Universidade de Lisboa

Faculdade de Farmácia

The Role of Beclin-1 deacetylation on Autophagic Flux in Alzheimer's disease

Filipa Isabel Parreiras Filipe

Dissertação de Mestrado orientada pela Professora Doutora Sandra Isabel Morais Cardoso e coorientada pela Professora Doutora Adelaide Maria Afonso Fernandes Borralho

Mestrado em Ciências Biofarmacêuticas

2018

Universidade de Lisboa

Faculdade de Farmácia

The Role of Beclin-1 deacetylation on Autophagic Flux in Alzheimer's disease

Filipa Isabel Parreiras Filipe

Dissertação de Mestrado orientada pela Professora Doutora Sandra Isabel Morais Cardoso e coorientada pela Professora Doutora Adelaide Maria Afonso Fernandes Borralho

Mestrado em Ciências Biofarmacêuticas

2018

ABSTRACT

Alzheimer's disease (AD) is a devastating neurodegenerative disorder, characterized by neuronal loss and gradual cognitive impairment, a serious public health problem, affecting more than 30 million people worldwide. The presence of two well-known abnormal protein aggregates in cerebral cortex and hippocampus characterize AD pathologically: senile plaques in specific areas of the brain, extracellular, and composed of insoluble Aβ peptides; and neurofibrillary tangles, intracellular aggregates, mostly consisted by hyperphosphorylated Tau, a microtubule-associated protein localized in axons. Several authors have described for decades that protein aggregation process can induce toxicity for neurons causing synaptic dysfunction, neuroinflammation and oxidative stress. One major aspect of AD pathology, that is observed in both humans and mouse models of the disease is the accumulation of senile plaques, containing Aβ peptides, leading to a neuronal dysfunction and cell death. Neuronal cell survival depends on a health and effective mitochondrial quality control, but also a balance between autophagic and lysosomal pathways. Data has demonstrated the crucial role of both macroautophagy (referred to here as autophagy) and lysosomal pathways in maintaining cellular homeostasis, as well in neuronal survival, degrading and decreasing the amount of misfolded proteins and impaired organelles, like that preventing the accumulation of toxic protein aggregates. Beclin-1 is a protein involved in several biological functions so relevant in several human diseases, such as heart disease, pathogen infection, development and neurodegenerative disorders. However, as one of the main proteins responsible of autophagy regulation, it has been shown that Beclin-1 levels are reduced in AD patient's brain. For several years, a lot of research has been focused on a family of protein deacetylases, Sirtuins (SIRTs), and its crucial role in a variety of cellular biological systems, including neuroinflammation, melanocortin system, energy balance, the ubiquitin-proteasome system; and central nervous system regulation. SIRT1's activity can influence autophagic pathway, acting on components of the autophagic machinery. Despite of this, it has not been described in neuronal cells the effect of SIRT1 on deacetylation of Beclin-1, which can result in deregulation of the autophagic pathway. Autophagy impairment plays a key role in sporadic Alzheimer's disease (sAD) neurodegenerative process. Nevertheless, the mechanism(s) that lead to a deficiency in autophagy in AD remains elusive. In this work we identify, for the first time, that Beclin-1 acetylation status is responsible for autophagosomes maturation and is implicated in the alterations in autophagy observed in AD neurodegeneration. We observed that Beclin-1 is deacetylated by SIRT1 and acetylated by p300. In addition, Beclin-1 acetylation inhibits autophagosomes maturation, leading to impairment in autophagic flux. We also analyzed some proteins, known to be involved in the maturation of autophagosomes, such as Rab 7 that

participates in the fusion step with lysosomes. We observed that an overexpression of Rab 7 and the formation of large perinuclear lysosome clusters are in accordance with an increase in lysosomal biogenesis determined by an increase in LAMP-2A and Cathepsin D expression in sAD cells. Thus, our data provide strong evidence that Beclin-1 acetylation impairs the autophagic flux and despite lysosomal biogenesis is triggered as a compensatory response, autophagosome fusion with lysosomes is compromised contributing to AD neurodegeneration.

Keywords: Alzheimer's disease, Autophagic-Lysosomal Dysfunction, Beclin-1, SIRT1 and Deacetylation.

RESUMO

A doença de Alzheimer (DA) é um distúrbio neurodegenerativo devastador, caracterizado por uma perda de neurónios e por um comprometimento gradual cognitivo, um grave problema de saúde pública, afetando mais de 30 milhões de pessoas em todo o mundo. A acumulação não normal de duas proteínas específicas no córtex cerebral e no hipocampo, caracteriza a DA patologicamente: as placas senis em áreas específicas do cérebro, depósitos extracelulares, e constituídos por peptídeos de β-amilóide insolúveis; e as tranças neurofibrilares, agregados intracelulares, constituídos principalmente pela Tau hiperfosforilada, uma proteína associada aos microtúbulos, localizada nos axónios. Por várias décadas, diversos autores têm descrito que o processo de acumulação proteica, pode induzir toxicidade aos neurónios, levando a uma disfunção sináptica, a uma neuroinflamação e a um estresse oxidativo. Uma das características mais importantes da patologia DA observada quer em humanos, quer em modelos de ratinho que apresentam a doença, é a acumulação das placas senis, as quais contêm peptídeos ricos em proteína β-amilóide, levando a uma disfunção neuronal e morte celular. A sobrevivência neuronal, depende tanto de um ótimo bem-estar e de um controlo efetivo de qualidade a nível mitocondrial, mas também de um equilíbrio entre as vias autofágica e lisossomal. Dados científicos têm demonstrado o papel fundamental de ambas as vias, a via da macroautofagia (designada aqui como autofagia) e a via lisossomal, na manutenção da homeostasia celular, bem como na sobrevivência neuronal, na degradação e diminuição da quantidade de proteínas disfuncionais e organelos deficientes, prevenindo assim a acumulação de agregados proteicos tóxicos. A Beclin-1 é uma proteína envolvida em várias funções biológicas e importante em diferentes patologias, como por exemplo, nas doenças cardíacas, na infeção por patógenos, no desenvolvimento e na neurodegeneração. No entanto, como uma das principais proteínas responsáveis pela regulação da via autofágica, foi demonstrado que os níveis de Beclin-1 estão reduzidos em cérebros de doentes com DA. Durante vários anos, a ciência focou-se numa família de proteínas de deacetilase de histonas, as Sirtuínas (SIRTs), e no seu papel de muito importância em diversos processos biológicos e celulares, incluindo na neuroinflamação, no sistema de melanocortina e no balanço energético, e no sistema proteossómico; e na regulação do sistema nervoso central. A atividade da SIRT1 pode influenciar o processo autofágico, atuando sobre os seus componentes presentes na maquinaria autofágica. No entanto, ainda não foi descrito o seu efeito de deacetilação na Beclin-1, em neurónios, podendo levar a um comprometimento do próprio processo autofágico. Um comprometimento por parte da autofagia vai desempenhar um papel crucial no processo neurodegenerativo da doença de Alzheimer do tipo esporádico (DAs). Contudo, o (s) mecanismo (s) responsável (eis) pela incapacidade da via autofágica na DA permanece inconclusivo. Neste trabalho identificámos, pela

primeira vez que o estado de acetilação da Beclin-1 é responsável pela maturação dos autofagossomas, e que está implícito nas alterações da via autofágica observada na neurodegeneração da DA. Verificámos que a Beclin-1 é deacetilada pela SIRT1 e acetilada pela p300. Para além disso, a acetilação da Beclin-1 inibe a maturação dos autofagossomas, levando a um comprometimento do fluxo autofágico. Também analisámos algumas proteínas, bastante conhecidas por estarem envolvidas na maturação dos autofagossomas, tais como a Rab7, a qual participa na etapa de fusão com os lisossomas. Observámos que uma sobre-expressão da Rab7 e a formação de grandes aglomerados lisossomais perinucleares estão de acordo com um aumento da biogénese lisossomal, determinada por um aumento na expressão de LAMP-2A e da Cathepsin D em células com DAs. Assim, os nossos resultados mostram fortes evidências de que a acetilação da Beclin-1 compromete o fluxo autofágico e, apesar da biogénese lisossomal ser desencadeada como uma resposta compensatória, a fusão dos autofagossomas com os lisossomas é prejudicada, contribuindo para a neurodegeneração da DA.

Palavras-chave: Doença de Alzheimer, Disfunção Autofágica-Lisossomal, Beclin-1, SIRT1 e Deacetilação

ACKNOWLEDGMENTS

During all this year, a lot of people were crucial for the success of my journey and for this reason I would like to thank to each single person. Firstly, I would like to express my gratitude to my supervisor, Professora Doutora Sandra Morais Cardoso from the Center for Neurosciences and Cell Biology (CNC), at Faculty of Medicine of the University of Coimbra, who encouraged me to pursue this project, for accept me in her group without hesitation, as well for believing in my potential from the beginning, and for her guidance, expertise, support and friendship.

I am also grateful to my second supervisor, Professora Adelaide Fernandes from the Department of Biochemistry and Human Biology, at Faculty Pharmacy of University of Lisbon for being an extraordinary person, supportive, present and kind all the way through.

I am especially grateful for our hard-workers and fun group members from our Lab.

To Ana Raquel Esteves, a crucial piece during my project journey, my enormous gratitude for all support, help, patient, knowledge, advises, corrections, listener, and friend.

To Diana Silva, Emanuel Candeias and Daniel Santos for all the good times, jokes, advises and imaginable team-work spirit at the lab.

I would like also to thank all my friends, who were so important during this year. To Mafalda Costa and Ana Maranha, for being the funniest and supportive friends. To my besties of long date, Cátia Mesquita and Inês, thank you just for being there, sometimes distance is not easy to deal, but I know in the end we are always there for each other.

A huge thank you to Luciano de Andrade Gomes, for all the precious moments and for being my unquestionable soulmate-there are no words.

Finally, I would like to thank to my family. To my parents, Helena and Carlos; and younger sister, Rafaela, my very profound gratitude for all their support, opportunities and attention.

I would like to dedicate this work, along with my past and future achievements, to my grandparents, Leonilde, Filipe, Josefina and Manuel, which are the most intelligent, wise, supportive and kind persons, for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. I am grateful that they have been such model teachers, demonstrating how important education and knowledge is in our lives. This accomplishment would not have been possible without them. Thank you for being the most amazing grandparents in the world.

.

SUMMARY

ABSTRACT v
RESUMO vii
ACKNOWLEDGMENTS ix
SUMMARY xi
LIST OF FIGURES xiii
LIST OF TABLES xvi
ABBREVIATIONS xvii
1 INTRODUCTION 1
1.1 Aging: a risk factor for neurodegenerative disorders *1*
1.2 Alzheimer's disease *2*
1.2.1 Neuropathology 3
1.3 Genetically *5*
1.4 Amyloid-β Protein (Aβ) *6*
1.5 Tau Protein *8*
1.6 Cellular Proteolysis *10*
1.7 Autophagic-Lysosomal Pathway *12*
1.7.1 An Overview 12
1.7.2 Autophagosomes Biogenesis 13
1.7.2.1 Initiation 13
1.7.2.2 Vesicle Nucleation 15
1.7.2.3 Vesicle Elongation 15
1.7.2.4 Autophagosome maturation and fusion 17
1.7.2.5 Lysosomal Pathway 18
1.7.2.5.1 The Lysosome *18*
1.8 The Role of Autophagy in the Brain *20*
1.9 Autophagy in Neurodegenerative Disorders *22*
1.9.1 Autophagy in AD 22
1.10 Beclin-1 ..... *24*
1.11 Beclin-1 and Neurodegenerative Disorders *26*
1.11.1 The connection between Beclin-1 and AD 26
1.12 Sirtuins: a key in Neurodegenerative diseases *27*
1.12.1 The History of Sirtuins: A Family of Proteins 27
1.12.1.1 The Basics 27
1.12.1.2 SIRTs subcellular localization 28

1.12.1.3 SIRTs enzymatic activity 28
1.12.1.4 SIRTs function 29
1.12.2 SIRT1: Biological Function and Targets 31
1.12.3 The Role of SIRT1 in CNS 33
1.12.3.1 Brain- Can SIRT1 protect the brain? 33
1.12.3.2 SIRT1: Role in Neurogenesis 34
1.13 SIRTs and Neurodegenerative disorders *35*
1.13.1 The Link between SIRT1 and AD 36
2 AIMS 39
3 MATERIALS AND METHODS 41
3.1 Reagents *41*
3.2 Antibodies *41*
3.3 Cell Culture *41*
3.3.1 Human Subjects 41
3.4 Creation of Cybrid Cell Lines *42*
3.5 Cell Media and Treatments *43*
3.6 Cell Proliferation Assay *44*
3.6.1 MTT Reduction Test 44
3.7 Preparation of Cellular Extracts *44*
3.7.1 Preparation of Whole Cellular Extracts 44
3.7.2 Preparation of Cytosolic and Nuclear Cellular Extracts 44
3.8 SDS-PAGE and Immunoblotting *45*
3.9 Immunoprecipitation assay *45*
3.10 Immunofluorescence and Confocal Microscopy *46*
3.11 Data acquisition and Analysis *47*
4 RESULTS 49
4.1 Effect of NAM and C646 on toxicity in cybrid cell lines *49*
4.2 SIRT1 and p300 roles in acetylation of Beclin-1 in cybrid cellular model *50*
4.3 Autophagic flux- a link to pathophysiological processes in AD *57*
4.4 Lysosomes localization and function might be affected *61*
4.5 Endo-lysosomal fusion: an essential key on autophagic flux *66*
5 DISCUSSION 71
6 CONCLUSION AND FUTURE PERSPECTIVES 77
REFERENCES 79

LIST OF FIGURES

Figure 1:1 - **Neuropathological Hallmarks of AD pathology.** SPs- Senil plaques (arrows) and NFTs- Neurofibrillary tangles (arrowheads) Filipe, 2018. Immunohistochemistry stain Tau-C3 from a patient with AD (adapted from Binder *et al*., 2005) 3

Figure 1:2 – **The pathology of Alzheimer's disease. a**, Brain of a healthy person. **b**, Brain of an Alzheimer's disease patient, which exhibits clearly marked shrinkage of gyri in the temporal lobe (lower part of the brain) and frontal lobes (left part of the brain). **c,** and **d**, Positron Emission Tomography (PET) scans images showing glucose uptake (red and yellow indicate high levels of glucose uptake) in a living healthy person and a normal control subject. The Alzheimer's patient exhibits large decreases in energy metabolism in the frontal cortex (top of brain) and temporal lobes (sides of the brain). Adapted from Mattson 2004. 4

Figure 1:3 - **The amyloid (or Aß) cascade hypothesis.** Gradual changes in the steady-state levels of Aß in the brain are thought to initiate the amyloid cascade. Adapted from Haass & Selkoe 2007. 8

Figure 1:4 - **Pathological aggregation of Tau.** A schematic representation of the different stages of the formation of pathological Tau aggregates. **a.** Abnormal disengagement of Tau from the microtubules and a concomitant increase in the cytosolic concentration of Tau are likely to be the key events that lead to Tau-mediated neurodegeneration. Direct causes of abnormal disengagement of Tau from the microtubules, include an imbalance of Tau kinases and/or phosphatases, mutations of the *Tau* gene, covalent modification of tau causing and/or promoting misfolding, and possibly other causes such as other post-translational modifications. **b.** Once Tau is unbound from microtubules it becomes more likely to misfolded. This is thought to be a stochastic phenomenon that is more likely at higher cytosolic Tau concentrations. **c.** Early deposits of Tau, called 'pretangles', are not stained by Congo Red or Thioflavine T, indicating that these intermediate forms of aggregated Tau do not exhibit the pleated ß sheet structure typically found in amyloid aggregates. **d.** A structural transition leads to this more organized aggregate and the eventual development of NFTs. **e.** Such transitions may be facilitated by heterogeneous interactions with membranous structures. Adapted from Ballatore et al. 2007. 10

Figure 1:5- **Scheme of Autophagy-lysosomal pathway.** Autophagy is regulated by multiple signalling pathways. Autophagic stimuli, including nutrient deprivation, or low energy, activate the ULK1/2 complex, which leads to increased activity of the Beclin-1–Vps34 complex through phosphorylation of Beclin-1. PI3P produced by Vps34 recruits effector proteins, such as DFCP1 and WIPI, to promote autophagosome formation. Elongation of the autophagosome is mediated

by two conjugated systems comprising Atg12– Atg5–Atg16L and LC3–PE. After formation of complete autophagic vesicles, the mature autophagosome becomes fused with a lysosome to create an autolysosome, where sequestered molecules and organelles are degraded (Filipe, 2018). 17

Figure 1:6 - **The structure of Beclin-1.** Schematic representations of domains of human Beclin-1. Beclin-1 has a Bcl-2 homology-3 (BH-3) motif at the N terminus (residues 114-123), a central coiled-coil domain (CCD) in the middle (residues 144-244) and an evolutionarily conserved domain (ECD), at C-terminal (residues 244-337). Adapted from Fu, Cheng and Liu, 2013. 25

Figure 1:7 - **Biological functions of SIRT1 protein in different physiological and pathological conditions.** Adapted from (Nogueiras et al. 2012) 33

Figure 3:1 - **Cybrid results from the repopulation of the Rho 0 cell line with mtDNA exogenous.** Rho 0 cell line results from the elimination of mtDNA of immortalized cell lines by the presence of ethidium bromide. Rho 0 cells are then fused with patient platelets, which contain mitochondria but not nuclei. In this case, mtDNA exogenous is derived from platelets isolated from different individual disease-free CT, MCI and AD subjects. So, differences in function between CT, MCI and AD cybrid cell lines likely arise through differences in their mtDNA. Adapted from Arduino 2012 43

Figure 4:1 – **Effects of NAM and C646 on MTT reduction test.** Cybrid cell lines were incubated with NAM at concentrations 0.5 mM, 1mM, 5mM and 10mM **(A)**. Cybrid cell lines were incubated with concentrations 1 µM, 2.5 µM, 5 µM, 10 µM and 20 µM of C646 **(B)**. 49

Figure 4:2 - **SIRT1 and p300 roles in acetylation of Beclin-1 in cybrid cellular model.** Immunoblotting for phospho-SIRT1 protein levels for each separate cybrid group treated with NAM and C646 (**A**). Immunoblotting for nuclear and cytosolic SIRT1 levels for each separate cybrid group treated with NAM (**B**). Immunoblotting for Beclin-1 protein levels for each separate cybrid group treated with NAM and C646 (**C)**. Immunoprecipitation for Acetylated-Beclin-1 basal levels and for total Lysine-Acetylated basal protein levels (**D**). Immunoprecipitation for acetylated-Beclin-1 and for total Lysine-Acetylated protein levels for each separate cybrid group treated with NAM and C646 (**E**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line. Data were analyzed by Student's T-test and are presented as mean ± S.E.M. 56

Figure 4:3 -**Autophagic flux- a link to pathophysiological processes in AD.** Immunoblotting for LC3II protein levels for each separate cybrid group, untreated and treated with NH_4CL/Leupeptin-NAM and NH_4CL/Leupeptin-C646 (**A**). Immunoblotting for p62 protein levels for each separated

cybrid group, untreated and treated with NH_4CL/Leupeptin-NAM and NH_4CL/Leupeptin-C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line. Data were analyzed by Student's T-test and are presented as mean ± S.E.M. 60

Figure 4:4 - **Lysosomes localization and function might be affected.** Immunoblotting for LAMP-2A protein levels for each separate cybrid group, untreated and treated with NH_4CL/Leupeptin-NAM and NH_4CL/Leupeptin-C646 (**A**). Immunoblotting for CatD protein levels for each separated cybrid group, untreated and treated with NH_4CL/Leupeptin-NAM and NH_4CL/Leupeptin-C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line ; # indicates that the ANOVA calculation itself was not statistically significant. Data were analyzed by Student's T-test and are presented as mean ± S.E.M. 65

Figure 4:5 - **Endo-lysosomal fusion: an essential key on autophagic flux.** Immunoblotting for Rab7 protein levels for each separate cybrid group, treated with NAM and C646 (**A**). Immunofluorescence for LAMP-1 protein levels for each separated cybrid group, treated with NAM and C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** and # indicates that the ANOVA calculation itself was not statistically significant. Data were analyzed by Student's T-test and are presented as mean ± S.E.M. 70

LIST OF TABLES

Table 1-1 - SIRTs Subcellular Localization, Enzymatic Activity and Functions. 30

ABBREVIATIONS

ABCA7-Adenosine triphosphate-binding cassette subfamily A member 7;

Aβ- β Amyloid Protein;

AD- Alzheimer's disease;

ADP- Adenosine diphosphate;

ALS- Amyotrophic Lateral Sclerosis;

AMBRA1- Beclin-1-regulated autophagy;

AMP- Adenosine monophosphate;

AMPK- Adenosine monophosphate activated protein kinase;

ANOVA- Analysis of variance;

Atg- Autophagy-related;

Atg1- Autophagy-related 1;

Atg3- Autophagy-related 3;

Atg4- Autophagy-related 4;

Atg5- Autophagy-related 5;

Atg6- Autophagy-related 6;

Atg7- Autophagy-related 7;

Atg8- Autophagy-related 8;

Atg9- Autophagy-related 9;

Atg13- Autophagy-related 13;

Atg14- Autophagy-related 14;

Atg14L- Autophagy-related 14-like;

Atg16L- Autophagy-related 16-like;

Atg17- Autophagy-related 17;

Atg101- Autophagy-related 101;

ATP- Adenosine trisphosphate;

APOE- ApolipoproteinE;

APP- β-Amyloid Precursor Protein;

AVs- Autophagic vesicles;

Bax- B-cell lymphoma-2- associated x;

Bcl-2- B-cell lymphoma-2;

BBB- Blood-brain barrier;

BH-3- Bcl-2-homology-3;

Bif1- Bax-interacting factor 1;

BSA- Bovine Serum Albumin;

Ca^{2+}- Calcium;

c-AMP- cyclic AMP;

CatD- Cathepsin D;

CCD- Central coiled-coil domain;

Cdk5- Cyclin-dependent kinase 5;

CMA- Chaperone -mediated autophagy;

CNS- Central nervous system;

CR- Caloric restriction;

CREB- Cyclic AMP response element-binding protein;

CSF- Cerebrospinal fluid;

CT- Cognition-control status;

DFCP1- Double FYVE domain-contain 1;

DMEM- Dulbecco's modified Eagle's medium;

DNA- Deoxyribonucleic acid;

DR- Dietary restriction;

DS- Down's Syndrome;

DTT- Dithiothreitol;

ECD- Evolutionarily conserved domain;

EDTA- Ethylenediamine Tetraacetic Acid;

EGTA- Ethylene Glycol-bis (β-aminoethyl Ether)- N,N,N',N'-tetraacetic Acid;

ER- Endoplasmic reticulum;

ESCRT- Endosomal sorting complex required for transport;

FBS- Fetal Bovine Serum fetal;

FAK- Focal adhesion kinase;

FIP200- Focal adhesion kinase family interacting protein of 200 kDa;

FOXO- Forkhead-O-box;

HCl- Hydrogen Chloride;

HD- Huntington's Disease;

HDACs- Deacetylases of histones;

HEPES- 4-(2-hydroxyethyl)-1- Piperazineethanesulfonic Acid;

Hps70- Heat-shock protein of 70 kDa;

Hsc70- Heat-shock cognate protein of 70 kDa;

H_2O_2- Hydrogen peroxide;

LAMPs- Lysosomal membrane proteins;

LAMP-1- Lysosomal-associated membrane protein type 1;

LAMP-2A- Lysosomal-associated membrane protein type 2A;

LC3- Microtubule-associated protein 1A/1B-light chain 3;

LEs- Late endosomes;

KFERQ- Amino acid sequences;

MAPs- Microtubule-associated proteins;

MCI- Mild cognitive impairment;

$MgCl_2$- Magnesium Chloride;

MJ- Machado-Joseph;

mRNA- Messenger ribonucleic acid; m

M6P- Mannose-6-phosphate;

MPR- Mannose-6-phosphate receptor;

mtDNA- Mitochondrial DNA;

MTOC- Microtubule-organizing center;

mTOR1- mammalian target of rapamycin 1;

mTORC1- mammalian target of rapamycin complex1;

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

MVBs- Multivesicular bodies;

NAD^+- Nicotinamide adenine dinucleotide;

NaF- Sodium Fluoride;

NAM- Nicotinamide;

Na_3VO_4- Sodium Orthovanadate;

NES- Nuclear export signal;

NF- κB- Nuclear factor kappa Beta;

NFTs- Neurofibrillary Tangles;

NH_4Cl- Ammonium Chloride;

NSCs- Neural stem cells;

OAADPr- 2'-O-acetyl-ADP ribose;

PBS- Phosphate-Buffered Saline;

PD- Parkinson's disease;

PE- Phospholipid phosphatidylethanolamine;

Pen-Strep- Streptomycin;

PET- Positron Emission Tomography;

PHFs- Paired helical filaments;

PI3K- Phosphatidylinositol 3-kinase;

PI3P- Phosphatidylinositol-3-phosphate;

PMSF- Phenylmethane Sulfonyl Fluoride;

POMC- Pro-opiomelanocortin;

PPARα- Peroxisome proliferators-activated receptor alpha;

PPARγ- Proliferator-activated receptors gamma;

PSEN1- Presenilin 1;

PSEN2- Presenlin 2;

PVD- Polyvinylidene Difluoride;

RBCC1- Retinoblastoma 1- Inducible Coiled-Coil 1;

RER- Rough Endoplasmic reticulum;

RNA- Ribonucleic acid;

ROCK1- Rho-activated kinase 1;

ROS- Reactive oxygen species;

rRNA- Ribosomal ribonucleic acid;

RSV- Resveratrol;

sAD- sporadic Alzheimer's disease;

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis;

Sir2- Silent information regulator 2;

SIRTs- Sirtuins;

SIRT1- Sirtuin 1;

SEM- Standard Error of the Mean;

SGZ- Subgranular zone;

SNARE- Soluble N-ethylmaleimide-sensitive factor activating protein receptor;

SNC- Suprachiasmatic nucleus;

SORL1- Sortilin-related receptor-1;

SPs- Senile Plaques;

SVZ- Subventricular zone;

TBS- Tris-Buffered Solution;

TGN- *trans*-Golgi network;

UCP2- Uncoupling protein 2;

ULK1- Uncoordinated (UNC)-51-like kinase 1;

ULK2- Uncoordinated (UNC)-51-like kinase 2;

UPS- Ubiquitin-proteasome system;

UVRAG- Ultraviolet radiation resistance-associated gene;

V-ATPases- Vacuolar H+ ATPases;

Vps15- Vesicular protein sorting 15;

Vps34- Vesicular protein sorting 34;

WIPI- WD-repeat domain phosphoinositide-interacting protein.

1 INTRODUCTION

1.1 Aging: a risk factor for neurodegenerative disorders

Neurodegenerative diseases are a heterogeneous group of age-related disorders, which can be defined as hereditary or sporadic conditions (Peden and Ironside, 2012). Traditionally, these disorders are characterized by a selective loss of specific neuronal cells and aggregation, as well as deposition of misfolded proteins, a common mechanism shared by these types of maladies, leading to a chronic and progressive nervous system dysfunction (Skovronsky, Lee and Trojanowski, 2006).

Aging, a time-dependent functional decline, results from the accumulation of damage molecules, cells and tissues during the lifetime, which often leads to a progressive loss of physiological integrity, preceded of an impaired function and an increased vulnerability to death (López-Otín *et al.*, 2013). At first sight, aging is the biggest risk factor for many diseases, such as cancer, cardiovascular diseases and neurodegenerative disorders.

Over the last century, life expectancy of the human population has increased and as a reflection the possibility of developing these specific disorders will increase exponentially over time (Walker and Jucker, 2015; Wyss-Coray, 2016).

Numerous studies indicate that changing life-style factors, including a healthy diet, a low-calorie diet (caloric restriction), and specific micro/macronutrients as unsaturated fatty acids, might have beneficial effects on the aging process (Witte *et al.*, 2009). Additionally, caloric restriction (CR) is a reduction of food intake without malnutrition, a key anti-aging intervention, that extends life-span in most animals so far tested, such as rhesus monkeys (Mattison *et al.*, 2017), reducing the incidence of diabetes, cardiovascular disease, cancer, and brain atrophy (Rubinsztein, Mariño and Kroemer, 2011).

Furthermore, it was shown in animal models of aging and neurodegenerative diseases, that CR could protect hippocampal, striatal and cortical neurons, and at same time ameliorated functional decline (Witte *et al.*, 2009). At the same time, some epidemiologic and short-term human studies have supported CR health benefits, specifically the CR diet consumed by centenarians in Okinawa, Japan, which have been used as an argument to support the CR hypothesis in humans for contributing to healthy aging and longevity (Willcox *et al.*, 2007).

