sometimes transported through long axonal projections and be degraded in the proper structures before they can induce cell death. Besides keeping homeostasis, autophagy is also important in the reshaping of neuronal terminals, thus contributing to neuronal plasticity (Komatsu & Yue, 2007).

It has been proven in the last years that autophagy loss in neurons is capable of inducing neurodegeneration in mice, and that lysosomal dysfunction can occur in every stage of human life with disastrous consequences (Komatsu & Tanaka, 2006) (Nixon & Lee, 2008). This is possibly the result of an inefficient clearance of misfolded and aberrant proteins inside neurons that can promote aggregation, as well as the sequestering of malfunctioning organelles like mitochondria (Wong & Cuervo, 2010). Among the diseases associated with pathological autophagic flux we can include Parkinson's, polyglutamine disorders (such as Huntington), as well as amyotrophic lateral sclerosis (ALS), besides AD (DiFiglia & Kegel, 2000) (Ravikumar & Rubinsztein, 2002) (Webb & Rubinsztein, 2003).

In AD brains a high concentration of autophagosomes and autolysosome has been observed, suggesting a pathological autophagy process. This could be due to an excessive formation of

autophagosomes induced by a higher concentration of PHF and NFTs, or on the other hand it could be related with a decrease in the autophagosome degradation (Lee, Lee, & Rubinsztein, 2013).

Interestingly, reports suggest that the lysosomal protease cathepsin D is responsible for the degradation of Tau and NFTs, and that the inhibition of the lysosomal activity is responsible for an increase in the aggregated forms of Tau (Kenessey, Nacharaju, Ko, & Yen, 1997). Another important point is the idea that the degradation could be related with Hsp70 which implies Tau degradation via the CMA pathway. This hypothesis is strengthened by the fact that this protein has two motifs which are believed to be involved in CMA targeting - ^{336}QVEVK^{340} and ^{347}KDRVQ^{351}- in the C-terminus region (Ciechanover & Kwon, 2015). Recent studies indicate that beside macroautophagy being responsible for Tau degradation and its aggregates, the CMA system can be targeted in the early stages. Moreover, it was surprisingly found that the cleavage of this protein by cathepsin L can produce two fragments (F1 and F2) that are highly amyloidogenic and have a seeding effect in Tau aggregation (Wang, Martinez-Vicente, & Kruger, 2009).

1.5 Current research on Tau

On the next chapters a quick and general overview will be made regarding the existing models for the study of new therapies for the Tau hypothesis, as well as the potential targets for new drug development.

1.5.1 Cellular and animal models

One of the most recent feature with higher potential in model developing for the study of tauopathies is the ability of a seed to enter a cell and to modulate the conformational state of Tau protein (Guo & Lee, 2011). This process, also known as seeding, is particularly important and has caught the attention of neuroscientist, since it can be applied in the development of new models of the disease in both cell culture and animals. Even more, not only it can be applied to study the progression of Tau pathology, but also in the identification and development of drugs that inhibit the disease progress. Since the early studies in the field, scientists have identified some mutations and conformational changes that seem to be involved in the pathology, mainly correlated with familiar forms of tauopathies.

The first studies on FTDP-17 have identified more than 42 mutations in Tau with possible relation with its aggregation. Some of them were inserted in transgenic animal models, like: N279K, ΔK280, P301L, P301S, V337 and R406W (Götz & Ittner, 2011). The first model to be developed was a transgenic mouse that over-expressed the longest isoform of human Tau. This model shows a higher rate of phosphorylation and a small portion of pre-aggregates formation (Götz , Probst , & Spillantini , 1995).

The second model to be design was the transgenic mice for the mutation P301L, which is nowadays the most generally used in tauopathies research (Lewis, McGowan, Rockwood, & Melrose, 2000). This specimen has a switch in the proline amino acid 301 to a leucine that localizes in the MBD and is responsible for a higher phosphorylation rate via GSK-3 (Rizzu, Joosse, & Ravid, 2000). Nowadays this is considered the strongest model not only because it correlates well with observations in patients mutations, but also because it is highly effective in promoting hyperphosphorylation and Tau aggregation. Therefore, it has been used not only in *in vivo* studies but also as a primary cell culture model. Since these early developments, other changes have been used, mainly the P301S, which showed increased neuronal death and microglia activation (Allen, Ingram, & Takao, 2002).

Other models have been developed to scan a possible relation between Aβ and Tau protein. Transgenic mice's for mutant APP are used to test possible effects of Aβ over expression on Tau aggregation, and the results suggested a possible relation between both hypothesis (Roberson, Scearce-Levie, & Palop, 2007). Furthermore, studies to explore the effects of the co-existence of both mutated APP and Tau have been made, thus showing a higher neurodegeneration and toxicity (Lewis, Dickson, & Lin, 2001).

Recent advances have been made in the field of prion-like propagation of Tau protein in the last years. One of the main obstacles is the fact that the prolonged exposure and expression of higher concentration of the full length Tau is toxic to cells, mostly in dividing cells where the higher microtubule stabilization prevents the normal mitoses. To avoid this problem, neuronal cells can be manipulated to express only the MBD that represents the principal region of hyperphosphorylation and consequent aggregation (Wischik & Novak, 1988). Experiments with this strategy shown to be sufficient to induce Tau aggregation in other cell lines, as long as the MBD regions inserted processed a β-sheet conformation. Furthermore, it was proven that Tau strains originated from these molecules were able to propagate to distant but interconnected regions (Sanders, Kaufman, DeVos, & Diamond, 2014).

Other studies with wild type Tau pre-formed fibrils demonstrated the same recruitment of endogenous Tau proteins to structures similar to NFTs, which could prove that the seeding effect could be present *in vivo* conditions. Nevertheless, the aggregation rate was proven to be considerably higher when mutations associated with tauopathies are present. Even more, it was also proven that Tau intake is mediated by endocytosis (Guo & Lee, 2011). To complete, the ability to "seed" NFTs could represent a strong and reliable instrument for further studies. Based on this

model several therapeutic targets have been identified, thus showing a true propose and applicability

Despite the existence of many models we only focus on the principal ones. All of them have a high relevance in the study of tauopathies, and have an even higher importance in the research and finding of possible therapies.

1.5.2 Potential drug targets for the treatment of tauopathies

As it was already focused during this work, tauopathies, and in particularly AD, comprehend a set of harmful effects not only for the patient but also for relatives and society. With projections of increasing aging population worldwide the challenge to develop an effective treatment or cure are higher than ever. With the model development advance in the last 10 years, our comprehension of the disease as grown exponentially and so as the drug development.

Most of these new targets that have been identified refer to mechanisms or pathways already discussed in this work. These strategies could be related with: phosphorylation inhibition, increase in degradation, inhibition of aggregation and cytoskeleton stabilization, blocking of the spreading [Figure 9]. The different approaches will be discussed next.

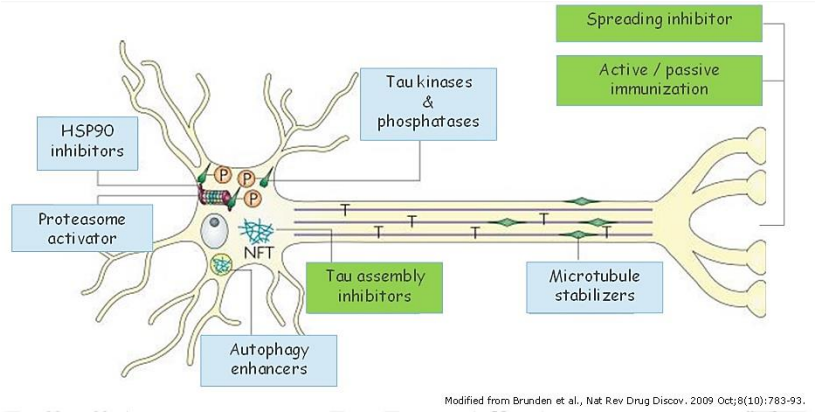

Figure 9 – Some of the mechanisms identified for drug search and development in new therapies. Adapted from *(Brunden, Trojanowski, & Lee, 2009)*.

1.5.2.1 Kinase inhibition

The three main kinases in Tau, GSK-3, CDK5 and MARK are responsible for the regulation of Tau binding to microtubules in physiological condition. However the pathological over activation of this enzyme is one of the responsible for the detachment from microtubules and subsequent aggregation of Tau proteins that causes the already identified LOF and GOF (Brunden, Trojanowski, & Lee, 2009).

The development of kinase inhibitors is particularly challenging. The most used strategy has focused on the development of small molecules that can bind to the ATP domain without functional activity. The inhibition of the ATP binding site is based on the fact that kinases use the

phosphate group of ATP or GTP. Although the antagonist effect is relatively easy to achieve in drug discovery, the main problem is related with the lack of specificity for GSK-3 and CDK5. Even more it must be taken into account that reducing the activity of this kinases could have other side effects with impact on the organism since this enzymes play important roles in cellular metabolism, immune response, neuronal maturation, and synaptic vesicle exocytosis. For instance, there are more than 90 kinases just for the tyrosine residue in proteins, all of them with critical significance in cell survival. Since all the tyrosine kinases bind ATP which has a high concentration inside the cell, the affinity and specificity become a major key player and an obstacle to screening process (Ghoresch, Laurence, & O'Shea, 2009).

It´s also important to note that strategies are exclusive for kinase specific for tyrosine residues, which account only for a small percentage of the amino acids that usually are phosphorylated, especially in Tau where only 5 of these elements are present. We also have to have in account the threonine and serine residues that have their own kinases. Therefore special concern must be taken in the development of new drugs, namely the characteristics of the enzyme.

Besides the development of small molecules for the inhibition of the principal kinases involved in Tau phosphorylation, other strategies have been explored. One of this new approaches consist in identify the most important kinases in Tau hyperphosphorilation. By using high-throughput RNAi technics, 572 enzymes where identified as possible responsible for phosphorylation on S262residue, a critical region for the regulation in microtubule binding. In a second phase, siRNA was used to see the effects of knock-down the protein expression, resulting in the identification of potential targets that could inhibit the phosphorylation of the 12E8 epitope (pS262/pS356), like: the eukaryotic translation initiation factor 2 α kinase 2 (EIF2AK2), dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), and α-kinase anchor protein 13 (AKAP13) proteins. From this, EIF2AK2 has sowed higher potential in the treatment of tauopathies, based on the ability to not only reduce phosphor-Tau but also total Tau (Azorsa, Robeson, Frost, & Hoovet, 2010). The total reduction of Tau is highly important, since it won't only block the conformational alteration and aggregation by phosphorylation, but also it will reduce the amount of Tau inside the cell that could be altered by the prion-like behaviour.

Although these new approaches based on EIF2AK2 knock-down seem really promising, there are still some questions that need to be solved. First off all it's necessary to perform the same experiments in other models that simulate the prion-like activity or in more complex conditions to see if siRNA present the same efficacy. Secondly, it's also necessary to evaluate the impact on cell viability, since Tau complete inhibition could disrupt some of the physiological functions like

microtubule stabilization. Finally there is no evidence that Tau depletion leads to a decrease in its aggregation or in the formation of tangles.

1.5.2.2 Increase in degradation

Proteins degradation, among others, is performed by two cellular systems: the ubiquitin-protease system and the autophagy-lysosome system. Both of them are altered in AD neuronal cells and both are used to degrade hyperphosphorylated Tau and NFTs (Nixon, Wegiel, Kumar, & Yu, 2005) (Keller, Hanni , & Markesbery , 2000). In theory, by all that was covered in sections 1.2.2.1 and 1.2.2.2, the increase in hyperphosphorylated Tau and NFTs clearance could represent an effective strategy.

The chaperone molecules Hsp70/90 and CHIP are among the most well-known and explored pathway to increase the specific Tau degradation in pathological conditions. The efforts in drug research applied in this mechanism are based on the fact that Hsp are found up-regulated in AD brains and for instance on the results of experiments that showed that Hsp90 is capable of binding to misfolded Tau proteins thus promoting its correct refolding or degradation via proteasome. It can also form a complex with CHIP that is responsible for the degradation of phosphorylated Tau. (Dickey, Kama, Lundgren, & Klosak, 2007)

Nevertheless most of the strategies for new drugs screenings have focused on the inhibition of Hsp90, not only for neurodegenerative disorders, but also in cancer therapies (Hong, Banerji , Tavana, & George, 2013). In tauopathies, the inhibition of Hsp9 showed to decrease hyperphosphorylated Tau and consequently the formation of NFTs (Luo, Duo, Rodina, & Chip, 2007). Moreover it was proven to be an increase in Hsp70 and CHIP when Hsp90 is inhibited, also demonstrating an increase of Tau ubiquitination that targets the protein to autosomes or proteasomes (Salminen, Ojala, & Kaarniranta, 2011). This concept has been proven and explored for drug discovery in many studies (Dickey, Eriksen, Kamal, & Burrows, 2005) (Zhao, Michaelis, & Blagg, 2012).

1.5.2.3 Microtubule stabilizer

The treatments proposed for microtubule stabilization and aggregation inhibition are design to attend the LOF seen in tauopathies. Although they aren't fit to reverse the disease, its application could be responsible for attenuating the harmful toxic effects usually observed by the pathology.

Referring to the first strategy, taxols are considered the best strategy to study the effects on microtubule stabilization to restore some of the lost function like axonal transport. Taxols have been used in medicine as anticancer drug by its ability to stop mitosis through microtubule

stabilization. The research made with small concentrations of this drug (to avoid blocking cell division) showed positive effects on vesicles and organelles transport. Nevertheless we must take in to account that the presence of hyperphosphorylated Tau is still responsible for organelles disruption and mitochondria dysfunction that are not prevented by this treatment (Zhang, Maiti, Shively, & Trojanowski, 2004). Besides small molecules, peptides have also been developed to stabilize microtubules with results in promoting cognitive function and reducing the pathology associated with Tau (Matsuoka, Jouroukhin, & Gray, 2008).

1.5.2.4 Aggregation inhibitors

For the aggregation inhibition, the main goal focus on avoiding the establishments of interactions between Tau proteins to prevent formation of oligomers and tangles. The main challenge is to develop a small molecule that is capable to bind to residues that are responsible for these interactions. Some of these regions have been already identified by evaluating the changes in amino acids sequence (Li & Lee , 2006). By targeting these regions in high-throughput screenings there were 285 compounds identified from different pharmacological groups, with demonstrated efficacy in inhibiting the Tau-Tau interactions (Brunden, Ballatore, Crowe, Lee, & Trojanowski, 2010). Further studies should be made to evaluate the ability of these small molecules to pass the blood-brain barrier (BBB), the capacity to induce this same inhibition *in vivo* in doses which can be administered in, and other secondary effects.

1.5.2.5 Blocking Tau spreading

Tau seeding and spreading are one of the new characteristics found to contribute to the pathology of Alzheimer that possibly can be target as a therapy. To avoid the propagation of aggregates between neurons, Tau targeted molecules have been developed and tested to prevent the spreading process inside the cell and antibodies to block the spread to other cells. Anti-bodies to extracellular Tau have demonstrated a significant reduction in Tau hyperphosphorylation, aggregation, and insoluble tangles formation. Furthermore it was also proven a block in Tau spreading to other parts of the brain that resulted in better cognitive function and a reduction in microglia activation (Yanamandra, Kfoury, Jiang, & Mahan, 2013) (Golde, Lewis, & McFarland, 2013). The results obtained so far with this approach has demonstrated interesting results. Also it as demonstrated the importance of Tau spreading and seeding in the pathological process.

To conclude, many targets have been identify and possible therapeutic interventions have been developed in the last years based on the scientific advances made in cellular and animal models as well as the techniques. Nevertheless further investigation is needed not only to develop the

existing experimental treatments, but also to try and integrate them as one with capacity to act on the diverse existing mechanisms.

1.6 Objectives

As it was covered in the last chapters, loss of function of Tau protein and its consequent aggregation into PHFs and NFT is considered one of the main hallmarks of AD and other Tauopathies. Special effort is being placed to develop strategies that inhibit the building-up of protein aggregates or increase the degradation of this. Recently, the discovery of the CLEAR network and improved techniques to measure degradation inside the cell has confirmed the autophagy and lysosomal pathway as potential targets in neurodegenerative disorders and proteinopathies.

With this work, we aim to validate some of the hit compounds picked in the screening assays developed in house. Our goal is not to only confirm that this molecules are able to decrease the amount of aggregated Tau in our cells, but also which mechanism is triggered and how it affects cell survival. By disclosing the pathway responsible for the decrease in Tau aggregation, we hope to find a new target and develop new strategies to trigger it. To achieve this, we intend to use biochemical and imaging technics.

Another goal of this project is to study how induction of the autophagy pathway or disabling the lysosomal degradative capacity by validated molecules can affect the amount of aggregated Tau in our model. Finally, we aim to over express the transcription factor EB to induce the CLEAR network in our cell model and evaluate its impact in aggregated protein clearance and its potential as a target for AD.

Chapter 2 – Materials and Methods

2.1 Materials and reagents

Company	Product
Alpha Aesar	Bafilomycin A1 (J61835)
DB Falcon	Tissue culture flasks (353028)
	Conical Tubes Screw Caps of 50mL(358206), 15mL(352097)
Bio-Rad	Criterion ™ XT Precast Gel 4-12% Bis-Tris 18 wells (345-0123)
	Criterion ™ XT Precast Gel 4-12% Bis-Tris 26 wells (345-0125)
	Trans-Blot® Turbo™ Midi 0.2µM Nitrocellulose Transfer membrane (170-4159)
	Trans-Blot® Turbo™ Midi 0.2µM PVDF Transfer membrane (170-4157)
	Trans-Blot® Turbo™ Transfer System #170-4150
Santa Cruz	Non-fat dry milk (NFDM) (sc-2325)
Enzo – Life sciences	Cyto-ID® Autophagy detection kit
Eppendorf	Eppendorf tubes of 1.5mL (022363212) and 2 mL (022363344)
Eurogentec	SmartLadder (MW-1700-10)
Falcon	Multiwell plates of 96 (655946), 24 (354414) and 6 (354515) wells
Greiner bio-one	Cell culture microplate, 384 wells, PS, F-bottom, µclear, Black (#781091)
HyClone	Research Grade Fetal Bovine Serum(FBS) (SV30160.03)
Life technologies	DMEM - Dulbecco's modified Eagle's medium (1956)
	Dulbecco's phosphate-buffered saline (D-PBS) (1X) (-/- CaCl2; -/- MgCl2) (14190-094)
	E-Gel® Single Comb (G5018-08)
	HCS LipidTox ™Green Neutral Lipid Stain (H34475)
	Lipofectamine® RNAiMAX transfection reagent (13778-075)
	LysoTracker® and LysoSensor™ Probes (L7528)
	MagicMark™ XP Western Protein Standard (LC5602)
	NuPAGE® LDS Sample Buffer (NP0007)
	NuPAGE® MES SDS Running Buffer (NP0002)
	One Shot® TOP10 Chemically Competent *E. coli* (C4040-10)
	Opti-MEM® Reduced-Serum Medium (11058-021)
	Penicillin-Streptomycin (10,000 U/mL) (15140-122)
	Premo ™ Autophagy Sensors (LC3B-FP) BacMam 2.0 (P36235)
	Premo ™ Autophagy Sensor (p62-FP) BacMan 2.0 (P36240)
	Premo ™ Autophagy Tantedm Sensor RFP-GFP-LC3B Kit (P36239)
	UltraPure™ 5 M Sodium chloride (NaCl) (24740)
	UltraPure™ 1M Tris-HCl, pH 8.0 (15568-025)
	0.05% Trypsin-EDTA (1X) (25300-054)
Perkin Elmer	AlphaLISA immunoassay buffer (AL000F)
	OptiPlate-384, White opaque 384-MicroPlate (6007299)
	EnVision® Multilable Reader
Promega	FuGENE® 6 Transfection Reagent (E2691)
	CellTiter-Glo® Luminescent Cell viability assay (G7572)

QiaGene	QIAGEN Plasmid Maxi Kit (12162)
	QIAprep Spin Miniprep Kit (27104)
Roche	cOmplete mini EDTA-free Mini Protease Inhibitor Cocktail Tablets (0469319001)
	PhosphoSTOP Phosphatase Inhibitor Cocktail Tablets (04906837001)
Sigma-Aldrich	Amiodarone hydrochloride (A8423)
	Bicinchoninic Acid Kit for Protein Determination (BCA1-1KT)
	D-(+)-Trehalose dehydrate (T9531)
	LB Broth-Liquid Medium (L2542)
	Leupeptine Hemisulfate from microbial source (L2884)
	Pepstatin A (P4265)
	TWEEN® 20 (P1379)
	Triton™ X-100 (T8787)
	Rapamycin from Streptomyces (R0395)
Thermo Scientific	SuperSignal* West Dura Chemiluminescent Substrate (34076)
	SuperSignal* West Femto maximum sensitivity substrate (34095)
	Restore ™ Plus Western Blot Stripping Buffer (46430)

2.2 Antibodies

Antibody	Target	Host specie	Company	Dilution
Anti-Actin Antibody, clone C4 (MAB1501)	Actin	Mouse	Millipore	1:5000
Anti-LC3B antibody (L7543)	LC3B	Rabbit	Sigma-Aldrich	1:2000
Anti-mTOR antibody (T2949)	mTOR	Rabbit	Sigma-Aldrich	1:1000
Anti-Rab5 (R4654)	Rab5	Rabbit	Sigma-Aldrich	1:1000
Anti-Rab7 (R4779)	Rab7	Rabbit	Sigma-Aldrich	1:1000
ECL Mouse IgG, HRP-linked whole Ab (NA931V)	IgG	Sheep	GE Healthcare Life Sciences	1:20000
ECL Rabbit IgG, HRP-linked whole Ab (NA934V)	IgG	Donkey	GE Healthcare Life Sciences	1:10000
Hoechst 33342 (H21492)	Double-stranded DNA	Not applicable	Invitrogen	0.2 µg/mL

hTau10 biotinylated	Human Tau	Mouse	Inhouse	0.08 nM
hTau 10 conjugated acceptor beads	Human Tau	Mouse	Inhouse	10 µg/mL
LAMP-1/CD107a antibody	LAMP1	Rabbit	Thermo scientific	1:1000
p70 S6 Kinase antibody (#9202)	p70 S6 kinase	Rabbit	Cell Signalling	1:1000
Phospho-p70 S6 Kinase (Thr389) antibody (#9205)	Phosphorylated p70 S6 kinase on	Rabbit	Cell Signalling	1:1000

2.3 Cell culture and dose-response screening

Human embryonic kidney-derived QBI-HEK 293A cells (QBIogene) with a 2N4R Tau Tet-On inducible expression system by doxycycline (Clone 20) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) heat inactivated FBS (Fetal Bovine Serum) and 1% PenStrep (Penicillin-Streptomycin) in tissue cultured flasks at 37ºC in humidified atmosphere with 5% CO_2.