However, an excessive caloric intake, which is related to obesity has been associated with changes in brain structure, cognitive deficits, dementia, Alzheimer's disease and Parkinson's disease (PD) (Mazon *et al.*, 2017).

Therefore, brain function can be optimized by intermittent dietary energy restriction, as well as exercise, and these types of energetic challenges enhance adaptive cellular stress-response signaling pathways in neurons involving neurotrophic factors, protein chaperones, deoxyribonucleic acid (DNA)- repair proteins, autophagy and mitochondrial biogenesis (Mattson, 2012).

1.2 Alzheimer's disease

In 1906, Alois Alzheimer, a German clinical psychiatrist and neuroanatomist reported for the first time "A peculiar severe process of the cerebral cortex" to the 37th Meeting of South-West German Psychiatrists in Tübingen, Germany (Hippius and Neundorfer, 2003). Alzheimer described the long-term study of Auguste Deter, a "51 years old woman" patient with striking symptoms: reduced comprehension and memory, disorientation, paranoia, aphasia and auditory hallucinations (Maurer, Volk and Gerbaldo, 1997), and the occurrence of histological alterations on her autopsy in the cerebral cortex, later-on known as senile plaques and neurofibrillary tangles (Alzheimer, 1991).

Nowadays, Alzheimer's disease (AD) is one of the most frequent neurodegenerative disorder, currently without an effective cure, treatment or prevention, affecting people in worldwide (Citron, 2010). AD is characterized by neuronal loss and gradual cognitive impairment and considered a critical public health issue. AD, as the major cause of dementia, in the elderly (Vinters, 2015) is defined by a cognitive impairment affecting mostly memory, language, behavioral and motor disturbances (Kovacs, 2014), that predominantly affects more women than men (Candeias *et al.*, 2017), also number of AD cases expected to triple by the year 2050, without effective treatments (Huang and Mucke, 2012).

As a multifactorial disorder, many factors have been linked to AD, such as exacerbation of aging; genetic inheritance; environment factors (diet and malnutrition); head injury, or exposure to aluminum; infectious agents; degeneration of anatomical pathways; a compromised blood brain barrier (BBB); mitochondrial and immune system dysfunction, all may contribute to development and progression of this malady (Armstrong, 2013) .

Normally, patients with AD show an impaired ability to perform tasks on routine basis, and often experience psychiatric, emotional, and personality disturbances (Tarawneh and Holtzman, 2012).

1.2.1 Neuropathology

The two common and distinctive neuropathological "hallmarks" lesions present in selective brain regions of patients, the temporal and parietal lobes, as well in restricted regions within the frontal cortex and cingulate gyrus, characterize AD pathologically:

(1) the senile plaques (SPs) composed of extracellular deposits of abnormal fibrillar insoluble β-amyloid peptide (Aβ) in the brain parenchyma and in blood vessels of the brain; and

(2) the neurofibrillary tangles (NFTs) formed by accumulation of intracellular aggregates of hyperphosphorylated and glycosylated Tau protein in dystrophic neurites, as neuropil threads, or as massive NFTs in neuronal cell bodies (**Figure 1.1**), a microtubule-associated protein involved in the promotion and stabilization of microtubules (Serrano-Pozo *et al.*, 2011; Jack and Holtzman, 2013).

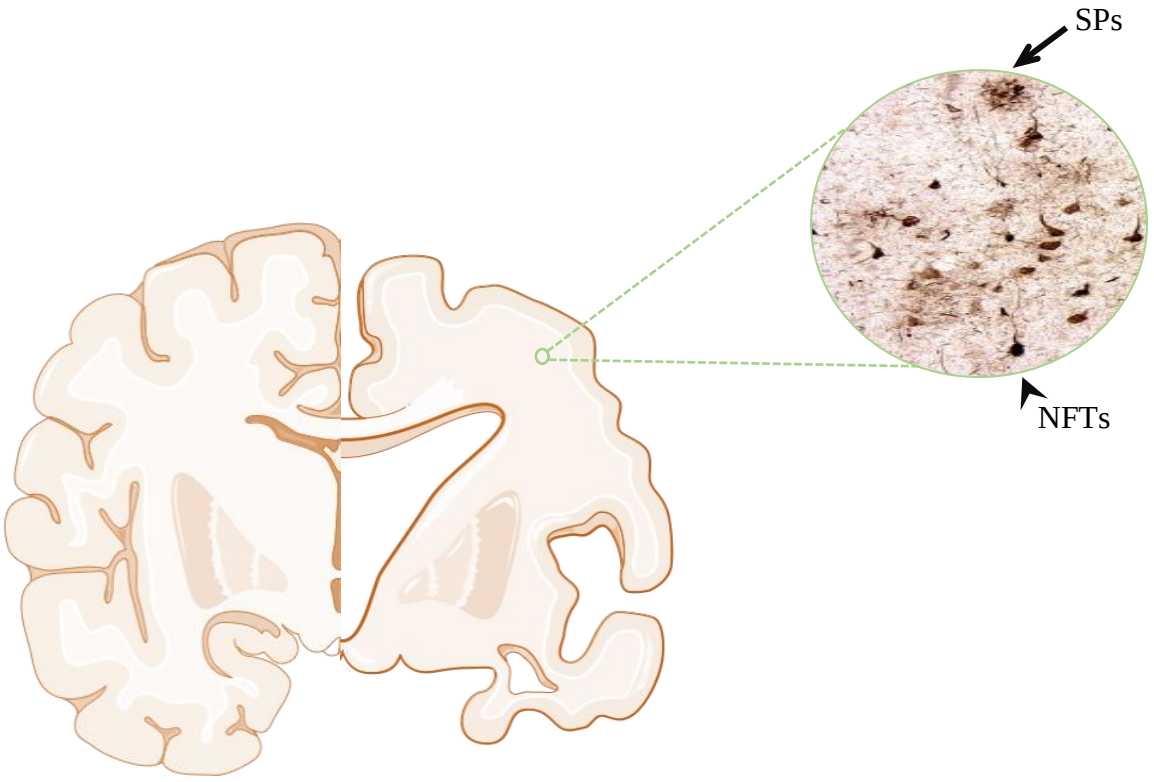


Figure 1.1 **Neuropathological Hallmarks of AD pathology.** SPs- Senile plaques (arrows) and NFTs- Neurofibrillary tangles (arrowheads) Filipe, 2018. Immunohistochemistry stain Tau-C3 from a patient with AD **(**adapted from Binder *et al.*, 2005)

Several special staining techniques are used to identify neurons and neurofibrils. However, the Bielschowsky silver method is the most useful technique in the identenfication of multiple plaques subtypes of SPs, that have been described over the years, such as diffuse, primitive, neuritic, compact cored, and cotton-wool. Anyway, the neuritic plaques, in particularly, have been the most significant plaque type for the diagnosis of AD during autopsies (Castellani, Rolston and

Smith, 2010). The SPs are normally interspersed among clusters of axons and dendrites (neurites), which are usually grossly swollen or atrophic (Nixon, 2007).

In contrast, NFTs are a massive fibrillar intracytoplasmic inclusions in neuronal cell bodies/proximal dendrites, dense aggregates of long unbranched filaments, paired helical filaments (PHFs), which become extracellular ghost tangles after the death of the neurons. Additionally, NFTs are present in the cytoplasm of neurons, such as cortical pyramidal cells, in the entorhinal cortex, hippocampus, amygdala and among other regions. These filaments, are composed of microtubule- associated protein Tau in an abnormally phosphorylated manner, that self-assembles to develop, the PHFs. Besides that, these are seen in dystrophic neurites of plaques, as fine neuropil threads, reinforcing the correlation between density of NFTs and severity of dementia (Longstaff, 2005).

Regularly, they are present mainly in brain regions involved in learning and memory and emotional behaviors (**Figure 1.2**), meaning that an abnormal excessive accumulation of proteins in the brain can lead to loss of neurons and synapses, causing an atrophy in different parts of the brain (Mattson, 2004).

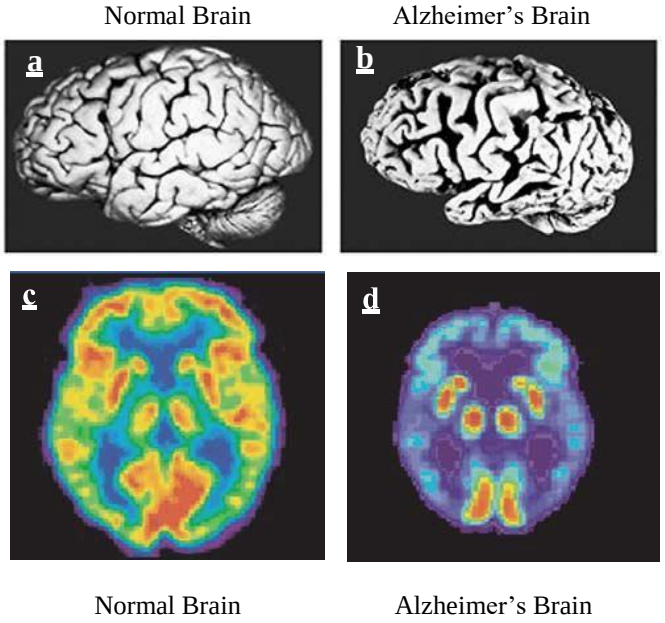


Figure 1:2 – **The pathology of Alzheimer's disease. a**, Brain of a healthy person. **b**, Brain of an Alzheimer's disease patient, which exhibits clearly marked shrinkage of gyri in the temporal lobe (lower part of the brain) and frontal lobes (left part of the brain). **c** and **d**, Positron Emission Tomography (PET) scans images showing glucose uptake (red and yellow indicate high levels of glucose uptake) in a living healthy person and a normal control subject. The Alzheimer's patient exhibits large decreases in energy metabolism in the frontal cortex (top of brain) and temporal lobes (sides of the brain). Adapted from Mattson, 2004.

Despite this, "plaques" and "tangles" are found in normal aged brains. When they are present as an abnormal excessive accumulation manner, this can play a critical role, affecting numerous cellular processes such as energy metabolism, synaptic transmission, generation of reactive oxygen species (ROS), cellular calcium and protein homeostasis; and then increasing the risk for developing AD pathology (Lu *et al.*, 2015).

1.3 Genetically

AD is normally divided into two forms: familial or early-onset AD (2-10% cases), where individuals typically develop the disease before 60 years; and the so-called "sporadic" or late-onset AD (non-hereditary), that occurs late in life, over 60 years (Bertram, Lill and Tanzi, 2010). Mutations in three genes were identified as autosomal dominant forms of AD (rare genetic mutations): β-Amyloid Precursor Protein (*APP*), Presenilin 1 (*PSEN1*) and Presenlin 2 (*PSEN2*), which are located on chromosomes 21, 14 and 1, respectively.

All three mutations in *APP*, *PSEN1* and *PSEN2* (explain 2-10% of the occurrence of early-onset AD) are involved in the overproduction of Aβ. As an example of the correlation between overproduction of Aβ and dementia is the Down's Syndrome (DS) or Trisomy 21, with a triplication of *APP* gene, where patients have an increased chance to develop early-onset AD (Head *et al.*, 2012).

On the other hand, late-onset AD pathology results from the interaction of environmental, genetic and epigenetic factors, indicating that gene polymorphisms may interact with environmental factors to predispose to AD (Van Cauwenberghe, Van Broeckhoven and Sleegers, 2016). Several genes variations are involved in development of the late-onset AD, including the ε4 allele of the apolipoprotein E (*APOE*) gene, sortilin-related receptor-1 (*SORL1)*, adenosine triphosphate (ATP)-binding cassette subfamily A member 7 (*ABCA7*) and among others.

The ε4 allelic variant of the *APOE* gene is the biggest risk factor for late-onset AD, located on chromosome 19 (Citron, 2010). The human *APOE* gene contains three polymorphic allelic variants at a single gene locus (ε2, ε3 and ε4) encoding for three ApoE isoforms (ApoE2, ApoE3 and ApoE4). Normally, ApoE is originated essentially by the liver and macrophages in peripheral organs, mediating cholesterol metabolism in an isoform-dependent manner. However, in the central nervous system (CNS), ApoE is synthetized locally in the brain, mainly by astrocytes, transporting cholesterol to neurons via ApoE receptors.

Therefore, ApoE plays a crucial role in the CNS, including in neuronal development, in regenerating and in deposition or clearance of Aβ by direct protein-to protein interaction (Posse de Chaves and Narayanaswami, 2008; Liu *et al.*, 2013).

1.4 Amyloid-β Protein (Aβ)

Over decades, studies have been supporting that an imbalance between the production and clearance of Aβ occurs in a very early AD stage. As a normal cellular metabolism product is necessary a homeostatic balance between Aβ production and clearance, crucial to maintaining a healthy brain (Wang *et al.*, 2017).

At physiologically level, Aβ (4 kDa) a hydrophobic 39-43 amino acid peptide, exists not only in the brain, including neurons, astrocytes, microglia and cerebrospinal fluid (CSF), but as well as in periphery regions, organs and tissues, such as adrenal gland, kidney, heart, liver, pancreas, spleen, muscles and various blood and endothelial cells (Pawlowski, Meuth and Duning, 2017). Aβ peptides are the main compounds of SPs and derives from the proteolytic cleavage of a larger glycoprotein named APP.

APP is an integral membrane protein, synthesized in the endoplasmic reticulum (ER), transported to the Golgi complex, and then glycosylated and further maturated (Toh *et al.*, 2017). After, transported to the plasma membrane, mature APP, best known as the precursor molecule is cut by two membrane-bound endoprotease, β-secretase and γ-secretase to produce Aβ residue peptide (Haass and Selkoe, 1993). Mutations in the human *APP* gene cause the development of amyloid plaques, seen especially in early-onset AD pathology. These mutations related to *APP* gene are close to the γ-secretase site and can increase the $A\beta_{42}/A\beta_{40}$ ratio. Also, other mutations can occur in the genes *PSEN1* and *PSEN2* (Chen *et al.*, 2017).

Accordingly, there are different Aβ species, but the two major isoforms of Aβ peptide in human brain are $A\beta_{1\text{-}40}$ (80-90%) and $A\beta_{1\text{-}42}$ (5-10%), differing from each other by two amino acids. The slightly longer forms of Aβ peptide, in particular $A\beta_{42}$ are more hydrophobic and fibrillogenic, and the main species accumulated in the brain (Murphy and Levine III, 2010).

Besides that, Aβ monomers aggregate into different forms of assemblies, such as oligomers, protofibrils and amyloid fibrils. These particular Aβ forms may be involved in neurodegeneration events at different stages of AD condition. In fact, Aβ aggregation in the brain, ultimately ends with the formation of insoluble protein fibrils, as a part of the components of amyloid plaques. Evidences suggest that neurotoxic species are named as soluble oligomers or protofibrils of Aβ, present on or off deposition pathways, leading to fibril formation (Dubnovitsky *et al.*, 2013).

Aβ was first isolated as the principal component of amyloid deposits in the brain and in cerebrovasculature from DS and AD patients, playing a key role in the pathogenesis of AD condition (Murphy and Levine III, 2010).

Usually, Aβ levels in both brain and peripheral tissues are known to be lower in

cognitively normal elderly individuals, comparing with AD patients (Wang *et al.*, 2017). Aβ has been described in various organelles connected with degradative pathways linked to lysosome compartment, including endosomes (Cataldo *et al.*, 2004), autophagic vesicles (AVs) (Yu *et al.*, 2004), late endosomes (LEs) or multivesicular bodies (MVBs) (Takahashi *et al.*, 2002) and lysosomes itself. Therefore, Aβ deposition may be the primary event, that occurs in SPs formation (Castellani, Rolston and Smith, 2010).

The Aβ production levels is balanced by the carrier- and receptor-mediated transport across the BBB by microglial cells and astrocytes. Moreover, the concentration of soluble Aβ in the CNS, central for the formation of protofibrils and vascular aggregated Aβ forms, is influenced by Aβ active transport exchange through BBB (Wilhelmus, De Waal and Verbeek, 2007).

This balance of Aβ levels is acerbated in AD brains, resulting in an extensive depositation and aggregation of Aβ peptides. Furthermore, Aβ aggregation relates to generation of Aβ oligomers protofribils, the most toxic forms, responsible for initiating the degeneration process in neurons, and mature fibrils (Selkoe, 2000). These apparent toxic species — soluble Aβ oligomers — and their subsequent ability to cause neuronal injury, depend on the precision of an intramembranous proteolytic cleavage (Haass and Selkoe, 2007).

In vitro, some evidences support that aggregation may be linked to pathogenicity and Aβ protofibril may be the pathogenic species (Caughey and Lansbury, 2003). Other studies *in vitro* seem to demonstrate that Aβ42 aggregates into both amyloid fibrils and soluble intermediates much more rapidly than does Aβ40 (Levites et al., 2006).

On the other hand, some studies *in vivo* demonstrated that Aβ42 is the predominant form of Aβ that accumulates in the AD brain, essential for seeding Aβ deposition (Younkin, 1998; Fryer and Holtzman, 2005). Literally, Aβ40 accumulates in AD brain, but the extent of Aβ40 accumulation relative to Aβ42 is highly variable and usually attributed to deposition of Aβ40 in cerebral vessels (Kim et al., 2007).

These findings support that an excessive Aβ accumulation and aggregation can trigger a complex downstream cascade, implicated in the symptoms of AD pathology. Amyloid cascade hypothesis proposes that gradual accumulation, as well as aggregation of Aβ peptide, can lead to a slow deadly cascade resulting in synaptic alterations, microglial and astrocytic activation, modifications of normal soluble Tau protein into oligomers and later into insoluble PHFs, and progressive neuronal loss (Hardy and Higgins, 1992).

This deadly cascade culminates with cell death, and cognitive failure resulting in a progressive dementia connected with Aβ and Tau pathologies (Haass and Selkoe, 2007) (**Figure 1.3**).

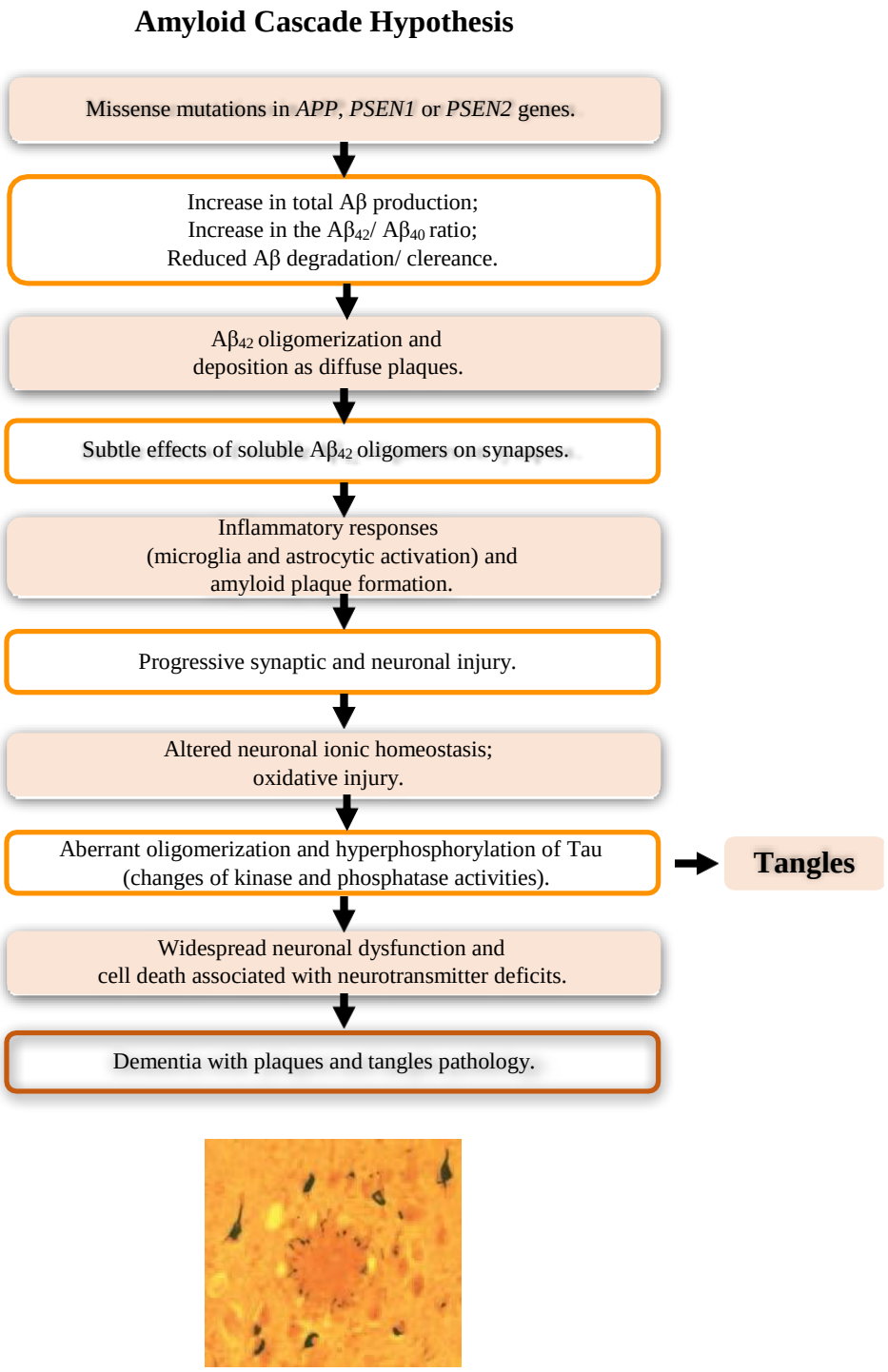


Figure 1:3 - **The amyloid or Aβ cascade hypothesis.** Gradual changes in the steady-state levels of Aβ in the brain are thought to initiate the amyloid cascade. Adapted from Haass and Selkoe, 2007.

1.5 Tau Protein

Microtubules are very dynamic structures, from cytoskeleton, responsible for the development of cell processes, establishment of cell polarity and intracellular transport. Some specific proteins are associated with its stabilization, including the microtubule-associated proteins (MAPs)- MAP1A, MAP1B, MAP2 and Tau protein. MAPs are seen specifically in mature neurons

and Tau protein is preferentially abundant in axons (Avila *et al.*, 2004). However, recent data suggest that Tau might play also a physiological role in dendrites (Mietelska-Porowska *et al.*, 2014).

Tau protein (50kDa) is a very hydrophilic, soluble and displays a "natively unfolded" structure, stabilizing microtubules in neurons (Mandelkow and Mandelkow, 2012). It can be phosphorylated at multiple sites, which some are responsible to regulate its microtubule-binding properties (Mandelkow and Mandelkow, 1998). Tau protein can be found in many animal species, including *Caenorhabditis elegans*, *Drosophila*, rodents, monkeys, bovines and humans. On the other hand, in human beings, Tau protein is present in neurons, as well as in glial cells, mainly in pathological circunstances, and in numerous peripheral tissues, such as heart, kidney, lung, muscle, pancreas, testis and in fibroblasts (Buée *et al.*, 2000).

Basically, Tau function depends on its phosphorylation state, where the incorporation of phosphate groups into Tau protein is determined by its conformation and by the balance between the activities of Tau kinases (responsible for phosphorylation state) and phosphatases (responsible for dephosphorylation state). The non-phosphorylated forms preferentially link to microtubules (Kolarova *et al.*, 2012).

Notably, during the development of fetal brain, normal phosphorylated Tau proteins seems to be substantially higher; however in the adult brain neurons are normally characterized by a lower Tau phosphorylation state (Ballatore, Lee and Trojanowski, 2007).

As a fact, several pathogenic phenomena may contribute directly or indirectly to Tau protein loses its biological activity and as consequence the capacity to bind to microtubules.

For example, hyperphosphorylation, misfolding and aggregation, such as imbalance in the activity levels or regulation of Tau kinases and phosphatases, Tau gene mutations and post-translational modifications may contribute to abnormal Tau protein accumulation in brain. Tau protein is a highly soluble cytoplasmic protein that binds to tubulin during its polymerization into microtubules in neurons (Jouanne, Rault and Voisin-Chiret, 2017). Normally, in pathological conditions Tau protein do not bind to microtubules, resulting in an increase of abnormal levels of free (unbound) Tau fraction. Additionally, in normal Tau protein, both amino terminal and the carboxyl terminal flanking regions to microtubule binding repeats, appear to inhibit its self-aggregation into filaments.

In AD ailment, abnormal hyperphosphorylation events, such as the phosphorylation of the amino terminal and the carboxyl terminal flanking regions occur, resulting in the formation of tangles of PHFS. The NFTs are mostly constituted by bundles of PHFs in AD pathology (Ballatore, Lee and Trojanowski, 2007) (**Figure 1.4**).

In addition, aggregated of Tau filaments cannot be degraded by host neurons and gradually

accumulate within the cytoplasm.

Thus, four Tau-based therapeutic approaches have been proposed: inhibition of Tau hyperphosphorylation; inhibition of Tau aggregation; clearance of Tau and Tau pathology; and rescue of neuronal plasticity by stabilizing the microtubule network or promoting neuroregeneration (Iqbal, Liu and Gong, 2016).

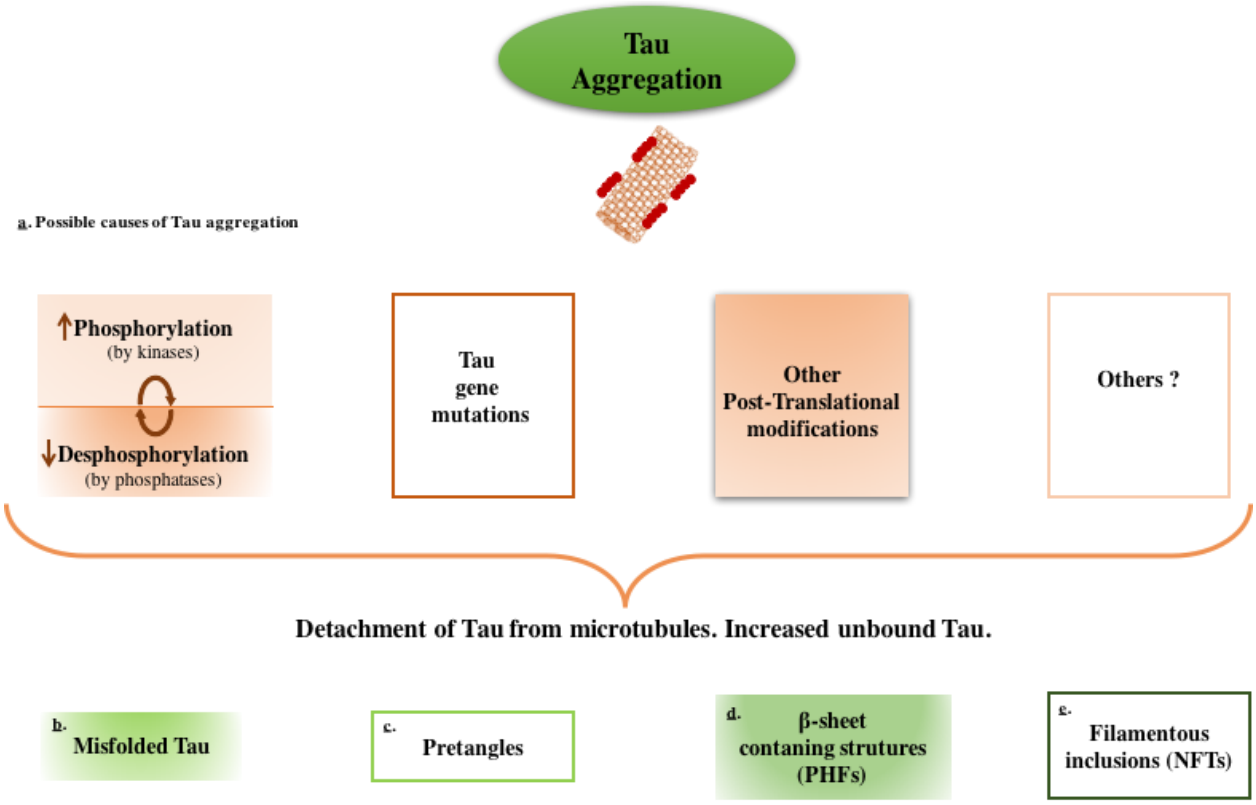


Figure 1:4 - **Pathological aggregation of Tau.** A schematic representation of the different stages of the formation of pathological Tau aggregates. **a.** Abnormal disengagement of Tau from the microtubules and a concomitant increase in the cytosolic concentration of Tau are likely to be the key events that lead to Tau-mediated neurodegeneration. Direct causes of abnormal disengagement of Tau from the microtubules, include an imbalance of Tau kinases and/or phosphatases, mutations of the Tau gene, covalent modification of Tau causing and/or promoting misfolding, and possibly other causes such as other post- translational modifications. **b.** Once Tau is unbound from microtubules it becomes more likely to be misfolded. This is thought to be a stochastic phenomenon that is more likely at higher cytosolic Tau concentrations. **c.** Early deposits of Tau, called 'pretangles', are not stained by Congo Red or Thioflavine T, indicating that these intermediate forms of aggregated Tau do not exhibit the pleated β sheet structure typically found in amyloid aggregates. **d.** A structural transition leads to this more organized aggregate and the eventual development of NFTs. **e.** Such transitions may be facilitated by heterogeneous interactions with membranous structures. Adapted from Ballatore, Lee and Trojanowski, 2007.

1.6 Cellular Proteolysis

A fine maintenance of proteome integrity is necessary for a proper balance of protein homeostasis, cell function and viability, cellular control between synthesis, organelle biogenesis, trafficking and degradation of proteins. All these are crucial for a good cellular development, differentiation, maintenance and cell survival in a changing extracellular environment (Moreira et al., 2010). Accordingly, cells have an elaborate enzymatic machinery, named molecular chaperones that bind non-native polypeptides and promote their folding to the native state in an ATP- dependent manner (McClellan et al., 2005).

Besides that, diverse cellular events can influence protein misfolding during cell life, such as stochastic fluctuations, stress conditions or just metabolic changes, which particularly happen during aging and cancer (Chen et al., 2011). Failure to eliminate misfolded proteins can result in potentially toxic aggregates, inactivating functional proteins and finally leading to a cell death.

Usually, cells require a continuous recycling of their cytoplasm to create macromolecular building blocks and energy, both under physiological and stress conditions (Nikoletopoulou, Papandreou and Tavernarakis, 2015). However, protein aggregation is a continuously ongoing cellular process, where some cellular vital functions require the aggregation of specific proteins. In other cases, protein aggregates generate by numerous stressors are a prerequisite to develop many pathologies, including neurodegenerative diseases (Hayat, 2013).

The two distinct proteolytic systems in eukaryotic cells responsible for the complete degradation of intracellular proteins and recycling pathways are the ubiquitin-proteasome system (UPS), important for the degradation of 80-90% of proteins (short-lived, abnormal, denatured or in general, damaged proteins) and the autophagy-lysosomal pathway, capable of degrading most long-lived proteins, aggregated proteins, as well as cellular organelles (Lilienbaum, 2013). Focusing on autophagy-lysosomal pathway, autophagy (from Ancient Greek *autófagos*, meaning "self- eating") was first described morphologically over 50 years ago, as a constitutive homeostatic process conserved from yeast to mammals (Coutts and La Thangue, 2016). In autophagy pathway cellular components, such as damaged organelles, long-lived or aberrant proteins and superfluous or aged portions of cytoplasm, are engulfed by autophagosomes and recycled through the lysosomal component without compromising cellular functions and tissue homeostasis (Caballero and Coto-Montes, 2012).

Under physiological conditions, autophagy bears several vital roles, including maintenance of the amino acid pool during starvation, preimplantation development (oocyte fertilization) and differentiation (erythrocytes, lymphocytes and adipocytes) (Ravikumar *et al.*, 2010), as well as prevention of neurodegeneration, anti-aging, tumor suppression, clearance of intracellular microbes and homeostasis of innate and adaptive immunity system (Mizushima, Yoshimorim and Levine, 2010).

At the first sight, autophagy appears to be active at a low basal levels in most of the cells in our body, reflecting its main role on regulate the turnover of long-lived proteins and eliminate damaged structures (Codogno and Meijer, 2005). This autophagy- dependent quality control is also crucial to restrict the production of ROS (Beau, Mehrpour and Codogno, 2011).

Even so, in different circumstances, autophagy can be stimulated during pathological and physiological states by nutrient deprivation (Cardoso, 2015), both in cultured cells and in intact organisms, from yeast to mammals. Besides starvation, responsible for playing a cytoprotective

effect by blocking the induction of apoptosis upstream of mitochondrial phenomes (Boya *et al.*, 2005), autophagy can be induced by other physiological stress stimuli, such as low oxygen levels, or decreased energy supply, ER stress, high temperature, hormonal stimulation, pharmacological agents (rapamycin), innate stimulation signals, among others (Mizushima, Yoshimorim and Levine, 2010). Bergamini and colleagues (Bergamini *et al.*, 2007) have suggested that autophagy can be stimulated by CR extending lifespan in rat (Beau, Mehrpour and Codogno, 2011), through activation of deacetylase Sirtuin1 (Wang, 2014).

On the other hand, a malfunction of autophagy causes several human diseases, where autophagy may be dysregulated, including liver, muscle and infectious diseases, as well as cancer and neurodegenerative disorders (Levine and Kroemer, 2008).

1.7 Autophagic-Lysosomal Pathway

1.7.1 An Overview

Based on the type of cargo delivery in mammalian cells, there are three main forms of autophagy: Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy (CMA).

a. **Macroautophagy:** Macroautophagy is a conserved intracellular degradation pathway mediated by the autophagosome, a double membrane-bound vesicle, that traffics substrates engulfing cytoplasmic proteins and organelles, including ribosomes, mitochondria, ER, part of nucleus and peroxisomes, for delivery to the lysosome compartment to be degraded (Mizushima, Yoshimori and Ohsumi, 2011).

b. **Microautophagy:** A non-selective lysosomal degradative process, engulfing soluble intracellular substrates directly by invagination, protrusion or septation through the lysosomal membrane under nutrient depletion that are subsequently degraded by lysosomal proteases. Microautophagy is induced by nitrogen starvation in yeast and rapamycin, as occurs in macroautophagy (Hayat, 2013).

c. **Chaperone-Mediated Autophagy (CMA):** A uniquely selective form of autophagy, where only proteins are selected through a recognition motif in their amino acid sequences (KFERQ). The proteins are target with a specific cytosolic member of chaperone protein family-heat-shock protein of 70 kDa (Hps70), the heat-shock cognate protein of 70 kDa (Hsc70) to the lysosomal membrane protein, lysosomal-associated membrane protein type 2A (LAMP-2A) receptor and then delivered into lysosomes lumen for degradation by the hydrolases (Cuervo and Wong, 2014). Furthermore, CMA and macroautophagy can act in a sequential manner during starvation process, where macroautophagy is activated and, if the

starvation persists, cells switch from this bulk degradation to CMA, targeting non-essential proteins for degradation to obtain the amino acids required for the synthesis of essential proteins (Orenstein and Cuervo, 2010).

1.7.2 Autophagosomes Biogenesis

The process of mammalian macroautophagy (henceforth autophagy) is divided into six principal steps: initiation, nucleation, elongation, closure, maturation and degradation or extrusion (Kang *et al.*, 2011; Kim and Lee, 2014) **(Figure 1.5).**

1.7.2.1 Initiation

The first step of autophagosome formation is characterized ultrastructurally by the surrounding and sequestration portions of cytoplasmic organelles and proteins at the phagophore assembly site (also known as the isolation of membrane) (Kaur and Debnath, 2015). While discrete regions of ER enriched in phosphatidylinositol-3-phosphate (PI3P), called omegasomes (due to their Ω shape), may serve as the nucleation for the formation of autophagosomes in mammalian cells (Roberts and Ktistakis, 2013); many other evidences support that to create the membrane, which contribute to phagophore formation and elongation, different potential sources are requested: the Golgi complex, ER, ER-Golgi intermediate compartment, recycling endosomes, mitochondria and the plasma membrane (Rubinsztein, Bento and Deretic, 2015).

The coordination of the membrane rearrangements that enhance autophagosome development, and their subsequent delivery to the lysosomal compartment, is stimulated by multiple autophagy- related (Atg) proteins. Several *ATG* genes, more than 30, have been identified in yeast genetic screenings. Most of these genes are conserved in humans and have orthologues in higher eukaryotes. The molecular composition of the autophagy-related 1 (Atg1), a serine/threonine kinase, complex appears to differ between yeast and mammals (Hosokawa *et al.*, 2009).

In 2007, two mammalian homologues of Atg1 were discovered to be involved in autophagy, uncoordinated (UNC)-51-like kinase 1 and 2 (ULK1 and UKL2) complex. There is some redundancy between these two ULKs, with UKL1 having the main role. ULK1 was identified as the mammalian homolog of *Caenorhabditis elegans* UNC-51, being originally characterized as essential for neuronal axon guidance (Russell, Yuan and Guan, 2014).

Autophagy is initiated by the ULK complex, composed by autophagy-related 13 (Atg13), autophagy-related 101 (Atg101) and scaffold protein, focal adhesion kinase (FAK)-family

interacting protein of 200 kDa (FIP200) or autophagy-related 17 (Atg17) (Mcknight, Mizushima and Yue, 2012).

Basically, there are some autophagy inducers (cellular stress signals), including lowered concentrations of specific amino acids, growth factors or ATP, hypoxia, certain types of protein aggregates, ER stress and inhibition of mammalian target of rapamycin 1 (mTOR1), responsible for activating ULK complex, and then essential for autophagosome formation.

Under nutrient-rich conditions ULK complex interacts directly with mTOR, however, during nutrient deprivation ULK complex dissociates from mTOR, being recruited to the site of autophagosome composition. This physically disengagement from mTOR, a series of phosphorylation/ dephosphorylation events involving UKL1, Atg13 and FIP200 ensures, that the complex achives at the autophagosome formation site is competent for the downstream steps (Nixon, 2013; Roberts and Ktistakis, 2013).

Further, adenosine monophosphate (AMP)-activated protein kinase (AMPK) has been reported to play a role in the regulation of ULK during autophagy. In fact, various subunits AMPK complex may directly interact with the ULK1 network and phosphorylates different sites essential for ULK activation.

Although, AMPK and the mammalian rapamycin-sensitive mTOR complex 1 (mTORC1) can oppositely regulate ULK1/ULK2 kinase activity by direct phosphorylation (Alers et al., 2012). Under these circunstances, a dephosphorylation of UKL1/UKL2 occurs, and the UKL complex comprising UKL1, Atg13, Atg101 and FIP200 dissociate from mTORC1. Then, UKL1 is rendered enzymatically active and phosphorylates Atg13 and FIP200, leading to subsequent localization of the activated ULK network to the phagophore, beginning the process of autophagy (Kim and Lee, 2014).

Additionally, FIP200 also known as *Retinoblastoma 1*- Inducible *Coiled-Coil 1 (RBCC1)* is a multifunctional protein that has several interaction partners and regulates numerous cellular processes, such as cell growth, proliferation, cell spreading, and migration and it has been proposed to be a functional orthologue of yeast autophagy-related 17 (Atg17). FIP200 promotes ULK1 kinase activity, translocate to the pre-autophagosomal membrane after starvation, occurs autophagosome induction and its role is extremely important for the stability and phosphorylation of ULK1 (Alers *et al.*, 2012).

On the other hand, Atg101, is a protein conserved in various eukaryotes, important for the stability and basal phosphorylation of Atg13 and UKL1 (Hosokawa *et al.*, 2009). However, under nutrient rich conditions, the ULK complex interacts with mTORC1 and remains inactivated by mTORC1-mediated phosphorylation of ULK1/ULK2.

1.7.2.2Vesicle Nucleation

The first step of autophagosome formation, autophagosome nucleation requires Beclin-1 protein or autophagy-related 6 (Atg6). At this nucleation stage, the activated ULK kinase complex targets, a class III phosphatidylinositol 3- kinase (PI3K) containing, a vesicular protein sorting 34 (Vps34), p150 a mammalian homolog of yeast vesicular protein sorting 15 (Vps15) and autophagy-related 14 (Atg14)-like (Atg14L) protein or Barkor (Funderburk, Wang and Yue, 2010). All of these elements are associated with Beclin-1 protein to generate the pre-autophagosomal structure (Wong *et al.*, 2013).

Various additional components of Beclin-1 complex are also needed such as Ultraviolet radiation resistance-associated gene (UVRAG) and Beclin-1-regulated autophagy (AMBRA1), necessary to influence its functions and responsible for the induction of autophagy network (Liang *et al.*, 2008).

UVRAG is known to regulate autophagosome formation, binding to Beclin-1 during autophagy pathway. The B-cell lymphoma-2 (Bcl-2)- associated x (Bax)-interacting factor 1 (Bif1) interacts with Beclin-1 complex through UVRAG protein, and positively regulates autophagy and suppress tumorigenesis (Takahashi *et al.*, 2007).

Besides that, AMBRA1 is a positive regulator of Beclin-1-dependent autophagy and regulates the development of the nervous system.

Furthermore, recruitment of other proteins required for the autophagosome formation, including WD-repeat domain phosphoinositide-interacting protein (WIPI) and double FYVE domain-containing protein 1 (DFCP1), both PI3P effectors, localized in ER and Golgi apparatus membranes, and though to help to drive progression of the omegasome to the isolation membrane (Axe *et al.*, 2008).

Another protein essential for the initiation of autophagy is autophagy-related 9 (Atg9), recruited to the autophagosome formation site. This protein is localized at the *trans*-Golgi network (TGN) and LEs, and during autophagy trafficked to endosomal membranes. Atg9 is involved in the early stages of autophagosome formation, leading to autophagosome expansion (Coutts and La Thangue, 2016).

1.7.2.3 Vesicle Elongation

The expansion of the isolation membrane is basically the simultaneous elongation and nucleation of little cistern. There are two ubiquitin-like complexes, involved in autophagosome elongation and maturation. The first ubiquitin-conjugation system, the autophagy-related 16-like

(Atg16L) complex is combined with the autophagy-related 12 (Atg 12) protein, and autophagy-related 5 (Atg5) protein, essential for the formation of pre-autophagosomes, controlling autophagy pathway (Pyo, Nah and Jung, 2012).

Atg12 is a 186 amino acid protein conjugated to Atg5 protein in a reaction that requires autophagy-related 7 (Atg7) protein, ubiquitin-activating-enzyme (E1)-like and autophagy-related 10 (Atg10) protein, ubiquitin-conjugating-enzyme (E2)-like (Mizushima *et al.*, 1998). Atg12-Atg5 conjugate interacts non-covalently with Atg16L protein, ubiquitin-activating-enzyme (E3)-like to form Atg12-Atg5-Atg16L complex (Mizushima *et al.*, 2004).

The second ubiquitination-like protein conjugation system involves the lipidation of microtubule- associated protein 1A/1B-light chain 3 (LC3) or also autophagy-related 8 (Atg8).

Autophagy-related 4 (Atg4) protein cleaves pro-LC3 to form cytosolic LC3I, which is covalently conjugated to phosphatidylethanolamine (PE), via autophagy-related 3 (Atg3)-autophagy-related 7 (Atg7) and Atg 5-12 to form membrane associated LC3II (Mizushima *et al.*, 2004). This allows the association of LC3II with the autophagosome, where it remains bound until it is recycled during lysosomal degradation (Coutts and La Thangue, 2016).

Atg5-Atg12 conjugate seems to modulate LC3I conjugation to PE by acting in an E3-like fashion manner (Hanada *et al.*, 2007), and the Atg5-Atg12-Atg16L complex, determines the sites of LC3 lipidation. In this way, LC3 is specifically targeted to Atg5-Atg12-associated membranes, that expanded phagophores.

Besides that, Atg5-Atg12 conjugate is lost upon completion of autophagosome formation, and LC3II remains associated with autophagosomes even after fusion with lysosomes, then LC3II on the cytosolic face of autolysosomes may be dilapidated and recycled to LC3I.

Conjugated LC3 is a substrate for Atg4, which dilapidates LC3II, removing it from the membrane. Furthermore, the production of ROS; specifically, hydrogen peroxide (H^2O^2), during starvation results in oxidation and inactivation of Atg4 protein. This inhibits LC3II dilapidation and might promote autophagosome formation (Scherz-Shouval et al., 2007). LC3II is the only known protein that specifically associates with autophagosomes and not with other vesicular structures.

Therefore, LC3I levels correlate with autophagic vacuole numbers, that can also be assessed by scoring LC3-positive vesicle numbers (Kabeya *et al.*, 2000). LC3II can mediate membrane tethering and hemifusion, and these functions might be crucial for the expansion of autophagosome membranes. These data also raise the possibility that LC3 assists the final fusion of the pre-autophagosomal double-membrane ‘cups’ into fused vesicles (Nakatogawa, Ichimura and Ohsumi, 2007).

1.7.2.4Autophagosome maturation and fusion

Autophagosomes form randomly in the cytoplasm, and once formed, autophagosomes traffick along microtubules in a dynein-dependent manner to lysosomes, which are clustered around the microtubule-organizing center (MTOC), located in the peri-nuclear region of the cell.

There, autophagosomes tether, dock and fuse with lysosomes, and the contents of the two vesicles are released and mixed, generating a now mature autolysosome. The details of this aspect of mammalian autophagy are still unclear. However, the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins have been characterized and are involved in yeast autophagosome-vacuole fusion (Reggiori and Ungermann, 2017).

In the route of fusion with lysosomes, autophagosomes can fuse with endosomes to form amphisomes. It is currently not clear whether amphisome formation is a prerequisite for autophagosome-lysosome fusion. Reduced activity of some endosomal sorting complex required for transport (ESCRT) proteins disrupts autophagosome-lysosome fusion, resulting in the accumulation of autophagosomes (Skibinski *et al.*, 2005; Kim and Lee, 2014) (**Figure 1.5**).

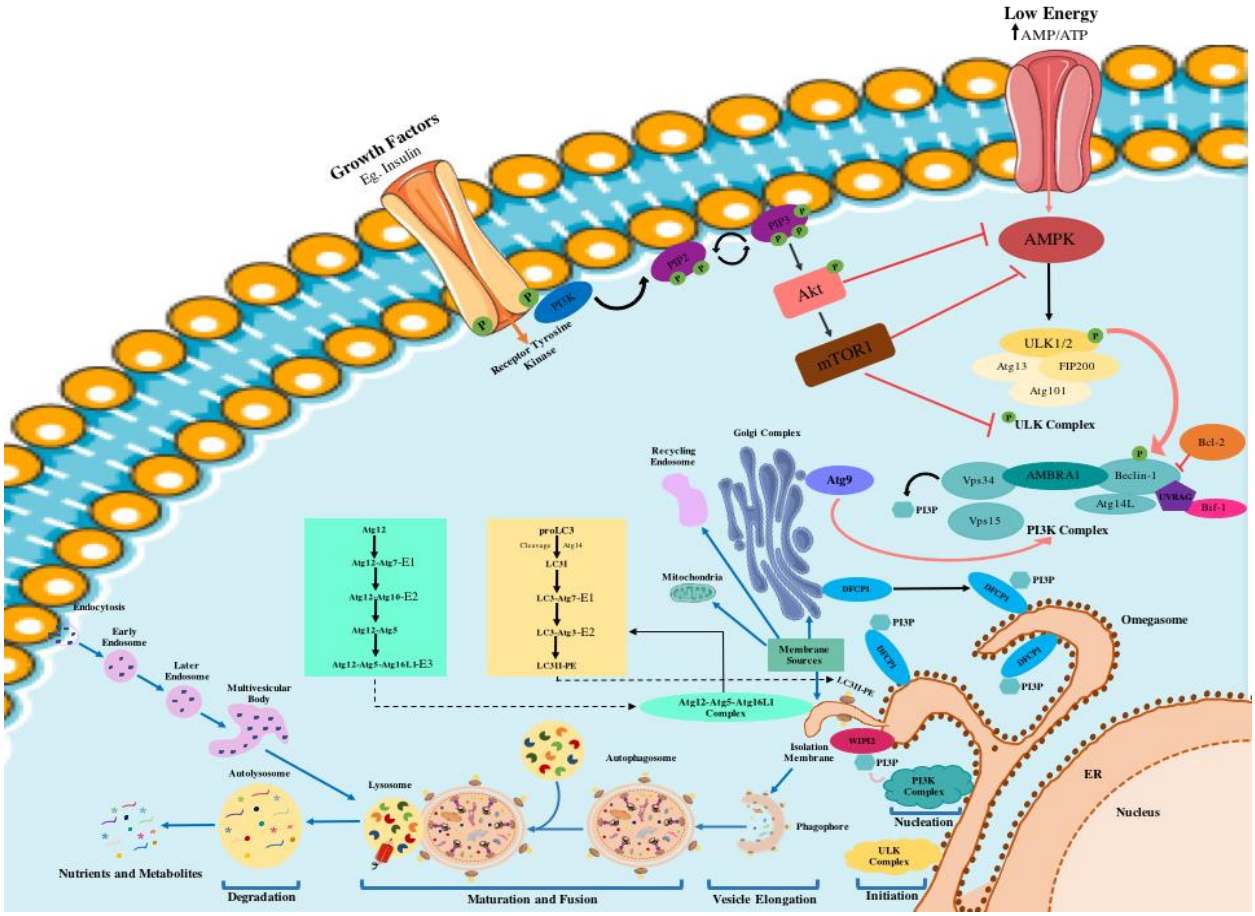


Figure 1:5 – **Scheme of Autophagy-lysosomal pathway.** Autophagy is regulated by multiple signalling pathways. Autophagic stimuli, including nutrient deprivation, or low energy, activate the ULK1/2 complex, which leads to increased activity of the Beclin-1–Vps34 complex through phosphorylation of Beclin-1. PI3P produced by Vps34 recruits effector proteins, such as DFCP1 and WIPI, to promote autophagosome formation. Elongation of the autophagosome is mediated by two conjugated systems comprising Atg12– Atg5–Atg16L and LC3–PE. After formation of complete autophagic vesicles, the mature autophagosome becomes fused with a lysosome to create an autolysosome, where sequestered molecules and organelles are degraded (Filipe, 2018).

1.7.2.5 Lysosomal Pathway

1.7.2.5.1 The Lysosome

"Lysosome" term was originally coined by Christian de Duve (de Duve et al., 1955) to describe a newly discovered organelle, that housed a pool of soluble hydrolases, capable of degrading proteins, nucleic acids, carbohydrates, lipids and cellular debris (Lim and Zoncu, 2016). Firstly, characterized as granules, and long considered as "suicide bags", lysosomes are catabolic membrane-bound organelles, the hub for proteostasis, present in all eukaryotic cells. Still, their size, shape, number and function differ greatly depending on species, cell type and context. These dynamic organelles function at the end of the endocytic, autophagic and phagocytic pathways (Wartosch, Bright and Luzio, 2015). In fact, lysosomal network, a degradation center and amino-acid recycling within lysosomes, is responsible to degrade complex biological molecules (e.g., proteins and lipids) into simpler components (e.g., amino acids and free fatty acids), being recycled to the cytosol and reused either as a source of energy or as building blocks for the biosynthesis of new molecules (Fraldi et al., 2016).

Lysosome, the incinerator of the cell, is a crucial regulator for cellular homeostasis events and can receive its substrates by multiple routes. In general, extracellular or cell surface cargos are delivered to lysosome through the endocytic pathway and the digestion of the intracellular components, occurs via a self-catabolic process known as autophagy (Saftig and Klumperman, 2009).

Lysosomes interact with LEs, also known as MVBs by direct fusion or 'kiss-and-run' events, and lysosomes fusing with endosomes form endolysosomes. Similialry, fusion of lysosomes with autophagosomes results in the formation of autolysosomes, and as well as fusion with phagosomes creates phagolysosomes (Wartosch, Bright and Luzio, 2015).

The limiting membrane of the lysosome is composed of a single phospholipid bilayer, containing more than 100 transmembrane proteins, and the most abundant proteins the lysosomal membrane proteins (LAMPs) type 1 (LAMP-1) and type 2 (LAMP-2), are estimated to constitute 80% of all proteins of the lysosome membrane protein content (Perera and Zoncu, 2016). One isoform of LAMP-2, the LAMP-2A is responsible to transport 30% of cytosolic proteins, which contain a KFERQ motif into lysosomes for degradation via CMA (Saftig, Schröder and Blanz, 2010).

Once inside the lysosome, substrates are degraded by aproximadetly 60 resident acid hydrolases, including proteases, sulfatases, glycosidases, peptidases, phosphatases, lipases, and

nucleases.

These proteins are synthesized at the rough endoplasmic reticulum (RER), traveling along the secretory pathway until reach the TGN, where are sorted and delivered to pre-lysosomal compartments. Additionally, mannose-6-phosphate (M6P) is the sorting signal for the majority, not all, of proteins designed to lysosomes. In fact, M6P groups have been found also in proteins with no lysosomal localization. Contrarily, proteins not bearing the M6P group can be targeted to the lysosome and proteins bearing the M6P group can be delivered to the lysosome independently of this group (mannose-6-phosphate receptor (MPR)-independent transport) (Castino and Isidoro, 2008).

Furthermore, these enzymes are turned to function optimally at the acidic pH 4.6-5.0 within the luminal environment of the lysosome, which is required for its internal hydrolytic activities, generated by the activity of lysosomal ion channels, ATP-dependent proton pumps (H^+ ions), the vacuolar H^+ ATPases (V-ATPases). Indeed, these proton pumps are essential to pH homeostasis in organelles. Proton gradient provides the driving force for the proton-coupled transport of metabolites, ions and soluble substrates into and out of the lysosomal lumen, necessary for a proper targeting of new synthesized lysosomal enzymes from the Golgi apparatus to the lysosome (Forgac, 2007; Lim and Zoncu, 2016).

Among the 60 resident lysosomal hydrolases, the best known of these enzymes are the cathepsin family of proteases (Appelqvist *et al.*, 2013). Cathepsins are crucial to regulate different physiological processes, and show to be dysregulated in some diseases, thereby represent viable targets for therapeutic intervation (Kramer, Turk and Turk, 2017).

A diverse human cathepsins have been identified, which can be subdivided into three distinct groups, according to the amino acid found in the active site, responsible for their catalytic activity; serine (A and G), cysteine (B, C, F, H, K, L, O, S, V, W and X), and aspartic cathepsins (D and E) (Zhang, Sheng and Qin, 2009).

Cathepsin D (CatD) is one the most studied aspartic proteins, involved in diverse biological processes. CatD is synthesized in the RER as a preproenzyme (inactive zymogen). After removal of signal peptide, the proCatD (52 kDa) is targeted to endosomes to generate an active single-chain intermediate, active form (48 kDa), and then to the lysosomes to create the fully active mature protease composed by a heavy chain (34 kDa) and a light chain (14 kDa) (Domenico, Tramutola and Perluigi, 2016). It is widely known that the major function of CatD is its involvement in general protein degradation and turnover within the lysosome. Also, CatD appears be associated with different physiological functions and pathological scenarios, such as cancer, AD, atherosclerosis and inflammatory disorders (Pereira *et al.*, 2015).

Furthermore, during the last decades, more studies have demonstrated some abnormalities

of endo-lysosomal pathway at early and late stages of AD pathogenesis, related to lysosomal hydrolases (Cataldo *et al.*, 1995).

Lysosomal functions can be divided into three main types: degradation, secretion and signaling. As the last step of the conserved cellular process of autophagic pathway, lysosomal degradation is essential to preventing the accumulation of toxic or exhausted molecules and damaged organelles in the cell, as well known in neurodegenerative disorders. This particularly function is extremely important in neurons, nondividing cells, which are not capable of dilute cytosolic material during mitotic episodes (Settembre *et al.*, 2013). Finally, cargoes are delivered to lysosomes basically through their fusion with endocytic vesicles and AVs, requiring the coordinated activityof three principal classes of proteins: (i) motor and adaptor protein complexes that regulate the vectorial movements of vesicles along microtubules (Ravikumar *et al.*, 2005; Fraldi *et al.*, 2016); (ii) membrane-associated RAB GTPases, such as Rab7, which control organelle trafficking and fusion (Kümmel and Ungermann, 2014) and (iii) lysosomal membrane proteins, LAMPs (Eitan *et al.*, 2016).

1.8 The Role of Autophagy in the Brain

Brain is the most complex organ of human body, a temporal and spatially multiscale structure, responsible for many different purposes, including cognition, emotion, circadian behaviors and autonomic functions (Satoh, Imai and Guarente, 2017). It communicates with each part of the body through a network of interconnected neurons, the nervous system. During of aging process some alterations occurs in the brain, including homeostasis changes, cognitive skills failure and the risk to develop dementia or neurodegenerative disorders increases exponentially (Przedborski *et al.*, 2008). The most widely noted cognitive adjustment relate to the process of aging is memory, especially episodic memory of hippocampus in medial temporal lobe region, a characteristic of the memory loss, typically seen in AD pathology (Peters, 2006). These events may exacerbate some negative effects, such as decrease production of new cells, low levels of neurogenesis, as well increase cellular damage, neuroinflammation, BBB disruption, reduced synaptic densities, ROS production and mitochondrial dysfunction (Camandola and Mattson, 2017), autophagy and lysosomal pathways impairment (Carmona-Gutierrez *et al.*, 2016). As a sequel, such events may result in neuronal dysfunction and neuronal cell death, which are seen in neurodegenerative disorders.