On the first day, the test compounds were diluted to the desired concentration in OptiMEM containing 1 µg/mL Dox at the desired concentration, after which 5 µL was added to the bottom of an empty 384 multi-well plate. Fully confluent cells were trypsinized and seeded at a density of $3x10^3$ cells in 15 µL of OptiMEM per well. Before the seeding of the cells, K18:P301L fibrils (40 µM) in sodium acetate was sonicated on ice, with 30 pulses of 2 seconds and 15% of amplitude. Three hours after plating the cells, K18:P301L fibrils were diluted to 2 µM in OptiMEM and 20 µL was added to each well. After three hours another 20 µL of OptiMEM supplemented with FBS and PenStrep was added and cells were left for incubation through 2 days before being analysed by alphaLISA

2.4 Primary neuronal culture and AAV6-hTauP301L transduction of cortical neuron primary cultures

E17-E18 Wistar WT rat embryos were used for primary neuronal culture. The brains were dissected and kept in pre-warmed HBSS/Hepes solution (7 mM Hepes in HBSS and PenStrep) at 37ºC. The cortices were manually removed and dissociated using chemical approach by trypsinization (4.5 mL of HBSS/Hepes, 500 µL of trypsin) during 15 minutes at 37ºC. Cortices were washed three times in MEM-horse medium with 10% horse serum and 0.6% (v/v) glucose. Mechanical dissociation was performed using Pasteur pipettes in 3 mL of MEM-Horse medium.

The cells were centrifuged for 5 minutes at 450 xg and resuspended in 3 mL of MEM-Horse medium, after which $2x10^4$ cells were plated per well in 96 multi-well plates. After 4 hours the medium was changed to serum free medium supplemented with B27 (2% B27 and 10 mM L-glutamine in Neurobasal medium)

Three days after plating (DIV3), cells were transduced with AAV6-hTauP301L at a multiplicity of infection (MOI) of 100, diluted in Neurobasal medium with B27. At DIV8, K18:P301L fibrils were diluted in Neurobasal medium with B27 and added to the neurons so that a fibrils final concentration of 25 nM was used per well.

2.5 Plasmid and siRNA transfection of Clone 20 cells

For plasmid transfection, one day before the experiment, fully confluent Clone 20 cells were trypsinized and seeded at $5x10^4$ cells per well in 24 multi-well plates. On the next day, 3 µL of Fugene® 6 was diluted in 97 µL of OptiMEM and incubated for 5 min at room temperature (RT). At the same time, TFEB-pCMV6, PTEN-pCMV6 or the empty pCMV6 vector plasmids were diluted to 10 ng/µL in OptiMEM, and 1 µg was added to the diluted FuGene 6. This was left to incubate for 15 minutes at RT to allow the formation of complexes between Fugene 6 and the different plasmids. Finally the cells were washed with antibiotic free DMEM and the Fugene:plasmid complex solution was added to 400 µL of the last medium.

For the siRNA transfection, we used the protocol for reverse transfection supplied by the manufacture. Briefly, 6 pmol of each siRNA was diluted in 100 µL of OptiMEM in the 24 well tissue culture plate. 1 µL of Lipofectamine RNAiMAX was diluted in the siRNA mixtures and after a gentle mix was incubated for 10-20 minutes at RT. Cells were diluted to $5x10^4$in 500 µL of DMEM without antibiotics and added to each well and left to incubate with the siRNA:Lipofectamine mix. On the next day, cells were harvested and plated in 384 multi-well plates at $3x10^3$ in OptiMem for seeding and alphaLISA analysis, like described previously.

2.6 Imaging after using the BacMam system.

The BacMam system was selected for its convenience and efficiency in transfecting any kind of cell in an easy way. On the day prior to transfection, $2x10^4$ QBI cells per well were plated in 96 well, µclear plates, in order to achieve 70% confluency on the next day. Cells were then transfected with the BacMam system at a MOI of 30 and were left to incubate for 24 hours. On the third day, the respective treatment was applied and after an incubation period of 16hours, the cells were washed with pre-warmed PBS and fixed with 4% paraformaldehyde. Cell were washed again in PBS and then observed under a confocal microscope with the appropriate channels.

2.7 Western Blot

After the treatment, cells were washed twice with pre-warmed PBS and lysed for 15 minutes with ice-cold lysis solution (1% TritonX-100 in PBS, complemented with PhoshphoSTOP Phosphatase and cOmplete mini EDTA-free Mini Protease inhibitors). Cells were scraped and transferred to pre-chilled Eppendorf tubes to be centrifuged at 21000 xg for 10 minutes at 4º. The supernatant was collected and a BCA protein quantification assay was carried out. 10 to 15 µg of protein was loaded in Criterion ™ XT Precast Gel 4-12% Bis-Tris and run in MES SDS running buffer. The gel was then transferred onto a nitrocellulose or PVDF membrane (depending on the protein studied) using the Trans-Blot®Turbo™ system. After transferring, the membranes were blocked with 5% non-fat dried milk (NFDM) in TBS-T (1M Tris, 150 nM NaCl and 0.05 % (v/v) Tween-20, pH 8.5) for 1 hour at RT. The membranes were then incubated overnight at 4ºC with the respective primary antibody diluted in 5% NFDM in TBS-T. On the next day, membranes were washed 4 times in TBS-T for 5 minutes before being incubated either with sheep anti-mouse IgG-horseradish peroxidase or donkey anti-rabbit IgG-horseradish peroxidase in TBS-T for 1 hour at RT. Membranes were again washed 4 times with TBS-T, after which they were incubated either with SuperSignal® West Dura or the SuperSignal® West Femto and exposed on the Lumi-Imager. For reprobing, the membranes were stripped with the Restore™ Western Blot Stripping Buffer for 15 minutes at room temperature, followed by a washing step with TBS-T and blocking again with 5% NFDM for 1 hour before incubating with another primary antibody. Western blot bands were quantified with the GeneTools (Syngene) software.

2.8 alphaLISA

Medium from the 384 multi-well plate was removed and replaced with 45 µL of ice-cold lysis solution (1% TritonX-100 in PBS, complemented with phosphatases and proteases inhibitors). To quantify Tau aggregation, two antibodies hTau10 conjugated either with an acceptor bead or biotin were used diluted in AlphaLisa immunoassay buffer. 20 µL of this mix was incubated for 2 hours at RT with 5 µL of sample, after which 25 µL of streptavidin coated donor beads diluted in the same buffer were added to each well in light reduced condition. After incubating the samples in the dark for 30 minutes, the plates were read on an EnVision® Multilable Reader.

2.9 Cell viability assay

The same sample as those obtained for the alphaLISA was applied, taking care to use it within 1 hour after lyses to prevent ATP depletion. Briefly, for each well, 12.5 µL of the CellTiter Glo solution was mixed with 7.5 µL of OptiMEM in the 384 Opti plate. To each well we added 5 µL of sample

and let incubate at RT for 15 minutes, after which luminescence was read in the EnVision® Multilable Reader

2.10 Statistical analysis

Statistical analysis was performed with the GraphPad Prism software. Results are expresses as mean ± SD. Power analysis was performed using a confident interval of α=0.05. Comparisons between two groups were analysed with a two-tailed Student's *t*-test. Three or more groups comparisons were performed with an one-way ANOVA *post-hoc* Dunnett's multiple comparison test. P-values bellow or equal to 0.05 were considered statistically significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Chapter 3 - Results

3.1 Screening assay and phenotype identification

Recently Guo and Lee (2011) described a cellular model in which *in vitro* pre-aggregated Tau fibrils were applied to cells to induce intracellular Tau aggregation. This model was adopted in house to a high content assay and was used to screen a large collection of molecules. Compounds that reduce build-up or stimulate the break-down of intracellular Tau aggregates in this model are potential drug candidates for the treatment of AD. We first tried to confirm a subset of the identified hits using a biochemical assay in the same model. This is a QBI cell line, a derivative of HEK 293 cells that expresses 2N4R-TauP301L under a Dox inducible promoter (Clone 20).

Clone 20 cells were incubated one week without Dox to allow the clearance of all Tau proteins and possible aggregates that are formed during this time. Tau expression is first induced by culturing the cells in the presence of Dox (1 µg/mL) when the cells are seeded in 384 multi well plates, followed by the treatment with compounds for 3 hours. After this incubation period, 2 µM of pre-aggregated and sonicated K18:P301L seeds were added in order to induce Tau aggregation. Cells are then incubated for a period of 2 days at 37ºC 5% CO_2, after which they are lysed with 45 µL of 1% Triton in PBS with phosphatase and protease inhibitors. Finally, 5 µL of the lysates were used for a cell viability assay with CellTiter Glo and another 5 µL were used to assess Tau aggregation by alphaLISA.

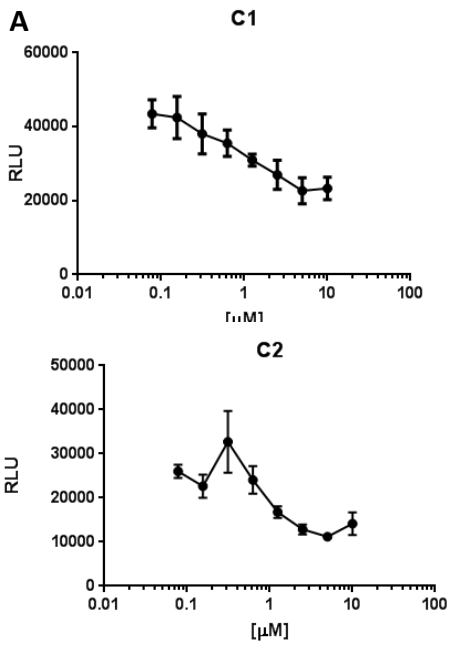

Figure 11 – **Dose-response curves of hit compound**. A, Two compounds that reduce Tau aggregation in the cellular model were confirmed by alphaLISA

All compounds identified as hits in the high content screen were tested using this method. Figure 11 illustrates the behaviour of most compounds that were validated as hits. Most of the molecules showed a higher efficiency when in concentrations of 2.5 to 5 µM. This results were always compared with cell viability assays in order to prove that the observable decrease is not a result of toxicity caused by the compound.

Active compounds identified in the HTS and tested throughout this thesis are referred as C1, C2, C3 and C4 due to confidentiality. These molecules were chosen based on their safer and more efficient profile.

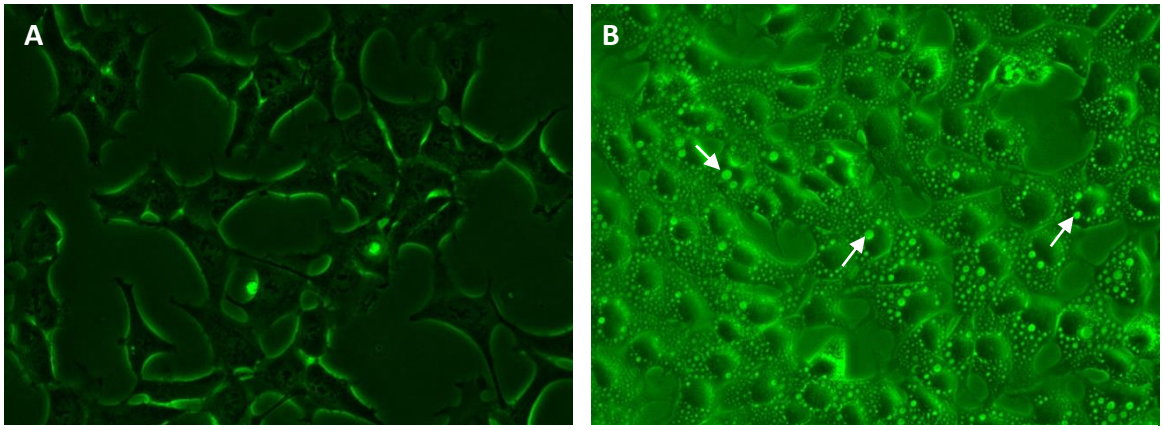

Figure 12 – **Identified compounds induced vesicle formation.** A, QBI cells treated with 0,1% DMSO. B, QBI cells treated with C3 [5 µM] after 9 hours. White arrows exemplify the larger vesicles, even though the phenotype is spread through the cells.

Interestingly, cell treatment with some of the hit compounds was responsible for the development of a vesicle phenotype (white arrows) [Figure 12]. Not all hit molecules positively confirmed were systematically tested for the morphological change. However, at least 9 compounds showed different stages of vesicles development in QBI cells. For some, this phenotype was also observed in a comparable Tau aggregation model in rat primary neurons. Changes in the sub-cellular structures were observed in some cases after only one or two hours of incubation. After 9 hours, different stages of vesicles development were identified with different compounds, implying different potencies. The fact that multiple active molecules presented an observable phenotype in our cell model, suggests a shared mechanism for Tau aggregation clearance related with the vesicles formation.

Taking into account the importance of these hits, special effort was put into the identification of the vesicles in order determine the mechanism by which aggregated Tau decreases in our model. The next chapters will focus on the identification of the phenotype origin

3.1.1 Compound treatment does not induce phospholipidosis but increases lipid structures

Phospholipidosis is a lysosomal storage condition responsible for an abnormal accumulation of phospholipids inside the cells due to the inability of the lysosome to degrade this molecules. This condition is known to be induced by a series of drugs (usually cationic amphiphilic molecules) currently approved in the market, such as anti-depressants, anti-anginal and antimalarial. Even though it does not provide an explanation for the decrease in Tau aggregation, the fact that this pathology is related with the degradation system merits further investigation.

To test if phospholipidosis could be the responsible for the vesicle formation we used a commercially available lipid based reagent with a dye, the HCS LipidTOX (Invitrogen), which

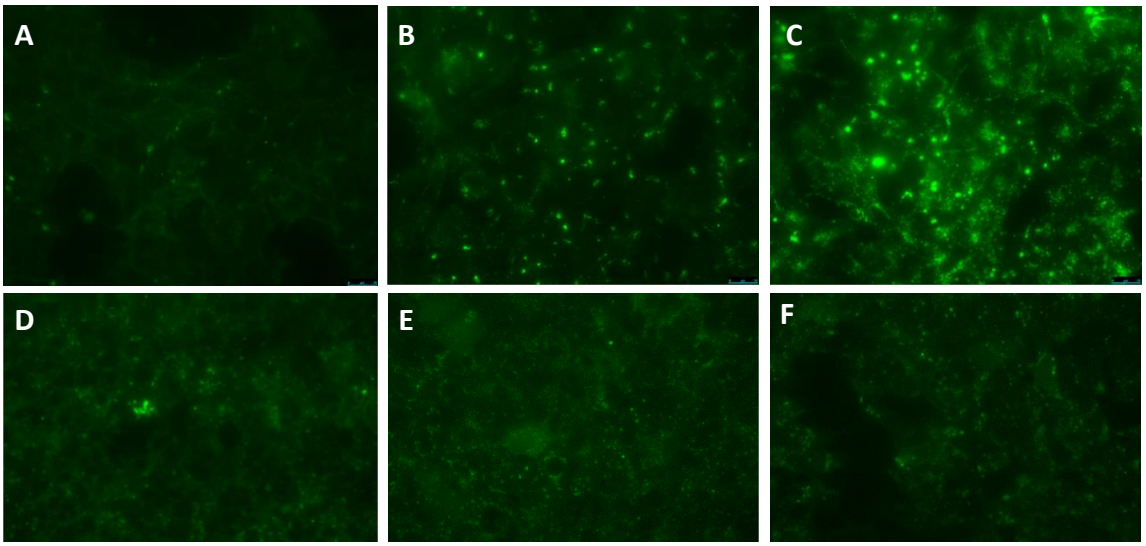

Figure 13 – **The vesicles are not the result of impaired lipid metabolism, but rather show its lipidic nature.** QBI cella treated over night with A, Vehicle (0,1% DMSO); B, Amiodarone [12,5 µM]; C, Fluoxetine [5 µM]; D, C1 [5 µM]; E, C2 [5 µM]; and F, C3 [5 µM]and stained with LipidTOX.

fluorescence can be imaged at 488 nm. This allowed us to see if there is accumulation of stained vesicles determined by a disorder in lipid degradation.

When compared with Amiodarone and Fluoxetine, respectively an antiarrhythmic and an antidepressant known to induce phospholipidosis, none of our compounds showed an increase in the intensity of the staining resembling phospholipidosis [Figure 13]. On the other hand, there seems to be an overall increase in the background signal that could be explained by the lipid nature of the vesicles instead of phospholipid deposition. This could mean that even though there are more lipidic structures inside the cell, the degradation pathway is working properly.

3.1.2 Identification of autophagosomes

Considering our first results that showed a decrease in the amount of aggregated Tau and the possible lipidic nature of the vesicles, we hypothesized that the compounds could be acting upon the cellular autophagy system.

To test this theory we used another commercial kit supposedly designed to identify autophagosomes and autophagic flux by immunofluorescence, the Cyto-ID green detection reagent (Enzo life sciences). The dye was incubated in combination with our compound and

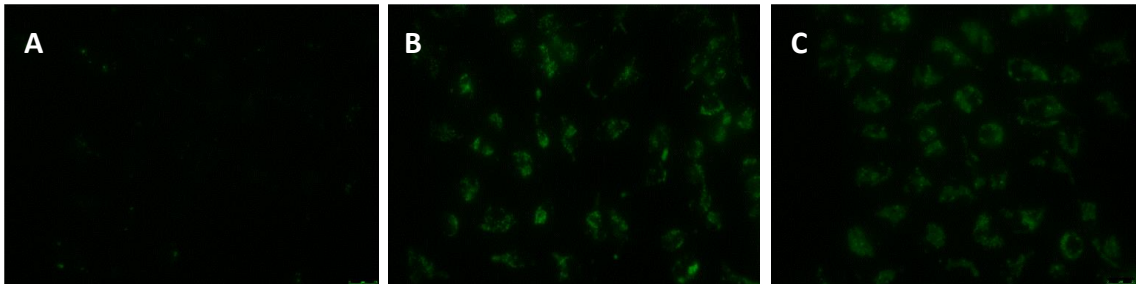

Figure 14 – **Compound treatment increases the amount of autophagosomes in the cells.** QBI cells stained with Cyto-ID after overnight treatment with A, vehicle (0,1% DMSO); B, Chloroquine [30 µM] and C, C1[5 µM].

chloroquine, an antimalarial that increases lysosomal pH, was used as a control to block the autophagic flux and arrest autolysosome degradation [Figure 14]. Our data showed that compound treatment was responsible for a clear increase in the Cyto-ID staining, that is also coherent with the morphology of the vesicles observed in a normal microscope. This confirms our initial suspicion that the vesicles could be somehow related with the autophagy process.

3.1.3 Neurons share the same phenotype as QBI cells after treatment with the compounds.

The vesicles identified in QBI cells after treatment with the active compounds were also identified in primary neuronal cultures and we decided to evaluate if they share the same nature when submitted to the same conditions. This was important to stablish, since our main focus is to find new molecules active in decreasing Tau aggregation in neurons. QBI cells were used to allow screening of larger numbers of compounds in faster and simpler protocols. Furthermore we decided to test if compound induced vesicles formation could be related with Tau expression or Tau aggregation through seeding with K18:P301L [Figure 15].

Not surprisingly, rat cortical neurons submitted to treatment with the active compounds (A-D) resulted in the development of the vesicle phenotype regardless other variables tested [Figure 15]. Images from the fluorescence microscope of neurons treated with compounds and stained with the Cyto-ID reagent kit showed an increase in vesicles formation in all four conditions, especially when neurons were seeded with K18:P301L fibrils, with and without Tau expression (A and C) and when Tau expression was induced with an AAV without seeding (B). Interestingly, and even though a high number of vesicles can be identified, compound treatment in the absence of Tau expression and K18:P301L treatment (D) led to some decrease in vesicle staining compared to the other three conditions.

On the other hand, the cells that were not treated with the compound (E-H) show little or absence of vesicles staining when compared to the compound treated ones. Neurons that had Tau expression induced by an AAV (E, F) present a residual signal despite seeding by K18:P301L. This could mean that the expression of a mutant form of Tau is sensed by cells as a harmful situation, thus activating cellular mechanisms responsible for the response and clearance of aberrant structures such as autophagy. Finally, no signal from Cyto-ID was detected in conditions where there is no Tau expression (G and H).

We theorise that although the vesicles are the result of the compound treatment, autolysosomes formation and autophagy stimulation can be activated by the expression of a mutant form of Tau.

This could mean that neurons can detect and degrade aggregates in pathological conditions, as has been previously reported (Lee, Lee, & Rubinsztein, 2013).

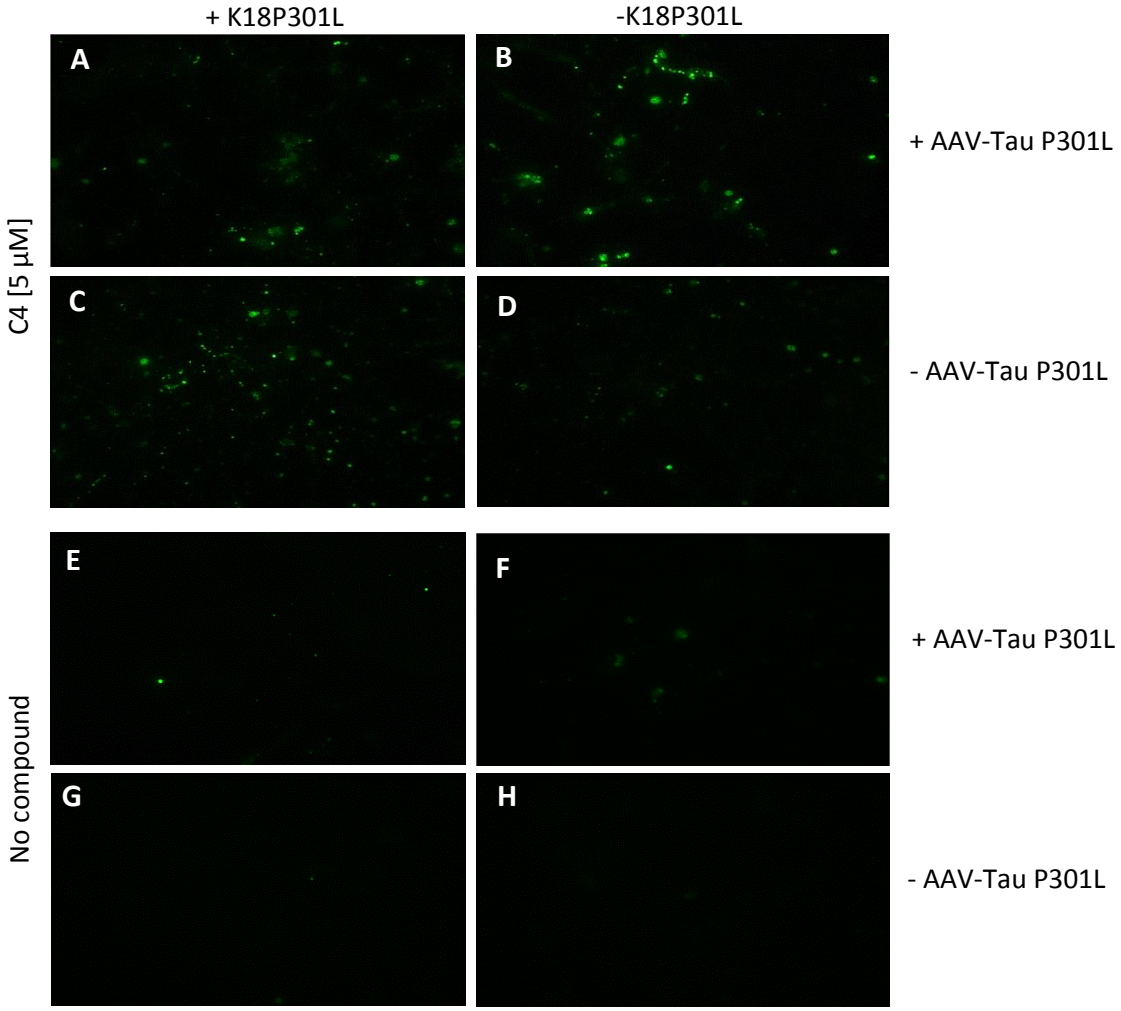

Figure 15 – **Vesicles formation is not dependent on Tau seeding, but rather of the compound treatment and Tau expression.** Rat primary cortical neurons with (A, B, E, F) or without (C, D, G, H) Tau P301L overexpression were treated with (A, C, E, G) or without (B, D, F, H) 25 nM of K18:P301L to induce aggregation of the expressed Tau P301L. Neurons were then treated with either C4 [5 µM] (A, B, C, D) or with vehicle (DMSO, 0,1%) (E, F, G, H). The cells were stained with Cyto-ID and imaged by fluorescent microscopy.

3.2 Autophagy identification and confirmation

After we had confirmed that the group of active compounds induced vesicle formation in neurons and that the vesicles share the same nature, we decided to continue our research using QBI cells. This was to keep the model simple and generate results faster. We also decided to use other strategies to validate the results obtained with Cyto-ID. Although proven to be a simple, direct and cheap method for our first experiments, the kit had some limitations. For example, the lack of precise, validated information on the specificity for autolysosomes. Therefore, using this method no conclusion can be made on the specificity of the compound induced vesicles.

Taking into consideration the latest results, we decided to evaluate if the compound treatment could promote any changes in the lysosomes morphology. This structure was chosen based on the fact that it constitutes one of the key stages of autophagy and is responsible for the degradation process. Therefore, we overexpressed the lysosome associated membrane protein 1 (LAMP1) tagged with RFP, using a commercially available virus, the BacMam virus (LifeTechnologies). Cells were incubated with the virus for 24h to allow a robust protein expression after which the compound treatment was added. After an overnight incubation, cells were washed and fixed to be analysed under a fluorescence microscope [Figure 16].

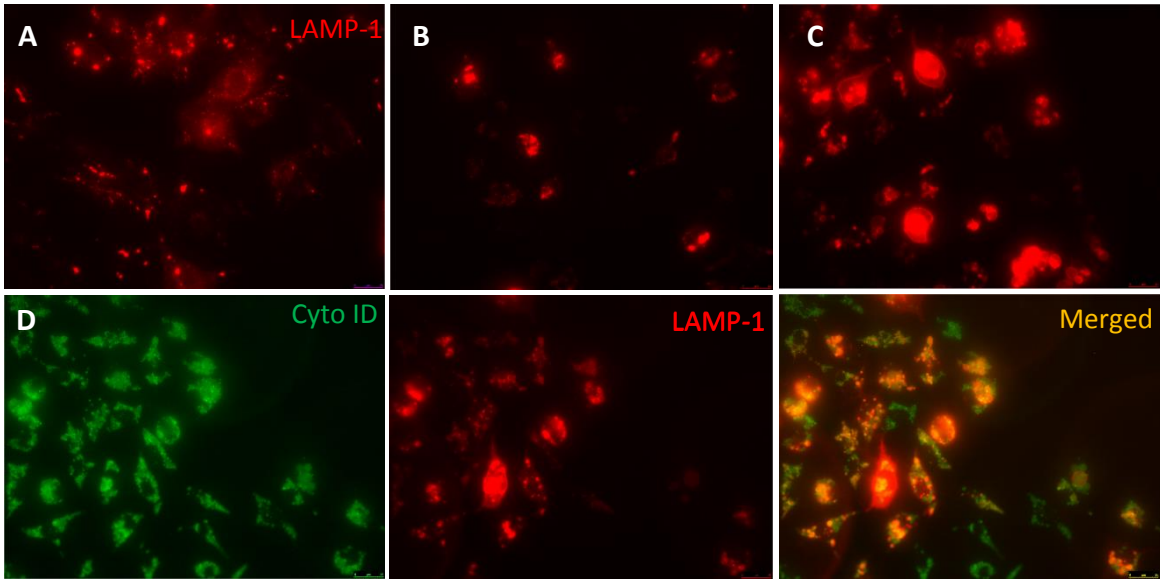

Figure 16 – **Compound treatment is responsible for morphological changes in lysosomes.** QBI cells were transfected with LAMP1-RFP and submitted to an overnight treatment with A, vehicle (0,1% DMSO); B, chloroquine [30 µM] and C, C1 [5 µM]. D. Co-staining of QBI cells treated overnight with C1 [5 µM] with Cyto-ID staining and LAMP1-RFP.

While in untreated condition lysosomes appeared as small dots spread through the cell with sporadic larger structures (A), chloroquine treatment blocked lysosomal degradation and was responsible for the sequestration of LAMP1 positive vesicles that tend to expand (B). The most surprizing result however was achieved compound treatment (C). This condition not only showed a higher staining with LAMP1-RFP, but also induced changes in vesicles morphology. To assess if the lysosomes identified by LAMP1 transfection are different or co-localize with the ones identified by the Cyto-ID experiment, we decided to co-stain using both probes (D). Interestingly, a lot of vesicles showed to be co-stained with both the Cyto-ID dye and LAMP1. This indicates that the Cyto-ID is not a specific marker for autophagosomes but rather for vesicular structures associated with autophagy.

Biochemical confirmation of LAMP1 increase

To confirm previous results that compound treatment induced vesicles might be lysosomes, we decided to confirm that there is indeed an increment in the lysosomal markers when cells are

submitted to the compound treatment. To study this, we decided to treat cells with one of the compounds for different times and analyse LAMP1 expression by Western blot [Figure 17].

Although protein quantification (B) did not show a significant increase in LAMP1 expression, there is a trend for an increment at longer incubation period times after treatment with one of the compounds (24 hours). This is supported by the visualization of the Western blot (A) and comparing the vehicle and the 24 hours condition.

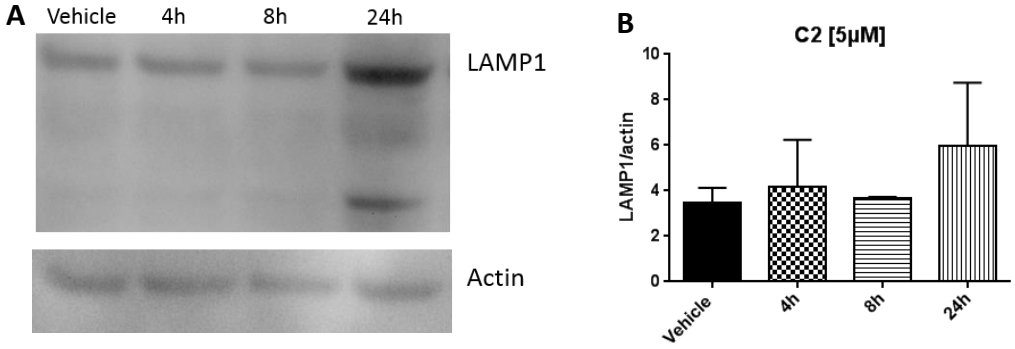

Figure 17 – **Compound treatment appears to increase LAMP1 in longer periods.** A, Western blot for LAMP1 in cells treated with 5 µM of the compound C2 for 4, 8 and 24 hours. B, Quantification of LAMP1 western blot.

3.2.1 Immunofluorescence assay does not show modification in autophagy markers

The imaging experiments showed that the phenotype initially observed is consistent with lysosomal vesicles. Whether the increase of these structures was the result of a higher production of autophagosomal vesicles or a stimulus in lysosomal biogenesis is still unclear. Therefore, we decided to investigate if the autophagy pathway was a target for our compounds using imaging and biochemical techniques. As we had achieved robust results with the BacMam viral expression system, we choose to start by transducing a tagged p62 protein and the LC3-Tandem probe.

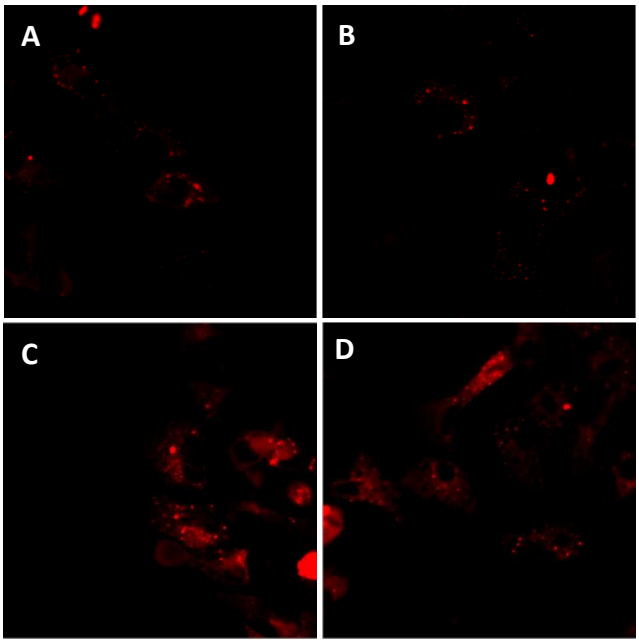

Figure 18 – **Compound treatment is responsible for cytoplasmatic accumulation of p62.** QBI cells were transfected with p62-RFP and treated overnight with A, Vehicle (0,1% DMSO); B, rapamycin [500 nM]; C, rapamycin [500 nM] + chloroquine [30 µM] and D, C1 [5 µM].

p62

p62 is a small protein known to mediate the interaction between LC3 and ubiquitinated proteins, thus facilitating the degradation of aberrant proteins. With autophagy induction, p62 tends to change its localization from the cytoplasm to small vesicles, or autophagosomes, and general staining tends to decrease through a higher degradation (Pircs & Juhasz, 2012). To compare the results we used rapamycin, a known autophagy inducer through the mTOR pathway as a control.

We observed that rapamycin treatment alone (Fig. 18, B) is not enough to increase the overall expression of p62, even though more vesicle staining events per cell are observed and less cytoplasmatic staining is detected, consistent with our predictions. On the other hand, the combined treatment of rapamycin and chloroquine, an autophagy inducer via mTOR and an lysosome- autophagosome fusion inhibitor respectively, seems to be responsible not only for increasing the number of vesicles positively stained with p62, but also, by the general increase in the cytoplasm. This could be explained by the stimulating effect of rapamycin on p62 expression but at the same time the blockage of its degradation by chloroquine. Finally, our compound treatment shows a general staining of p62 in the cytoplasm and some vesicles formation (D). This suggests a higher accumulation of vesicles in the cytoplasm due to an impaired fusion of autophagosomes with lysosomes rather than a stimulus in autophagy.

Tandem probe

To better understand and comprehend the effect of the compound treatment in autophagy, we decided to use the LC3 – Tandem probe. This is constituted by an LC3B protein tagged with RFP and GFP that is sensitive to acidic environments. In theory, autophagosomes fusion with lysosomes will appear as red dots without green because the drop in the pH decreases the GFP intensity. Unfused autophagosomes will stain both red and green.

Our first experiment with a negative control (0,1% DMSO) shows that the GFP staining is relatively higher in the centre of the cell, while the RFP one is much more spread(A) [Figure 18]. Interestingly, when we merge the two channels into one single picture, we see a lot of yellow around the nucleus, whereas small red puncta are present in the surrounding areas of the cell, this suggesting that autolysosomes tend to be present in the surroundings of the cell. When we used rapamycin at mild concentrations [500 nM] (B), the RFP staining increased substantially when compared with the control. As for GFP expression, this appears to remain constant in the nucleus while decreased in the cytosol. Nevertheless it is possible to see LC3 accumulation in larger vesicles, most likely autophagosomes. The difference was more pronounced when we evaluated the merged channels, where it's possible to see a great deposition of red marked structures in the

surrounding areas of the cell (white arrows). This results prove the theoretical mechanism of the tandem probe, since the activation of autophagy is responsible for the increase in autolysosomes and other related vesicles. Finally, when we used our compound, C1, in the standard concentrations [5 µM] we saw a great increase in LC3B all over the cell. Besides the increased expression of the LC3B protein, we also observed the development of large vesicles co-stained with both GFP and RFP (white arrows) that possibly represent autophagosomes. This points out that compound treatment is responsible for a higher LC3B expression, either by more synthesis or less degradation that transduce in more production of autophagosomal vesicles. To clarify, our active molecules are responsible for changes in the autophagy pathway, increasing the markers related with it, but with a different morphology of the one seen with a direct stimulus by mTOR.

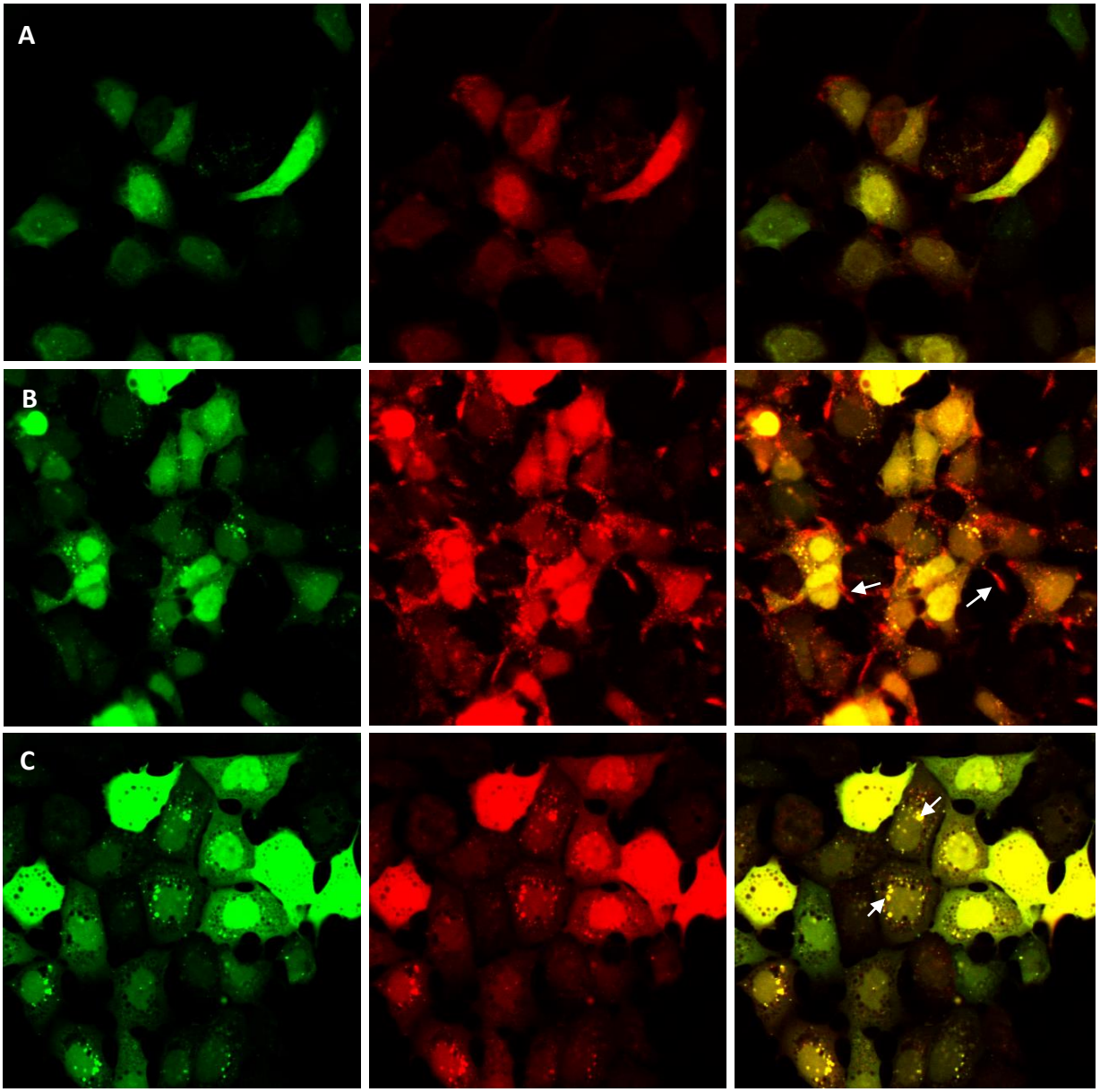

Figure 19 – **Compound treatment increases LC3 expression and changes the morphology of autophagosomes.** QBI cells transfected with the LC3B –Tandem probe treated overnight with A, vehicle (0,1% DMSO); B, rapamycin [500 nM]; and C, C1 [5 µM].

3.2.2 Compound treatment is not responsible for a direct stimulus in autophagy

After we confirmed through imaging techniques that the compounds effect on the phenotype might have an impact on the autophagy mechanism, we decided to corroborate this using biochemical techniques.

LC3

Based on our previous results using imaging technices, and based on the general acceptance of LC3 as autophagy marker, we decided to quantify this protein in a compound dose and time dependent manner.

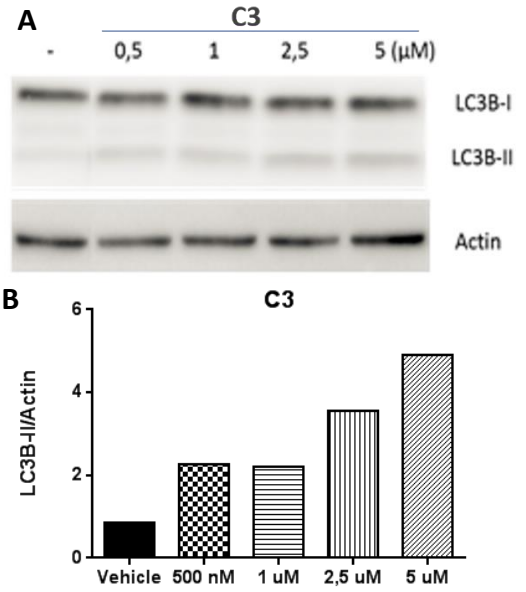

Figure 20 – **C3 treatment induces upregulation of LC3B expression in a dose-response manner.** QBI cells were treated with different concentrations of the C3 compound for 4 hours and analysed by Western Blot (A) and quantified using actin as a load control (B)

In the first experiment we decided to study the conversion response of LC3B-I (18 kDa) into LC3B-II (16 kDa) using different concentration of the compound, starting with an initial concentration ten times lower (500 nM) than the one we had been using [Figure 19]. Interestingly even at low concentrations it's possible to observe a higher conversion of LC3 into the lipidated form when normalized for actin (A and B), as well as a higher percentage of LC3B-II in the total LC3B (data not shown). The fact that LC3B-II conversion is dependent on the dose, indicates a direct relation between the compound treatment and autophagy activation.

Since we observed in previous experiments that a small incubation period was enough to cause the phenotype development, we decided to set an experiment to evaluate how time influences the expression of LC3B and its turnover to LC3B-II [Figure 20].Not surprisingly even the 1 hour incubation period has proven to be enough to stimulate an increase in LC3B-II conversion, showing once again that the vesicles observed in early stages are probably related with the autophagy pathway. As time increases, so does the percentage of LC3B-II/total LC3B. At 24 hours, almost all LC3B is in the lipidated form and little amounts of LC3B-I are detected, suggesting that the effect of the compound affects LC3B expression, as well as its conversion.

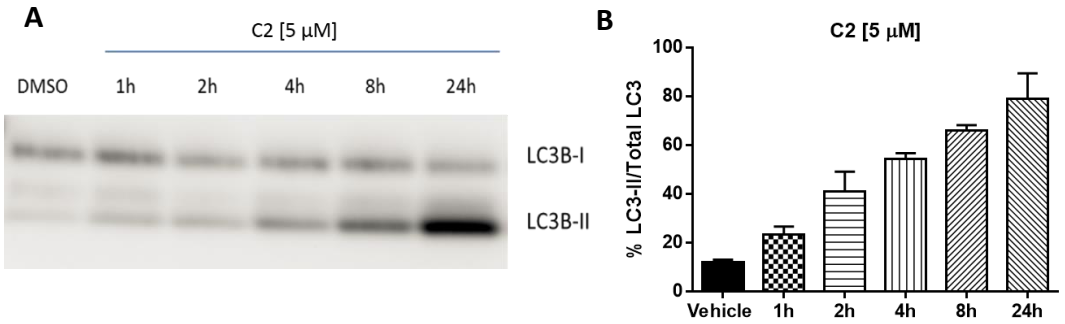

Figure 21 – **C2 treatment induces LC3B conversion into the lapidated form in a time-dependent manner.** QBI cells were treated with the C2 [5 µM] compound for different time points and analysed by Western Blot (A) followed by a quantification of the lapidated form regarding the total LC3B (B).

Autophagic flux

Our results show the involvement of the autophagy system in the lysosomal vesicles formation, supporting the data obtained with the imaging techniques. This helps to understand how different concentrations of the compounds influence the autophagosomal markers as well as how treatment time might be crucial for the next experiments. Despite all this, we still do not know at this stage if the higher expression of LC3B-II is the result of increased autophagic flux, meaning either more LC3B-I is being produced and converted in the lipidated form, or if on the other hand there is a blockage in the degradation of this protein by impaired fusion between autophagosomes and lysosomes. To answer this question we decided to use the method described by Rubinsztein, *et al* (Rubinsztein, Cuervo, & Klionsky, 2009). In brief, we used an autophagy blocker in combination with the treatment, and compared LC3B-II levels of this with the blocker and the treatment separately. Since we needed a strong inhibitor, we decided to use bafilomycin A1 in saturating concentrations. Bafilomycin is a vacuolar H+ ATPase (V-ATPase) inhibitor that interrupts vesicles acidification, therefore blocking vesicle maturation and the fusion process between autophagosomes and lysosomes.

Indeed, bafilomycin has proven to be a robust autophagy inhibitor by showing a constant increase in LC3B-II over time at the saturating concentration of 50 nM (A) [Figure 21]. Simultaneously administration of bafilomycin A1 and one of the compounds (B) is responsible for a pattern similar to the one seen when only the compound is administered (A from Figure 20).

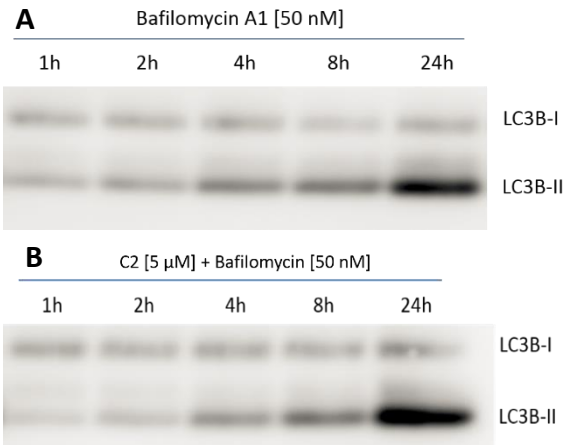

Figure 22 – **Bafilomycin A1 treatment blocks lysosomal action and increases the amount of LC3B-II.** QBI cells were treated either with bafilomycin A1 [50 nM] (A) or the compound C2 [5 µM] + bafilomycin A1 [50 nM] (B) during different time points. LC3B was analysed by Western Blot and quantified.