Neurons are highly differentiated structures for intercellular communication with soma, dendrites, axons and synapses. As postmitotic cells, do not replicate in general and, it is believed that neurons have permanently blocked their capacity to proliferate, when differentiated, being

typically found in a quiescent state in the adult nervous system (Frade and Ovejero-Benito, 2015). For this reason, neurons have a predisposition for the accumulation of toxic proteins and damaged organelles inside of them, that could be diluted during cell division in replicating cells (Son *et al.*, 2012). Cell cycle reactivation in adult neurons is an early hallmark of neurodegeneration and CNS injury.

Also, it has been reported that a specific disruption of autophagy in neuronal cells, results in neurodegeneration, reflecting a deficiency of basal autophagy levels in the mouse brain (knockout of either Atg5 or Atg7), which leads to an accumulation of ubiquitinated protein aggregates (Lenk *et al.*, 1992).

Autophagy activity is maintained at a low level in normal brain. Therefore, is essential for a normal function of the nervous system, playing a pivotal role in neuronal homeostasis during development, guarantee the shaping and function of the nervous system. Homeostasis in neurons include a vast synthesis and anterograde axonal transport of proteins, which must be balanced by a similar rate of retrograde transport and clearance by autophagy network (Kundu and Thompson, 2008).

In neurons is vital a constitutive active and highly efficient basal autophagic activity, characterized by particularly high levels of both ATP demand and protein synthesis (Lumkwana *et al*., 2017), maintaining the cell clean, and playing a neuroprotective role in synaptic neuronal plasticity anti-inflammatory function in glia cells, oligodendrocyte development, and myelination process (Martinez-Vicente, 2015; Nah, Yuan and Jung, 2015).

Genetic studies have firmly established that the loss of basal autophagy by either deletion of *ATG* gene or inhibition of autophagic clearance in neurons originated disruption of axonal transport of vesicles containing substrates degraded in lysosomes and axonal swelling, leading to axonal dystrophy (Lee, 2012).

However, autophagy can play also a deleterious role, promoting neuronal damage and loss. For example, inhibition of autophagy during excitotoxicity stress can be protective and potentially promote recovery. This dichotomy suggests that the impact of autophagy can be influenced not only by neuronal subtype, but by the neuron's age. Moreover, the yet unknown contribution of glial autophagy may also be a key factor in how autophagy affects neural health (Yamamoto and Yue, 2014). Evidences of an impairment in autophagy network have been reported in different neurological disorders such as in AD, where is noted to occur an abnormal amount of autophagosomes in the affected neurons (Wong and Cuervo, 2010).

Basically, a failure of any molecule involved in autophagic process may lead to neurodegeneration. Additionally, the abnormal accumulation of autophagosomes in neurons imprint the first sign of deficits in autophagy pathway. Besides that, autophagosomes and their

presence in cells depends on both rate of formation and rate of clearance by lysosomal network degradation.

An example of this, is the observation in cultured primary neurons that blocking autophagosome clearance by inhibiting cathepsins causes rapid AVs accumulation without altering autophagy induction (Bolanda *et al.*, 2008). The impairment of later digestive steps in the autophagy pathway, as well as, a slow rate of autophagosome formation combined with insufficient lysosomal fusion and digestion, reflects a deficiency in autophagy network in neurodegenerative disorders (Viscomi *et al.*, 2017).

Other evidences about the critical role of autophagy in the basal turnover of diffuse cytosolic proteins in neurons, suggest that large inclusion bodies themselves might not be pathogenic, but mutant proteins present diffusely in the cytosol could be the primary source of toxicity. For this reason, it is possible that autophagosomes can selectively recognize abnormal soluble proteins or micro-aggregates on the membrane surface (Mizushima and Klionsky, 2007). However, the exact mechanisms that leading to this autophagic dysfunction and how a dysfunctional autophagy may lead to neuronal death is still not well understood.

Summarizing, basal autophagy is extremely important for good manner function of neurons, playing a cytoprotective role, and preventing build up proteins aggregates, as well as damaged mitochondria (Murrow and Debnath, 2013).

1.9 Autophagy in Neurodegenerative Disorders

Some abnormal accumulation of Avs, including autophagosomes or autolysosomes has been described in affected neurons of brain, reflecting an autophagy network dysfunction in several neurodegenerative disorders such as AD, PD, Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), among others (Frake *et al.*, 2015).

Even some studies from *post-mortem* brains with these neurological disorders, have reported how autophagy may contribute to neurodegeneration. For this reason, in a nearly future autophagy system may be a potential therapeutic avenue for neurodegenerative maladies (Ciechanover and Kwon, 2015).

1.9.1 Autophagy in AD

Nowadays, some increasing evidences support that autophagy pathway dysfunction is implicated in AD pathogenesis. The first evidence that autophagic activity was associated with AD pathogenesis theory, came from the observation of AVs in biopsy specimens of neocortex

from brains of AD patients, where AVs proliferation is unexpected robust in dystrophic neurites, including grossly abnormal accumulations of immature forms of AVs (autophagosomes) (Nah, Yuan and Jung, 2015). Basically, AVs are uncommon in neurons of a healthy brain. The results from Nixon and colleagues proved that lysosomal system mobilization, what occurs in AD, is connected in somehow, with the induction of autophagy in affected neurons within neuritic processes, as synapses regions. This suggests that while autophagy may be induced in AD condition, AVs transport and maturation can be also impaired in damaged neurons (Nixon *et al.*, 2005).

Additionally, data already reported that the amount of AVs are much more present in neurodegenerative diseases brain than in health controls, indicating strongly evidences the failure maturation of autophagosome to autolysosome step (Guo *et al.*, 2017). Even more, studies have identified abundant accumulation of bodies as early or late AVs, or autophagosomes and autophagolysosomes, which are intermediate structures of autophagy network (Funderburk, Marcellino and Yue, 2010).

Also, depletion of key autophagy genes such as *Atg5* and *Atg7*, that are essential for autophagosome evolution, may lead to neurodegeneration in mouse CNS (Komatsu *et al.*, 2006).

On the other hand, Hara *et al.* (Hara *et al.*, 2006) described in theirs studies that inclusions bodies, proteins aggregates, appeared in later phases of autophagy deficiency, implying that the primary role of autophagy process under normal conditions is the turnover of diffuse cytosolic proteins and not the direct elimination of inclusion bodies (Hara *et al.*, 2006).

All these data, support abnormalities in autophagy induction, as well as, in autophagosome formation, which in somehow contribute to AD pathogenesis. Besides, it is important to refer that a selective transport deficit, compromising in general autophagy-related compartments, have been described in mouse and cell models of AD pathology (Nixon and Yang, 2011).

For example, axonal transport is focally interrupted by nerve ligation or toxic agents, which often accompanies neurodegeneration. In addition, this axonal disrupted transport has been described in live-imaging studies of cortical neurons, when lysosomal proteolysis is inhibited, leading to an axonal dystrophy, characterized by a selective AVs accumulation, as believed to occur in AD condition (Sooyeon, Sato and Nixon, 2011).

Although, a recent study has stablished that in contrast to degradative role of autophagy ending in lysosomal degradation already described, autophagy pathway may influence the secretion of Aβ. Particularly in this study, Nilsson and colleagues hypothesizes (Nilsson *et al.*, 2013) that, autophagy may directly affect intracellular Aβ accumulation and extracellular Aβ plaque generation. Autophagosomes are sites of Aβ formation that can fuse with endosomes at the late stage of autophagy pathway, and Aβ-containing endosomes may potentially be the source of

Aβ delivered to extracellular milieu. This releasement of Aβ to extracellular space may be influenced by autophagy network as a general excretory mechanism for cellular waste, independent of the regulated secretory pathways as well-known (Nilsson *et al.*, 2013).

At the other sight, Beclin-1, a crucial key in autophagosome generation, has been shown to be transcriptionally suppressed in AD brains. Under pathogenic conditions, Caspase-3 as an essential component in apoptosis pathway, may cleave Beclin-1 protein, leading to autophagy disruption. The cleaved form of Beclin-1 is therefore regarded as a common in vitro marker for apoptosis in AD pathogenesis (Kang *et al.*, 2011). Therefore, pharmacological targeting the induction of autophagy can have potential therapeutic benefits, reducing neurodegeneration in AD.

At this regard, autophagic activity induction before the development of AD-like pathology can reduce the levels of soluble Aβ and Tau proteins, whereas autophagic activity induction after the formation of mature plaques and tangles installed, may not have effect on AD-like pathology or cognitive deficits (Viscomi et al., 2017). Thus, an accumulation of AVs is likely arising from impaired clearance rather than the induction of autophagy pathway itself, suggesting the modulation of late steps of autophagy as a possible therapeutic strategy for AD malady.

1.10 Beclin-1

Beclin-1, a coiled-coil protein was first identified as a direct interactor of the Bcl-2 protein in a yeast two-hybrid screen (Liang *et al.*, 1998). Beclin-1, also named Atg6 is a highly conserved in eukaryotes and can restore starvation-induced autophagy in *ATG6*- disrupted yeast strains, regulating positively autophagic activity (Hayat, 2013).

Atg6 has been well-characterized to play a crucial protective role in autophagy network, for reducing protein aggregates, also as a fundamental regulator of autophagy process in the nucleation of the autophagosomes, as well as for the maturation step of the pathway (Cao and Klionsky, 2007).

Recently, another member of Atg6/Beclin-1 family, Beclin-2 was discovered and demonstrated to bear an important role in two different lysosomal degradation pathways, essential for cellular homeostasis, autophagy and endolysosomal trafficking of G protein-coupled receptors (Kang *et al.*, 2011). Nowadays, studies have described that Beclin-1 is a haploinsufficient tumor-suppressor gene that is either monoallelically deleted or display reduced expression in various cancers, such as human breast, ovarian and prostate cancer (Zhu and He, 2015).

Moreover, Beclin-1 is involved in numerous biological processes, as well as in human conditions, including adaptation to stress, development, endocytosis, tumorigenesis, pathogen infection and neurodegeneration (Hayat, 2016).

Struturally, Beclin-1 is a 450-amino acid protein that contains three distinct functional domains: a Bcl-2 homology-3 (BH-3) motif at the N terminus (residues 114-123), a coiled-coil domain (CCD) in the center (residues 144-244) and an evolutionarily conserved domain (ECD), at C-terminal (residues 244-337), which allows multiple protein interactions (Fu, Cheng and Liu, 2013).

Bcl-2 directly bind the BH-3 domain of Beclin-1 and effectively counteract Beclin-1 dependent autophagy. The anti-apoptotic role of Bcl-2 can suppress autophagy pathway. Even phosphorylation and ubiquitination events of Beclin-1 and Bcl-2 may either stabilize or dissociate the Beclin-1-Bcl-2 complex, leading to inhibition or initiation of autophagy pathway, respectively (Wirawan *et al.*, 2012).

The CCD domain is involved in heterodimerization with UVRAG, but can function independently to Beclin-1 (Sinha and Levine, 2008). Beclin-1 can interact with other proteins, such as Bif-1. Additionally, through its ECD and CCD, Beclin-1binds PI3K-III complex, which regulates autophagosome formation.

Likewise, Beclin-1 contains a short leucine-rich amino acid sequence, a nuclear export signal (NES), located within the CCD domain (Zhu and He, 2015).

The NES is responsible for the predominant cytoplasmic localization of Beclin-1, and within cytoplasmic structures Beclin-1 exists primarily to ER, mitochondria, TGN and perinuclear membrane (Kang *et al.*, 2011; Fu, Cheng and Liu, 2013; Zhu and He, 2015). (**Figure 1.6**).

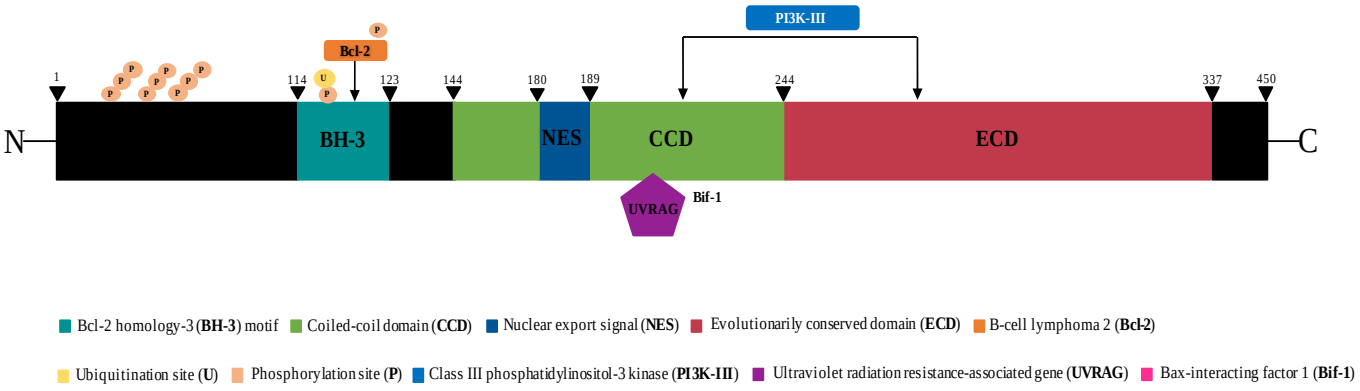


Figure 1:6 - **The structure of Beclin-1.** Schematic representations of domains of human Beclin-1. Beclin-1 has a Bcl-2 homology-3 (BH-3) motif at the N terminus (residues 114-123), a central coiled-coil domain (CCD) in the middle (residues 144-244) and an evolutionarily conserved domain (ECD), at C-terminal (residues 244-337). Adapted from Fu, Cheng and Liu, 2013.

Beclin-1 is expressed in many human and mouse tissues, including in brain, heart, liver, spleen, ovary, among others (Liang *et al.*, 1998). Beclin-1 protein is a part of a lipid kinase complex, imperative for autophagy pathway, involved in different scenarios, including tumor suppression, longevity, innate immunity, neuroprotection and cell death (Funderburk, Wang and Yue, 2010). Beyond autophagy, Beclin-1 functions are evident in other membrane-trafficking pathways, such as endocytic and phagosomal maturation.

Therefore, Beclin-1 is a central hub that regulates environmental stimuli with downstream physiological outputs, when different signaling path, as oncogenic/tumor- suppressive, immune, and stress-responsive signals converge on Beclin-1 to regulate autophagy network (Levine *et al.*, 2015).

1.11 Beclin-1 and Neurodegenerative Disorders

In mammalian adult brain, Beclin-1 is expressed in neurons and glia cell population, specially astrocytes from different regions, cerebral cortex, hippocampus and cerebellum (Erlich, Shohami and Pinkas-Kramarski, 2006). During of aging process, Beclin-1 levels decline, where at same time the levels of aggregate-prone proteins accumulate, and some collapsing effects at give point on the system of autophagic homeostasis may happen, leading to irreversible dysfunction and/or death of neurons.

Besides, different studies have been observed a decreased of Beclin-1 levels aged brains in various neurodegenerative disorders, such as AD (Pickford *et al*., 2008), HD (Shibata *et al*., 2006) and Machado-Joseph (MJ) (Nascimento-Ferreira *et al*., 2011).

1.11.1 The connection between Beclin-1 and AD

Beclin-1 and its interacting proteins are important in aging and age-related neurodegenerative pathologies. It is been described that Beclin-1 levels decline with age in the human brain, confirming that decreased Beclin-1 levels lead to an impairment of autophagy activity and at the same time, autophagy decline role is probably an important factor contributing to aging (Morris *et al.*, 2015).

The first article, reporting the protective effects of an overexpressed levels of Beclin-1 in neurodegenerative diseases was published in 2008. Pickford *et al.* (Pickford *et al.*, 2008) have been reported that endogenous protein levels of Beclin-1 were seen reduced in *post-mortem* brain samples of AD patients, specifically in gray matter. This overexpression of Beclin-1 levels was described to alleviate AD condition (Pickford *et al.*, 2008). Additionally, in subjects with mild cognitive impairment (MCI) or severe AD, they found that both messenger ribonucleic acid (mRNA) and protein levels of Beclin-1 were reduced only in affected brain parts, such as the entorhinal cortex (Lee and Gao, 2008). Also, in the same study, authors demonstrated that in transgenic mice deficient in Beclin-1 protein, a disruption of autophagic pathway, increased neurodegeneration and Aβ accumulation (Rohn *et al.*, 2011).

Besides, Beclin-1 levels were seen reduced in microglia from AD patients, reflecting a

reduced retromer trafficking, and suggesting deficits in receptor mediated Aβ phagocytosis (Wang *et al.*, 2018). Although, the mechanisms behind of this reduction are still not clear.

Furthermore, an activation of autophagy or an overexpression of Beclin-1 levels may prevent neuronal cell death and may benefit clearance of toxic protein aggregates. It is also reported that both Beclin-1 and Vsp34 protein levels are reduced in AD tissue, reinforcing one more, a compromising initiation of autophagy pathway (Jaeger and Wyss-Coray, 2010).

One hypothesis, that could explain the decline levels of Beclin-1 seen in AD malady is an impairment of autophagosome-lysosomal function, which may active a negative feedback loop, downregulating autophagy induction in response to abundant autophagosome numbers. This loop may be preventing an uncontrolled run-off activation of autophagy with potentially serious consequences for the cell (Jaeger *et al.*, 2010).

And may be an upregulation of Beclin-1 should compensate for this reduction instead of leading to an unphysiological excessive activation of autophagy but, since Beclin-1 function is tightly regulated by other proteins, such as Bcl-2, a permanent upregulation or overexpression should not lead to an excessive autophagy situation (Jaeger and Wyss-Coray, 2010).

1.12 Sirtuins: a key in Neurodegenerative diseases

1.12.1 The History of Sirtuins: A Family of Proteins

1.12.1.1 The Basics

The mammalian family of deacetylases of histones (HDACs) are involved in several biological processes and divided into four classes: class I- HDAC1, -2, -3, and - 8; class II- HDAC4, -5, -6, -7, -9 and -10; class III- Sirtuins; and class IV- HDAC11, based on sequence similarity and co-factor dependency (Yang and Seto, 2007).

Class III HDACs, also known as Sirtuins (SIRTs), constitute a special family of proteins because of their requirement for nicotinamide adenine dinucleotide (NAD^+) as a cofactor in their deacetylation reaction (Rothgiesser *et al.*, 2010) and for being involved in regulating a variety of pathways, including aging and longevity in lower eukaryotes such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Michishita *et al.*, 2005). During the deacetylation process, nicotinamide (NAM) is cleaved from NAD^+ and the acetyl group of the substrate is afterwards transferred to adenosine diphosphate (ADP) ribose, generating the novel metabolite 2'-O-acetyl-ADP ribose (OAADPr) (Denu, 2003).

The SIRTs family has high homology to the yeast silent information regulator 2 (*Sir2*) gene

and consists of seven isoforms in mammals (SIRT1-SIRT7), which vary in their subcellular localization, substrate specificity, enzymatic activity, targets and functions (Blander and Guarente, 2004) (**Table 1.1**).

1.12.1.2 SIRTs subcellular localization

SIRTs are ubiquitously expressed, containing a conserved catalytic core domain comprised of approximately 275 amino acids. SIRTs regulate a diversity of cellular functions, including genome maintenance, longevity and metabolism, exhibiting a different pattern in their subcellular localization from nucleus to nucleolus, and from cytoplasm to mitochondria (Rothgiesser *et al.*, 2010; Cantó and Auwerx, 2012).

SIRT1, the best characterized family member, is mainly located in the nucleus but also present in the cytosol. Under certain conditions, its nuclear export signal can shuttle from the nucleus to the cytosol, but the physiological relevance of this shuttling is still unclear (Houtkooper, Pirinen and Auwerx, 2012).

SIRT2 is predominantly cytoplasmic, but has also been found in the nucleus, modulating cell cycle control- G2 phase to M phase transition of the cell cycle (Chang and Guarente, 2014). SIRT3 (Ansari *et al.*, 2017), SIRT4 (Anderson *et al.*, 2017), and SIRT5 (Guedouari *et al.*, 2017) are considered mitochondrial proteins. Both SIRT6 (Tasselli, Zheng and Chua, 2017) and SIRT7 (Fukuda *et al*., 2018) are present in the nucleus. SIRT6 has essential roles in heterochromatin silencing and SIRT7 has been identified in the nucleolus, important to regulate RNA polymerase I transcription (Michan and Sinclair, 2007; Li *et al.*, 2016).

1.12.1.3 SIRTs enzymatic activity

Enzymatic activity by SIRTs requires NAD^+ as a co-substrate, distinguish them from the other classes of protein deacetylases. In addition, the concentration of each deacetylases is determined by the nutritional stated of the cell (Feldman, Dittenhafer-Reed and Denu, 2012). During this enzymatic reaction process catalyzed by SIRTs, NAD^+ is converted to NAM, also named vitamin B3, the first product released, followed by deacetylated lysine and OAADPr (Cantó and Auwerx, 2012). Indeed, NAM at higher concentrations can non-competitively bind and thereby feedback-inhibit SIRTs activity (Houtkooper, Pirinen and Auwerx, 2012).

Among the seven SIRTs, only SIRT1-3 display a robust deacetylase activity *in vitro*, whereas recent reports implicate SIRT5, a class III mitochondrial SIRT, as a protein desuccinylase and demalonylase (Teng *et al.*, 2015). SIRT2 possesses efficient demyristoylase activity. Moreover,

SIRT6-7 exhibit little or undetectable deacetylation activity *in vitro*. Although, SIRT4 and SIRT6 were described as ADP-ribosyltransferases. Finally, SIRT6 exhibits three distinct enzymatic activities, deacetylase, ADP-ribosyltransferase and defatty-acylase (Gomes, Outeiro and Cavadas, 2015).

1.12.1.4 SIRTs function

Different roles of SIRTs have been described in a great number of different conditions. SIRT1 is involved in a several physiological mechanisms and regulates numerous functions, which are going to be described in more detail later.

Additionally, SIRT2 regulates cell cycle and is associated with various mitotic structures, such as the centrosome, mitotic spindle and midbody to ensure normal cell division. SIRT2 overexpression occurs in the M phase of mitotic cycle (de Oliveira *et al.*, 2012). SIRT2 is responsible for transcription under regulatory pathways that control oligodendrocytes differentiation and myelin expression (Zhu *et al.*, 2012). Also, SIRT2 has been shown to deacetylate and activate FOXO3 a in response to oxidative stress (Gomes, Outeiro and Cavadas, 2015).

SIRT3 influences energy metabolism processes, including fatty acid β-oxidation, respiratory chain, ketogenesis and tricarboxylic acid cycle by targeting the responsible enzymes. It also controls the flow of mitochondrial oxidative pathways and, ultimately, the rate of ROS (Ansari *et al.*, 2017).

SIRT4 has been shown to repress glutamate dehydrogenase activity and suppresses insulin secretion in mouse models. Moreover, SIRT5 can promote in liver the detoxification of ammonia by deacylating and activating carbamoyl phosphatase synthetase 1, the first and rate-limiting enzyme in the urea cycle (van de Ven, Santos and Haigis, 2017). SIRT6 plays a role in genome integrity, metabolic regulation and controls flux of glycolysis (Pan *et al.*, 2011).

Finally, SIRT7 is associated with active ribosomal ribonucleic acid (rRNA) genes, interacting with ribonucleic acid (RNA) polymerase I. Its overexpression increases rRNA transcription, whereas its down-regulation decreases rRNA transcription (Yamamoto, Schoonjans and Auwerx, 2007).

Table 1-1 - SIRTs Subcellular Localization, Enzymatic Activity and Functions.

SIRTs	Class	Subcellular Localization	Enzymatic Activity	Functions
SIRT1	I	Nucleus, Cytosol	Deacetylation	Chromatin structure Cell cycle Glucose metabolism Insulin secretion Neuroprotection Fatty acid oxidation
SIRT2	I	Cytosol, Nucleus	Deacetylation, Demyristoylation	Cell cycle control Oligodendroglia proliferation Oxidative stress
SIRT3	I	Mitochondria	Deacetylation	Metabolic regulation Oxidative stress Acetate metabolism Insulin secretion β-oxidation
SIRT4	II	Mitochondria	ADP-ribosylation	Insulin secretion
SIRT5	III	Mitochondria	Deacetylation Demalonylation Desuccinylation	Urea cycle
SIRT6	IV	Nucleus	Deacetylation ADP-ribosylation Defaty-acylase	Glucose homeostasis Telomeric function DNA repair
SIRT7	IV	Nucleolus	Deacetylation	RNA pol I transcription

Summarizing, SIRTs are engaged in extremely complex cellular and physiological processes: proliferation, differentiation, stress response, genome stability, cell survival, metabolism and energy homeostasis and aging process (**Table 1.1**). Due to the multifaceted and ubiquitous nature of these class of proteins, in particularly of SIRT1, studies have described its role in chronic inflammatory diseases, diabetes mellitus type II, cardiovascular, neurodegenerative and kidney diseases (Michan and Sinclair, 2007). Therefore, it is believed that SIRTs, in a near future, will play a part in new therapeutics applicable to multiple pathophysiological states.

1.12.2 SIRT1: Biological Function and Targets

SIRT1, the mammalian ortholog most highly related to Sir2 is by far the best characterized family member of SIRTs. SIRT1 is involved in a wide range of cellular biological pathways, such as DNA damage repair, inflammation process, mitochondrial homeostasis, insulin secretion, stress response, apoptosis, genome stability, among others (Polito, Biella and Albani, 2017). SIRT1 is highly expressed in a wide range of tissues and organs including heart, liver, muscle, pancreas, brain and adipose tissue mice (Nogueiras *et al.*, 2012). (**Figure 1.7**). Moreover, SIRT1 has been shown to reduce age associated physiological changes in mice (Guarente, 2007).

Several lines of evidence, indicate numerous neuroprotective functions that SIRT1 plays, such as anti-oxidant and anti-inflammatory response, anti-apoptosis, regulation of insulin, gene transcription, autophagy and mitochondrial biogenesis (Braidy *et al.*, 2012). In fact, SIRT1 protein has been reported to play also, a role in a variety of pathophysiological processes of metabolic diseases, neurodegenerative disorders, cancer and aging (Zschoernig and Mahlknecht, 2008).

Therefore, because of SIRT1's ability to deacetylate both histones and non-histone substrates, to promote directly an activation or inhibition of transcriptional factors and modify their interaction profiles, SIRT1 became an important target to innovative molecular therapy strategies.

First, hepatic metabolic derangements are key components in the development of fatty liver, insulin resistance, and atherosclerosis. SIRT1 plays an important regulator of energy homeostasis in response to nutrient availability. SIRT1 deacetylase is expressed in the pancreatic β-cells, as well as glucagon-producing α-cells. In β-cells, SIRT1 enhances insulin secretion by suppressing the expression of uncoupling protein 2 (UCP2). Additionally, SIRT1 protein appears to have a protective effect against glucose-induced cytotoxic in pancreatic β-cells, causing β-cell degeneration, seen in diabetic patients with chronically high plasma glucose levels. These cytotoxic effects of increased glucose concentrations are associated to elevated mitochondrial oxidation rates, leading to an increase of ROS production (Anastasiou and Krek, 2006).

Another site of SIRT1 deacetylase protein function relates to genes that control mammalian energy and nutrient homeostasis. In liver, SIRT1 is known to control gluconeogenic activity by modulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Canto and Auwerx, 2009). Finkel's group identified the functional interaction and deacetylation of PGC-1α and SIRT1 (Nemoto, Fergusson and Finkel, 2005).

Other studies have been focus on the important role of hepatic SIRT1 deacetylase as a regulator of lipid homeostasis in response to nutrient availability by positively regulating peroxisome proliferators-activated receptor a alpha (PPARα), a nuclear receptor, responsible for mediating the adaptive response to fasting and starvation, whereas pharmacological activation of SIRT1 can prevent of obesity associated metabolic diseases (Purushotham *et al*., 2009). However, scientists have been study the effect of SIRT1 also in neurodegeneration, once its protective role as been shown in several models of neurodegenerative disorders, such as AD pathology.