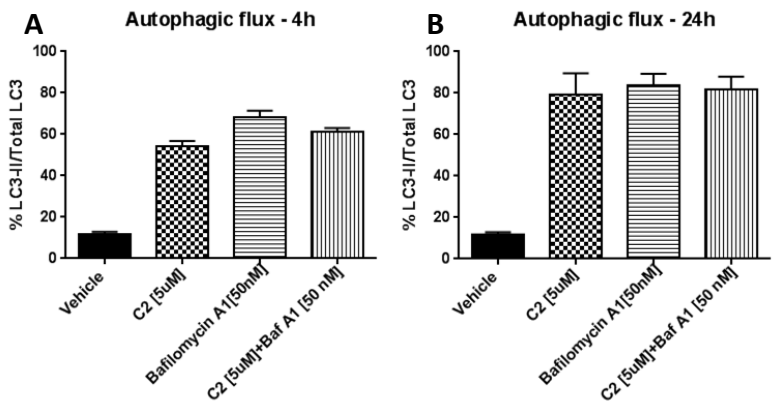

Figure 23 - **C2 does not induce autophagy in QBI cells.** QBI cells treated with a vehicle (0,1% DMSO), C2 compound [5 µM], bafilomycin [50 nM] and simultaneously bafilomycin A1 [50 nM] and C2 [5 µM] in different time points. LC3B expression was analysed through Western Blot and quantified using the proper software. LC3B-II was quantified and normalized to total LC3B at 4 hours (A) and 24 hours (B) of treatment.

Since we already knew that time could play a critical role in our experiments, we decided to use two time sets to evaluate the autophagic flux, 4 and 24 hours [Figure 22]. When treated with either the compound or bafilomycin, there is a substantial increase in LC3B percentage at 4 hours which is further increased at 24h. The simultaneous treatment of cells with our compound and bafilomycin did not translate to a significant increment of LC3B-II when compared with the compound condition alone. Rather, its levels stayed the same in both time points. Although we were expecting an increment in the autophagic flux to explain the decrease in aggregated Tau in the alphaLISA, this data supports the observations made with the imaging technics and LC3 quantification. This suggests that in our model the treatment with the compound is not responsible for the direct induction of the autophagic flux. This would explain why when we used the tandem probe the response was unlike the one seen with rapamycin. We used the same protocol for two more compounds from the list of active molecules known to cause the vesicle phenotype. The results obtained were similar to the ones presented here.

p70 S6 kinase

To confirm that the increment in LC3 expression and autophagy activation does not come from a direct stimulus to the autophagy pathway down-stream from autophagosome formation, we decided to study the only known pathway that plays a role in autophagy induction, the mammalian target of rapamycin (mTOR). Unfortunately, mTOR is a heavy protein with many regulating sites by phosphorylation, making it an extremely difficult protein to study. Therefore we decided to quantify the expression and phosphorylation stage of p70 S6 kinase, an upstream protein that is phosphorylated by mTOR and is responsible for autophagy activation and protein synthesis. In practical terms, autophagy induction by inhibition of mTOR (like the one seen with rapamycin) leads to a decrease in the phosphorylation of p70 S6 kinase, thus leading to the activation of autophagy and decreasing protein synthesis. We also decided to use three main active compounds studied throughout the project to determine whether they have different mechanisms of action

As we initially predicted, there was no significant change in the expression of p70 S6 in any of the conditions (A) [Figure 23]. There is however a tendency by the three compounds to have a slightly higher amount of the protein. This trend could be explained by a tendency of the cell to increase the expression of regulator proteins to counterbalance the intracellular changes.

When we analysed the phosphorylation state with a phospho-specific antibody (B), rapamycin was the only condition to have a significant decrease, as it was expected. All other conditions where a compound was used showed no difference when compared with the control.

Even though other unknown pathways for autophagy stimulation may exist, this results proved that our compounds do not act on downstream regulators of autophagosomes formation. Given this conclusion, we decided to focus our attention in the lysosomal formation and maturation.

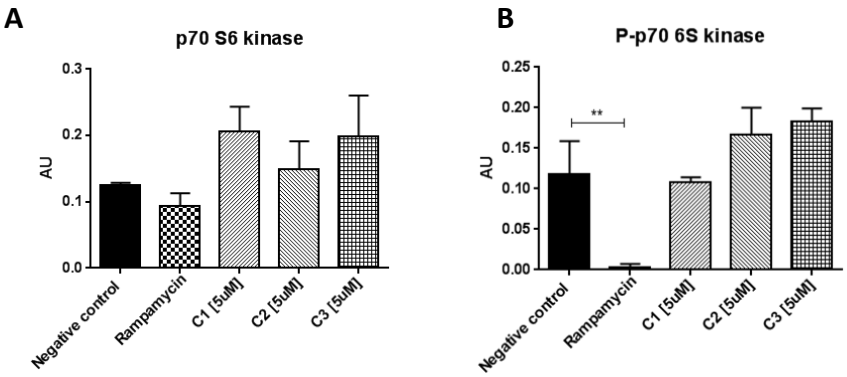

Figure 24 – **Induction of the vesicle phenotype by compounds treatment does not involve the mTOR pathway** QBI cells were treated overnight with a vehicle (0,1% DMSO), rapamycin [500 nM], and the compounds C1, C2, C3 [5 µM]. p70 S6 kinase expression (A) and phosphorylation at Thr389 (B) were analysed by western blot and quantified using the proper software after normalization by actin.

3.3 Role of the endocytic pathway in vesicles formation

During our experiments to assess the autophagic flux, we observed that when the active compounds and bafilomycin were added together, the vesicle phenotype does not develop even after extended times of incubation (more than 72 hours) [Figure 25]. This result can be explained by taking into account the lysosomal nature of the vesicles and the inhibitory effect of bafilomycin in its development and maturation process. Nevertheless it was interesting that concentrations as low as 10nM were enough to block the phenotype development. Additionally, after we induce

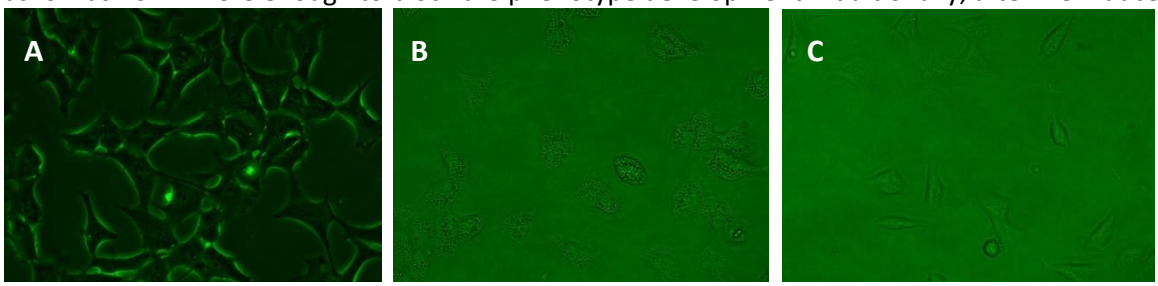

Figure 25 – **Vesicle formation induced by C1 treatment can be reversed with bafilomycin A1.** QBI cells were treated overnight by A, vehicle (0,1% DMSO); B, C1 [5 µM] and C, C1 [5 µM]+Bafilomycin [50 nM] simultaneously and were observed under a normal microscope. Cells in image C do not develop any vesicle phenotype, contrary to Image B that show the already described effect.

the vesicles formation with compound for 4 hours and then add bafilomycin, the induced vesicle phenotype receded after 24 hours (data not shown).

3.3.1 Immunofluorescence and biochemical assays confirm the effect of bafilomycin A1 over vesicle biogenesis

To assess if the change in the phenotype by simultaneous treatment with one of the compounds and bafilomycin also resulted in a change in the LAMP1 expression, we decided to test this hypothesis by using the BacMam system to transfect LAMP1-GFP. In addiction we used the lysosomal dye Lysotracker, a cathionic molecule that accumulates in acidic compartments. This would confirm the true nature of the vesicles, and to see if the compound treatment can be responsible for changes in the lysosomal pH [Figure 26].

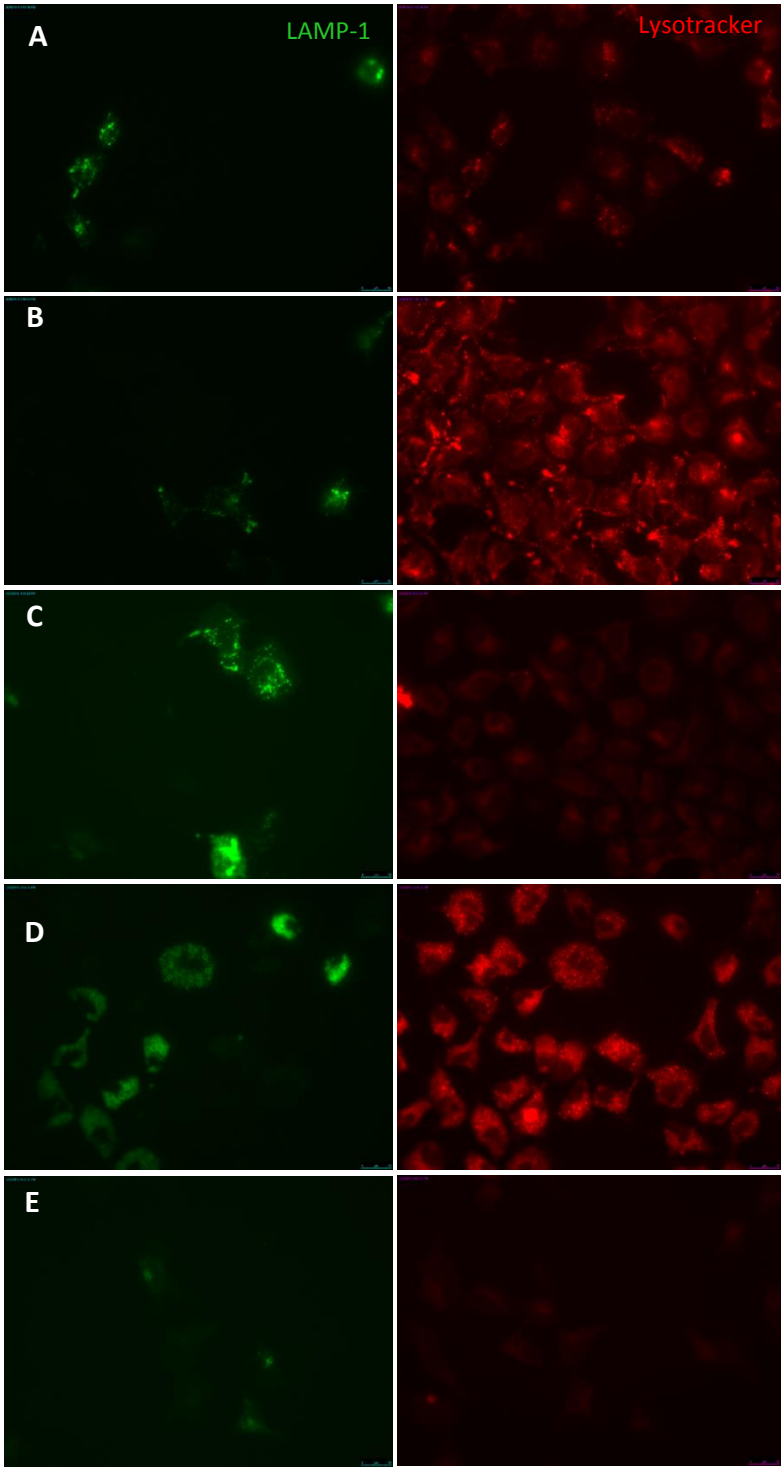

Figure 26 – **Compound treatment is responsible for an increase in lysosomal vesicles and decreased pH, while co-treatment with Bafilomycin reverses this effect.** QBI cells transfected with LAMP!-GFP where treated with A, vehicle (0,1% DMSO); B, rapamycin [500 nM]; C, bafilomycin A1 [50 nM]; D, C1 [5 µM]; and E, C1 [5 µM] + Bafilomycin [50 nM] overnight and incubated with Lysotracker.

In this experiment we observe that rapamycin (B) strongly induces the lysosomal acidification when compared with a negative control (A). As expected, the single administration of bafilomycin was responsible for small LAMP1 stained vesicle accumulation and a

complete depletion of the lysosomal pH (C).On the other hand, compound treatment not only produces the enlargement of lysosomes, like the one already described, but is also responsible for an increase in the Lysotracker staining similar to the one observed in the rapamycin condition (D). This result is extremely important as it proves that the active compounds are able to induce the expansion of the lysosomes. Also important, it showed that this lysosomes are functional. Finally, the combined treatment of compound and bafilomycin causes atrophy of lysosomal vesicles, as well as the complete pH depletion inside this vesicles (E), thus showing that the phenotype can indeed be blocked by bafilomycin treatment.

To confirm the previous results, we decided to do a biochemical quantification of lysosomal markers in the studied conditions. Figure 27 shows LAMP1 quantification by Western Blot.

As previously found, our compound can induce a mild increase in LAMP1 expression. Even though it is not statistically significant to be considered a positive result, we believe that this increase can explain the vesicles phenotype. As predicted, our results show that bafilomycin does not produce a change in LAMP1 in the different time points (A,C). We assume that by blocking the endocytic pathway completely, LAMP1 formation and degradation is stopped by bafilomycin. However, the combined treatment of cells by one of our compounds and Bafilomycin show a little increase at 4 hours that normalizes after 8 and 24 hours. This results proves again the importance of time in the vesicles formation and shows that the phenotype can be blocked by bafilomycin treatment.

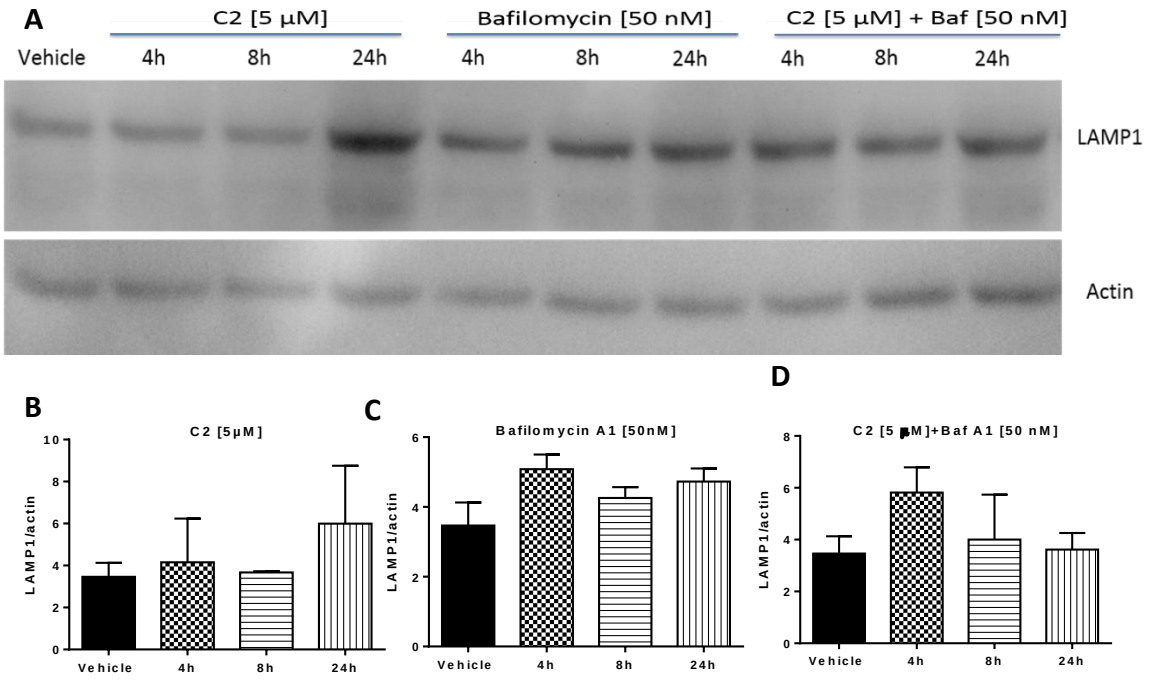

Figure 27 – **Compound treatment induces a mild, non-significant, increase in LAMP1, while Bafilomycin reverts this effect.** QBI cells were treated with C2 [5 µM], Bafilomycin [50 nM] or C2 [5 µM]+Bafilomycin [50 nM] in different time sets, after which they were analysed by Western Blot (A). The Western Blots were quantified with the proper software and normalized for actin (B-D). (Error bars: SD, n=2)

3.3.2 Compounds treatment increase early endosome positive vesicles

Our experiments show that our compounds can induce autophagy not by a direct stimulus downstream the pathway, but rather that it represents a collateral effect of an increment in the lysosomal system. To better understand the origin of the lysosomal vesicles we decided to study the importance of the endocytic pathway in the biogenesis of the phenotype.

We used the BacMam system to transfect LAMP1-RFP and Rab5-GFP to the cells treated with the compound. Rab5 was chosen since it's a marker for early endosomes and it could give is a clue on where to look in lysosomal biogenesis [Figure 28]. Interestingly we observed that with the compound treatment there is an increment on the number of vesicles stained with Rab5 (B,B'). Not only there is an enlargement of the early endosomes when compared with the negative control, but also the vesicles tend to locate near lysosomes (B').

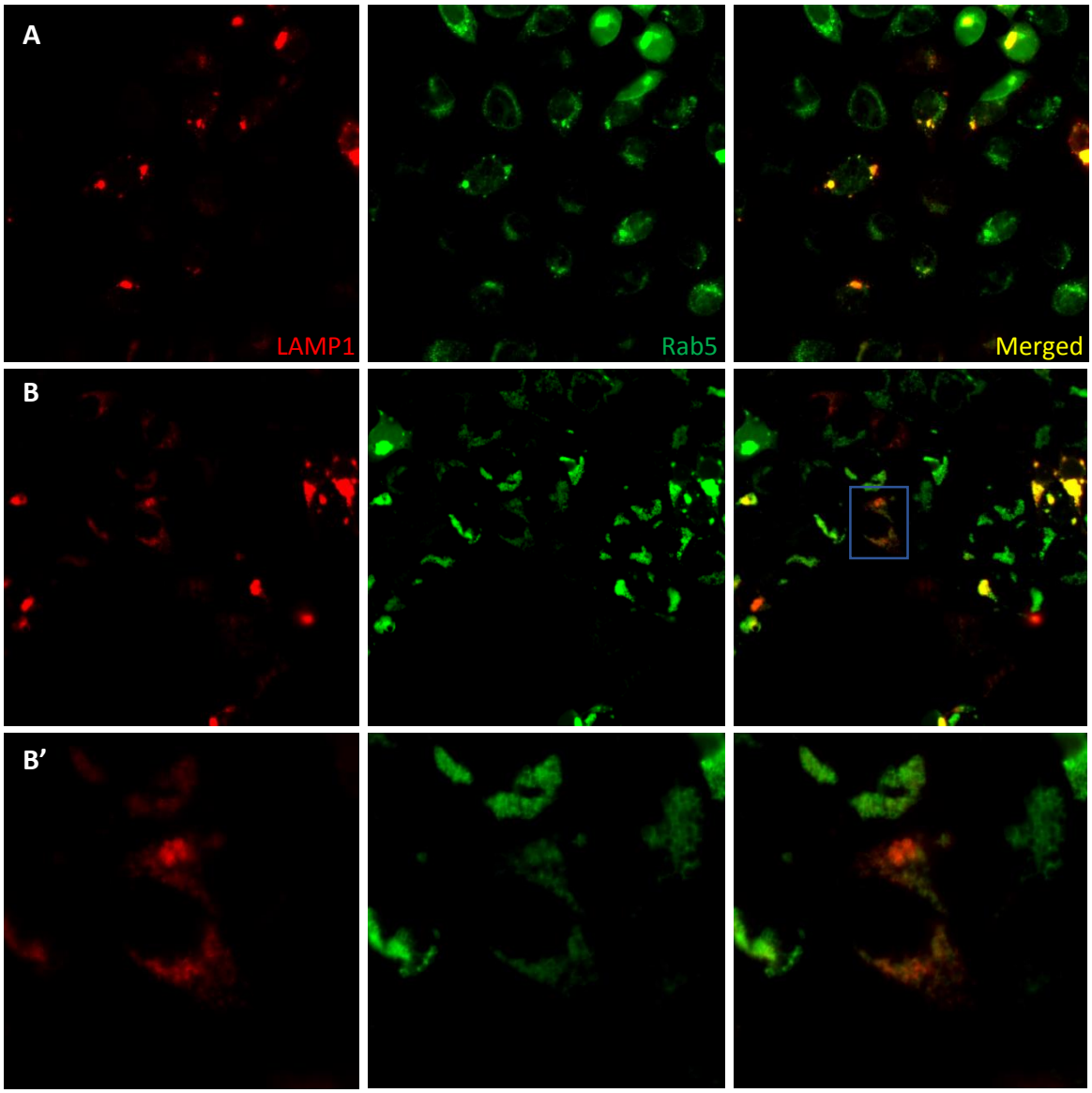

Figure 28 – **Compound treatment with the hit compounds increased early endosomes formation.** QBI cells were transfected with LAMP1-RFP and Rab5-GFP and were submitted to overnight treatment with A, vehicle (0,1% DMSO) and B, C2 [5 µM]. B', represents a closer lock to one of the cells in B. Compound treatment increased the amount of vesicles positively stained for Rab5 and LAMP1. Higher amplification showed that the staining does not co-localize.

3.3.3 Compounds treatment are responsible for changes in Rab7

The last experiment suggests that the lysosomal increment triggered by our active compounds are caused by modifications related with the endocytic pathway. To confirm this hypothesis we tried to quantify the expression of Rab7, a marker for late endosomes, using western blot [Figure 28]

We found that after treatment with one of the compounds there is a slight reduction in the expression of Rab7 in the first hours that is reversed at 24 hours (A,B). We assume that this can be explained by the fact that Rab7 is quickly used to by the cell to increase the delivery of late endosomes to lysosomes. After a certain period of time however, the flux is normalized and the expression of Rab7 is restored. As for the bafilomycin treatment (A, C), the levels remain stable until 24 hours where there is a mild increase, possibly provoked by the blocking of delivery of late endosomes to lysosomes and stopping its turnover. Finally, the treatment with bafilomycin and the compound did not change the overall expression of Rab7 at the different incubation times.

In conclusion, we believe that the origin of the lysosomes in the cells treated with the hit compounds are due to a stimulation of the endocytic pathway that increases the delivery of vesicles to the lysosomal system. Because of this, the whole autophagy pathway is activated as a collateral effect, increasing the degradation of aberrant proteins and culminating in the augmented clearance of Tau aggregates in our model.

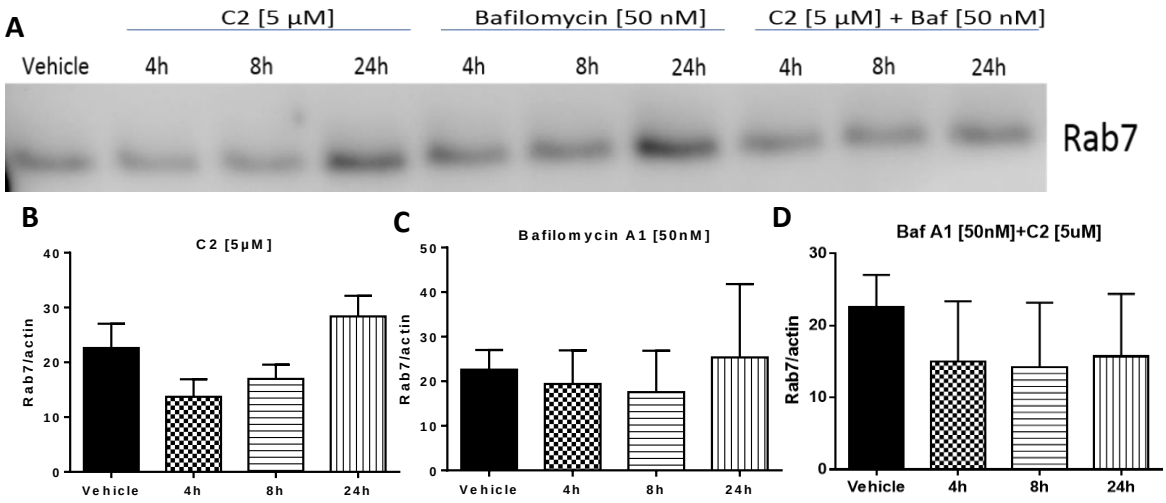

Figure 29 – **Compound treatment non-significantly change the expression of Rab7.** QBI cells were treated with C2 [5 µM], Bafilomycin [50 nM], and C2 [5 µM]+Bafilomycin [50 nM] in different times of incubation, and were analysed for Rab7 expression through Western Blot The Western Blots were quantified with the proper software and normalized for actin (B-D). (Error bars: SD, n=2)

3.4 Effects of autophagy modulation on Tau degradation

So far in this project we studied how the active compounds identified in the HTS can modify the lysosomal activity and the autophagy pathway in order to increase the degradation of Tau aggregates. To understand better how the modulation of this mechanisms can have repercussions in our model, we decided to use a set of compounds with known effect in the degradation system. Therefore, we use a set of activator and inhibitor molecules as well as knock-down technics to measure changes in Tau aggregates as well as cell viability.

3.4.1 Autophagy induction in Tau clearance

To first begin our study, we started our experiments by trying to induce autophagy via the most studied pathway we know: the mTOR. Since we had already used rapamycin to successfully induce mTOR and p70 S6 kinase inhibition, we decided to study how different doses of this compound could affect Tau aggregation [Figure 30].

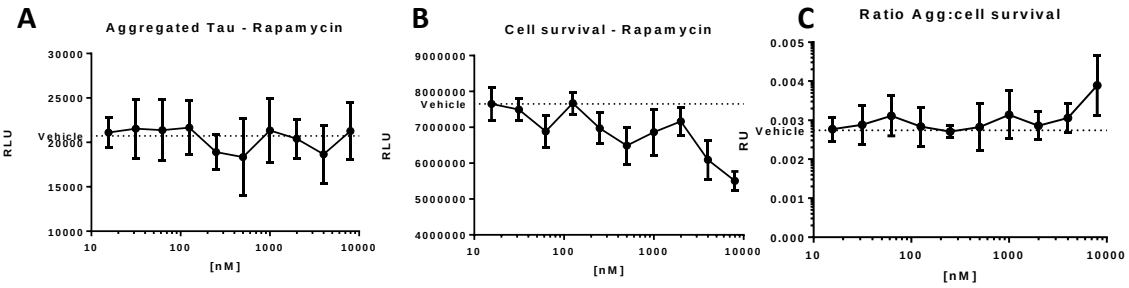

Figure 30 – **Rapamycin treatment does not change Tau aggregation.** Clone 20 cells were treated with different concentrations of rapamycin through 2 days after inducing Tau expression and aggregation with K18:P301L fibrils. Tau aggregation was measured using alphaLISA (A) and cell viability by luciferase (B). (C) Represents the ratio between aggregated Tau and cell survival values (error bars: SD, n=6)

Our results show that autophagy activation by rapamycin treatment (A) does not change the amount of aggregated Tau in our model. This results are particularly important since we saw through biochemical and imaging assays that a concentration of 500 nM is enough to trigger the autophagy pathway via mTOR. Even though in this experiment we used concentrations as high as 8 µM there was no change in the effective clearance of aberrant proteins. Also important is the fact that with large amounts of rapamycin there is a significant increase in cell death (B). In fact the ratio between the values for aggregated Tau and cell viability (C) shows that there is an increment in the amount of aggregation, most likely cause by failure in other degradation systems.

To further understand if mTOR inhibition could have a true impact on the amount of aggregated Tau, we decided to knock down the mTOR expression using three separate siRNA's for the FRAP1 gene (FK506 binding protein 12-rapamycin associated protein 1 or mTOR) and compare the results with two non-targeting negative control siRNA (SiNC) and two siRNA for Tau.

We found that all three siRNAs for mTOR have a significant decrease in the amount of aggregated Tau when compared to the two scrambled siRNAs after two days of Tau seeding (protocol time similar to the one used for the compound screening) [Figure 31]. Even more, mTOR knock-down did not produce cell death detectable by the CellTiter Glo assay (data not shown), even though this protein is known to regulate many cellular actions. Therefore autophagy induction via mTOR inhibition might be a relevant pathway for aggregates clearance, although it must be said that validation of the mTOR expression knock-down is necessary to confirm this results.

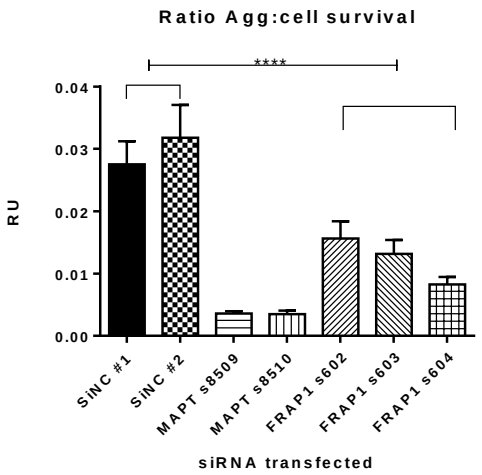

Figure 31 – **mTOR knock-down significantly decreases Tau aggregation.** siRNA transfection was done in Clone 20 cells, after which, Tau aggregation was induced with K18:P301L fibrils. Ratio between the alphaLISA values for aggregated Tau and cell survival detected by luciferase using CellTiter glo assay for the different siRNA's. (Error bars: SD, n=20,****p<0,0001)

This study shows that there might be a difference in the action of pharmacological methods for mTOR inactivation when compared with the direct knock down by siRNA's. We have proven that mTOR pathway inhibition could be a strong target when reducing Tau aggregation, but also that pharmacological approaches are significantly more difficult to obtain.

To complete our study we decided to use trehalose to see how the activation of other unknown autophagy pathways could affect the aggregation state of Tau. Trehalose is a natural, highly soluble disaccharide known to induce autophagy in neurons, that has been involved in the degradation of Tau (Krüger & Mandelkow, 2012). Even though the first results seemed promising, they were not reproducible in a satisfactory way (data not shown). In addition, only high doses (around the mM) of the molecule are known to induce autophagy, making it an undesirable control for our experiments.

Taken together these results showed that by one side the activation of autophagy may be a viable target for a future treatment, but on the other, efficient pharmacological targeting of the pathways could be difficult to achieve in suitable molecular concentrations.

3.4.2 Autophagy inhibition in Tau clearance

Recent papers have shown that in pathological conditions, Alzheimer's patients neurons may have compromised autophagy process when compared with normal aging people, adding to the fact that obsolete protein degradation could be compromised by faulty lysosomal degradation (Wong & Cuervo, 2010) (Menzies & Rubinsztein, 2015). One of the proposed hypothesis is that

autophagosomes accumulate inside the cell, not being able to deliver cargo to the lysosomes, therefor affecting the whole degradation pathway (Lee, Lee, & Rubinsztein, 2013).

To understand better how autophagy can be important in the degradation process of Tau, and also normal conditions for the model, we decided to inhibit this mechanism using some known compounds.

Bafilomycin was successfully used in previous experiments to inhibit lysosomal formation and was capable of blocking the phenotype formation. Therefore we decided to use it to see how lysosomal biogenesis and the endocytic pathway could impact Tau aggregation and cell viability [Figure 32]

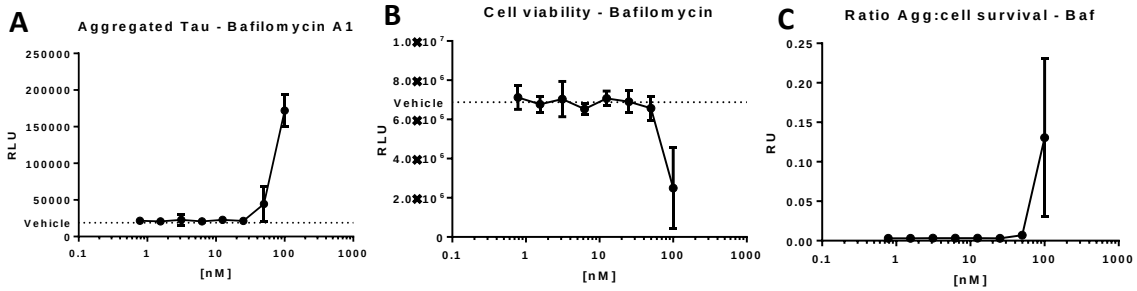

Figure 32 – **Bafilomycin A1 treatment is responsible for a significant increase in Tau aggregation and cell death.** Clone 20 cells were treated with different concentrations of bafilomycin A1 through 2 days after inducing Tau expression and aggregation with K18:P301L fibrils. Tau aggregation was measured using alphaLISA (A) and cell viability by luciferase (B). (C) Represents the ratio between aggregated Tau and cell survival values (error bars: SD, n=6)

Not surprisingly, bafilomycin treatment was responsible for a significant increase in Tau aggregation at high concentrations (A). More important, this increment was still observed in toxic condition when only around 35% of cell survival was observed (B,C). By showing an increment in the values of Tau aggregation in a dose response manner to lysosomal blockers after inducing aggregation by seeding with K18:P301L fibrils, we demonstrated that the degradation system is capable of clearing aberrant proteins, thus protecting the cells.

Since lysosomal pH is responsible for proteases activity and autophagosomes fusion with lysosomes, we thought about blocking the same using chloroquine. Chloroquine is a lysomotropic molecule, meaning it has the ability to accumulate in lysosomes and other acidic compartments, increasing their pH. In practical terms this means chloroquine administration simultaneously blocks proteases activity whilst inhibiting lysosomal fusion with autophagosomes in a pH dependent manner.

Once again our experiments show that extended stimulus of lysosomal dysfunction by increasing concentrations of a disturbing agent like chloroquine, is responsible for an increment in Tau aggregation values (A) [Figure 33]. Even more important, there is a clear increase in cellular death even with mild concentrations of chloroquine (B). Toxicity by this compound might be caused

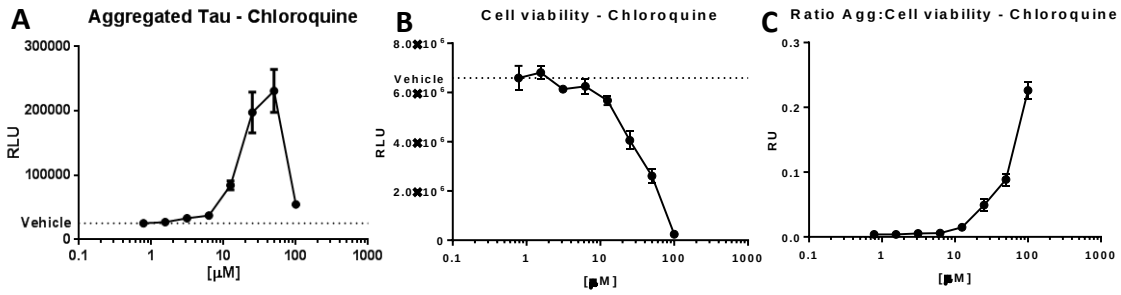

Figure 33– **Chloroquine treatment is responsible for a significant increase in aggregated Tau and cell death.** Clone 20 cells were treated with different concentrations of chloroquine through 2 days after inducing Tau expression and aggregation with K18:P301L fibrils. Tau aggregation was measured using alphaLISA (A) and cell viability by luciferase (B). (C) Represents the ratio between aggregated Tau and cell survival values (error bars: SD, n=6)

either by a deficient degradation process that affects all cellular metabolism, or by direct harmful action of the Tau aggregates that accumulate in the cell. Not surprisingly, the ratio between the aggregated Tau values and cell viability show a constant increment with chloroquine concentration (C).

After showing the relevance of lysosomal biogenesis and cargo delivery from the ealry autophagy process (autophagosomes) to the lysosomes in protein aggregates clearence, we decided to specificly block the enzime responsible for Tau degradation, cathepsin D (Kenessey, Nacharaju, Ko, & Yen, 1997). Pepstatin A was chosen based on its higlhy specific inhibitory activity against cathepsin D (k_i=10 µM), while not showing any effect against other lysosomal proteases like thiol, neutral, and serine proteases. In order to evaluate if cell death derives from general proteases inhibition or Tau aggregates acumulation, leupeptin was used as control. Leupeptin is known to specifically inhibit calpain, cathepsin B, H, J and tryspin among others, whereas no activity against cathepsin D is observed [Figure 34].

Our results show that leupeptin treatment does not produce any difference, neither in Tau aggregation nor in cell survival (A-C). This observation was consistent even at concentrations of 200 µM, significantly higher than the amount of compound estimated to inhibit most proteases of 10-50 µM. Therefore, we believe that the cell as mechanism that allow to bypass the inhibition of important lysosomal proteases and ensure cell survival in normal conditions. On the contrary, specific cathepsin D inhibition seems to be extremely important in situations where Tau is overexpressed and aggregated. The data presented show not only that there is a consistent significant increase in Tau aggregation with growing concentrations of pepstatin (D and G), but also that cell viability might be compromised with accumulation of aggregated Tau.

Summarizing, the data here presented shows that in pathological conditions of Tau overexpression and aggregation, lysosomal activity plays a critical role clearing obsolete proteins, even without external stimulus.

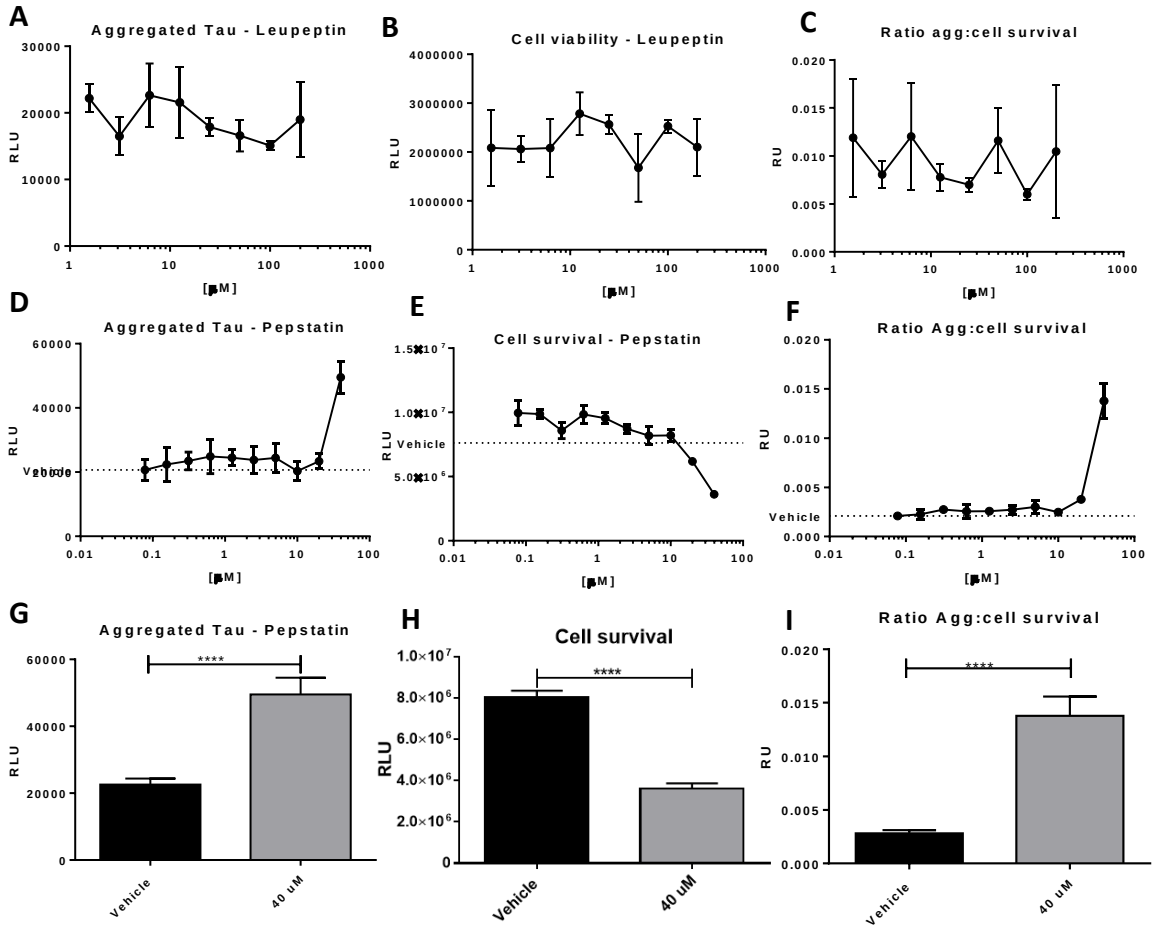

Figure 34 – **Specific inhibition of Tau protease is responsible for cell toxicity and increased Tau aggregation.** A and D, alphaLISA for aggregated Tau of clone 20 cells treated for 2 days with different concentration of leupeptin and pepstatin respectively. B and E, Luciferase values for the cell viability assay of the cells treated with different concentrations of leupeptin and pepstatine respectively. C and F, Ratio values between aggregated Tau values and cell viability of leupeptine and pepstatin treatments. G-I, comparison between the vehicle treatment and the highest concentration for the pepstatin treatment for aggregated Tau, cell viability and ratio between aggregation and cell viability values respectively. (Error bars: SD, n=4, ****p<0.0001).

3.5 Lysosomal biogenesis modulation in Tau clearance

So far we have showed that lysosomes formation and activity may play a more important role in Tau aggregates clearance than autophagy activation itself. How lysosomal biogenesis can be triggered by molecular stimulus still remains unclear, however our data suggests that our hit compounds may be responsible for this event by increasing endocytic vesicles delivery.

To better understand how lysosomal formation and the regulation of autophagy can interfere with protein and subcellular structures degradation, more specifically Tau aggregates, we decided to study the role of the transcription factors responsible for these processes regulation in our model. Transcription factor EB (TFEB) was recently proposed as a potential target in selective degradation

of aberrant Tau proteins as well as a player in the rescue of neurotoxicity (Polito & Zheng, 2014). TFEB is localized in the cytoplasm but after dephosphorylation is translocated into the nucleus where induces the expression of autophagy and lysosomal target genes via the CLEAR (coordinated lysosomal expression and regulation) element (Sardiello & Ballabio, 2009). Evidence also suggests that TFEB action is dependent on phosphatase and tensin homolog (PTEN) activity as an upstream target. Based on this information, TFEB and PTEN were chosen as targets for further investigation.

3.5.1 TFEB and PTEN are responsible for an increase in autophagy markers

The first question we decided to address was if TFEB and PTEN overexpression could be responsible for an increment in autophagy and lysosomal proteins expression in our model. To answer this question, two plasmids for TFEB and PTEN tagged with a myc-FLAG tag were transfected into QBI cells and results were compared with an empty vector transfection.

Contrary to what is in the literature, TFEB and PTEN overexpression were responsible for different outcomes when protein expression was analysed by Western Blot [Figure 35]. They seem to be responsible for changes in the different autophagy markers tested for. TFEB transfection induced the expression of proteins related with early (Rab5) and late endosomes (Rab7) caused by an increase in endosomes formation, whilst not changing lysosomal proteins (LAMP1) (B-D). This could be due either to a decrease in maturation to the lysosome, making endosomal vesicles to accumulate in the cytoplasm, or by a more efficient formation and degradation of lysosomes. On the other hand, PTEN transfection was responsible for a significant increase in LAMP1 but not in Rab5 and Rab7(B-D). In addition, the percentage of LC3B-II regarding total LC3B is also significantly increased when compared with empty vector, meaning that there is more conversion of this protein to the lapidated form (autophagosomes) (F). This does not mean necessarily that the endocytic pathway is not involved, but rather that there could be a higher delivery from late endosomes or autophagic vesicles into lysosomes, promoted by an activation of the system either down or upstream lysosomes.

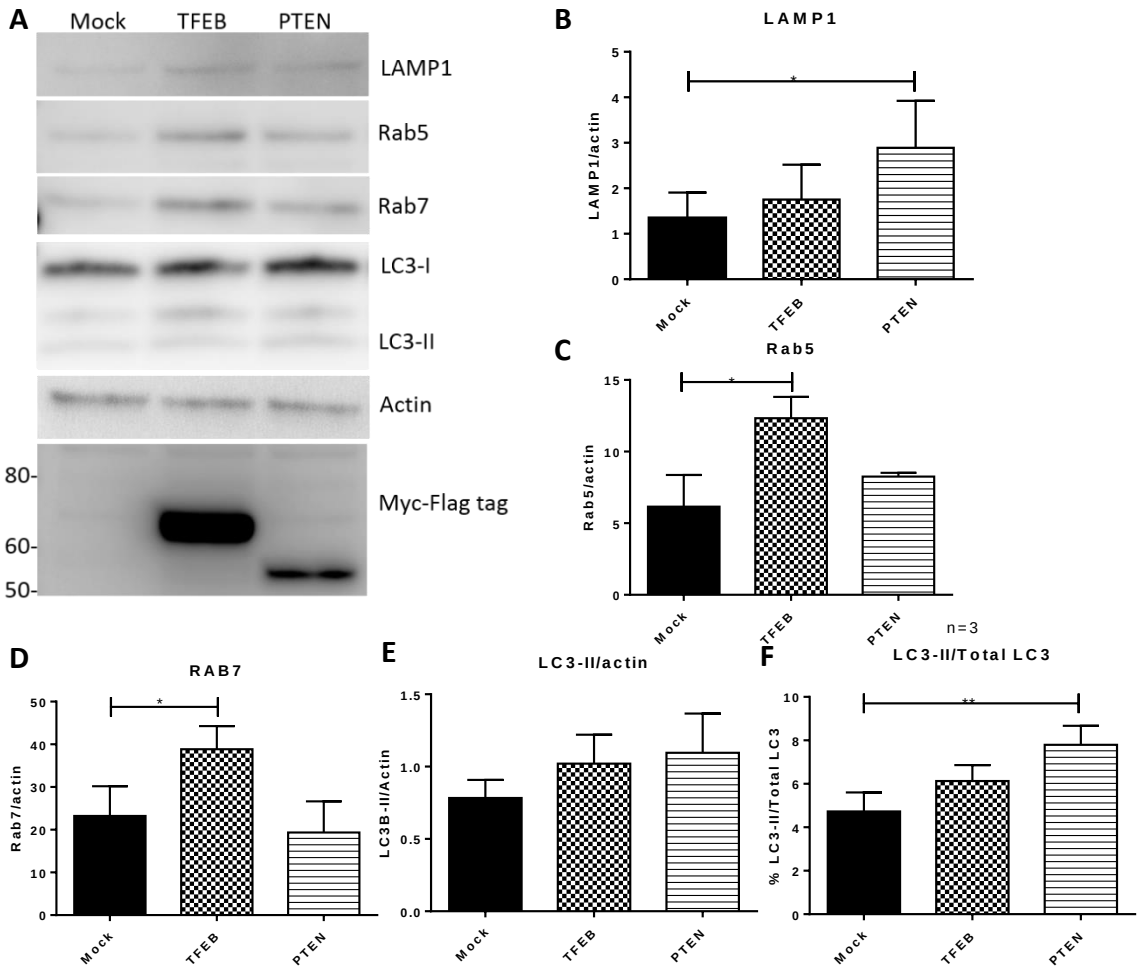

Figure 35 – **TFEB and PTEN over expression are responsible for independent changes in different markers.** A, WB for QBI cells transfected either with an empty vector (Mock), TFEB (predicted MW: 65-70 kDa) and PTEN (predicted MW: 54 kDa). B, WB quantification for LAMP1 in cells transfected with a Mock, TFEB and PTEN plasmid. C, WB quantification for Rab5 in cells transfected with a Mock, TFEB and PTEN plasmid. D, WB quantification for Rab7 in cells transfected with a Mock, TFEB and PTEN plasmid. E, WB quantification for LC3B-II in cells transfected with a Mock, TFEB and PTEN plasmid. F, Percentage of LC3B-II and Total LC3B in cells transfected with a Mock, TFEB and PTEN. (Error bars: SD, n=4 (except for Rab5, n=3), *p<0.05, **p<0.005)

Until this point we do not know why TFEB and PTEN transfections show different protein expressions regarding lysosomal biogenesis and autophagy regulation taking into account that both act in the same pathway. We hypothesize that since TFEB is a downstream transcription factor, other proteins that regulate late endosome maturation into lysosomes might be produced, thus affecting markers expression values. On the other hand, PTEN may have a mechanism of action more focused in autophagy vesicles delivery into degradative structures such as lysosomes.