Additionally, another target of SIRT1 is against chronic inflammatory effects in diverse tissues and cells models by controlling the acetylation of nuclear factor kappa Beta (NF-κB), a transcription signaling pathway involved in the innate immune response, by downregulating its transcriptional activity (Rahman & Islam 2011). It has been reported that SIRT1 protein is elevate in cancer, such as prostate and primary colon cancer, acute myeloid leukemia and in skin cancers, including squamous cell carcinoma, basal cell carcinoma and actinic keratosis (Zilfou and Lowe, 2009; Rahman and Islam, 2011). Another family of transcription factors that are deacetylated by SIRT1, the Forkhead-O-box (FOXO), play an important key as regulator of lipid metabolism, stress resistance and apoptosis. For instead, FOXO3 is an important factor on upregulation of genes, that are involved in the apoptosis-induced stress, whereas FOXO1 plays a different role on regulating the expression of metabolic genes (Giannakou and Partridge, 2004).

Therefore, SIRT1 activity involving the deacetylation of FOXO3 and FOXO1 occurs in situations of oxidative and energy stress, and fasting. Besides this SIRT1-mediated FOXO deacetylation can also activate autophagy pathway. Recently, some studies have shown that adipose tissue is an important organ in lifespan, where SIRT1 represses peroxisome proliferator-activated receptors gamma (PPARγ) inhibiting lipid accumulation in adipocytes.

For all these functions that SIRT1 deacetylase show in so many physiological and pathological conditions, scientists have been focus on the effects of SIRT1 in neurodegeneration because of its protective role in several models of neurodegenerative disorders, such as AD pathology.

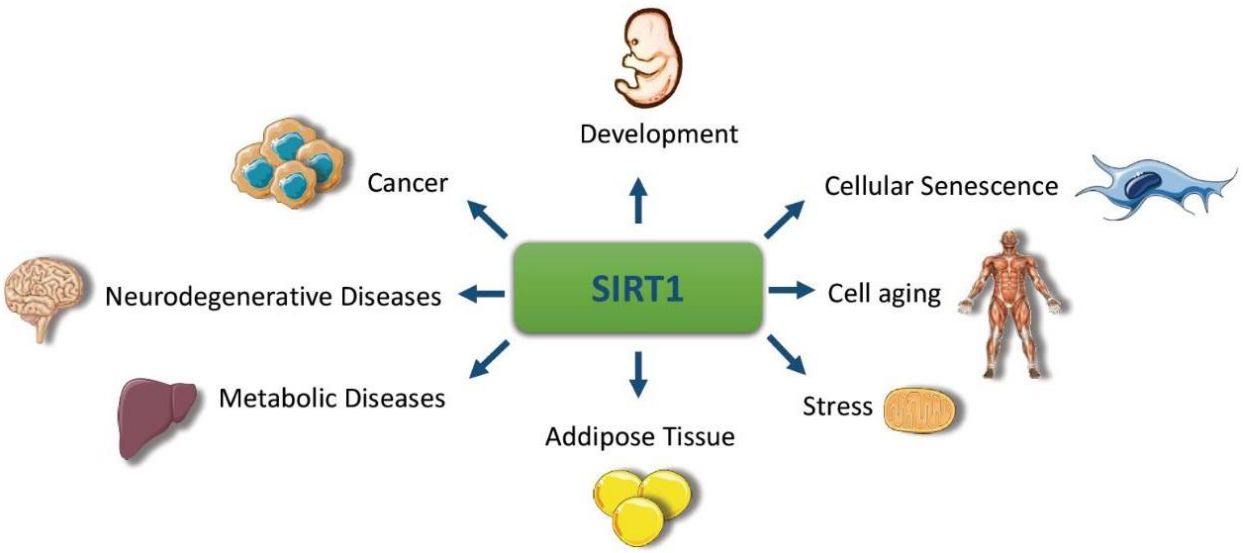


Figure 1:7 - **Biological functions of SIRT1 protein in different physiological and pathological conditions.** Adapted from (Nogueiras et al. 2012)

1.12.3 The Role of SIRT1 in CNS

1.12.3.1 Brain- Can SIRT1 protect the brain?

Several SIRTs are expressed in the mammalian brain, playing different roles on diverse brain functions and respond in dissimilar ways to stress and toxicity (Satoh and Imai, 2014). The best-characterized SIRTs family member, SIRT1 is ubiquitously present in areas of the brain, especially susceptible to age-related neurodegenerative states, including the prefrontal cortex, hippocampus and basal ganglia (Kelly, 2010). Furthermore, SIRT1 is also expressed in metabolic centers of the brain, such as the hypothalamic arcuate, ventromedial, dorsomedial, and paraventricular nuclei (Paraíso, Mendes and Santos, 2013). Also, it is involved in mechanisms of cellular protection against oxidative stress in different conditions from aging to neurodegeneration processes. For this reason, data have described that SIRT1 participates in the maintenance of the brain integrity, regulating activities like oxidative stress, neuronal differentiation and neurogenesis (Ramadori and Coppari, 2011; Herskovits and Guarente, 2014).

1.12.3.2 SIRT1: Role in Neurogenesis

Neurogenesis is the process of producing new neurons from neural stem cells (NSCs). In most brain regions, this process is restricted to a limited period during development, and ends shortly after birth. Although, neurogenesis is observed into the postnatal period and throughout adulthood (Götz and Huttner, 2005), a multistage process regulated by a delicate balance between undifferentiated proliferative neural precursors cells and differentiated neuronal progeny. There are two evolutionarily conserved neurogenic niches in the adult mammalian brain, the subventricular zone (SVZ) (Ming and Song, 2011), which lines the lateral wall of the lateral ventricle and the subgranular zone (SGZ), that lies within the dentate gyrus of the hippocampus. Numerous transcription factors regulators, associated in NSCs selfrenewal and fate regulation, have been described for both neurogenic niches (Saharan, Jhaveri and Bartlett, 2013).

The link between SIRT1 and neurogenesis came from the finding that it can mediate cellular responses to changes in redox state in several different cell types (Libert, Cohen and Guarente, 2008). As an example of these diversity of cell types, Prozorovski *et al.* (Prozorovski *et al.*, 2008) showed that SIRT1 is directed involved in neural progenitors, under oxidative conditions both *in vitro* and *in vivo*, by promoting an astroglial lineage (Prozorovski *et al.*, 2008). Also, SIRT1 has been shown to regulate pluripotency and differentiation genes in human embryonic stem cells.

Recently, data have reported that SIRT1 ubiquitously expression is linked with high levels of expression in the developing mouse CNS, adult mouse and human brain (Herskovits and Guarente, 2014). For this reason, some studies have stated that the inhibition or silencing of SIRT1 can promote or alter neuronal differentiation. In the adult brain regions, expression of SIRT1 is more noticeable in neurons of the hippocampus and hypothalamus, with a more nuclear localization. The hypothalamus region is an important area of the brain, responsible to control human physiology, including regulation of body temperature, hunger/satiety, circadian rhythms and on the control of the pituitary gland hormones (Herskovits and Guarente, 2014) .

Normally, brain SIRT1 levels, especially in the hypothalamus change in response to diet, and appear to mediate numerous aspects of hypothalamic control. Depending on the type of diet SIRT1 levels are downregulated by a high fat diet (Ramadori and Coppari, 2011). For example, absence of SIRT1 blocks the somatotropic axis, which plays an important role in the co-ordination of protein and energy metabolism. On the other side, during CR/dietary restriction (CR/DR) and physical exercise, SIRT1 levels may increase in neurons, and at the same time increase neural activation in the dorsomedial hypothalamic nucleus (DMH) and lateral (HL) (Satoh *et al.*, 2010). Moreover, in the anoxerigenic pro-opiomelanocortin (POMC) neurons, responsible for regulating the hunger and food intake, localized in the arcuate nucleus of the hypothalamus, SIRT1 is

essential for maintaining the normal energy level according to diet. Recently studies have shown the crucial role of SIRT1 in metabolic homeostasis through its activity in these neurons (Ng, Wijaya and Tang, 2015). It is also well known that SIRT1 regulation of energy (glucose and lipid) metabolism has been so far correlated with induction of hepatic gluconeogenesis, pancreatic insulin secretion, increased muscle insulin sensitivity and the "browning" of white adipose tissues. In support of this, studies in SIRT1-knockout mice showed POMC neurons are more susceptible to obesity. Besides, energy expenditure is reduced, as well as signaling processes induced by the satiety hormone leptin (Michan and Sinclair, 2007). In the CNS, SIRT1 levels decline with age and its overexpression in brain counters the aging-associated circadian function defects. Central control of the mammalian circadian rhythm exists in the hypothalamic suprachiasmatic nucleus (SCN), which is regulated by a feedback of transcription factor interactions. Furthermore, the overexpression of SIRT1 in transgenic mice could delay aging process and prolong their survival (Ramadori *et al.*, 2010; Ng, Wijaya and Tang, 2015).

A few articles have described that an optimal function of SIRT1 would be crucial for the maintenance of synaptic plasticity, learning and memory processes, necessary for a better development of cognition. Only recently was clarified the role of SIRT1 in the physiologically unperturbed versions of learning and memory processes. The SIRT1 hippocampal expression in mice also enhances learning and memory (Braidy *et al.*, 2012; Ng, Wijaya and Tang, 2015).

It was shown that SIRT1-deficient mice had a grossly normal brain anatomy, but in contrast it was noticed a deficient dendritic development phenotype. On these mice, some defects in synaptic plasticity, such as basal synaptic transmission were indistinguishable from wild type. In a few behavioral tests, short-term memory, long term associative memory, as well as spatial learning were all impaired in SIRT1 knock-outs compared to control (Michán *et al.*, 2010). These evidences show how important SIRT1 role plays on our nervous system.

1.13 SIRTs and Neurodegenerative disorders

Neurodegenerative disorders are characterized by a progressive nervous system dysfunction, associated with atrophy of the affected central or peripheral nervous system structures. For this reason, scientists have been focused to understand the mechanisms responsible for human neurodegeneration and the exact sequence of events that trigger the neurodegenerative processes leading to different disorders, including AD, PD, HD and ALS, among others (Woulfe, 2008).

Many research studies conducted and published have been already demonstrated the important role of SIRTs family, that are involved in numerous processes, including neuroinflammation (Vachharajani *et al.*, 2016), energy balance and melanocortin system (Toorie

and Nillni, 2014) and UPS (Sampaio-Marques and Ludovico, 2015); and the regulation of the CNS (Satoh, Stein and Imai, 2011).

Therefore, these many evidences show the greatest potential of SIRTs as therapeutic candidates for intervention in various diseases, especially in neurodegeneration and neurodegenerative disorders.

1.13.1 The Link between SIRT1 and AD

SIRT1 deacetylase is often involved with a range of neuropathological conditions. In human AD brains, SIRT1 levels appear to be decreased in parietal cortex, which may correspond to some brain regions affected during AD progression from early to late stages (Wong and Tang, 2016). In addition, some studies have mentioned that a lower cortical SIRT1 was correlated with the duration of symptoms, reduced global cognition scores, and accumulation of Aβ and Tau proteins in the cerebral cortex.

For this reason, multiple evidences suggest that an upregulating SIRT1 in the brain in mouse models of AD, and these increasing SIRT1 levels may promote neuronal survival, playing a neuroprotector role in both AD condition and dementia (Ma *et al.*, 2014).

Data have described that SIRT1 activation by resveratrol (RSV), a SIRT1 activator, in transgenic mice models, could cross the BBB, protecting against toxicity and promoting neuroprotection in general (Della-Morte *et al.*, 2009). RSV improves neuronal cell survival in response to oxidative stress events and protects also neuronal cells from ischemic insults. These protect responses are involved with a RSV-induced increase in hippocampal SIRT1 activity (Raval, Dave and Pérez-Pinzón, 2006).

At the other sight, other studies have noticed that in AD mice model transgenic, an overexpression of SIRT1 decreases amyloid plaques accumulation, and at same time decline learning and memory capabilities, whereas brain SIRT1 specific knock-out had attenuated lifespan (Qin *et al.*, 2006).

These studies have been focused on Aβ formation, where an overexpression of SIRT1 in primary neurons,decreased Aβ secretion and diminished Rho-activated kinase 1 (ROCK1) expression.

Moreover, another pathway where SIRT1 plays a neuroprotection role is through the NF-κB route preventing Aβ neurotoxicity due to decreased microglial expression of NF-κB (Chen *et al.*, 2005).

It is believed that higher concentrations of Aβ lead to modifications of Tau protein. Evidences have been suggested that conversion from normal Tau protein into insoluble PHF shares

common mechanism pathways with the decrease in SIRT1 in AD pathology, linking SIRT1 deacetylase concentration to the severity of the disease (Julien *et al.*, 2009).

SIRT1 has also been shown to ameliorate Tau pathology in the p25 mouse model, affecting cognitive impairment and mortality. Cyclin-dependent kinase 5 (Cdk5) and its regulatory subunit p35 are integral players in the proper development of the mammalian CNS. The p25 results from the proteolytic cleavage of p35 subunit leading to an aberrant Cdk5 activation. When the plasticity is compromised, the formation of p25 occurs as a compensatory mechanism at an early stage of AD ailment (Michán *et al.*, 2010).

Finally, a recent approach important on AD malady is the autophagy-lysosomal pathway, directing the modified-release proteins, as well as dysfunctional organelles, involving several proteins, which in somehow depend on the regulation of SIRT1 activity. More studies are needed to understand the influence of SIRT1 in both Aβ and Tau proteins, and in this specifically network, autophagy-lysosomal pathway.

2 AIMS

According to World Health Organization (WHO), neurologic disorders are a serious public health problem in our society, affecting millions of people worldwide. Population is ageing rapidly and as an example, the total number of people with dementia is expected to increase to 82 million in 2030 and 152 million in 2050 (WHO, 2017).

One of the most prevalent malady among elderly adults, included in these complex group of diseases, responsible for memory impairment and dementia is AD, which still have not an effective medicine to treat the symptoms, slow, prevent or reverse the progressive deterioration of neuronal structures, occurring specifically in CNS. A vital homeostatic mechanism in healthy cells, an important cytoprotective response in a scenario of aging- and disease-related metabolic challenges is autophagic pathway, that has been implicated in the pathogenesis of major neurodegenerative disorders.

Impairment at different stages of autophagy leads to the buildup of pathogenic proteins and damaged organelles. At the beginning stage of autophagy, Beclin-1, one of the starters and essential proteins is decreased in AD patients, leading for an eventual impairment on autophagic flux.

Therefore, bearing in mind (i) The impact of brain disorders as one of the most serious problems facing modern society, (ii) the unpaired homeostasis process responsible for degradation of damaged proteins and organelles in neurodegenerative diseases, (iii) the unclear cause of decreased Beclin-1 levels in brain of AD patients and (iv) considering the use of cybrids as a cellular model for this study, we aimed:

1. To evaluate Beclin-1 acetylation levels in MCI and AD;
2. To understand the impact of (de)acetylation of Beclin-1 on autophagic flux;
3. To evaluate how (de)acetylation will affect lysosomal function and localization.

3 MATERIALS AND METHODS

3.1 Reagents

NAM, Leupeptin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ammonium Chloride (NH_4Cl) was obtained from Merck KGaA (Darmstadt, Germany) and C646 was purchased from Calbiochem, Merck KGaA (Darmstadt, Germany).

3.2 Antibodies

For Immunoblotting analysis, the following secondary antibodies were used and the working dilutions are given in brackets: Goat anti-mouse IgG alkaline phosphatase conjugated antibody (1:10,000) from Thermo Fisher Scientific Inc. (Rockford, IL, USA) and Goat anti- rabbit IgG alkaline phosphatase conjugated antibody (1:20,000) was from GE Healthcare (Bio- Sciences, Uppsala, Sweden).

The following primary antibodies were used and the working dilutions are given in brackets: mouse monoclonal anti-Cathepsin D (C-5) antibody (1:1000) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); rabbit polyclonal anti-SIRT1 (D739) antibody (1:1000), rabbit polyclonal anti-Phospho-SIRT1 (Ser47) antibody (1:2000), rabbit polyclonal anti-Beclin-1 antibody (1:1000), rabbit polyclonal anti-Rab7 antibody (1:1000), rabbit polyclonal anti-LC3B antibody, mouse monoclonal anti-α-tubulin (11H10) antibody (1:1000), rabbit polyclonal anti-Acetylated-Lysine antibody (1:1000) all were from Cell Signaling Technology, Inc. (Danvers, MA, USA); rabbit polyclonal anti-p62 antibody (1:1000), mouse monoclonal anti-β-Actin antibody (1:5000), all from Sigma (St. Louis, MO, USA). LAMP-2A (1:100) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). For Immunoprecipitation, Acetylated-Lysine antibody (1:500) was used from Cell Signaling Technology, Inc. (Danvers, MA, USA), and for Immunofluorescence was used LAMP-1 antibody (1:100) from Hybridoma Bank, USA.

3.3 Cell Culture

3.3.1 Human Subjects

Subject participation was approved by the Kansas University Medical Center's Institutional

Review Board. For this study subjects were recruited from the University of Kansas Alzheimer's disease Center (KU ADC). Additionally, each subject was determined, based on cognitive testing and by a memory disorders subspecialist clinician, to meet criteria for normal cognition-control status (CT), MCI, or sporadic AD (sAD). After providing informed consent, sAD (n=8), MCI (n=7), and CT (n=7) subjects underwent a 10 ml phlebotomy using tubes containing acid-citrate-dextrose as an anticoagulant. The age of the sAD subject platelet donors was 71.5 ± 9.7 years, the age of the MCI platelet donors was 72.3 ± 6.6, and the age of the CT subject platelet donors was 73.9 ± 7.7 (Silva et al. 2016).

3.4 Creation of Cybrid Cell Lines

Cytoplasmic hybrid (cybrid) technique was first described by King and Attardi, that consists in the transfer of mitochondrial (mtDNA) exogenous to mtDNA-depleted recipient cells (Rho 0 line cells), generating cybrids (King and Attardi, 1989). The cybrids were created from a SH-SY5Y cell nuclear background by the KU ADC Mitochondrial Genomics and Metabolism Core (Miller *et al.*, 1996). To generate the cybrid cell lines used in this study, we used platelets from human subjects, as already referred, CT, MCI and sAD, which were mixed with SH-SY5Y cells previously depleted of endogenous mtDNA (Rho 0 cells) as previously described (Swerdlow *et al.*, 1996; Arduino, 2012) (**Figure 3.1**).

During the overall cybrid generation procedure, several different types of media were used. Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/ F-12) was obtained from Gibco-Invitrogen (Life Technologies Ltd, UK), while non- dialyzed FBS was obtained from Sigma (St. Louis, MO, USA). SH-SY5Y Rho 0 cell growth medium consisted of DMEM/F-12 supplemented with 10 % non-dialyzed FBS, 200 μg/ml sodium pyruvate obtained from Sigma (St. Louis, MO, USA), 150 μg/ml uridine obtained from Sigma (St. Louis, MO, USA), and 1 % penicillin–streptomycin (pen-strep) solution. The SH-SY5Y cybrid selection medium consisted of DMEM/F-12 supplemented with 10 % dialyzed FBS and 1 % pen-strep solution. The selection process lasted 6 weeks. After cell line selection was completed, each line was continuously maintained in a cybrid growth medium containing DMEM/F-12 supplemented with 10 % non-dialyzed FBS and 1 % pen-strep solution for over 2 months prior to biochemical and molecular assays (Silva *et al.*, 2016).

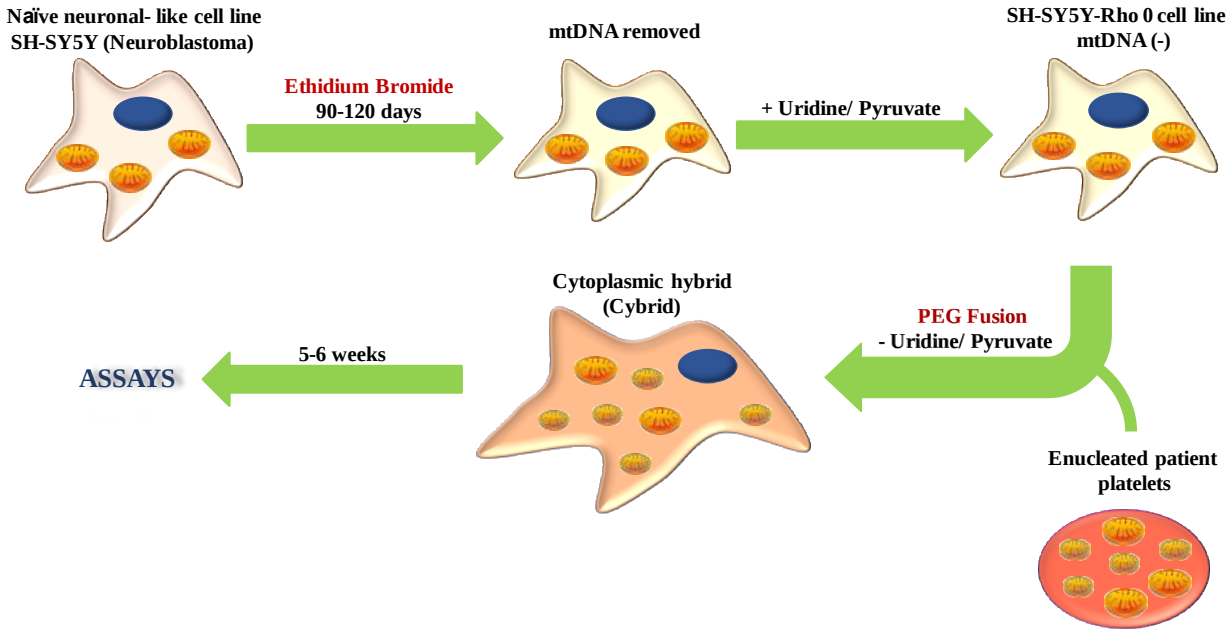


Figure 3.1 - **Cybrid results from the repopulation of the Rho 0 cell line with mtDNA exogenous.** Rho 0 cell line results from the elimination of mtDNA of immortalized cell lines by the presence of ethidium bromide. Rho 0 cells are then fused with patient platelets, which contain mitochondria but not nuclei. In this case, mtDNA exogenous is derived from platelets isolated from different individual disease-free CT, MCI and sAD subjects. So, differences in function between CT, MCI and sAD cybrids cell lines likely arise through differences in their mtDNA. Adapted from (Arduino, 2012)

3.5 Cell Media and Treatments

Cells were grown in 75 cm^2 tissue culture flasks maintained in a humidified incubator at 37 ºC and 5 % CO^2. For the MTT assay, cells were plated in 24-well plates at a density of 0.2 × 10^6 cells/ml. For Western blot analysis and Immunoprecipitation assay cells were plated in 6- well plates at a density of 0.25 × 10^6 cells/mL or in petri-dishes (10 cm). For immunocytochemistry analysis, cybrid cell lines were grown on coverslips in 12-well plates at a density of 0.1 × 10^6 cells/ml.

Prior to experiments, cell lines were maintained in the cybrid growth medium, cybrids containing DMEM supplemented with 10 % non-dialyzed FBS and 1 % pen–strep solution.

Cybrid cell lines were incubated for 6 h with 5 mM NAM, a SIRT1 inhibitor or with 2,5µM C646, a p300 inhibitor. Autophagy modulation consisted of the treatment of the respective cell line with 20 mM NH_4Cl and 10 mM Leupeptin for 4 h. For all experimental procedures, controls were performed in the absence of those agents.

3.6 Cell Proliferation Assay

3.6.1 MTT Reduction Test

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability (Mosmann, 1983).

Cells proliferation was determined by this colorimetric method, MTT. In viable cells, the enzyme succinate dehydrogenase metabolizes MTT into a formazan that absorbs light at 570 nm. Following the cell treatment protocol the medium was aspirated and 0.5 ml MTT (0.5 mg/ml) was added to each well. The plate was then incubated at 37 °C for 1 h 30 m protected from light. At the end of the incubation period the formazan precipitates were solubilized with 0.5 ml of acidic isopropanol (0.04 M HCl/ isopropanol). The absorbance was measured at 570 nm (Esteves *et al.*, 2010). Cell reduction ability was expressed as a percentage of the CT cybrids.

3.7 Preparation of Cellular Extracts

3.7.1 Preparation of Whole Cellular Extracts

Individual cells lines were washed with Phosphate-Buffered Saline 1 × (PBS), lysed on ice in a hypotonic lysis buffer containing 1 % Triton X-100, 25 mM 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES), pH 7.5, 2 mM Magnesium Chloride ($MgCl_2$), 1 mM Ethylenediamine Tetraacetic Acid (EDTA), 1 mM Ethylene Glycol-bis (β-aminoethyl Ether)-N,N,N',N'-tetraacetic Acid (EGTA) and supplemented with 2 mM Dithiothreitol (DTT) and protease inhibitors [0.1 mM Phenylmethane Sulfonyl Fluoride (PMSF), and a 1:1000 dilution of a commercial protease inhibitor cocktail]. Cell were then scrapped on ice. Subsequently, cell suspensions were frozen three times in liquid nitrogen and centrifuged at 20,000 × g for 10 min. The resultant supernatant was removed and stored at -80 °C. Protein content was determined using Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

3.7.2 Preparation of Cytosolic and Nuclear Cellular Extracts

Cell lines were washed with PBS (1 ×), lysed on ice in a first buffer, Buffer I containing 10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.5, supplemented with 0.1 % Triton X-100, with 1 mM DTT, with protease inhibitors [1 mM DTT, 1 mM PMSF and 1:1000 dilution of a commercial protease inhibitor cocktail] and with phosphatase inhibitors [2 mM Sodium

Orthovanadate (Na_3VO_4) and 50 mM of Sodium Fluoride (NaF)]. Cells were then scraped on ice. Afterwards, cell suspensions were centrifuged at 2,300 × g for 10 min at 4 ºC. The resulting supernatant was the cytosolic fraction. Then, the resulting pellet was resuspended in a second buffer, Buffer II containing 20 mM HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 20 % Glycerol, pH 7.5, supplemented with 1 mM DTT, 1 mM PMSF and 1:1000 dilution of a commercial protease inhibitor cocktail. Cell suspensions were centrifuged at 12,000 × g for 20 min at 4 ºC. The resulting supernatant was the nuclear fraction. Both fractions were stored at -80 °C. Protein content was determined using Bradford Protein Assay (Bio- Rad, Hercules, CA, USA).

3.8 SDS-PAGE and Immunoblotting

For Western Blot analysis samples were resuspended in 6 × sample buffer (4 × Tris. HCl /SDS; pH 6.8, 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue) under reducing conditions. Depending on the protein molecular weight, samples containing 30 μg of protein were loaded onto accordingly percent SDS- Polycrylamide (SDS-PAGE) gels. Specifically, for the analysis of LC3, LAMP-2A and Cathepsin D, samples were separated by electrophoresis on a 15 % gel and all the other protein samples were separated by electrophoresis on a 10 % gel. After electrophoresis, gels were transferred to Polyvinylidene Difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific binding was blocked by gently agitating the membranes in 3 % Bovine Serum Albumin (BSA) for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4°C with gentle agitation. The next day, membranes were washed in Tris-Buffered Solution (TBS) contain 0.1 % Tween, three times, each time for 5 min, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature with gentle agitation. After, three washes specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare). Fluorescence signals were detected using a Biorad Versa-Doc Imager.

3.9 Immunoprecipitation assay

Cells were scraped in buffer containing 20 mM Tris, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA (pH 7.0), and protease inhibitors (100 mM PMSF and a commercial protease inhibitor cocktail). CT, MCI and sAD cybrids cell suspensions were centrifuged at 20,000 × g for 10 min at 4 °C. Supernatants were collected and stored at -80 °C. Protein content was determined using Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) and 500 μg of cell lysates were incubated

with the Acetylated-Lysine (1:500) primary antibody overnight at 4 °C, and with gentle agitation. Lysates were then incubated with 100 μL of protein-A beads for 2 h at 4 °C and with gentle agitation. After completing this incubation, lysate tubes were centrifuged at 65 × g for 5 min at 4 °C, the supernatant was removed, and the beads were washed buffer five times (each time centrifuging at 4 °C and removing the supernatant). For the two first washes, the buffer was supplemented with 1 % Triton X-100. For the next two washes, the buffer was supplemented with 1 % Triton X-100 and 5 mM NaCl. The two final washes were performed with unsupplemented buffer. Finally, the last supernatant was removed, and 25 μL of sample buffer (2 ×) was added. The sample was boiled at 95-100 °C for 5 min to denature the protein and to separate it from the protein-A beads. The boiled proteins were centrifuged at 20,000 × g for 5 min at room temperature, and the supernatants were collected. The resulting immunoprecipitated samples were separated by SDS-PAGE and subjected to Western Blot analysis using anti-Beclin-1 antibody and anti-Acetylated-Lysine antibody, as previously described.

3.10 Immunofluorescence and Confocal Microscopy

Cybrid cells were washed with PBS (1 ×) and fixed for 30 min at room temperature using 4 % paraformaldehyde. Fixed cells were then washed again with PBS (1 ×), permeabilized with methanol at -20 °C for 30 min and blocked with 3 % BSA for 30 min.