3.5.2 TFEB, but not PTEN, significantly reduce Tau aggregation

To conclude our project, we decided to see if the changes observed in the protein expression of cells transfected with both TFEB and PTEN transduce in an effective clearance of Tau without affecting cell viability as it was previously proposed [Figure 36]. As a comparison, we used an empty vector without (no fibrils) and with K18:P301L fibrils seeding (Mock).

Our data shows that TFEB transfection was responsible for a significant decrease in the amount of aggregated Tau, while also increasing cell viability when compared with the "No fibrils" group, but especially with the mock plus fibrils condition (A,B). We found that the ratio between the signals for aggregated Tau and the cell viability were reduced by 49% when compared to mock transfected cells (C). In contrast, PTEN over expression did not cause a significant reduction in Tau aggregation, but did increase cell viability when compared with the mock condition. Because of this, the normalization of the aggregated Tau values significantly decreases when compared with the Mock condition. Therefor we assume that PTEN effect could be more focused on cell survival than in Tau clearance itself.

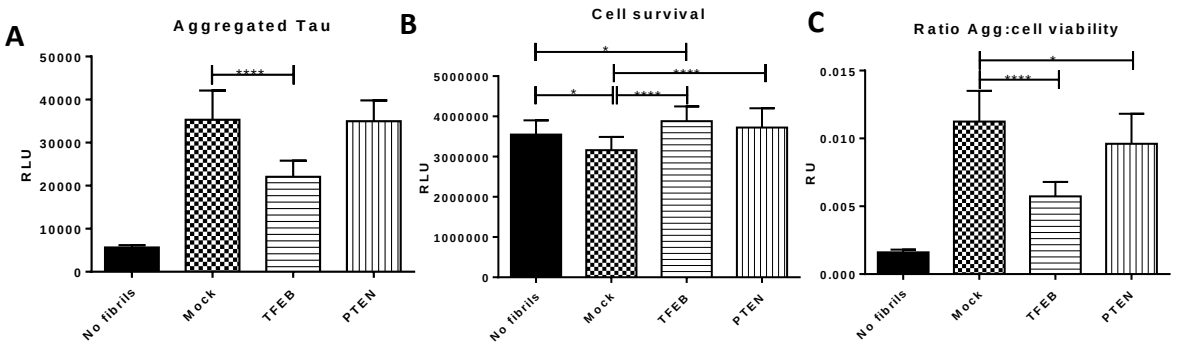

Figure 36 – **PTEN over expression is responsible for a significant decrease in Tau aggregation even though some cell viability is lost.** Clone 20 cells were transfected with an empty vector (Mock), TFEB and PTEN and were incubated for 2 days after inducing Tau expression and aggregation with K18:P301L fibrils except for the no fibrils condition that was just transfected with an empty vector. Tau aggregation was measured using alphaLISA (A) and cell viability by luciferase (B). (C) Represents the ratio between aggregated Tau and cell survival values (Error bars: SD, n=20, ****p<0.0001)

In conclusion, these results show that TFEB may be a good target for increased Tau clearance. The fact that this transcription factor was responsible for a higher degradation of the aberrant protein was probably also responsible for a better cell viability. As for PTEN, its action in Tau clearance was not as good as initially predicted. Nevertheless, the cell viability results show that it could be a viable target when trying to stimulate cellular survival.

Chapter 4 – Discussion and concluding remarks

Many late-onset neurodegenerative disorders like Alzheimer's diseases, Parkinson's, polyglutamines disorders and tauopathies share the same intra-cytoplasmic protein misfolding, aggregation and accumulation features. A great focus has been placed trying to identify new strategies that either block the build-up of this proteins aggregates, or enable the degradation of the same. Recently, our lab successfully identified a family of compounds responsible for the reduction of aggregated Tau in a cellular model based on the work of Guo and Lee (2011) via an unknown mechanism [Figure 11]. Here we found that some of these compounds affect aggregated Tau by targeting the autophagy and lysosomal biogenesis pathway. We were also able to show that lysosomal biogenesis and autophagy activation through TFEB overexpression may be a viable target in the future.

The compounds identified were part of a molecule library, produced in house, without any shared feature that would predict a molecular target. Surprisingly though, the incubation of some of this molecules for a short period of time was enough to induce the development of vesicles all over the cell [Figure 12]. We believed that the vesicles could be somehow related with the effects seen in Tau clearance. The first assays using commercial kits showed that the vesicles not only had a lipidic nature, but also that it could be related with an increment in autophagy related structures [Figure 13 and 14]. One of the main criticisms made to the kits used were related with the lack of independent and systematic validation in the literature, meaning no proper conclusions could be drawn at this point. Nevertheless, new studies have been published using these assays (Guo & Sheng, 2015) allowing us to have an idea where to look and confirm our first ideas.

Although not all of the active compounds were extensively tested, some of them showed the same phenotype in primary neuronal cultures, a more relevant cellular model for AD that the QBI cells used for screening [Figure 15]. By using this model we confirmed that the vesicle phenotype was reproducible on neurons and that this was a result of the active compounds treatment. In addition, by using the Cyto-ID kit, we showed that the vesicles phenotype was not related to mutant Tau expression or aggregation induction by K18:P301L fibrils. Nevertheless, the presence of one or both condition seems to slightly increase the development of autophagosomes. This could be caused by the fact that Tau expression constitutes a substrate for autophagy, thus stimulating the pathway. On the other hand, neurons treatment with the vehicle (DMSO) only showed a background signal that we attribute to basal autophagy. Interestingly though, mutant Tau overexpression by an AAV also increased the Cyto-ID staining. This result could be explained by the fact that the proteasome cannot degrade Tau in neurons in an efficient manner (Brown & Geddes, 2005). Therefore, we assume autophagy is activated in response to Tau overexpression.

Besides the results in the QBI cell model showed in this project, our group validated the active compounds identified in the HTS in neuronal primary cultures to see if they are active in this type of cells as well (data not shown). Most of the tested molecules, including the ones that develop the phenotype, have proven to be efficient in decreasing Tau aggregation. The results observed in the neurons are particularly important for us, as they prove that the mechanisms for the vesicles formation and the impact on aggregated Tau is conserved between the two types of cells. This enable us to use the QBI Clone 20 cells in our studies, allowing simpler and faster protocols.

Since the exact cellular target of the Cyto-ID kit is not known, we decided to identify the nature of the vesicles using proper, validated markers. First we studied the effect of the compounds on the lysosome formation as it represents the endpoint of the degradative pathways. We used LAMP1 as a marker for lysosomes, a highly specific protein of these structures. Using imaging technics, we confirmed that most of the vesicles developed after the active compound treatment were lysosomes [Figure 16]. Unfortunately LAMP1 quantification through Western Blot did not show any significant changes over time, most likely due to the constant degradation and turnover of this structure. Nevertheless, we were able to see a mild, non-significant, increase in this protein expression after 24 hours of treatment [Figure 17]0. To biochemically confirm the increase of lysosomes after our compound treatment, more repeats have to be done, preferentially with a more sensitive technique, such as ELISA. Altogether, this data shows that the compounds are most likely affecting the formation of lysosomes.

Even though increasing number of lysosomes and its enlargement does not represent a direct increment in protein degradation, this observation strengthens our initial idea that the autophagy pathway could be triggered by our compounds, and that this is in fact the main reason why Tau aggregation decreases. By increasing the size of the lysosomes, the cell is most likely increasing its capability to answer to harmful events. Based on the information and evidence available in the literature we decided to focus our attention on the process that could explain the large aggregates clearance in the cell: macroautophagy.

To properly address autophagosome formation through imaging technics we used p62 transfection with a tag for RFP [Figure 18]. p62 is an important protein in the autophagosome development since it promotes the link between ubiquitinated proteins and LC3, meaning it is responsible for mediating the targeting of outdated proteins for degradation. We found that our compounds did not promote the translocation of p62 from the cytoplasm to small vesicles in the same extend as with rapamycin, a known activator of autophagy via mTOR inhibition. Rather, the compounds induced both an increase of p62 levels in the cytoplasm and in smaller vesicles, which

could mean that there is either a blockage in autophagosomal fusion with lysosomes, or that there is an increment in the expression of the protein that is not being degraded at a satisfactory level. This pattern is similar to the one observed when rapamycin is co-administered with chloroquine, a lysomotropic molecule that inhibits binding of the autophagosomes and lysosomes.

To further complete our imaging studies, we decided to use the LC3 tandem probe (LC3 tagged with both GFP and RFP) [Figure 19]. This allowed us not only to see the expression level of the protein, but also to distinguish if autophagosomes mature into autolysosomes after fusion with lysosomes. Not surprisingly, rapamycin treatment at normal concentrations [500 nM] showed a clear increment in the number of vesicles marked as autolysosomes, product of the autophagy activation. As for the hit compound treatment, we predicted that with the increment in lysosomal structures there would be a significant staining only for red, despite the fact that p62 seems to suggest the opposite. Surprisingly though, there were few vesicles stained as autolysosomes, while some bigger ones were marked as autophagosomes. On the other hand, the treatment showed a higher expression of LC3 stained for both channels all over the cell. This could be explained either by a blockage in the fusion between the autophagosomes and lysosomes or by a quick degradation of LC3 upon the merging of both structures due to higher activity of lysosomal proteases. None the less a higher expression of LC3 was detected.

Our imaging assays to assess autophagy markers have confirmed that indeed there is a modification on this pathway upon compound treatment. Although our first idea was confirmed, we observed that most likely the mechanism by which changes occur is not dependent on downstream regulators of the pathway. To gain a better understanding on how the hit molecules affect autophagy, we decided to use biochemical technics to quantify protein expression.

We decided to study LC3 behaviour, based on the data obtained with the imaging technics. We observed a direct relation between the concentration of compound used and the amount of LC3B-II expressed in the cells [Figure 20]. Even more important for us, concentrations ten times lower than the ones used for the imaging assay (500 nM) showed to have an impact only 4 hours after the treatment. On another experiment taking into account different incubation times with our molecules, we have showed that LC3B conversion to the lapidated form is time dependent, with differences being detected even after 1 hour of treatment [Figure 21]. Also important, we observed that after longer periods, such as 24 hours, there was some decrease in the amount of LC3B-I, while the lipidated form was almost oversaturated. This results confirmed our initial observation with the LC3 tandem probe that the compounds cause an overexpression of this protein. In addition, the expression variation with time suggests that this could be a critical

variable when analysing data. In practical terms, the results here presented could imply that there is either a higher amount of autophagosomes or that this structures present a bigger size. Nevertheless, a higher amount of autophagosomal markers point to a better capacity of the cell to “arrest” Tau aggregates through vesicles formation and deliver them to the proper structures for degradation.

The fact that we saw a dose and time dependent response in LC3B-II expression after compounds treatment supports our initial hypothesis that the active molecules responsible for the phenotype development are able to induce modifications in the autophagy pathway. Despite this fact, neither the imaging our biochemical assay are able to determine satisfactorily if there is a change in the autophagic flux. In fact, inability to measure autophagic flux is pointed as one of the main limitations in the field (Klionsky & Zuckerbraun, 2012). This is caused by the fact that autophagy is a continuous process of formation and degradation of vesicles, and the methods used to quantify are static, meaning they can only measure the protein existence in a certain time point. On another words, the increase in LC3 lipidation could be the result of higher expression through a stimulus or impaired fusion of autophagosomes with lysosomes. In the same way, stable levels of this protein could be caused by a more efficient degradation of autophagosomes.

To properly measure autophagic flux, we decided to use the methodology proposed by Rubinsztein and colleagues (Rubinsztein, Cuervo, & Klionsky, 2009). Briefly, this method measures the conversion of LC3 through Western Blot of cells treated with one treatment and compares it with a bafilomycin condition, as wells as with the treatment and bafilomycin simultaneously. In theory, a treatment that induces the autophagic flux should produce an increment in LC3B-II expression when used in combination with bafilomycin as compared with the other two conditions separately. In our case, the combined treatment of the compound with bafilomycin did not show any statistical difference when compared with the other conditions alone, for shorter and longer time points [Figure 22 and 23]. This observation suggests that the active compounds treatment does not stimulate the autophagic flux.

Data collected until this point did confirm the alternative hypothesis that the effect of the compounds on autophagy targeting is not due to a stimulus of the pathways responsible for the autophagosomes formation. To be completely sure, we decided to check for differences in the expression and phosphorylation state of p70 S6 kinase for all the three of the active compounds we have used during this project [Figure 24]. p70 S6 kinase is a protein from the mTOR pathway that is directly regulated by phosphorylation promoted by the mTOR complex itself (Maiese , 2014). Our data showed that despite a slight increase with the compounds, none of our conditions

produces significant changes in the kinase expression. On the other hand, only rapamycin was responsible for a significant decrease in p70 S6 kinase phosphorylation, as it was predictable taking into account its action on mTOR. It has to be highlighted however that at least two more pathways are known to interfere with autophagy regulation (the AMPK and p53) in ways still unclear.

Interestingly, when we tried to assess autophagy flux by using one of our active compounds, we observed that the vesicles development was completely blocked by bafilomycin, even after long periods of pre-incubation with the compound or low concentration of bafilomycin [Figure 25]. Although we were not expecting this impact on the phenotype, the result can be explained taking into account that we identified most of the structures as lysosomes. Lysosomal biogenesis is carried by a complex system that involves the endocytic pathway, the Golgi and the ER. To occur, the vesicles formed in the endocytic pathway from the plasma membrane need to mature into other structures, namely early and late endosomes, followed by a successive acidification of these structures (Ng & Tang, 2012) (Hirota & Tanaka, 2007). By using bafilomycin in saturating concentrations, the V-ATPase activity is inhibited, thus disabling endosomal maturation by decreasing their pH, culminating in the blockage of lysosomes formation (Sobota & Mains, 2009).

Since our previous experiments have shown that the vesicles increment was not due to a stimulus in the autophagy pathway and that the phenotype could be blocked by bafilomycin, we decided to change the focus on the structures origin and study if the compounds could somehow interfere with lysosomal biogenesis and function. By using the pH sensitive dye Lysotracker we were able to observe a significant increase in acidification of subcellular compartments with the active molecules treatment [Figure 26]. Not only this helps proving that the compounds action is responsible for the increment in lysosomal structures, but also shows that this structures are most likely functional. Not surprisingly, rapamycin also created the same result by autophagy activation, while bafilomycin had the opposite effect by reducing the signal to background levels. Moreover, the simultaneous use of the compound with bafilomycin caused the same pH depletion as bafilomycin alone. This shows that despite the strong compounds action, the development of lysosomes can be blocked by pH regulation.

To confirm this observations we also used LAMP1 quantification by biochemical assay to see how the previous conditions can limit lysosomes formation [Figure 27]. Once again bafilomycin did not produced any change in LAMP1 expression. We attribute this to the fact that while no new lysosomes were produced by inhibition of the endocytic pathway, there was also the blockage in degradation by pH depletion (Yamamoto & Tashiro, 1998). As for the combined use of the

compounds and bafilomycin, we observed that despite the slight increment at 4 hours of incubation, with time there was a normalization of the values. This is probably caused by the stimulating effect of the compound in the induction of a higher expression of lysosomal proteins in the first hours, after which, the blockage of new endosomes delivery stabilizes the values. Once again it has to be highlighted that this quantification of LAMP1 by Western Blot lacks sensibility and other methodologies should be applied in the future assays.

Our latest data strongly suggest that the origin of the vesicles could be attributed to an increment of the endocytic pathway activity that culminate in the increase of lysosomal structures. As a follow up of this hypothesis we decided to use imaging assays to identify early endosomes via Rab5 expression [Figure 28]. Images showed that there is a modification on the morphology of the vesicles stained with this protein upon treatment with the hit compounds when compared with our control. Not only there was an enlargement of this structures but we also observed an increment in the number of the same. In addition, we observe that in this conditions, early endosomes localize close to lysosomes, enhancing the idea of the close proximity between this two systems (Rink & Zerial, 2005).

Once again we choose to confirm the imaging results by quantifying protein markers in a time dependent manner, since, as we have noticed before, time could play a critical role when studying protein expression with compounds treatment. This time however we used Rab7 to assess late endosomes formation once this are closer in the pathway to lysosomes formation [Figure 19]. Quantification for the hit compounds condition showed that Rab7 expression non-significantly decreased in the first 4 to 8 hours of treatment, while slightly increasing after 24 hours. We attribute this change to higher maturation rate of late endosomes into lysosomes that cause a quick turnover in Rab7 expression. This could explain a short term depletion of the protein that is compensated after 24 hours by either higher expression to compensate the imbalance or by a decrease in the delivery of endosomes by saturation of the system (Ng & Tang, 2012). In this same experiment, we also observed that bafilomycin did not change the expression of Rab7 in the first 8 hours, and at 24 there was only a slight increment, possibly caused by the accumulation of vesicles due to blockage of the path. As for the combined treatment, no modification in the markers expression was observed regardless the time set considered. It should be highlighted however that these quantifications were not significant and that changes in Rab5 and Rab7 do not necessarily imply changes in the endocytic pathway. Future experiments have to be done in order to properly measure changes in the endocytic pathway upon the compound treatment

Concluding, we believe that some the active compounds identified in the HTS are capable of inducing degradation of Tau aggregates by increasing lysosomes development via the endocytic pathway stimulus. Also important, we have shown that even without the direct stimulus of the autophagic initiation machinery it is possible to induce LC3B expression and protein degradation. Until this point, we still do not know if the effect on autophagy is a collateral event from lysosomal and endosomal stimulation or on the other hand can be the result of other mechanisms affected by the compounds.

In an attempt to better understand how regulation of the autophagy and lysosomal systems activity could influence the clearance of Tau aggregation we have decided to stimulate or inhibit this pathways using validated control molecules. Contrary to what we were expecting, autophagy stimulation by rapamycin has proven to be inefficient in increasing aggregated Tau degradation [Figure 30]. This was true event at high concentration (8 μM) when some cell toxicity starts to be evident, which contrary most of the data found in the literature (Williams & Rubinsztein , 2006) (Richardson & Oddo, 2014) (Wyttenbach & Tolkovsky, 2008). The results were even stranger if one takes into account that concentrations 16 times lower (500 nM) than the ones used for this study were potent enough to induce autophagy activation in our imaging studies with the LC3 tandem probe and the biochemical quantification of phosphorylated p70 S6 kinase. However, when we used siRNAs transfection to knock-down the mTOR gene we observed a significant decrease in Tau aggregation, which oppose to the results obtained with rapamycin [Figure 31]. Furthermore we did not detect any significant difference in cellular toxicity in the knock-down cells.

The data collected demonstrated that even though mTOR inhibition could be considered a potential approach in aggregated Tau clearance, pharmacological induction of autophagy can be much more difficult to achieve in viable doses and in the proper time sets. This could be explained by the fact that inhibition by rapamycin has different potencies in the mTOR complexes 1 and 2. While the effect is highly potent in mTORC1, the inhibition of mTORC2 seems to be much more complex, depending on a more prolonged treatment with higher doses and on the type of cell studied (Barquilla & Navarro, 2008) (Sarbassov & Sabatini, 2006). We hypothesise that the different targeting of rapamycin could interfere with the specificity of autophagy for protein aggregates, since the two complexes regulate different pathways. This does not explain however the contradictory effects on aggregated Tau found in the current literature. Other hypothesis to explain the lack of activity could evolve modification of other pathways not related with mTOR that counter autophagy in the time set. Moreover, when we used trehalose, a disaccharide that has been correlated with autophagy activation and decrease in Tau aggregation in neurons (Krüger & Mandelkow, 2012), we did not observe any difference. Therefor we conclude that at least in our

model, targeting autophagy initiation through pharmacological strategies does not necessarily lead to a decrease in protein aggregates.

After trying to increase Tau clearance through the autophagy pathway without success, we tried to obtain the opposite effect by blocking lysosomal activity. Bafilomycin was the first compound chosen since we had previously shown that it could block lysosomal biogenesis by depletion of the vesicular acidification, also inhibiting the fusion between autophagosomes and lysosomes (Yamamoto & Tashiro, 1998) (Xie & Dong, 2014). Not surprisingly Tau aggregation suffered a significant increase of almost 10 times when compared with the vehicle condition [Figure 32]. Even more, this values were observed when a high amount of cellular viability was lost. This constitutes the first evidence that lysosomal activity plays a critical role keeping the homeostasis inside the cell and clearing potential toxic elements such as Tau aggregates in our model.

As a follow up, we used chloroquine to increase the pH inside lysosomal vesicles and at the same time block fusion between autophagosomes and lysosomes (Harhaji-Trajkovic & Trajkovic, 2012) (Thomé & Verinaud, 2013) [Figure 33]. Once again there was a significant increase in Tau aggregation values and consequent increase in cell toxicity. This time however, we used enough chloroquine to completely kill all the cells, again showing the importance of the lysosomal structure in keeping the homeostasis and ensuring cell survival in stress conditions.

Whether the cell death was caused by the loss of lysosomal function or by the toxic effect of more aggregated Tau was still a topic of discussion. To overcome this, we used Pepstatin A1, a specific inhibitor for cathepsin D, the lysosomal protease responsible for Tau degradation, and compared with another inhibitor for a more broad set of proteases (Leupeptin) [Figure 34]. As expected, Leupeptin treatment did not cause any increment in aggregated Tau and was not responsible for any significant change in the cell viability, even at concentrations substantially higher than the ones predicted to inhibit most of the lysosomic proteases. On the other hand, the use of pepstatin was responsible for a major increase in Tau aggregation followed by increased cell death at higher concentrations. To confirm if the toxic effects were a consequence of Tau aggregates accumulation or other metabolic failure caused by pepstatin, we intend to do the same experiment without the seeding process in the future.

We conclude that the inhibition of a significant part of lysosomal proteases can be bypassed by the cell in short periods of time, probably by proteasome preferential degradation (Ihara & Nixon, 2012). Also important, these results show that the inhibition of the mechanism responsible for the degradation of Tau may cause a fateful event for the cells that overexpress this protein and are exposed to aggregate-prone conditions. Confirmation on the true toxic effect of Tau

aggregates accumulation after degradation blockage is still necessary. We hypothesise that by knocking down the main pathway for Tau degradation, other proteases may interfere with more harmful effects. One of this enzymes could be asparagine endopeptidase (AEP), a thiol protease associated with the biogenesis of cathepsins and lysosomal degradation (Shirahama-Noda & Hara-Nishimura, 2003), that has been described as one of the responsible for neurofibrillary pathologies in AD through generation of neurotoxic fragments (Zhang & Ye, 2014) and Tau hyperphosphorylation (Basurto-Islas & Iqbal, 2013). Besides AEP, there are other proteases in the lysosome that play a more secondary role that may be responsible for the increase in toxicity through formation of Tau motifs with β-sheet structure more prone to induce aggregation (Wang & Mandelkow, 2010) (Liu & Eisenberg, 2012) (Patterson & Binder, 2011). Finally, it is possible that by blocking its specific degradation, Tau monomers and seeds accumulate in the cell leading to an increase in the amount of aggregates that cause higher toxicity (Kumar & Mandelkow, 2014).

During our project we have successfully shown that lysosomal biogenesis and proteases activity can promote the cellular clearance of Tau aggregates, while the pharmacological stimulus of the autophagy pathway seems to be insufficient for our pretentions. To our knowledge, so far no one was able to induce lysosomal biogenesis through pharmacological treatment, therefore we had to use TFEB transfection to stimulate the CLEAR network. PTEN was also used based on previous work of Polito *et al.* that shown that TFEB's effect might be mediated through this protein (Polito & Zheng, 2014).

Our data showed that overexpression of both proteins can induced molecular changes in proteins usually used as markers for autophagic, lysosomal and endosomal vesicles [Figure 35]. Although we proved that both proteins can induce modifications in the degradative machinery, we also observed differences in the structures targeted depending on the plasmid used. PTEN transfection caused an increase in LC3B-II and LAMP1, markers for autophagy and lysosomes respectively. This result showed that PTEN as an effect more focused in the autophagy initiation, complying with the known role in the AMPK pathway (Brenman & Williams , 2013). It has to be pointed that the increase in these markers does not necessarily mean an increment in the autophagic flux, as well as it does not imply an increase in the degradation of protein aggregates. As for TFEB, we observed an increment in the endosomal proteins, which was expected taking into account the known mechanism and role of this transcription factor in the formation of lysosomes (Settembre & Ballabio, 2014) (Peña-Llopis & Brugarolas, 2011). Once again, the fact that no significant changes were detected in the LC3B and LAMP1 proteins does not imply less degradation, as the flux can be upregulated and with it the recycling of cellular elements and structures.

Having shown that overexpression of both proteins affect the lysosomal and autophagic pathways, we decided to test the effect of their overexpression on Tau aggregate clearance [Figure 36]. As expected, TFEB transfection showed a significantly decrease in Tau aggregation while increasing cell viability when compared with cells transfected with an empty vector and seeded with K18:P301L fibrils. This could be explained by the fact that lysosomal biogenesis via the CLEAR network is responsible by increasing the clearance of protein aggregates like Aβ (Xiao & Lee, 2014), Tau (Polito & Zheng, 2014), α-sinuclein (Wang & Liu, 2015), huntingtin (Tsunemi & La Spada, 2012), and other toxic proteins (Pastore & Brunetti-Pierri, 2013). Due to the reduction of stress levels promoted by a higher clearance of obsolete and dysfunctional structures, there is an increase in cellular viability in the cells transfected with TFEB when compared with the empty vector, as supported by research in cancer and others (Emanuel & Razani, 2014) (Marchand & Boucher, 2015).

As for PTEN transfection, we did not observe a significant decrease in the total values of aggregated Tau. However, the overexpression of this protein was responsible for a higher cell survival when compared with the empty vector, culminating in a reduction of the ratio between the aggregated Tau values and cell viability. This could be explained by the fact that PTEN intervenes in the AMPK pathway, being responsible for the activation of autophagy via mTOR (Roe & Ren, 2015) (Chen & Zhu, 2015). Like we have shown before, simple induction of autophagy, as the one we have tried with rapamycin, does not seem to be enough to reduce protein aggregates at a satisfactory level, being compliant with results we had before. On the other hand, it has been showed that PTEN can regulate cell cycle, inducing cellular proliferation and cell survival even in neurons, therefor explaining the results with CellTiter glo (Morani & Isisdoro, 2014) (Goh & Tan, 2014). We hypothesise that while PTEN may be essential in TFEB action, the protein by itself is not capable of inducing an effective clearance of protein aggregates, once again showing that lysosomal biogenesis via the endocytic pathway may be more relevant for Tau clearance that simple autophagy activation.

Concluding remarks

In this study we have shown how screening assays have helped finding new molecules with the ability to decrease Tau aggregation via targeting of lysosomal biogenesis and the endocytic pathway. Even though the work of many groups point autophagy as the desirable target in future proteinopathies treatment, we have proven that simple manipulation of this pathway with classical inducers may not be enough. On the other hand, we showed that inhibition of lysosomes

formation and function has the opposite effect by inducing Tau aggregation and cell death, thus demonstrating the relevance of this structures in pathological conditions.

In an attempt to manipulate lysosomal biogenesis, we were able to not only change some of the vesicular markers, but we were also able to induce Tau aggregates clearance via TFEB transfection. In fact, and taking into account recent evidence in many neurodegenerative disorders, TFEB seems to be a more desirable target to induce degradation of protein aggregates and increase cell survival.

Further studies will be necessary to understand how TFEB can be pharmacologically modulated and how aggregates clearance is efficiently promoted by it. We also intend to expand our knowledge on the mechanism of the active compounds action by studying the vesicles nature. The development of new molecules with the capability to induce protein aggregates clearance could represent a new set of therapies not only for AD and tauopathies, but also to other neurodegenerative disorders such as PD and Huntington's.

Chapter 5 - References

Glockshuber, R., & Finder, V. (2007). Amyloid-β aggregation. *Neurodegenerative Diseases*, 13-27.

Lau, K.-F., Howlett, D. R., & Kesavapany, S. (2002). Cyclin-Dependent Kinase-5/p35 Phosphorylates Presenilin 1 to Regulate Carboxy-Terminal Fragment Stability. *Molecular and Cellular Neuroscience*, 13–20.

Stork , B., & Alers, S. (2012). Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Molecular cellular biology*, 2-11.

Aguzzi, A., & Rajendran, L. (2009). The Transcellular Spread of Cytosolic Amyloids, Prions, and Prionoids. *Neuron*, 783–790.

Allen, B., Ingram, E., & Takao, M. (2002). Abundant Tau Filaments and Nonapoptotic Neurodegeneration in Transgenic Mice Expressing Human P301S Tau Protein. *The Journal of Neuroscience*, 9340–9351.

Amadoro, G., Corsetti, V., & Calissano, P. (2011). Endogenous A causes cell death via early tau hyperphosphorylation. *Neurobiology of Aging*, 969–990.

Andreadis, A., William, B. M., & Kosik, K. S. (1992). Structure and Novel Exons of the human tau gene. *Biochemestry*, 10626-10633.

Avila, J., de Barreda, E. G., & Engel, T. (2010). Tau phosphorylation in hippocampus results in toxic gain-of-function. *Biochemical Society Transactions*, 977-980.

Azorsa, D. O., Robeson, R. H., Frost, D., & Hoovet, B. M. (2010). High-content siRNA screening of the kinome identifies kinases involved in Alzheimer's disease-related tau hyperphosphorylation. *BMC genomics*, 11-25.

Ballabio, A., & Fraldi, A. (2010). Lysosomal fusion and SNARE function are impaired by cholesterol accumulation in lysosomal storage disorders. *The EMBO journal*, 3607–3620.

Ballabio, A., & Settembre, C. (2011). TFEB Links Autophagy to Lysosomal Biogenesis. *Science*, 1429–1433.

Barquilla, A., & Navarro, M. (2008). Rapamycin inhibits trypanosome cell growth by preventing TOR complex 2 formation. *Proc Natl Acad Sci U S A*, 14579-84.

Basurto-Islas, G., & Iqbal, K. (2013). Activation of asparaginyl endopeptidase leads to Tau hyperphosphorylation in Alzheimer disease. *Journal of Biological Chemestry*, 17495-507.

Baumann, K., Mandelkow, E., & Biernat, J. (1993). Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. *FEBS letters*, 417-424.

Bhat, R., Leonov, S., & Lee, C. (2002). Interactions between deathGSK3beta and caspase signalling pathways during NGF deprivation induced cell. *Journal of Alzheimer's Disease*, 291–301.

Braak, E., & Braak, H. (1991). Neuropathological staging of Alzheimer related stages. *Acta Neuropathol*, 239–259.

Brenman , J., & Williams , T. (2013). Basal autophagy induction without AMP-activated protein kinase under low glucose conditions. *Autophagy*, 1155-65.

Breteler, M. M. (2000). Vascular risk factors for Alzheimer's disease:: An epidemiologic perspective. *Neurobiology of Aging*, 153-160.

Bright, N., & Paul Luzio, J. (2007). Lysosomes: fusion and function. *Nature rewies*, 622-632.

Brown , M., & Geddes, J. (2005). Proteasome or calpain inhibition does not alter cellular tau levels in neuroblastoma cells or primary neurons. *Journal of Alzheimer disease*, 15-24.

Brunden, K. R., Ballatore, C., Crowe, A., Lee, V. M.-Y., & Trojanowski, J. Q. (2010). Tau-directed drug discovery for Alzheimer's disease and related tauopathies: A focus on tau assembly inhibitors. *Experimental Neurology*, 304–310.

Brunden, K. R., Trojanowski, J. Q., & Lee, V. M.-Y. (2009). Advances in Tau-focused drug discovery for Alzheimer's disease and related tauopathies. *Nat Rev Drug Discov*, 783–793.

Bullmann, T., Holzer, M., Mori, H., & Arendt, T. (2009). Pattern of tau isoforms expression during development in vivo. *International Journal of Developmental Neuroscience*, 591–597.

Cardenas, A. M., Ardiles, A. O., Barraza, N., & Caviedes, P. (2012). Role of Tau Protein in Neuronal Damage in Alzheimer's Disease and Down syndrome. *Archives of Medical Research*, 645-654.

Chang, C., Wang, Y., Lu, C., & Huang, C. (2009). Elevated basal cortisol level predicts lower hippocampal volume and cognitive decline in Alzheimer's disease. *Journal of Clinical Neuroscience*, 1283-1286.

Chasseigneaux, S., & Allinquant, B. (2012). Functions of Aβ, sAPPα and sAPPβ : similarities and differences. *Journal of Neurochemestry*, 99-108.

Chen, J.-H., & Zhu, X.-F. (2015). ATM-mediated PTEN phosphorylation promotes PTEN nuclear translocation and autophagy in response to DNA-damaging agents in cancer cells. *Autophagy*.

Chirita, C. N., Congdon, E. E., Yin, H., & Kuret, J. (2005). Triggers of Full-Length Tau Aggregation: A Role for Partially Folded intermediates. *Biochemestry*, 5862-5872.

Ciechanover, A., & Kwon, Y. T. (2015). Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. *Experimental & Molecular Medicine*, 47.

Clavaguera, F., Tolnay, M., & Grueninger, F. (2014). Intercellular transfer of tau aggregates and spreading of tau pathology: Implications for therapeutic strategies. *Neuropharmacology*, 9-15.

Coleman, P. D., & Yao, P. J. (2003). Synaptic slaughter in Alzheimer's disease. *Neurobiology of Aging* , 1023–1027.

Colombo, M., & Zoppino , F. (2010). Autophagosome formation depends on the small GTPase Rab1 and functional ER exit sites. *Traffic*, 12461261.

Crespo-Biel, N., Theunis, C., & Borghgraef, P. (2014). Phosphorylation of protein Tau by GSK3β prolongs survival of bigenic Tau.P301L × GSK3β mice by delaying brainstem tauopathy. *Neurobiology of Disease*, 119–132.

Cripps, D., Thomas, S., Jeng, Y., & et al. (2006). Alzheimer disease-specific conformation of hyperphosphorylated paired helical filament-Tau is polyubiquitinated through Lys-48, Lys-11, and Lys-6 ubiquitin conjugation. *Journal of Biological Chemestry*, 10825-10838.

Cruz, J. C., Tseng, H.-C., & Goldman, J. A. (2003). Aberrant Cdk5 Activation by p25 Triggers Pathological Events Leading to Neurodegeneration and Neurofibrillary Tangles. *Neuron*, 471–483.

Cuervo, A. M. (2011). Chaperone-mediated autophagy: Dice's 'wild' idea about lysosomal selectivty. *Nature reviews*, 535-541.

de Duve, C. (1963). The lysossome. *Scientific American*, 64-73.

de Leon, M. J., Rusinek, H., & Mosconi, L. (2007). Early detection of Alzheimer's disease using neuroimaging. *Experimental Gerontology*, 129–138.

De Strooper, B., Delacourte, A., Papadopoulou, A. S., Horré, k., & Hébert, S. S. (2009). MicroRNA regulation of Alzheimer's Amyloid precursor protein expression. *Neurobiology of Disease*, 422–428.

DeKosky, S. T., Schef, S. W., & Styren, S. D. (1996). Structural Correlates of Cognition in Dementia: Quantification and Assessment of Synapse Change. *Neurodegeneration*, 417–421.

Delorme-Axford, E., & Klionsky, D. J. (2015). The yeast Saccharomyces cerevisiae: An overview of methods to study autophagy progression. *Methods*, 3-12.

Dickey, C. A., Eriksen, J., Kamal, A., & Burrows, F. (2005). Development of a high throughput drug screening assay for the detection of changes in tau levels -- proof of concept with HSP90 inhibitors. *Current Alzheimer Research*, 231-238.

Dickey, C. A., Kama, A., Lundgren, K., & Klosak, N. (2007). The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins. *Journal of Clinical investigaion*, 648-58.

DiFiglia, M., & Kegel, K. (2000). Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *Journal of neuroscience*, 7268-7278.

Ding, W.-X., Ni, H.-M., & Gao, W. (2007). Linking of Autophagy to Ubiquitin-Proteasome System Is Important for the Regulation of Endoplasmic Reticulum Stress and Cell Viability. *The American Journal of Pathology*, 513–524.

Dou, F., Netzer, W. J., Takashima, A., & Xu, H. (2003). Heat shock proteins reduce aggregation and facilitate degradation of tau protein. *International Congress Series*, 383 – 393.

Drewes, G., Ebneth, A., & Preuss, U. (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell*, 297–30.

Dupuis, L. (2013). Mitochondrial quality control in neurodegenerative diseases. *Biochimie*, 177e183.

Duran, R., & Hall, M. (2012). Regulation of TOR by small GTPases. *EMBO reports*, 121-128.

Duthey, B. (2013). *Background Paper 6.11 Alzheimer Disease and other Dementias.* WHO.

Dutschmann, M., Menuet, C., & Stettner, G. M. (2010). Upper Airway Dysfunction of Tau-P301L Mice Correlates with Tauopathy in Midbrain and Ponto-Medullary Brainstem Nuclei. *Neurobiology of Disease*, 1810 –1821.

Ebneth, A., Godemann, R., Stamer, K., & Illenberger, S. (1998). Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *The Journal of Cell Biology*, 777–794.

Emanuel, R., & Razani, B. (2014). Induction of lysosomal biogenesis in atherosclerotic macrophages can rescue lipid-induced lysosomal dysfunction and downstream sequelae. *Arterioscler Thromb Vasc Biol*, 1942-52.

Fang, C., Gao, C., Xu, Z., Zhang, M., Wang, Y., & Li, J. (2011). Vascular risk factors promote conversion from mild cognitive impairment to Alzheimer disease. *Neurology*, 1485-1491.

Feinstein, S. C., & Wilson, L. (2005). Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediat neuronal cell death. *Biochimica et Biophysica Acta*, 268– 279.

Feldman, D. E., & Frydman, J. (1995). Protein folding in vivo: the importance of molecular chaperones. *Current Opinion in Structural Biology*, 92–102.

Frost, B., & Diamond, M. (2010). Prion-like mechanisms in neurodegenerative diseases. *Nature Review Neuroscience*, 155–159.

Frost, B., Jacks, R. L., & Diamond, M. I. (2009). Propagation of Tau Misfolding from the Outside to the Inside of a cell. *The fournal of biologycal chemestry*, 12845–12852.

Fukuda , M., & Itoh, T. (2008). Direct link between Atg protein and small GTPase Rab: Atg16L functions as a potential Rab33 effector in mammals. *Autophagy*, 824-826.

Fukutani, Y., Kobayashi, K., & Nakamura, I. (1995). Neurons, intracellular and extracellular neurofibrillary tangles in subdivisions of the hippocampal cortex in normal ageing and Alzheimer's disease. *Neuroscience Letters*, 57-60.

Funk, K., & Kuret, J. (2011). Granulovacuolar degeneration (GVD) bodies of Alzheimer's disease (AD) resemble late-stage autophagic organelles. *Neuropathol Appl Neurobiol*, 295-306.

Ghoresch, K., Laurence, A., & O'Shea, J. J. (2009). Selectivity and therapeutic inhibition of kinases: to be or not to be? *Nature Immunology*, 356–360.

Glickman, M., & Ciechanover, A. (2002). The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. *Physiological Reviews*, 373–428.

Goate, A. M., & Karch, C. M. (2014). Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biological Psychiatry*, in press.

Goedert, M., Clavaguera, F., & Tolnay, M. (2010). The propagation of prion-like protein inclusions in neurodegenerative diseases. *Trends in Neurosciences*, 317–325.

Goh, C.-P., & Tan, S.-S. (2014). Nuclear trafficking of Pten after brain injury leads to neuron survival not death. *Experimental Neurology*, 37–46.

Golde, T. E., Lewis, J., & McFarland, N. R. (2013). Anti-Tau Antibodies: Hitting the Target. *Neuron*, 254–256.

Götz , J., Probst , A., & Spillantini , M. (1995). Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *The EMBO journal*, 1304-1313.

Götz, J., & Ittner, L. M. (2008). Animal models of Alzheimer's disease and frontotemporal dementia. *Nature Reviews Neuroscience*, 532-544.

Götz, J., & Ittner, L. M. (2011). Animal models of Alzheimer's disease and frontotemporal dementia. *Nature reviews Neuroscience*, 532-544.

Guan, K.-L. (2013). ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nature Cell biology*, 741-750.

Guo, J. L., & Lee, V. M. (2011). Seeding of Normal Tau by Pathological Tau Conformers Drives Pathogenesis of Alzheimer-like Tangles. *The journal of biological Chemestry*, 15317–15331.

Guo, S., & Sheng, Z. (2015). A rapid and high content assay that measures cyto-ID-stained autophagic compartments and estimates autophagy flux with potential clinical applications. *Autophagy*, 560-572.

Haines, J., Cupples, L., & Farrer, L. (1997). Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. *JAMA*, 1349-1356.

Hanger, D. P., Anderton, B. H., & Noble, W. (2009). Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends in molecular medicine*, 112–119.

Hanger, D. P., Anderton, B. H., & Noble, W. (2009). Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends in Molecular Medicine*, 112-119.

Hanger, D. P., Anderton, B. H., & Noble, W. (2009). Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends in Molecular Medicine*, 112-119.

Harhaji-Trajkovic, L., & Trajkovic, V. (2012). Chloroquine-mediated lysosomal dysfunction enhances the anticancer effect of nutrient deprivation. *Pharm Res.*, 2249-63.

Heppner, F. L., Prinz, M., & Aguzzi, A. (2001). Pathogenesis of prion diseases: possible implications of microglial cells. *Progress in Brain Research*, 737–750.

Hernández, F. (2010). GSK3: A possible link between beta amyloid peptide and tau protein. *Experimental Neurology*, 322–325.

Hernández, F., de Barreda, E. G., & Avila, J. (2010). GSK3: A possible link between beta amyloid peptide and tau protein. *Experimental Neurology*, 322–325.

Hirota, Y., & Tanaka, Y. (2007). A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments. *Biochem Biophys Res Commun.*, 40-7.

Hong, D. S., Banerji , U., Tavana, B., & George, G. C. (2013). Targeting the molecular chaperone heat shock protein 90 (HSP90): Lessons learned and future directions. *Cancer Treatment Reviews*, 375–387.

Hope, J. (2000). Prions and neurodegenerative diseases. *Current Opinion in Genetics & Development*, 568–574.

Ichimura, Y., & Komatsu, M. (2012). p62/SQSTM1/A170: Physiology and pathology. *Pharmacological Research*, 457-462.

Ihara, Y., Nukina, N., Miura , R., & Ogawara, M. (1986). Phosphorylated tau protein is integrated into paired helical filaments in Alzheimer's disease. *Journal of Biochemestry*, 1807-1810 .

Ihara, Y., & Nixon, R. (2012). The Ubiquitin–Proteasome System and the Autophagic–Lysosomal System in Alzheimer Disease. *Cold Spring Harb Perspect Med*, 2 (8).

Illenberger, S. (1998). The endogenous and cell Cycle-dependent Phosphorylation of tau protei in living cells: Implications for Alzheimer's disease. *Molecular Biology of the Cell*, 1495-1512.

Inestrosa, N. C., Nunez, M. T., Colombres, M., & Morgan, C. (2004). Structure and function of amyloid in Alzheimer's disease. *Progress in Neurobiology*, 323–349.

Itakura, E., & Mizushima, N. (2012). The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell*, 1256–1269.

Ittner, L. M., & Götz, J. (2011). Amyloid-β and tau — a toxic pas de deux in Alzheimer's disease. *Nature Reviews Neuroscience*, 1-6.

Ittner, L., Ke, Y., Delerue, F., & Wölfing, H. (2010). Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. *Cell*, 387–97.

Janssens, V., & GORIS, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochemical journal*, 417–439.

Kaneko, K., Ball, H. L., & Wille, H. (2000). A synthetic peptide initiates Gerstmann-Straussler-Scheinker (GSS) disease in transgenic mice. *Journal of molecular biology*, 997±1007.

Keck, S., Nitsch, R., Grune, T., & Ullrich, O. (2003). Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. *Journal of Neurochemistry*, 115–122.

Keller, J., Hanni , K., & Markesbery , W. (2000). Impaired proteasome function in Alzheimer's disease. *Journal of Neurochemestry*, 436-439.

Kenessey, A., Nacharaju, P., Ko, L.-w., & Yen, S.-H. (1997). Degradation of tau by lysosomal enzyme cathepsin D: implication for Alzheimer neurofibrillary degeneration. *Journal of Neurochemistry*, 2026–2038.

Klein, W. L., Viola, K. L., & Lacor, P. N. (2007). A-beta Oligomer-Induced Aberrations in Synapse Composition,Shape, and Density Provide a Molecular Basis for Loss of Connectivity in Alzheimer's Disease. *The Journal of Neuroscience*, :796–807.

Klionsky, D. J., & Zuckerbraun, B. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*, 445–544.

Ko, L.-w., DeTure, M., Sahara, N., & Chihab, R. (2005). Recent advances in experimental modeling of the assembly of tau filaments. *Biochimica et Biophysica Acta* , 125 – 139.

Komatsu, M., & Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*, 880-884.

Komatsu, M., & Yue, Z. (2007). Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *PNAS*, 14489-94.

Kominami, E., & Sou, Y.-s. (2005). Phosphatidylserine in Addition to Phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. *The journal of Biological Chemestry*, 3017-3024.

Kominami, E., & Tanida, I. (2000). The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. *The journal of Biological Chemestry*, 1701-1706.

Kominami, E., & Tanida, I. (2008). LC3 and autophagy. *Methods on Molecular Biology*, 77-88.

Kopito, R., & Brundin, P. (2010). Prion-like transmission of protein aggregates in neurodegenerative diseases. *Nat Rev Mol Cell Biol.*, 301-307.