Afterwards, cells were incubated with primary antibodies (1:100 anti-LAMP-1 (H4A30) from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA) overnight in a wet chamber at 4 °C. Later, cells were incubated 1 h with the appropriate secondary antibody (1:250 AlexaFluor 488 or 594 from Molecular Probes, Eugene, OR, USA). Finally, cells were washed in PBS (1 ×), incubated for 5 min with Hoechst (15 μg/μL) in the dark. Cells were then washed twice in PBS (1 ×) and the coverslips were immobilized on a glass slide with mounting medium DakoCytomation (Dako, Glostrup, Denmark). Confocal images were obtained using a Plan-Apochromat/1.4 NA 63 x lens on an Axio Observer. Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software.

LAMP-1-positive clusters per cell were quantified using the “analyze particle” function of the ImageJ, as previously described by Bandyopadhyay and colleagues (Bandyopadhyay *et al.*, 2014). Punctate lysosomes were selected with a size ≤ 0.2 μm, whereas lysosomes clusters were selected with a size between 0.2 μm and 5 μm. LAMP-1 fluorescence intensity was quantified using ImageJ v1.39 k (National Institute of Health, USA) program.

3.11 Data acquisition and Analysis

All data result was expressed as mean ± Standard Error of the Mean (SEM) in at least three independent experiments. Statistical analyses were performed using one-way Analysis of Variance (ANOVA) followed by Bonferroni Multiple-Comparisons Procedure as post-hoc test. Analyses and graphical presentation were performed with the GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA, USA). To compare means between two groups we used two-way, unpaired Student's T-tests, with p-values lower than 0.05 ($p < 0.05$), which were considered significant.

4 RESULTS

4.1 Effect of NAM and C646 on toxicity in cybrid cell lines

We used in our cellular model, as a parameter of cell viability, the MTT assay to establish nontoxic concentrations of NAM, a SIRT1 deacetylase inhibitor (Wang *et al.*, 2013) and C646, a relatively potent, selective, cell-permeable small molecule inhibitor of p300 lysine acetyltransferase (Shrimp *et al.*, 2015).

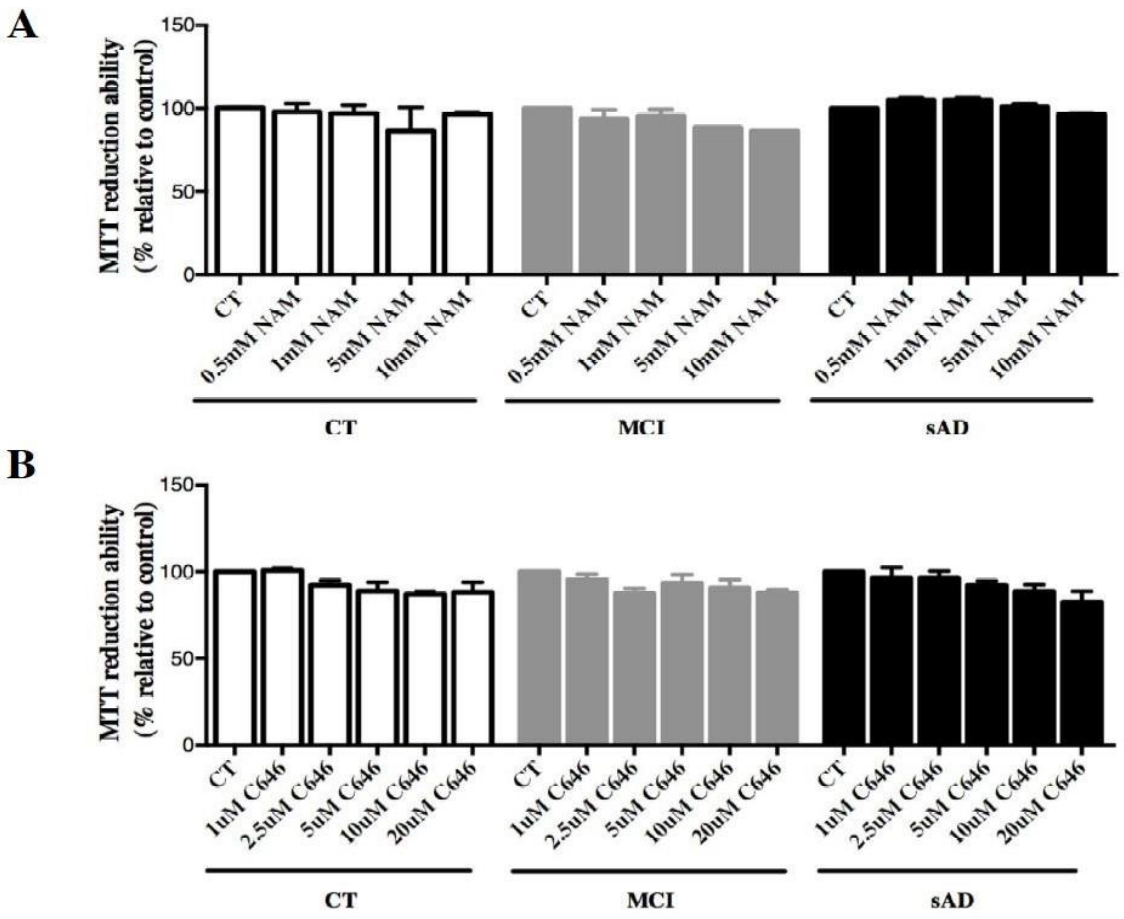


Figure 4:1 – **Effects of NAM and C646 on MTT reduction test.** Cybrid cell lines were incubated with NAM at concentrations 0.5 mM, 1mM, 5mM and 10mM **(A)**. Cybrid cell lines were incubated with concentrations 1 µM, 2.5 µM, 5 µM, 10 µM and 20 µM of C646 **(B)**.

4.2 SIRT1 and p300 roles in acetylation of Beclin-1 in cybrid cellular model

SIRT1, a phylogenetically conserved NAD^+ - dependent histone deacetylase has been described to play a predominant role in autophagy induction under starved conditions, regulating the deacetylation of multiple essential proteins involved in autophagy network (Jazwinski, Belancio and Hill, 2017).

Lee *et al.* (Lee *et al.*, 2008) have described that SIRT1 deacetylase is clearly an essential regulator of autophagic degradation both *in vitro* and *in vivo* conditions (Lee *et al.*, 2008).

Besides cellular deacetylases, regulation of autophagy activity can be provided by additional enzymes, responsible for catalyzing the forward acetylation reaction. Acetylation process is increasingly recognized as one of the major post-translational mechanism for the regulation of multiple cellular functions in mammalian cells.

Acetyltransferase p300 is a critical regulator of eukaryotic gene expression, involved in several biological processes (Ghosh and Varga, 2007) and the related cyclic AMP (cAMP) response element-binding protein (CREB), serve as transcription coactivator by acetylating core histones and nuclear non-histones proteins. p300 is predominantly nuclear but can shuttle between the nucleus and cytoplasm and recently has been suggested to participate in the regulation of autophagy pathway. Various Atg proteins, such as LC3 and Atg5 are targeted by p300-mediated acetylation, leading to an inhibition of their autophagic activities (Wan *et al.*, 2017).

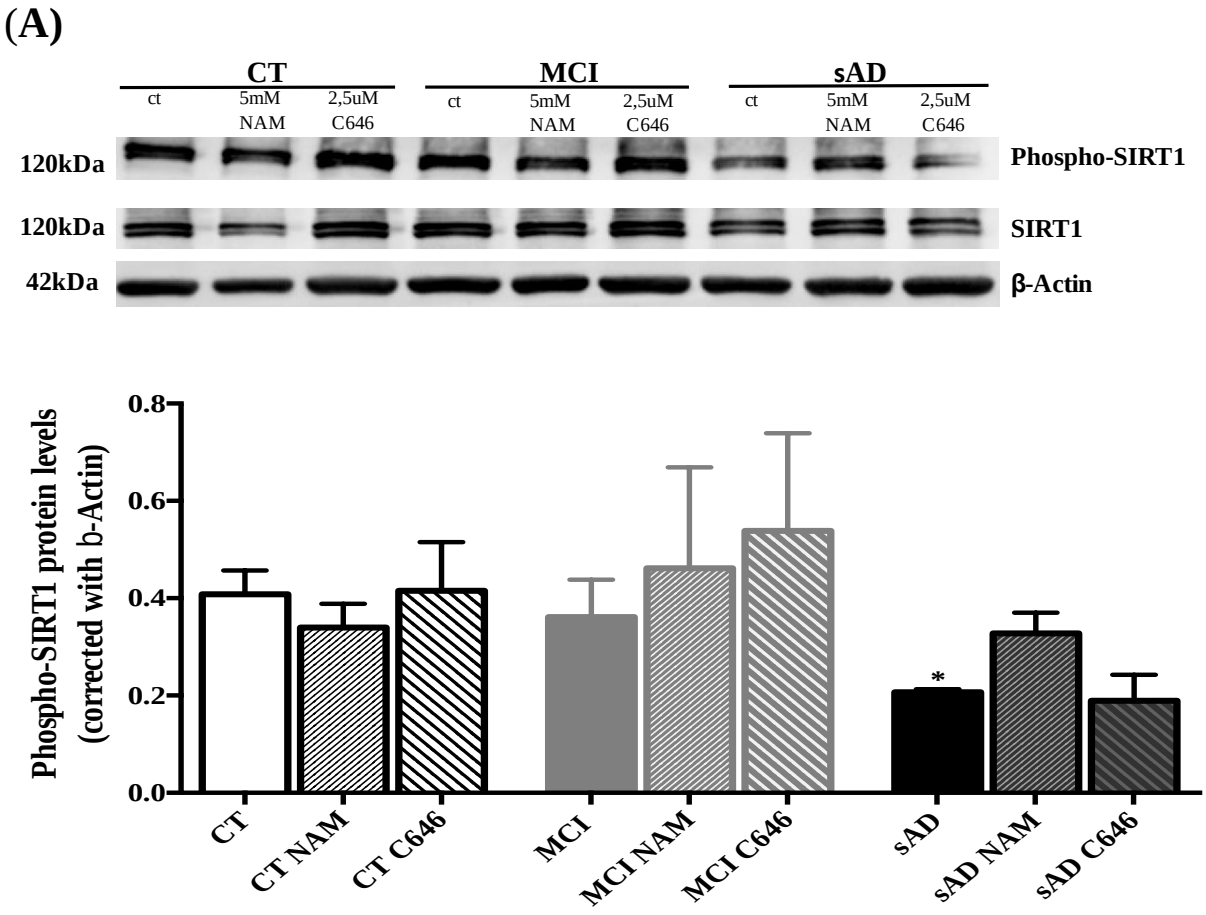
A dysregulation of histone acetylation has been described in a diversity of signal transduction pathways, including cell differentiation, cell apoptosis, vascular remodeling, inflammation reaction, immune responses, neuronal plasticity, among others (Lu *et al.*, 2015). An altered acetylation in non-histone proteins, nuclear and cytoplasmic, has been related to AD pathology, such as Tau protein (Irwin *et al.*, 2012), α-tubulin (Perez *et al.*, 2009; Silva *et al.*, 2016), p53 (Chang *et al.*, 2011) and NF-κB (Chen *et al.*, 2001). For this reason, we found interesting to understand the role of histone acetyltransferase p300 using a specific inhibitor-C646, as well an inhibitor-NAM for deacetylase SIRT1.

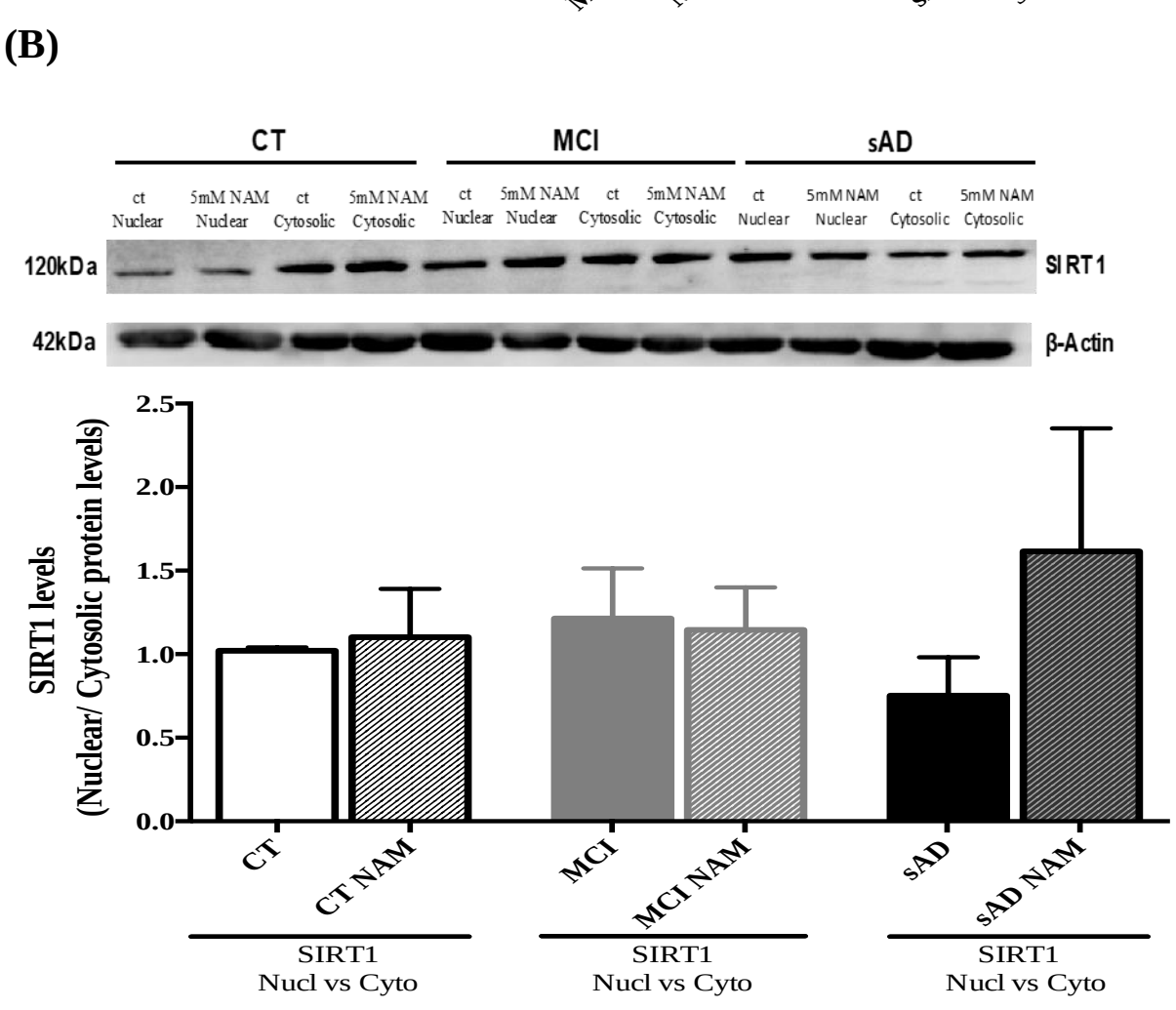
First, we evaluate the levels of phospho-SIRT1 (p-SIRT1), SIRT1 active form, in our cybrids cellular model. Our results point to a significantly decreased of basal p-SIRT1 in sAD cybrid cell line compared to MCI and CT cybrid cell lines (**Figure 4.2 A**), which is in accordance to previous results (Kumar *et al.*, 2013; Silva *et al.*, 2016). In fact, we observed that under basal conditions p-SIRT1 levels are very similar between MCI and CT groups, indicating what was described by Julien and colleagues (Julien *et al.*, 2009). This observation suggests that measurement of p-SIRT1 levels are of no value as a marker in the early stages of AD condition. Additionally, we did not see any significantly effect of NAM. When used C646, we noticed an increase of p-SIRT1levels in

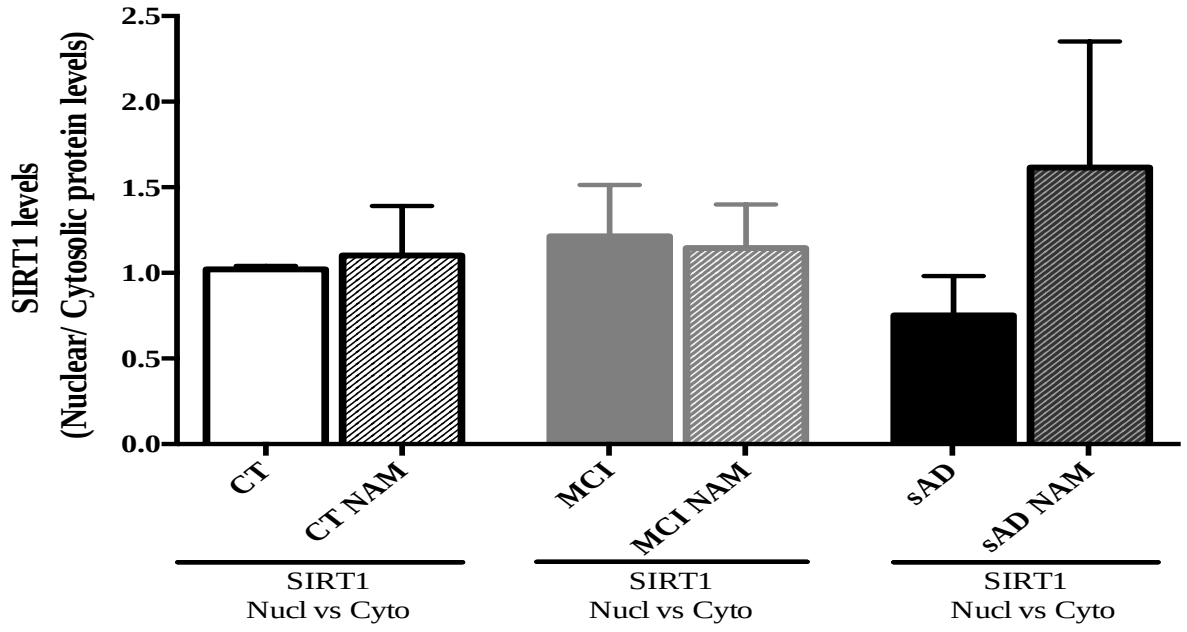
MCI cells, but a decrease in sAD cells. Moreover, we found a decreased ratio between nuclear/cytosolic SIRT1 in sAD cybrid cell line, similarly to what was observed by Silva and coworkers (Silva *et al.*, 2016) (**Figure 4.2 B**). These results indicate that the SIRT1 localization patterns differ between "normal cells" and "cells with disease".

Afterwards, we want to understand in our cybrids cellular model, how an inhibition of SIRT1 and p300 would affect Beclin-1 protein levels in our cell lines. Beclin-1 is an essential molecular anchor in autophagy pathway, regulating the initiation and nucleation of autophagosomes formation (McKnight and Zhenyu, 2013). Studies have described that Beclin-1 expression is significantly decreased in samples from AD brains, when compared with age- matched controls (Pickford *et al.*, 2008; Jaeger and Wyss-Coray, 2010). Moreover, Jaeger and colleagues (Jaeger *et al.*, 2010), have been described that a deficiency of Beclin-1 expression affects the degradation of autophagosomes in cultured cells. In our study using cybrid cells, Beclin-1 protein levels are reduced in basal sAD group, comparing with MCI and CT groups. At the same time, we see an increase of Beclin-1 in sAD cybrid cellular line when SIRT1 is inhibited, which may be related to altered acetylation leading to an impairment of autophagy pathway in AD pathology, described already (Uddin *et al.*, 2018). Although, MCI and CT groups show a slight decrease when treated with NAM, comparing with sAD group. Inhibiting p300, we noticed a decrease of acetylation levels in sAD cell line, as expected, but an increase in both MCI and CT cell lines (**Figure 4.2 C**). These evidences indicate that acetylation may play a role in autophagy regulation, namely alteration of Beclin-1 acetylation status in AD malady. Finally, with these interesting results we wanted to understand if acetylated Beclin-1 is in fact increased in our cellular model. We found that basal levels of acetylated Beclin-1 are significantly increased in sAD group, but not in MCI and CT groups. Curiously, the total of lysine-acetylation basal levels is in opposite way compared to basal acetylated Beclin-1 levels in sAD cybrid cells, significantly decreased compared with CT and MCI cybrid cells (**Figure 4.2 D**).

Afterwards, we observed that an inhibition of SIRT1 deacetylase increases significantly acetylated Beclin-1 levels in sAD group comparing to MCI and CT groups. These also interesting results, suggest the essential role played by SIRT1 in AD pathology. In addition, an inhibition of p300 acetyltransferase, leads to a slight decrease of acetylated Beclin-1 protein levels in MCI cybrid cell lines. When we used NAM, the total lysine-acetylation levels, showed a tendency to increase in CT, MCI and sAD groups. Plus, when we used C646, only in the CT cell line, the levels of the total lysine-acetylation were significantly decreased. (**Figure 4.2 E**).

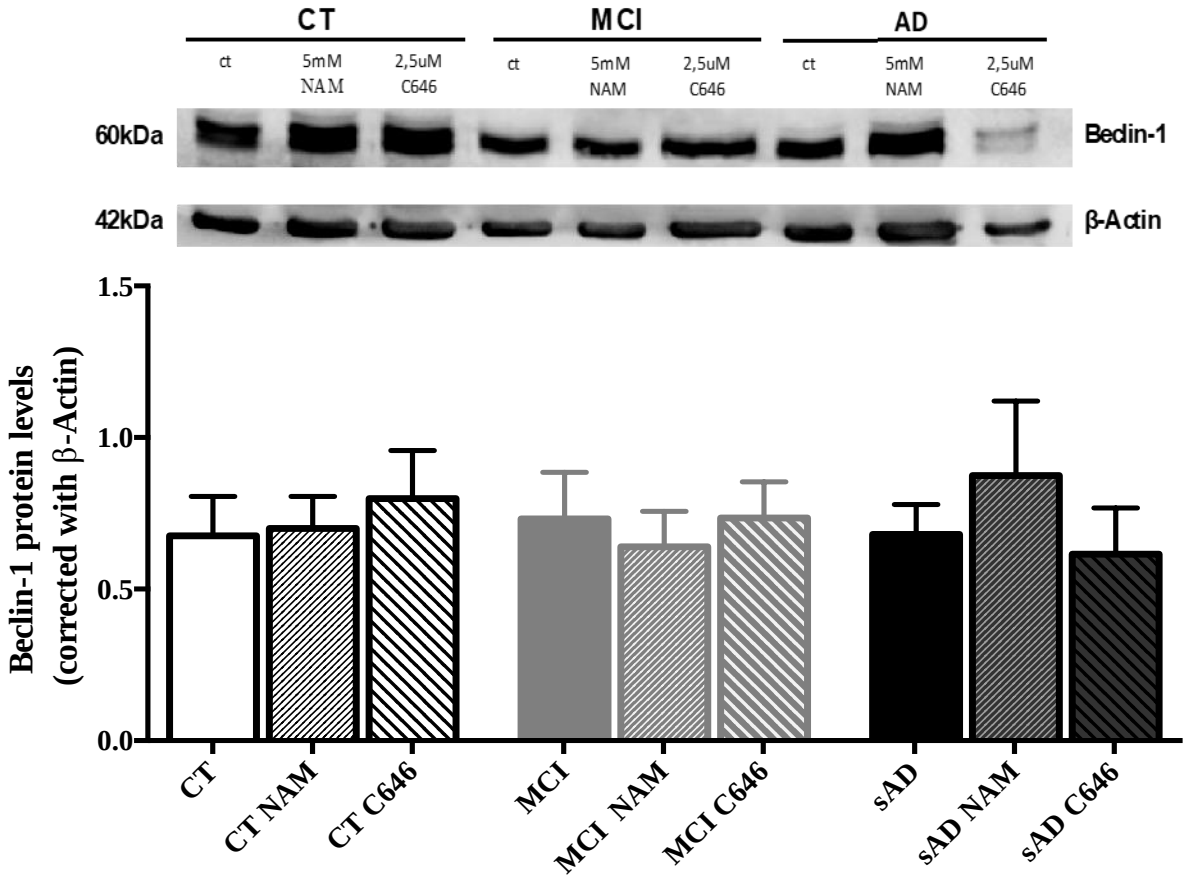

(A)
CT
MCI
sAD
ct
5mM NAM
2,5uM C646
120kDa
Phospho-SIRT1
120kDa
SIRT1
42kDa
β-Actin
Phospho-SIRT1 protein levels (corrected with b-Actin)
0.8
0.6
0.4
0.2
0.0
*
CT
CT NAM
CT C646
MCI
MCI NAM
MCI C646
sAD
sAD NAM
sAD C646


(B)
CT
MCI
sAD
ct Nuclear
5mM NAM Nuclear
ct Cytosolic
5mM NAM Cytosolic
120kDa
SIRT1
42kDa
β-Actin
SIRT1 levels (Nuclear/ Cytosolic protein levels)
2.5
2.0
1.5
1.0
0.5
0.0
CT
CT NAM
MCI
MCI NAM
sAD
sAD NAM
SIRT1 Nucl vs Cyto
SIRT1 Nucl vs Cyto
SIRT1 Nucl vs Cyto

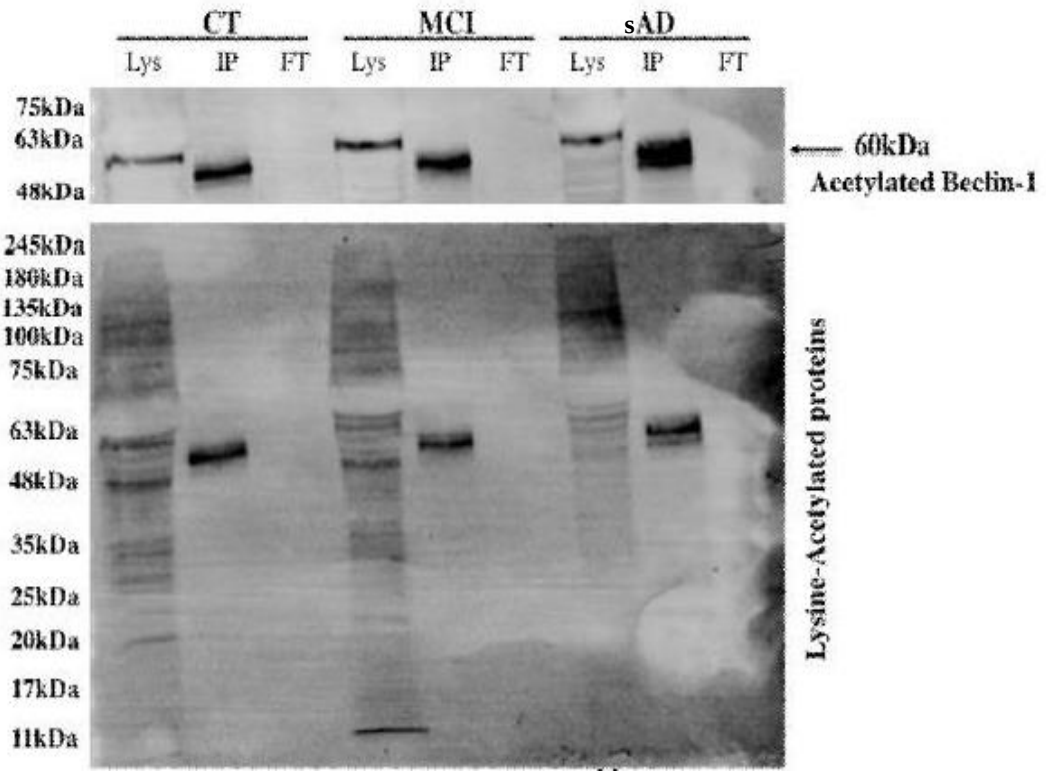

SIRT1 levels
(Nuclear/ Cytosolic protein levels)
2.5
2.0
1.5
1.0
0.5
0.0
CT
CT NAM
MCI
MCI NAM
sAD
sAD NAM
SIRT1
Nucl vs Cyto
SIRT1
Nucl vs Cyto
SIRT1
Nucl vs Cyto

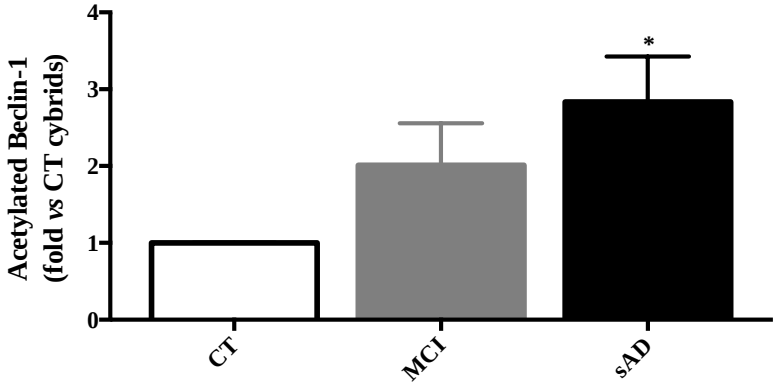
(C)