Kroemer, G., & Marino, G. (2014). Self-consumption: the interplay of autophagy and apoptosis. *Nature reviews Molecular Cell Biology*, 1-14.

Krüger , U., & Mandelkow, E. (2012). Autophagic degradation of tau in primary neurons and its enhancement by trehalose. *Neurobiology of aging*, 2291-305.

Krüger, U., & Mandelkow, E.-M. (2012). Autophagic degradation of tau in primary neurons and its enhancement by trehalose. *Neurobiology of aging*, 2291–2305.

Ktistakis, N., & Walker, S. (2008). Making autophagosomes: localized synthesis of phosphatidylinositol 3-phosphate holds the cue. *Autophagy*, 1093-1096.

Kumar, S., & Mandelkow, E. (2014). Stages and Conformations of the Tau Repeat Domain during Aggregation and Its Effect on Neuronal Toxicity. *Journal of Biological Chemestry*, 20318–20332.

Lambert, J. (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat Genet*, 1452–1458.

Lasagna-Reeves, C. A., Castillo-Carranza, D. L., & Sengupta, U. (2011). Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. *Molecular Neurodegeneration*, 39-53.

Lasagna-Reeves, C. A., Castillo-Carranza, D. L., & Sengupta, U. (2011). Tau oligomers impair memory and induce wild-type mice synaptic and mitochondrial dysfunction in. *Molecular Neurodegeneration*, 39-53.

Lee, M. J., Lee, J. H., & Rubinsztein, D. C. (2013). Tau degradation: The ubiquitin–proteasome system versus the autophagy-lysosome system. *Progress in Neurobiology*, 49-59.

Lee, S., Jung, C., & Hall, G. (2009). Exonic point mutations of human tau enhance its toxicity and cause characteristic changes in neuronal morphology, tau distribution and tau phosphorylation in the lamprey cellular model of tauopathy. *Journal of Alzheimer's Disease*, 99-111.

Lee, V. M.-Y., & Guo, J. L. (2011). Seeding of Normal Tau by Pathological Tau Conformers Drives Pathogenesis of Alzheimer-like Tangles. *Journal of Biological Chemestry*, 15317-15331.

Lewis, J., Dickson, D. W., & Lin, W.-L. (2001). Enhanced Neurofibrillary Degeneration in Transgenic Mice Expressing Mutant Tau and APP. *Science*, 1487-1491.

Lewis, J., McGowan, E., Rockwood, J., & Melrose, H. (2000). Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nature Genetics*, 402 - 405.

Li, W., & Lee , V. M.-Y. (2006). Characterization of two VQIXXK motifs for tau fibrillization in vitro. *Biochemestry*, 15692–15701.

Liberski, P. P. (2014). Prion, prionoids and infectious amyloid. *Parkinsonism and Related Disorders*, S80–S84.

Lippincott-Schwartz, J., & Hailey, D. W. (2010). Mitochondria Supply Membranes for Autophagosome Biogenesis during Starvation. *Cell*, 656-667.

Liu, C., & Eisenberg, D. (2012). Characteristics of amyloid-related oligomers revealed by crystal structures of macrocyclic β-sheet mimics. *J Am Chem Soc*, 6736–6744.

Lucas, J., Hernandez, F., & Avila, J. (2001). Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegenerin GSK-3beta conditional transgenic mice.ation. *EMBO*, 27–39.

Luo, W., Duo, F., Rodina, A., & Chip, S. (2007). Roles of heat-shock protein 90 in maintaining and facilitating the neurodegenerative phenotype in tauopathies. *PNAS*, 9511-9516.

Maeda, S., Sahara, N., & Saito, Y. (2006). Increased levels of granular tau oligomers: An early sign of brain aging and Alzheimer's disease. *Neuroscience Research*, 197–201.

Maiese , K. (2014). Taking aim at Alzheimer's disease through the mammalian target of rapamycin. *Annals of Medicine*, 587–596.

Mandelkow, E. M., Stamer, K., & Vogel, R. (2003). Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses. *Neurobiology of Aging*, 1079–1085.

Mandelkow, E., & Maldelvow, E. (1998). Tau in Alzheimer's disease. *Trens in Cell Biology*, 425-427.

Marchand , B., & Boucher, M. (2015). Glycogen synthase kinase-3 (GSK3) inhibition induces prosurvival autophagic signals in human pancreatic cancer cells. *Journal of Biological Chemestry*, 5592-605.

Marcusson, J., Granseth, B., Agholme, L., & Nath, S. (2012). Spreading of Neurodegenerative Pathology via Neuron-to-Neuron Transmission of beta-Amyloid. *The Journal of Neuroscience*, 8767– 8777.

Mari, M., & Reggiori, F. (2010). An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *Journal of cell biology*, 1005-1022.

Martin, L., Latypova, X., & Terro, F. (2011). Post-translational modifications of tau protein: Implications for Alzheimer's disease. *Neurochemistry International*, 458–471.

Masel, J., Jansen, V. A., & Nowak, M. A. (1999). Quantifying the kinetic parameters of prion replication. *Biophysical Chemistry*, 139-152.

Matenia, D., & Mandelkow, E.-M. (2009). The tau of MARK: a polarized view of the cytoskeleton. *Trends in Biochemical Sciences*, 332–342.

Matsuoka, Y., Jouroukhin, Y., & Gray, A. J. (2008). A neuronal microtubule-interacting agent, NAPVSIPQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease. *The Journal of Pharmacology and Experimental Therapeutics*, 146-153.

McNally , F. J. (1996). Modulation of microtubule dynamics during the cell cycle. *Current Opinion in Cell Biology*, 23-29.

Mellman, I., & Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. *Annual Reviews Biochemestry*, 663-700.

Menzies, F. M., & Rubinsztein, D. C. (2015). Compromised autophagy and neurodegenerative diseases. *Nature rewies Neuroscience*, 345-357.

Mizushima , N. (2010). Methods in Mammalian Autophagy Research. *Cell*, 313-326.

Mizushima, N., & Shen, H.-M. (2014). At the end of the autophagic road: an emerging understanding of lysosomal functions in autophagy. *Trends in Biomedichal Sciences*, 61–71.

Mizushima, N., Yoshimori, T., & Levine, B. (2010). Methods in Mammalian Autophagy Research. *Cell*, 313-326.

Morani, F., & Isisdoro, C. (2014). PTEN deficiency and mutant p53 confer glucose-addiction to thyroid cancer cells: impact of glucose depletion on cell proliferation, cell survival, autophagy and cell migration. *Genes cancer*, 226-39.

Morsch, R. B., Simon, W., & Coleman, P. D. (1999). Neurons May Live for Decades with Neurofibrillary Tangles. *Journal of Neuropathology & Experimental Neurology*, 188–197.

Mosconi, L., & Leon, M. (2007). Early detection of Alzheimer's disease using neuroimaging. *Experimental Gerontology*, 129-138.

Nacmias, B., Bagnoli, S., & Sorbi, S. (2001). Genetic risk factors in familial Alzheimer's disease. *Mechanisms of Ageing and Development*, 1951–1960.

Natalwala, A. (2013). Alzheimer's disease and co-morbidity: Increased prevalence and possible risk factors of excess mortality in a naturalistic 7-year follow-up. *European Psychiatry*, 40–48.

Nelson, P. N., Neltner, J. H., & Scheff, S. W. (2014). Is synaptic loss a unique hallmark of Alzheimer's disease? *Biochemical Pharmacology*, 517–528.

Ng, E., & Tang, B. (2012). Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes. *Cell Biochem Funct*, 515-23.

Nixon, R., & Lee, J. (2008). Neurodegenerative lysosomal disorders: a continuum from development to late age. *Autophagy*, 590-599.

Nixon, R., Wegiel, J., Kumar, A., & Yu, W. (2005). Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *Journal of Neuropathological experimental Neurobiology*, 113-122.

Norberg, H. V. (2015). Pharmacologic agents targeting autophagy. *The journal of Clinical investigation*, 5-13.

Nunomura , A., Perry, G., Aliev, G., & Hirai , K. (2001). Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol*, 759-67.

Oddo, S., Green, K. N., & LaFerla, F. M. (2007). Intracellular amyloid-bold beta in Alzheimer's disease. *Nature Reviews Neuroscience*, 499-509 .

Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol.* , 107-132.

Ohsumi, Y., & Yamamoto, Y. (2012). Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *The journal of Cell biology*, 219-233.

Olesen, J. (2011). Cost of disorders of the brain in Europe 2010. *European Neuropsychopharmacology*, 718–779.

Orgel , L. E. (1996). Prion replication and secondary nucleation. *Crosstalk* , 413-414.

Panda, D., Samuel, J. C., Massie, M., & Wilson, L. (2003). Differential regulation of microtubule dynamics by three- and four-repeat tau: implications for the onset of neurodegenerative disease. *Proc Natl Acad Sci USA*, 9548-53.

Pastore, N., & Brunetti-Pierri, N. (2013). Autophagy master regulator TFEB induces clearance of toxic SERPINA1/α-1-antitrypsin polymers. *Autophagy*, 1094-6.

Patterson, K. R., & Binder, L. I. (2011). Characterization of Prefibrillar Tau Oligomers in Vitro and in Alzheimer Disease. *Journal of Biological Chemestry*, 23063–23076.

Paudel, H., & Li, W. (1999). Heparin-induced conformational change in microtubule-associated protein Tau as detected by chemical cross-linking and phosphopeptide mapping. *The Journal of biological chemestry*, 8029-38.

Peña-Llopis, S., & Brugarolas, J. (2011). TFEB, a novel mTORC1 effector implicated in lysosome biogenesis, endocytosis and autophagy. *Cell Cycle*, 3987-8.

Pennanen, L., Schild, A., & Gotz, J. (2004). Amyloid-induced neurofibrillary tangle formation in Alzheimer's disease: insight from transgenic mouse and tissue-culture models. *Int. J. Devl Neuroscience*, 453–465.

Peskind, E., Breitner, J., & Kukull, W. (2007). Age-varying association between blood pressure and risk of dementia in those aged 65 and older: a community-based prospective cohort study. *55*(1161–1167).

Petkova, A. T., Leapman, R., & Guo, Z. (2005). Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. *Science*, 262-5.

Petrucelli, L., Dickson, D., & Kehoe, K. (2004). CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. *Human Molecular Genetics*, 703–714.

Pickart, C., & Cohen, R. (2004). Proteasomes and their kin: proteases in the machine age. *Nature Reviews Molecular Cell Biology*, 177–187.

Pircs, K., & Juhasz, G. (2012). Advantages and limitations of different p62-based assays for estimating autophagic activity in Drosophila. *PLoS One*, 7.

Polito, V., & Zheng, H. (2014). Selective clearence of aberrant tau proteins and rescue of neuotoxicity by transcription factor EB . *EMBO Molecular Medicine*, 1142-1160.

Pooler, A. M., Usardi, A., Evans, C. J., & Philpott, K. L. (2012). Dynamic association of tau with neuronal membranes is regulated by phosphorylation. *Neurobiology of Aging*, 431.e27– 431.e38.

Prusiner, S. B. (1984). Some Speculations about Prions, Amyloid, and Alzheimer's Disease. *New Ingland Journal of medicine*, 661–663.

Prusiner, S. B. (1986). Prions: Novel Infectious Pathogens. *Advances in virus research*, 1-56.

Rademakers, R., Sleegers, K., Theuns, J., & Van den Broeck, M. (2005). Association of cyclin-dependent kinase 5 and neuronal activators p35 and p39 complex in early-onset Alzheimer's disease. *Neurobiology of Aging* , 1145–1151.

Ravikumar, B., & Rubinsztein, D. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet*, 1107-1117.

Ravikumar, B., & Rubinsztein, D. (2010). Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nature Cell Biology*, 747-757.

Ribé, E. M., Perez, M., & Gómez-Islaa, T. (2005). Accelerated amyloid deposition, neurofibrillary degeneration and neuronal loss in double mutant APP/tau transgenic mice. *Neurobiology of Disease*, 814–822.

Richardson, A., & Oddo, S. (2014). How longevity research can lead to therapies for Alzheimer's disease: The rapamycin story. *Experimental Gerontology*.

Rink, J., & Zerial, M. (2005). Rab Conversion as a Mechanism of Progression from Early to Late Endosomes. *Cell*, 735-749.

Rink, J., & Zerial, M. (2005). Rab Conversion as a Mechanism of Progression from Early to Late Endosomes. *Cell*, 735–749.

Rink, J., & Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell*, 735-749.

Rizzu, P., Joosse, M., & Ravid, R. (2000). Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *Human molecular Genetics*, 3075-3082.

Roberson, E. D., Scearce-Levie, K., & Palop, J. J. (2007). Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. *Science*, 750-754.

Roe, N. D., & Ren, J. (2015). Targeted deletion of PTEN in cardiomyocytes renders cardiac contractile dysfunction through interruption of Pink1–AMPK signaling and autophagy. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 290–298.

Rubinsztein, D. C., Cuervo, A., & Klionsky, D. (2009). In search of an "autophagomometer". *Autophagy*, 585-589.

Saito, Y., Ruberu, N., & Sawabe, M. (2004). Staging of argyrophilic grains: an age-associated tauopathy. *Journal of Neuropathology*, 911-8.

Sala, G., & Ferrarese, C. (2012). A panel of macroautophagy markers in lymphomonocytes of patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler*, 119-124.

Salminen, A., Ojala, J., & Kaarniranta, K. (2011). Hsp90 regulates tau pathology through co-chaperone complexes in Alzheimer's disease. *Progress in Neurobiology*, 99-110.

Sanders, D. W., Kaufman, S. K., DeVos, S. L., & Diamond, M. I. (2014). Distinct Tau Prion Strains Propagate in Cells and Mice and Define Different Tauopathies. *Neuron*, 1-18.

Sarbassov, D., & Sabatini, D. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*, 159-68.

Sardiello, M., & Ballabio, A. (2009). A gene network regulating lysosomal biogenesis and function. *Science*, 473-477.

Schaffer, B., Wiedau-Pazos, M., & Geschwind, D. (2003). Gene structure and alternative splicing of glycogen synthase kinase 3 beta (GSK-3β) in neural and non-neural tissues. *Gene*, 73–81.

Scheltens, P., Feldman, H., & Dubois, B. (2007). Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS–ADRDA criteria. *Lancet Neurology*, 734–46.

Seamen, M. N. (2014). Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. *The Journal of Cell Biology*, 111-122.

Selgen, P. O. (2011). Seeing is believing: The impact of electron microscopy. *Autophagy*, 935-956.

Sennvik, K., Boekhoorn, K., & Van Leuven, F. (2007). Tau-4R suppresses proliferation and promotes neuronal differentiation in the hippocampus of tau knockin/knockout mice. *The FASEB Journal*, 2149–2161.

Settembre, C., & Ballabio, A. (2014). Lysosomal adaptation: how the lysosome responds to external cues. *Cold Spring Harb Perspect Biol*.

Shastry, B. (2013). Neurodegenerative disorders of protein aggregation. *Neurochemistry International*, 1-7.

Shen, H.-M., & Zhou, J. (2013). Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. *Cell Research*, 508–523.

Shirahama-Noda , K., & Hara-Nishimura, I. (2003). Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Journal of biological chemestry*, 33194-9.

Shoji-Kawata, S., & Levine, B. (2013). Identification of a candidate therapeutic autophagy-inducing peptide. *Nature*, 201-206.

Sigurdsson, E. M., Wisniewski, T., & Frangione, B. (2002). Infectivity of amyloid diseases. *TRENDS in Molecular Medicine* , 411-413.

Sleat, D., & Lübke, T. (2009). Proteomics of the lysosome. *Biochimica et Biophysica Acta*, 625–635.

Smith, M. A., Casadesus, G., Joseph, J. A., & Perry, G. (2002). Amyloid-β and τ serve antioxidant functions in the aging and Alzheimer brain. *Free Radical Biology and Medicine*, 1194–1199.

Smith, M. A., Nunomura, A., Moreira, P. I., & Lee, H.-g. (2005). Tau phosphorylation in Alzheimer's disease: pathogen or protector? *TRENDS in Molecular Medicine*, 164-169.

Sobota, J. A., & Mains, R. E. (2009). Inhibitors of the V0 subunit of the vacuolar H+-ATPase prevent segregation of lysosomal- and secretory-pathway proteins. *Journal of Cell Science*, 3542-3554.

Sontag, E., Nunbhakdi-Craig, V., & Lee, G. (1996). Regulation of the Phosphorylation State and Microtubule-Binding Activity of Tau by Protein Phosphatase 2A. *Neuron*, 1201–1207.

Soto, C., & Saborío, G. P. (2001). Prions: disease propagation and disease therapy by conformational transmission. *TRENDS in Molecular Medicine*, 109-114.

Soto, C., Estrada, L., & Castilla, J. (2006). Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. *TRENDS in Biochemical Sciences*, 150–155.

Spires-Jones, T. L., Stoothoff, W. H., & de Calignon, A. (2009). Tau pathophysiology in neurodegeneration: a tangled issue. *Trends in Neurosciences*, 150-159.

Stoothoff, W. H., & Johnson, G. V. (2005). Tau phosphorylation: physiological and pathological consequences. *Biochimica et Biophysica Acta*, 280– 297.

Sun, L., & Du, L. (2013). Global analysis of fission yeast mating genes reveals new autophagy factors. *PLoS Genet*, 1-16.

Takahashi, R. H., Almeida, C. G., & Gouras, G. K. (2005). *Neurobiology of Aging*, 1235–1244.

Takahashi, R. H., Capetillo-Zarate, E., & Gouras, G. K. (2010). Co.occurance of Alzheimer's disease beta-amyloid and tau pathologies at synapses. *Neurobiology of aging*, 1145–1152.

Takahashi, R. H., Capetillo-Zarate, E., & Gouras, G. K. (2010). Co-occurrence of Alzheimer's disease beta-amyloid and tau pathologies at synapses. *Neurobiology of Aging*, 1145–1152.

Tanaka, M., Collins, S. R., Toyama, B. H., & Weissman, J. S. (2006). The physical basis of how prion conformations determine strain phenotypes. *Nature*, 585-589.

Tee, A., & Dunlop, E. (2014). mTOR and autophagy: A dynamic relationship governed by nutrients and energy. *Seminars in Cell & Developmental Biology*, 121-129.

Thomé, R., & Verinaud, L. (2013). Chloroquine: modes of action of an undervalued drug. *Immunol Lett*, 50-7.

Tooze, S. A., & Chan, E. Y. (2009). Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *MOLECULAR AND CELLULAR BIOLOGY*, 157–171.

Tooze, S. A., & Simonsen, A. (2009). Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. *The journal of cell biology*, 773-782.

Tooze, S., & Lamb, C. (2013). The autophagosome: origins unknown, biogenesis complex. *Nature reviews Molecular Cell Biology*, 759-774.

Tooze, S., & Orsi A. (2012). Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. *Molecular Biology of the cell*, 1860-1873.

Triller, A., Klein, W. L., & Marianne Renner, M. (2006). Deleterious Effects of Amyloid b Oligomers Actingas an Extracellular Scaffold for mGluR5. *Neuron*, 739–754.

Trojanowski, J., Toledo, J., & Shaw, L. (2012). Cardiovascular risk factors, cortisol, and amyloid-β deposition in Alzheimer's Disease Neuroimaging Initiative. *Alzheimer's & Dementia*, 483–489.

Trojanowski, J., Shaw, L., Weiner, M., & Toledo, J. (2012). Cardiovascular risk factors, cortisol, and amyloid-β deposition in Alzheimer's Disease Neuroimaging Initiative. *Alzheimer's & Dementia*, 483-489.

Tsunemi, T., & La Spada, A. (2012). PGC-1α rescues Huntington's disease proteotoxicity by preventing oxidative stress and promoting TFEB function. *Sci Transl Med*, 142.

Van Broeckhoven, C., Sleegers, K., & Bettens, K. (2013). Genetic insights in Alzheimer's disease. *The lancet Neurology*, 92.104.

Vickers, J. C., Dickson, T. C., Adlard, P. A., & Saunders, H. L. (2000). The cause of neuronal degeneration in Alzheimer's disease. *Progress in Neurobiology*, 139–165.

Viles, J. H., Cohen, F. E., & Prusiner, S. B. (1999). Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A.*, 2042-7.

Voeltz, G., & Rowland, A. (2012). Endoplasmic reticulum-mitochondria contacts: function of the junction. *NBature review Molecular Cell Biology*, 607-625.

Wang, M., & Liu, C. (2015). TNF compromises lysosome acidification and reduces α-synuclein degradation via autophagy in dopaminergic cells. *Experimental Neurology*, In press.

Wang, Y., & Mandelkow, E. (2010). Proteolytic processing of tau. *Biochemical society transactions*, 955-61.

Wang, Y., Martinez-Vicente, M., & Kruger, U. (2009). Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. *Human Molecular Genetics*, 4153–4170.

Webb, J., & Rubinsztein. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *Journal of Biochemical Chemestry*, 25009-13.

Williams , A., & Rubinsztein , D. (2006). Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. *Current Topics in Developmental Biology*, 89-101.

Willis, A. (2013). The coming crisis - Obtaining care for the growing burden of neurodegenerative conditions. *Neurology*, 80:1989-1996.

WISCHIK, C. M., & NOVAK, M. (1988). Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. *Proc Natl Acad Sci USA*, 4506-10.

Wong, E., & Cuervo, A. (2010). Autophagy gone awry in neurodegenerative diseases. *Nature neuroscience*, 805-811.

Wyttenbach, A., & Tolkovsky, A. (2008). Amelioration of protein misfolding disease by rapamycin: translation or autophagy? *Autophagy*, 542-545.

Xiao, Q., & Lee, J. (2014). Enhancing astrocytic lysosome biogenesis facilitates Aβ clearance and attenuates amyloid plaque pathogenesis. *Journal of Neuroscience*, 9607-20.

Xie, Z., & Dong, Q. (2014). Bafilomycin A1 inhibits autophagy and induces apoptosis in MG63 osteosarcoma cells. *Mol Med Rep*, 1103-7.

Yamamoto, A., & Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct.*, 33-42.

Yanamandra, K., Kfoury, N., Jiang, H., & Mahan, T. E. (2013). Anti-Tau Antibodies that Block Tau Aggregate Seeding In Vitro Markedly Decrease Pathology and Improve Cognition In Vivo. *Neuron*, 402-414.

Yang, W. X., & Yamamoto, A. (2014). CLEARance wars: PolyQ strikes back. *Nature Neuroscience*, 1140-1142.

Yao, J., & Hall, G. F. (2005). Modeling tauopathy: a range of complementary approaches. *Molecular Basis of Disease*, 224–239.

Yoshiyama, Y., Higuchi, M., & Zhang, B. (2007). Synapse Loss and Microglial Activation Precede Tangles in a P301S Tauopathy Mouse Model. *Neuron*, 337-351.

Young, J., Budge, M., Cherbuin, N., & Anstey, K. (2011). Body mass index in midlife and late-life as a risk factor for dementia: a meta-analysis of prospective studies. *Obes Rev*, 426-437.

Zhang, B., Maiti, A., Shively, S., & Trojanowski, J. Q. (2004). Microtubule-binding drugs off set tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. *PNAS*, 227–231.

Zhang, Z., & Ye, K. (2014). Cleavage of Tau asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nature Medicine*, 1254-1262.

Zhao, H., Michaelis, M. L., & Blagg, B. S. (2012). Hsp90 modulation for the treatment of Alzheimer's disease. *Advances in Pharmacology*, 1-25.