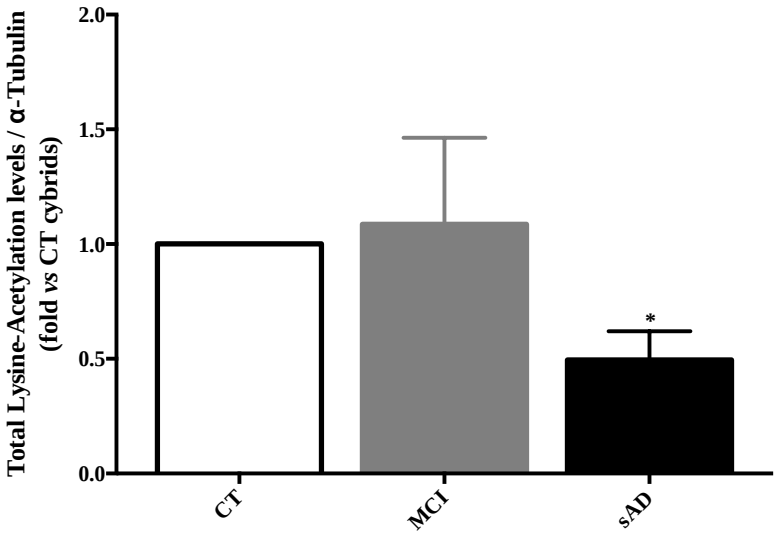
s


CT
MCI
AD
ct
5mM NAM
2,5uM C646
60kDa
Bedin-1
42kDa
β-Actin
Beclin-1 protein levels
(corrected with β-Actin)
1.5
1.0
0.5
0.0
CT
CT NAM
CT C646
MCI
MCI NAM
MCI C646
sAD
sAD NAM
sAD C646

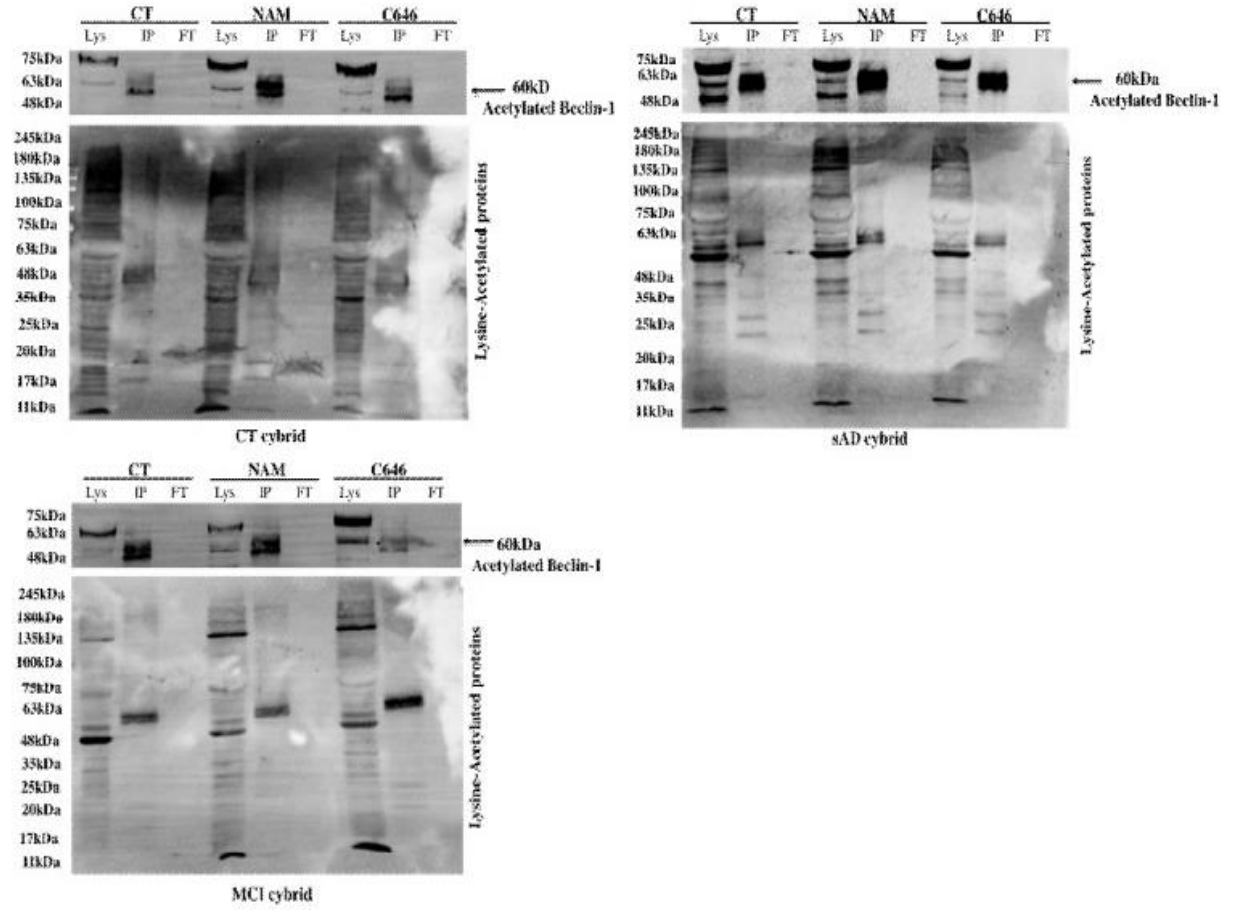
(D)

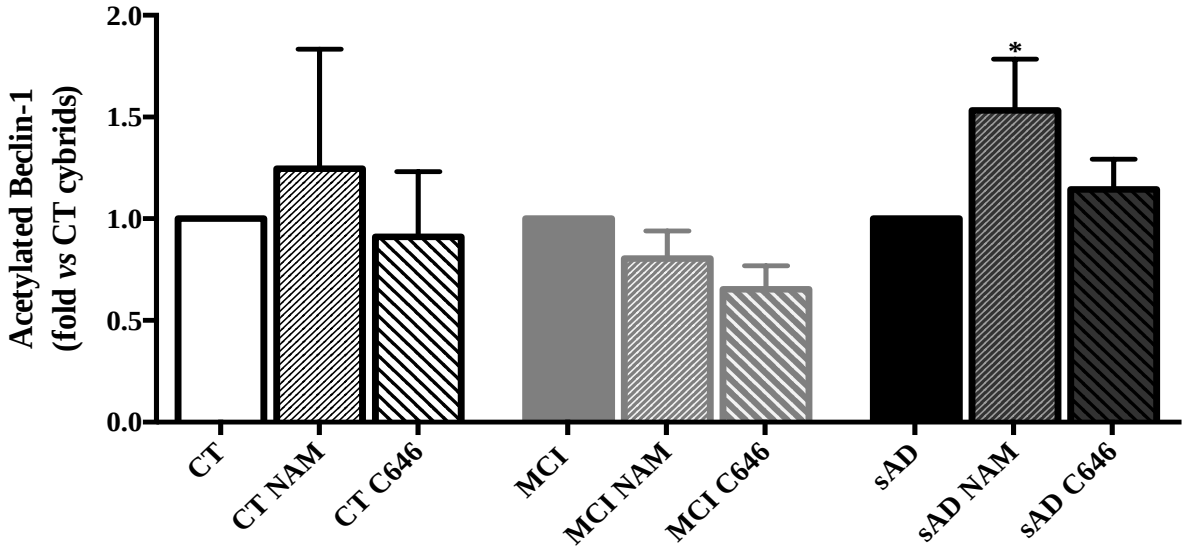

CT
MCI
sAD
Lys
IP
FT
75kDa
63kDa
48kDa
60kDa
Acetylated Beclin-1
245kDa
180kDa
135kDa
100kDa
75kDa
63kDa
48kDa
35kDa
25kDa
20kDa
17kDa
11kDa
Lysine-Acetylated proteins


Acetylated Beclin-1
(fold vs CT cybrids)
4
3
2
1
0
*
CT
MCI
sAD


Total Lysine-Acetylation levels / α-Tubulin
(fold vs CT cybrids)
2.0
1.5
1.0
0.5
0.0
*
CT
MCI
sAD

(E)


CT
NAM
C646
Lys
IP
FT
75kDa
63kDa
48kDa
60kD
Acetylated Beclin-1
245kDa
180kDa
135kDa
100kDa
75kDa
63kDa
48kDa
35kDa
25kDa
20kDa
17kDa
11kDa
Lysine-Acetylated proteins
CT cybrid
60kDa
sAD cybrid
MCI cybrid


Acetylated Beclin-1
(fold vs CT cybrids)
2.0
1.5
1.0
0.5
0.0
*
CT
CT NAM
CT C646
MCI
MCI NAM
MCI C646
sAD
sAD NAM
sAD C646

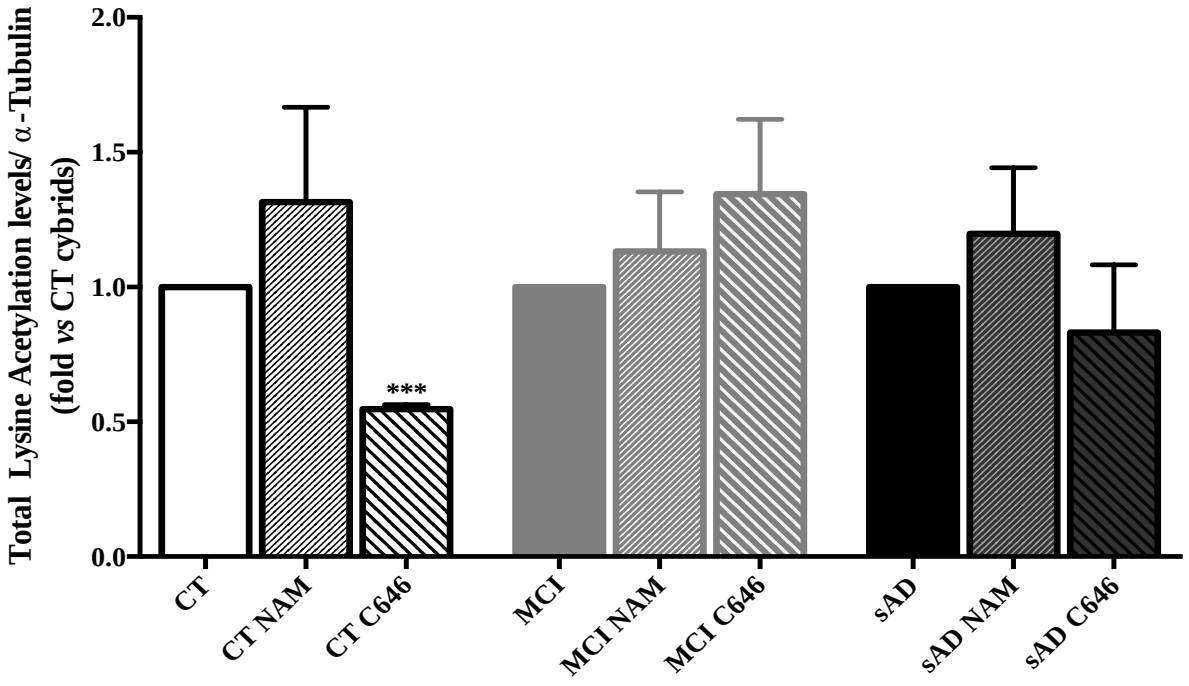


Figure 4:2 - **SIRT1 and p300 roles in acetylation of Beclin-1 in cybrid cellular model.** Immunoblotting for phospho-SIRT1 protein levels for each separate cybrid group treated with NAM and C646 (**A**). Immunoblotting for nuclear and cytosolic SIRT1 levels for each separate cybrid group treated with NAM (**B**). Immunoblotting for Beclin-1 protein levels for each separate cybrid group treated with NAM and C646 (**C)**. Immunoprecipitation for acetylated Beclin-1 basal levels and for total Lysine-Acetylated basal protein levels (**D**). Immunoprecipitation for acetylated Beclin-1 and for total Lysine-Acetylated protein levels for each separate cybrid group treated with NAM and C646 (**E**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line. Data were analyzed by Student's T-test and are presented as mean ± S.E.M.

4.3 Autophagic flux- a link to pathophysiological processes in AD

To elucidate further about autophagy network, we decided to analyze two important proteins in our cybrid cellular model, involved on autophagic flux.

The first one, LC3 protein has been widely used to monitor the number of autophagosomes, as well as autophagic activity, being responsible for controlling the major steps in the autophagy pathway, including the growth of autophagic membranes, recognition of autophagic cargoes, and the fusion of autophagosomes with lysosomes (Huang and Liu, 2015). LC3 is localized in both nucleus and cytosol, implying that the protein can shuttle between the two cellular compartments. Recent studies have described that during starvation, deacetylation by SIRT1 promotes LC3 redistribution from the nucleus to cytosol, where it associates with Atg7 protein, and with the rest of autophagy core machinery to form autophagosomes (Lee and Lee, 2016).

During the generation of autophagosomes membranes, cytosolic LC3I (resides in cytosol; free form) is conjugated to PE through two consecutive ubiquitylation-like reactions catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg3 to LC3II form (membrane bound). The intra-autophagosomal LC3II is degraded in autolysosomal lumen by lysosomal hydrolases during the fusion of autophagosomes with lysosomes.

For all these reasons, we decided to evaluate LC3II levels to monitor the autophagosomes membranes levels, as a purpose to investigate autophagic flux (Deretic, 2008) .

We observed that autophagic flow is significantly decreased in sAD group, when compared with MCI and CT groups. This decrease may represent an aberrant activation of autophagy pathway or a defective clearance of AVs. However, after SIRT1 deacetylase inhibition, we noticed a significantly decrease of autophagic flow in CT cybrid cell line compared with both MCI and sAD cybrid cell lines. These findings may be in accordance with the hypothesis that SIRT1 might deacetylate LC3 (Lee *et al.*, 2008). Deacetylation by SIRT1 is necessary for LC3 to shift its distribution from the nucleus to cytoplasm during cell starvation (Huang and Liu, 2015) and probably in pathological conditions, such as AD pathology. C646 increased significantly the autophagic flow in sAD cybrid cells (**Figure 4.3 A**).

These observations also may suggest that modulation of acetylation-deacetylation cycle of LC3 can affect the subcellular distribution of LC3 protein and the cytoplasmic redistribution of LC3 protein dependent on deacetylation event of LC3, leading to a possible dysfunction of autophagic flux. Furthermore, these notes can suggest that a Beclin-1 acetylation can promote Beclin-1-rubicon interaction, affecting or inhibiting autophagosome maturation and endocytosis, inducing to a decrease of autophagic flow in AD pathogenesis.

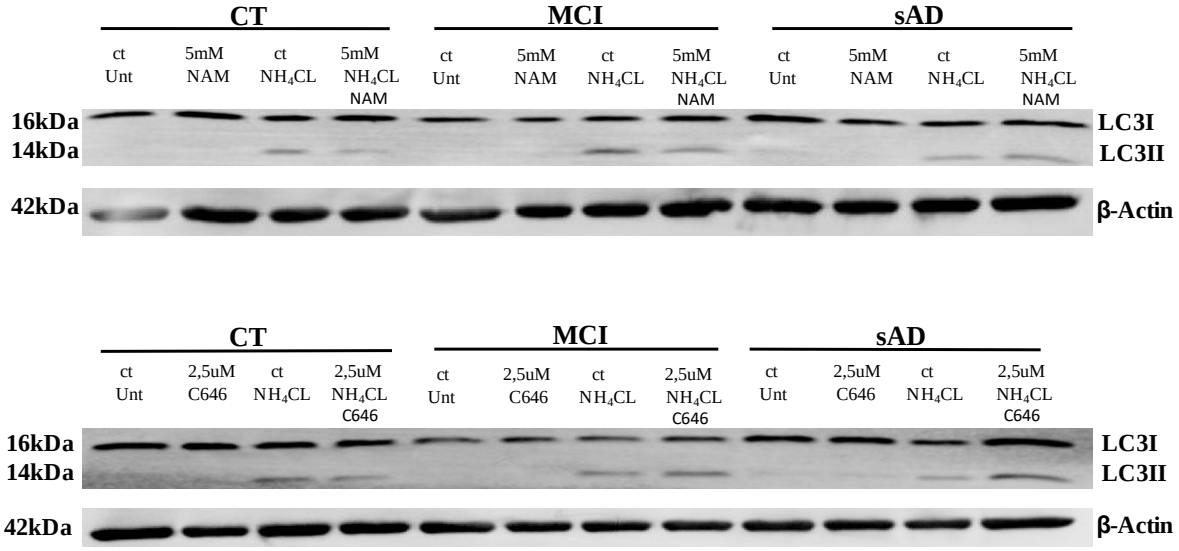
Other protein, extremely important in autophagy pathway is p62 involved in protein turnover, widely used as a marker for autophagic flux. p62 is a multifunctional protein, and plays some important functions through oxidative stress, autophagy and proteasome, as an adaptor protein to transport ubiquitinated and misfolded proteins for proteasomal and autophagic degradation (Caccamo *et al.*, 2017).

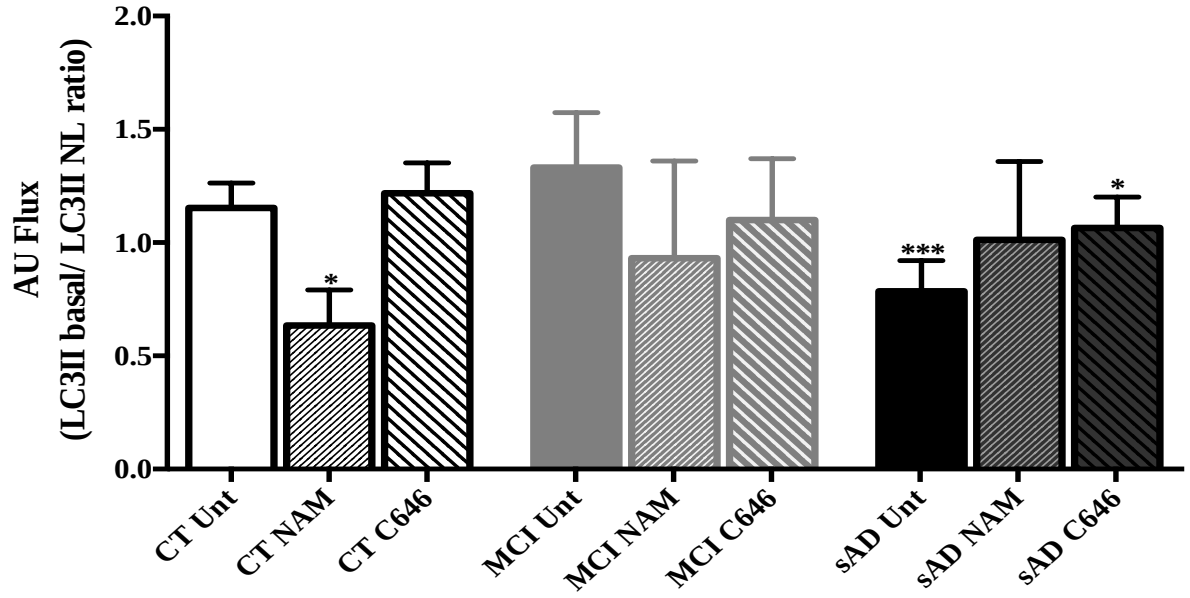
Also, p62 plays functions as an adaptor or cargo receptor in degradation of ubiquitinated proteins, and organelles, including mitochondria and peroxisomes. Additionally, a recent study has described that p62 self-oligomerization is important for its localization to the autophagosome formation site, being associated to ER, occurring in an independently LC3 process manner (Itakura and Mizushima, 2011). Importantly, p62 acts as a substrate during autophagy pathway degradation, and an increase levels of p62 protein suggests that autophagic flux may be impaired (Tanji *et al.*, 2014). In p62 knockout mice show age-dependent accumulation of NFTs and synaptic deficits underlying the role of p62 in Tau protein aggregation and degradation (Caccamo *et al.*, 2017). In AD condition, p62 is strongly bind to NFTs, mostly likely to target them for degradation. Thus, p62 is also related to deliver ubiquitinated proteins, such as Tau protein, to the proteasome for clearance (Liu *et al.*, 2016). Studies using AD brain tissue described that p62 protein levels are decreased, being directly associated with an increase of protein aggregation and deposition (Du *et al.*, 2009).

Therefore, we decided to evaluate p62 protein levels in our cybrid cellular model. First, we noticed that in both MCI and sAD untreated groups, the flux of p62 was significantly decreased compared to CT untreated group, as expected. These observations may indicate that p62 accumulation is due to inefficient autophagy network (Du *et al.*, 2009; Du, Wooten and Wooten, 2009). Although, treatment with C646 leads to an extreme increase of p62 flux in sAD cybrid cell line, comparing to both MCI and CT cybrid cell lines, indicating that an inhibition of acetylation, including of Beclin-1, may be in part benefic in AD condition (**Figure 4.2 B**). Indeed, Nixon and colleagues (Haung Yu *et al.*, 2005) showed in *post-mortem* human AD brains an accumulation of autophagosomes, suggesting deficits in autophagy flux in neurodegenerative disorders (Haung Yu *et al.*, 2005).

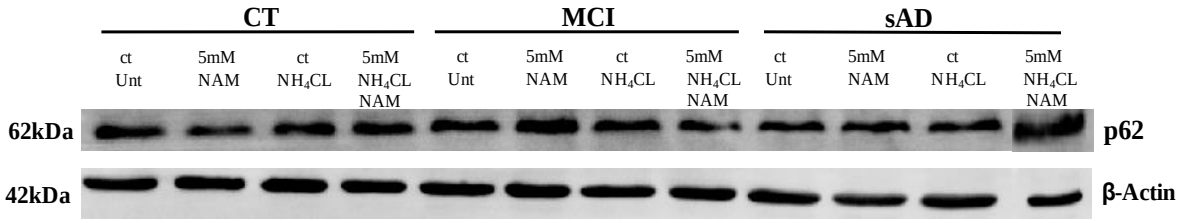
At the same time, these results may suggest that acetylation mechanism, specifically, involved with Beclin-1 protein, can influence both LC3 and p62 protein levels to be degraded after autophagosome maturation and fusion with lysosome, which might compromise the regulation of autophagic flux.

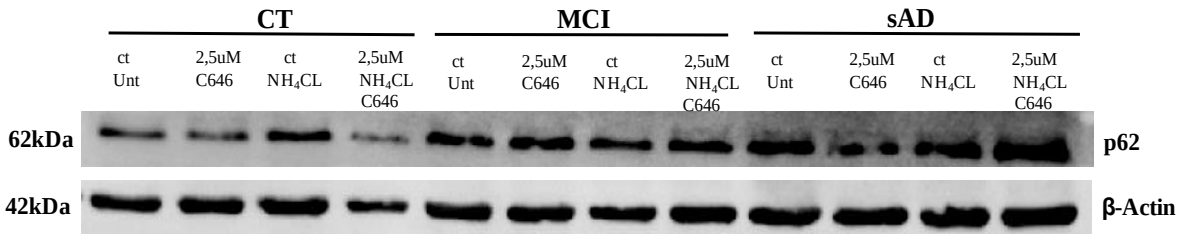
(A)


CT
MCI
sAD
ct Unt
5mM NAM
ct NH4CL
5mM NH4CL NAM
16kDa
14kDa
LC3I
LC3II
42kDa
β-Actin
2,5uM C646
2,5uM NH4CL C646


AU Flux
(LC3II basal/ LC3II NL ratio)
2.0
1.5
1.0
0.5
0.0
*
***
CT Unt
CT NAM
CT C646
MCI Unt
MCI NAM
MCI C646
sAD Unt
sAD NAM
sAD C646

(B)





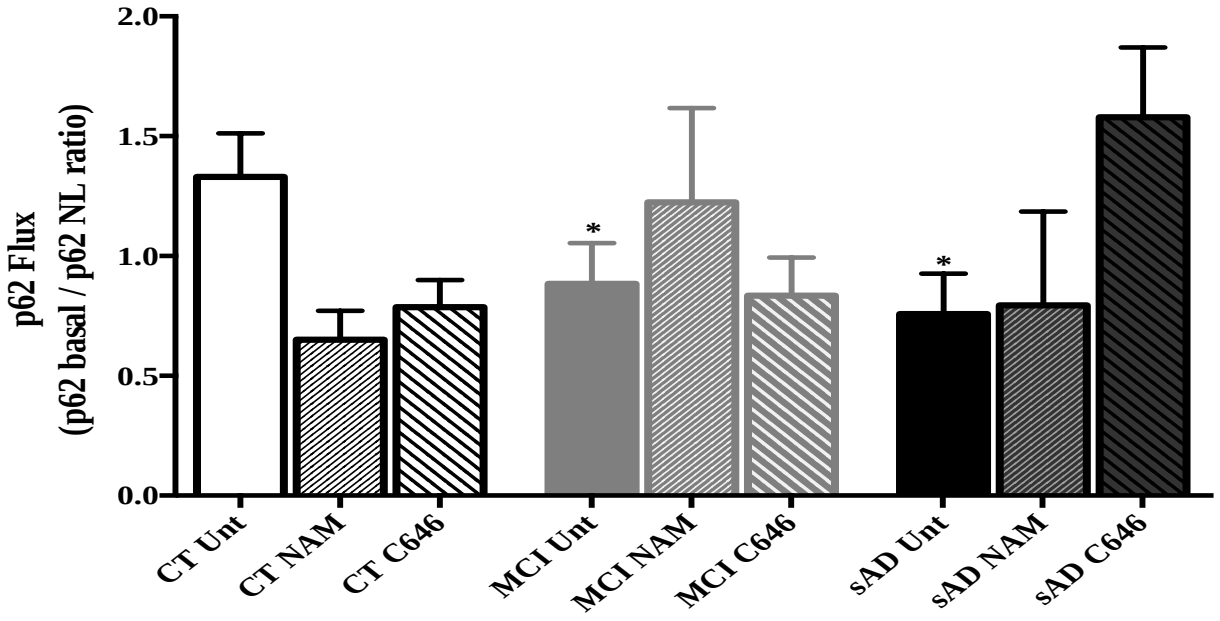


Figure 4:3 -**Autophagic flux- a link to pathophysiological processes in AD.** Immunoblotting for LC3II protein levels for each separate cybrid group, untreated and treated with NH4CL/Leupeptin-NAM and NH4CL/Leupeptin-C646 (**A**). Immunoblotting for p62 protein levels for each separated cybrid group, untreated and treated with NH4CL/Leupeptin-NAM and NH4CL/Leupeptin-C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line. Data were analyzed by Student's T-test and are presented as mean ± S.E.M.

4.4 Lysosomes localization and function might be affected

Another pathway, essential in all most cells of our body is lysosomal network, a sensitive system, important in neuronal cells, through of their extreme asymmetry, and the length of axons and dendrites (Pu *et al.*, 2016). Multiple evidences suggest that changes or even mutations in components of the lysosome-positioning machinery may cause neurological disorders (Ballabio, 2016). An imbalance of autophagic flux, can lead to some diverse events, such as cell death either from an increase of autophagosomes generation and/ or a decrease in degradation substrates; disruption of axonal transport in late endosomes; lysosomes and autolysosomes accumulation in neurons, resulting in dystrophic axonal swellings, typically seen in AD ailment; and defects in lysosomal hydrolases. All of these events are responsible for an autophagy-lysosome dysfunction contributing to develop of neurodegenerative diseases (Tammineni *et al.*, 2017).

For all these reasons, lysosomal dysfunction should be investigated as a potential risk factor in AD condition. Therefore, we found interesting to evaluate two important proteins involved in lysosome network, the LAMP-2A and CatD proteins.

The impairment of autolysosomal proteolysis system is mainly responsible for disruption of autophagy in early AD pathology. The progression of disease is accompanied by complex pathological changes, leading to a massive accumulation of LEs/MVBs, lysosomes, autolysosomes and autophagosomes (Nixon, 2016). The limiting membrane of lysosome contains more than a 100 proteins, and the most abundant are the LAMP-1 and LAMP-2, which together account 80% of lysosomal membrane protein content (Wartosch, Bright and Luzio, 2015). LAMPs are mainly localized to lysosomes, but can also be detected in lower amounts in endosomes and at the plasma membrane.

LAMP-2 is one of the two different classes of LAMPs proteins, expressed in different tissues and present mainly in the lysosomal lumen, as well as the lysosomal membrane. Additionally, LAMP-2A, one isoform of LAMP-2, is a receptor for substrates of CMA, when specific cytosolic proteins are directly transported through the lysosomal membrane within the lysosomal matrix to be degraded (Cuervo and Dice, 2000). This protein is one of the three splice variants encoded by the *LAMP-2* gene, which share identical regions and different transmembrane and cytosolic tails (Esteves, Oliveira and Cardoso, 2013). LAMP-2A is crucial for lysosomal protection and has been reported to be increased in several lysosomal storage disorders as a general response to decreased lysosomal clearance. However, little is known of how LAMP-2A is regulated. LAMP-2A have been described significantly increased in CSF from AD patients (Armstrong *et al.*, 2014). For all these facts, we found interesting to evaluate LAMP-2A levels in our cybrid cellular model.

We observed an increase of basal LAMP-2A levels in sAD group compared with CT and MCI groups, as expected. This result may indicate that an accumulation of both oligomeric Aβ1-42 and Tau protein may be responsible for an upregulation of LAMP-2A. It was described that Tau protein can associate with hsc70, the cytosolic chaperone responsible for targeting substrates to lysosomes (Wang *et al.*, 2009). Additionally, the presence of LAMP-2A is not sufficient to conclude that lysosome may be active for CMA, once not all LAMP-2A positive lysosomes can perform CMA pathway. An increase in LAMP-2A levels may indicate, an increase in lysosomal biogenesis or an upregulation to allow CMA via (Patel and Cuervo, 2015).

Interestingly, when we treated our cells with lysosomal inhibitors, such as NH_4CL/ Leupeptin, using NAM, we noticed that LAMP-2A levels in sAD cybrid cell line are much higher than in MCI and CT cybrid cell lines. This result indicates that an inhibition of SIRT1 deacetylase have a negative effect in CM pathway, and consequently in AD malady. However, when we used C646, is noticeable a decrease of LAMP-2A levels in sAD group but not in MCI group (**Figure 4.4 A**).

These observations may be associated with an acetylation of Beclin-1 by the acetyltransferase p300. An inhibition of p300 in sAD cybrid cell line decrease levels of LAMP-2A, not affecting the fusion of autophagosomes with lysosomes, and CMA via. In the other hand an acetylation of Beclin-1 can affect the LAMP-2A receptor levels, which can influence cytosolic proteins degradation and consequently CMA pathway. Therefore, a deacetylation of Beclin-1 has a protective effect in autophagy-lysosomal pathway, lysosomes and in AD pathology.

CatD is a soluble lysosomal aspartic endopeptidase, playing numerous physiological functions in the cells, such as metabolic degradation of intracellular proteins and activation of enzymatic precursors. Several proteins produced in neurons are physiologic substrates of CatD, and would be abnormally accumulate if not correctly degraded, such as APP, α-synuclein and huntingtin.

Actually, dysfunctions of CatD into the lysosomal system are associated to mechanisms of neurodegeneration (Domenico, Tramutola and Perluigi, 2016).

Cataldo and Nixon (Cataldo and Nixon, 1990), have described that damaged neurons may be the principal source of cathepsins in SPs, which explains the abnormal localization of these enzymes and the abnormal processing of APP (Cataldo and Nixon, 1990). Other studies have been reported in hippocampus of MCI patient's higher levels of CatD protein and an upregulation of its gene expression (Perez *et al.*, 2015). The essential role of CatD in AD pathology is in part the involvement of clearance of Aβ and Tau proteins through autophagy-lysosomal system. Therefore, we found interesting to analyze CatD protein levels in our cellular model.

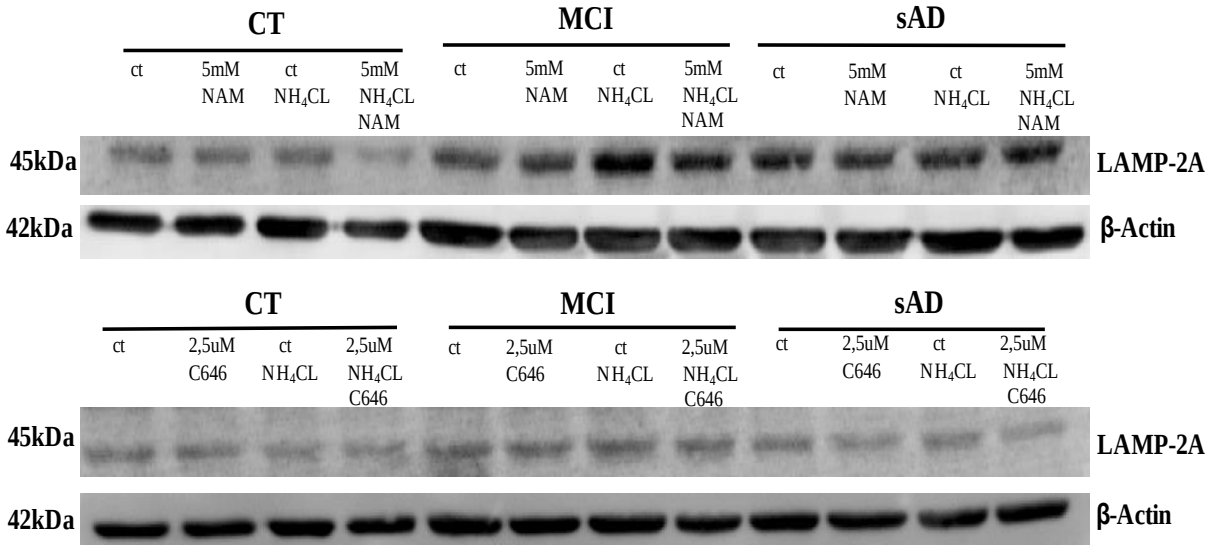
As expected, we observed a significantly increase of CatD protein levels in untreated sAD

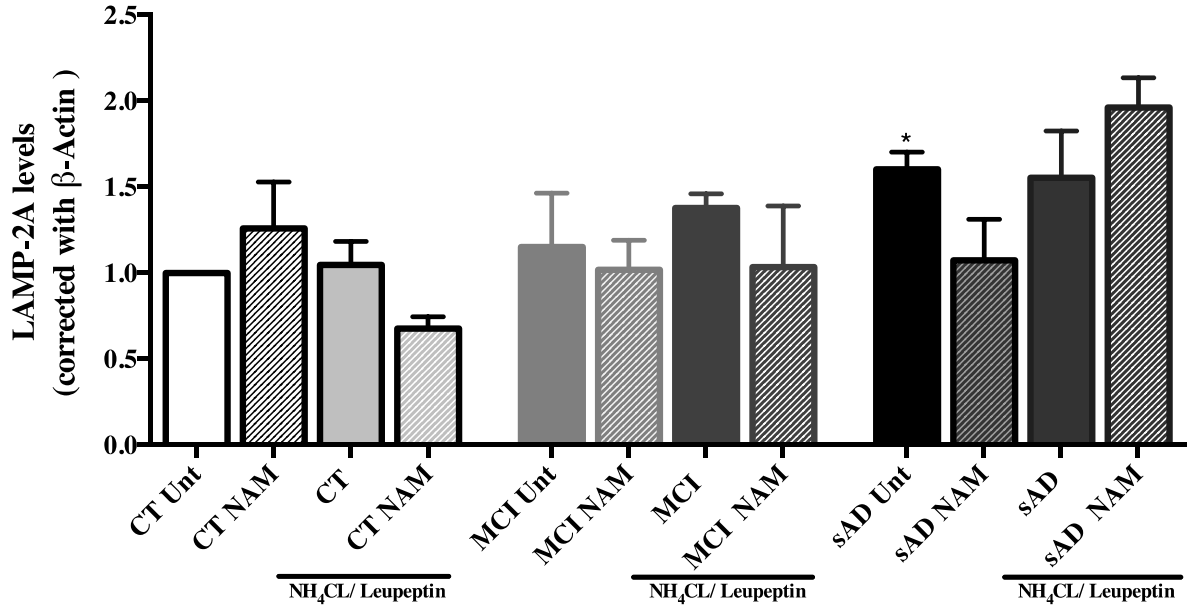
cells.

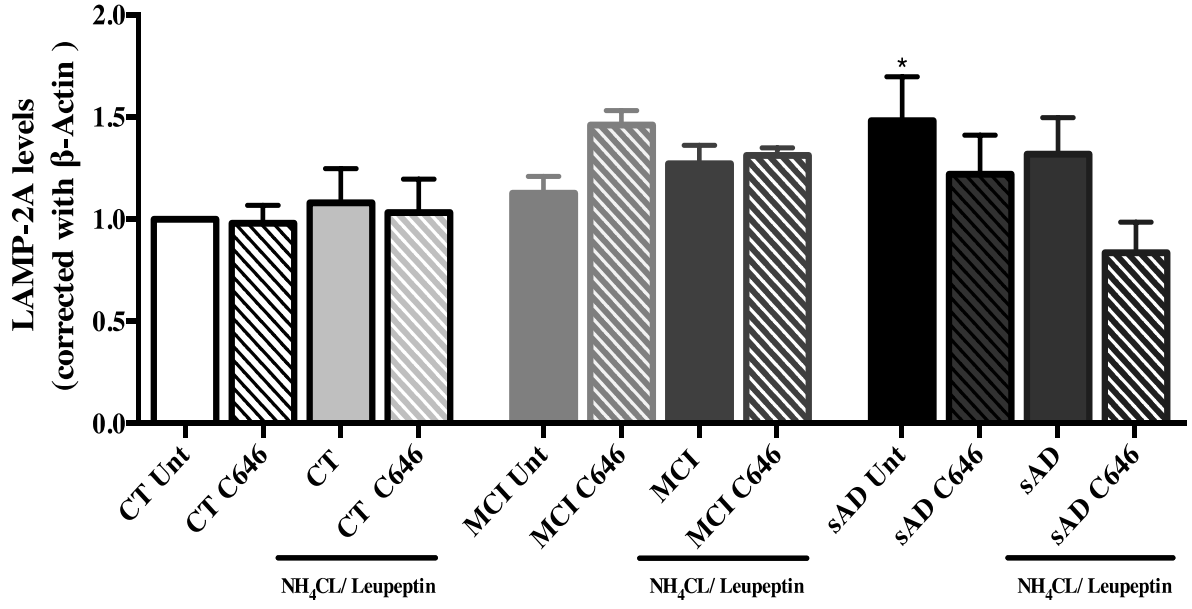
However, a noticeable decrease occurs in sAD group when treated with NAM. When treated with NH4CL/Leupeptin, a significantly decrease occurs in CT cybrid cell line. Also, an extreme increase of CatD levels is seen in sAD group, when treated with NH4CL/Leupeptin and NAM, comparing with both MCI and CT groups. This observation may suggest a compensatory protective mechanism when Beclin-1 acetylation is active blocking the elimination of protein aggregates.

When p300 and lysosomal function are inhibited a decrease of CatD protein levels occurs in both CT and sAD cybrid cell lines (**Figure 4.4 B**). These findings may indicate that SIRT1 deacetylase and a deacetylation of Beclin-1, can have a protective effect in autophagy-lysosomal network, leading to a decrease of CatD protein levels in AD condition.

(A)


CT
MCI
sAD
ct
5mM NAM
ct NH4CL
5mM NH4CL NAM
45kDa
LAMP-2A
42kDa
β-Actin
2,5uM C646
ct NH4CL
2,5uM NH4CL C646


LAMP-2A levels (corrected with β-Actin)
2.5
2.0
1.5
1.0
0.5
0.0
*
CT Unt
CT NAM
CT
CT NAM
MCI Unt
MCI NAM
MCI
MCI NAM
sAD Unt
sAD NAM
sAD
sAD NAM
NH4CL/ Leupeptin


LAMP-2A levels (corrected with β-Actin)
2.0
1.5
1.0
0.5
0.0
*
CT Unt
CT C646
CT
CT C646
MCI Unt
MCI C646
MCI
MCI C646
sAD Unt
sAD C646
sAD
sAD C646
NH4CL/ Leupeptin

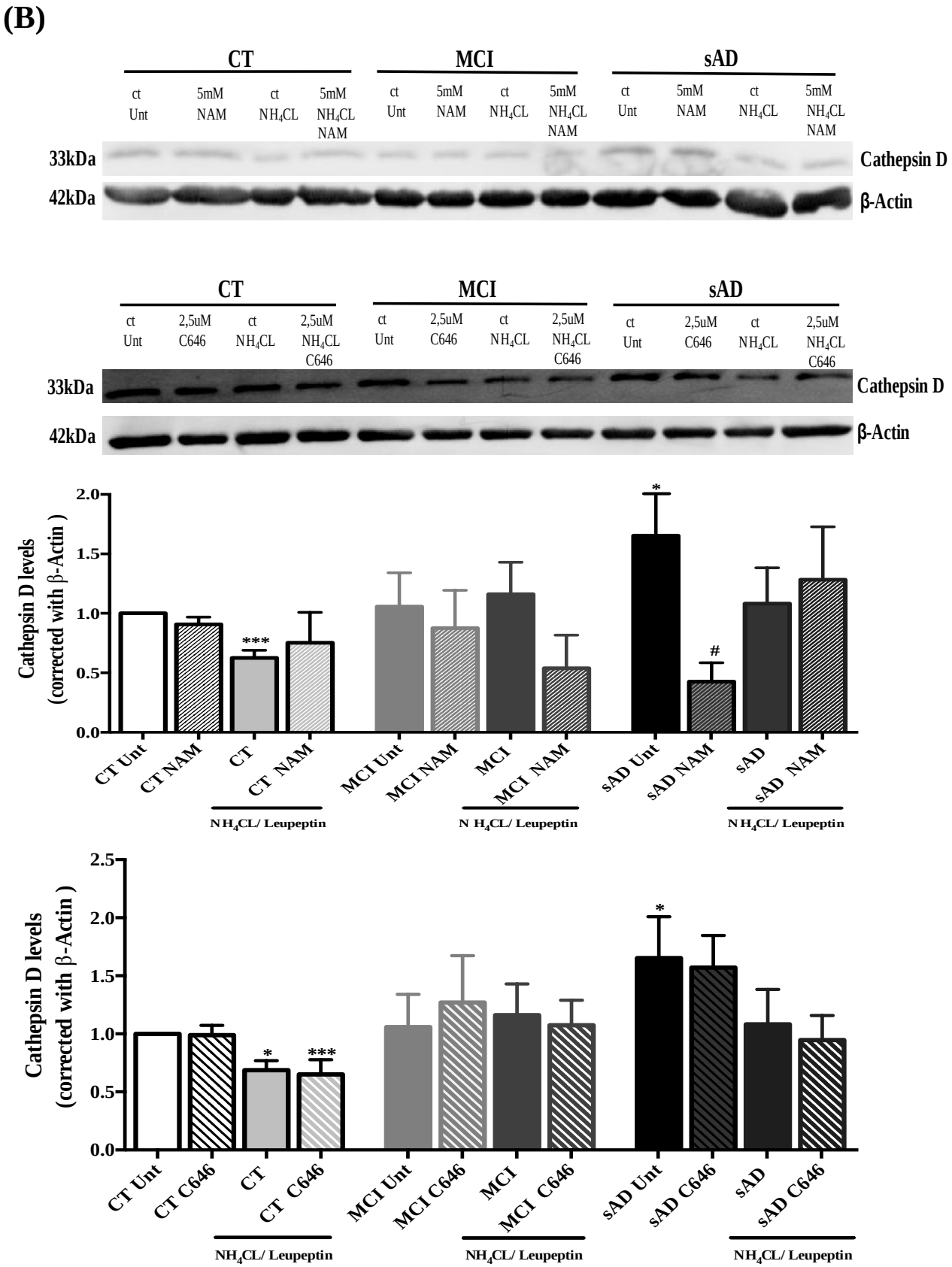


Figure 4:4 - **Lysosomes localization and function might be affected.** Immunoblotting for LAMP-2A protein levels for each separate cybrid group, untreated and treated with NH4CL/Leupeptin-NAM and NH4CL/Leupeptin-C646 (**A**). Immunoblotting for CatD protein levels for each separated cybrid group, untreated and treated with NH4CL/Leupeptin-NAM and NH4CL/Leupeptin-C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** indicates a $p < 0.001$ difference from the control CT cybrid cell line; # indicates that the ANOVA calculation itself was not statistically significant. Data were analyzed by Student's T-test and are presented as mean ± S.E.M.

4.5 Endo-lysosomal fusion: an essential key on autophagic flux

Finally, other pathway also extremely essential for cell biology is endo-lysosomal network, responsible for the transport and degradation of extracellular cargo. In mammalian, degradative endocytic pathway starts at the plasma membrane and ends in lysosomes. Endocytic pathway is responsible for controlling diverse cellular and physiological mechanisms, including the processing of extracellularly derived nutrients, regulation of activated surface receptors, maintenance of membranes homeostasis, as well as defense against external pathogens. Normally, internalized ligands are first delivered to endosomes and subsequently ligands or ligand-receptor complexes, are either delivered to lysosomes for degradation or recycled to the plasma membrane or Golgi complex (Cook *et al.*, 2014).

There are two important proteins frequently used to define LEs or MVBs and lysosomes, Rab7 and LAMP-1, respectively. Rab7, a small molecular weight G-protein, belongs to the Ras small GTPase superfamily and is involved in the maturation of autophagosomes directing the trafficking of cargos along microtubules, and in the fusion step with lysosomes (Vanlandingham and Ceresa, 2009).

Data have been suggesting that these proteins regulate not only membrane trafficking, but also cell signalling, cell growth, cell survival and development. Although Rab proteins and their associated regulators or effectors have been implicated in various disorders, including cancer, pigmentation and lipid metabolism disorders and neuropathy (Zhang *et al.*, 2009).

Rab7 expression has been described in hippocampal neurons during the progression of AD pathology, suggesting that dysregulation of a select Rab GTPase phenotype is a molecular pathogenic marker for neuronal dysfunction in other highly vulnerable regions of the brain, early in the disease process (Ginsberg, Alldred, *et al.*, 2010). Therefore, we decided first to evaluate the Rab7 protein levels in our cybrid cellular model. We observed an increase in sAD group comparing to MCI and CT groups, in accordance with what have been reported, an upregulation of Rab7 in AD ailment (Ginsberg, Mufson, *et al.*, 2010). We found that inhibitor NAM increases Rab7 levels only in sAD cybrid cell line. These findings suggest that an inhibition of SIRT1 deacetylase may lead to an increase of acetylation of FOXO1, a transcription factor important in the regulation of autophagy pathway, leading to an autophagy inhibition. FOXO1 and Rab7 interact with each other and an acetylation of FOXO1 can lead to higher levels of Rab7 protein (Bánréti, Sass and Graba, 2013). Although when p300 is inhibited occurs a decrease in sAD group comparing to CT and MCI groups (**Figure 4.5 A**). These results indicating that an acetylation of Beclin-1 may affect not only autophagy-lysosomal network, but also the endocytosis pathway, which compromise the degradation of toxic proteins, affecting neuronal cells, and leading to

neurodegenerative diseases.

LAMP-1 is a glycoprotein present in luminal side of lysosomes and serves as barrier to soluble cathepsins and hydrolases, preventing their liberation into cytoplasm. In fact, it is an abundant protein component of the lysosomal membrane bearing lysosomal properties such as enzyme activities, pH, osmotic stability, density, morphology, and subcellular distribution (Eskelinen, 2006).

Data have been reported that LAMP-1 mRNA expression levels and protein are increase in the cerebral cortex in AD pathology. Moreover, LAMP-1 protein is localized in the cytoplasm of neuronal cells, mainly those non-containing NFTs, and in glial cells surrounding SPs (Barrachina *et al.*, 2006). Additionally, it has been described that lysosomal disturbances may promote Aβ deposition in AD brains (Mathews *et al.*, 2002).

Therefore, we decided to analyze LAMP-1 protein levels in our cybrid cellular model.

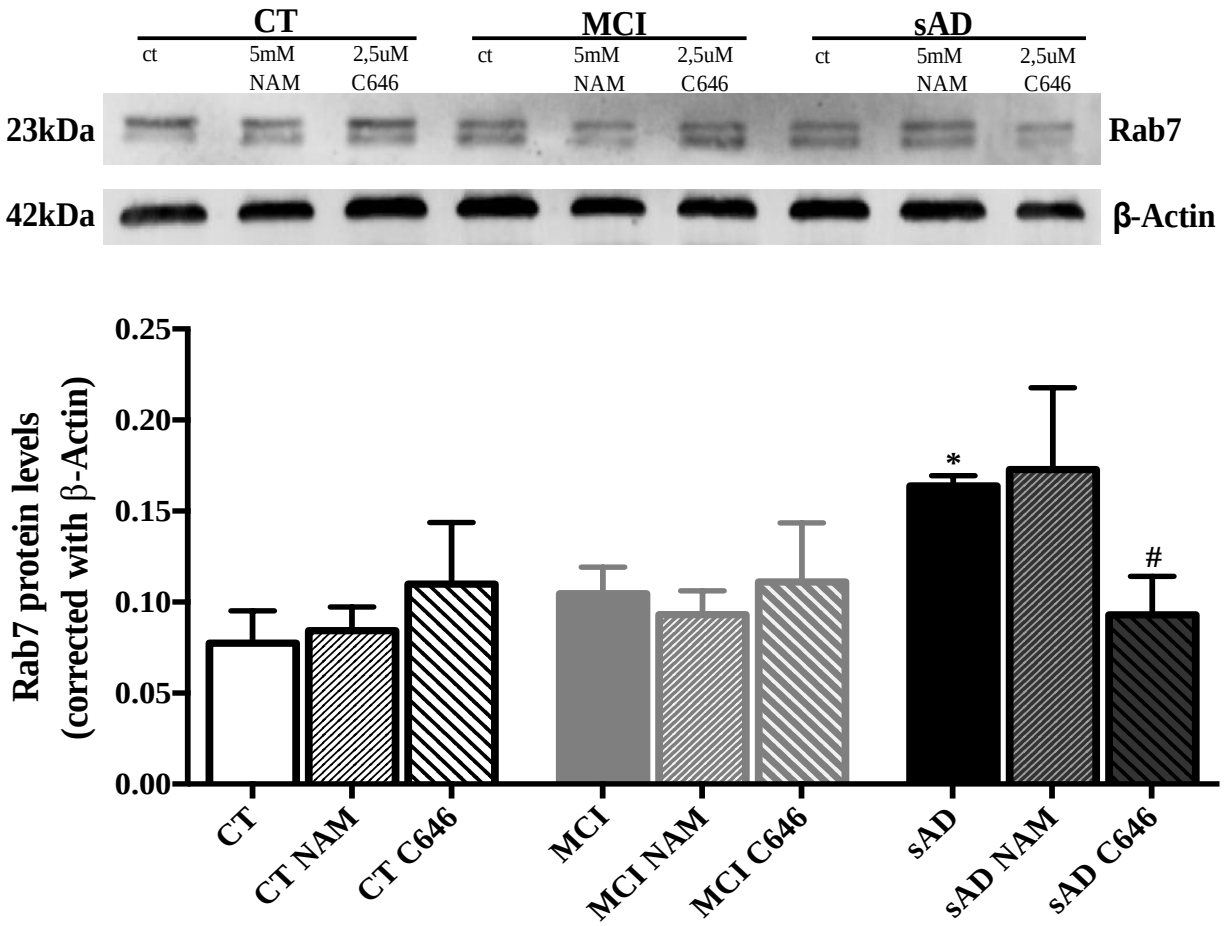
We observed that in sAD cells occurs an increase of basal LAMP-1 protein levels compared with MCI cells. These results suggest that a compensatory response occurs, in which cells are trying to eliminate AVs contents.

Additionally, an inhibition of SIRT1 deacetylase increases the formation of clusters in both MCI and sAD groups, showing once more the protective role of SIRT1 protein. However, an inhibition of p300 acetyltransferase, decreases the clusters size in both MCI and sAD cybrid cell lines (**Figure 4.5 B**).

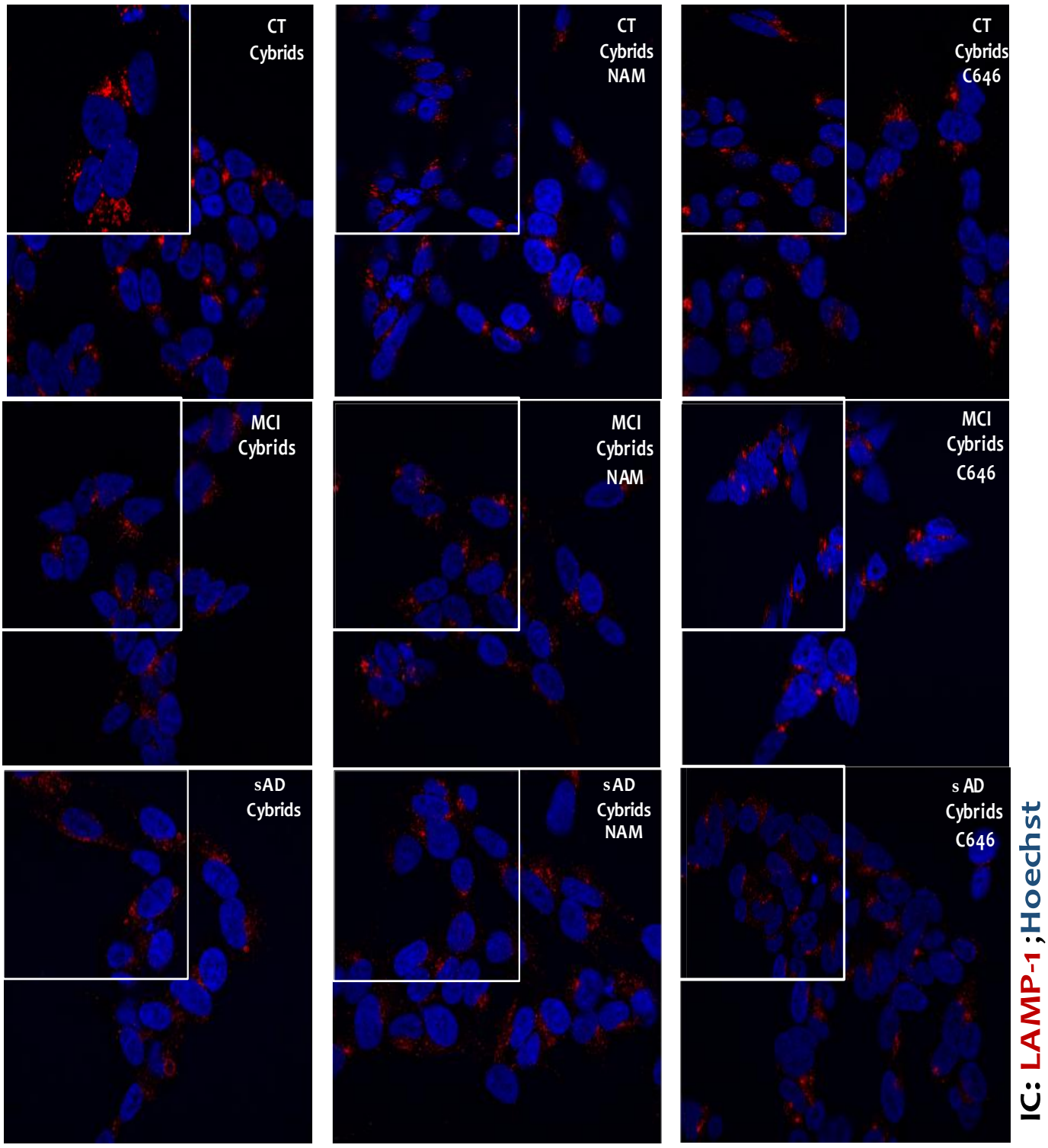
These observations are in accordance with our hypothesis, that acetylation event is not benefic in sAD cells, leading to a global impairment of autophagy-lysosomal and endocytic pathways.

Finally, is quite clear in our current study the effects of a deacetylated Beclin-1 by SIRT1 deacetylase, that plays a crucial protective role in both autophagy and lysosomal pathways in AD pathology.

(A)


CT
MCI
sAD
ct
5mM NAM
2,5uM C646
23kDa
Rab7
42kDa
β-Actin
Rab7 protein levels (corrected with β-Actin)
0.25
0.20
0.15
0.10
0.05
0.00
*
#
CT
CT NAM
CT C646
MCI
MCI NAM
MCI C646
sAD
sAD NAM
sAD C646

(B)


CT Cybrids
CT Cybrids NAM
CT Cybrids C646
MCI Cybrids
MCI Cybrids NAM
MCI Cybrids C646
sAD Cybrids
sAD Cybrids NAM
sAD Cybrids C646
IC: LAMP-1;Hoechst

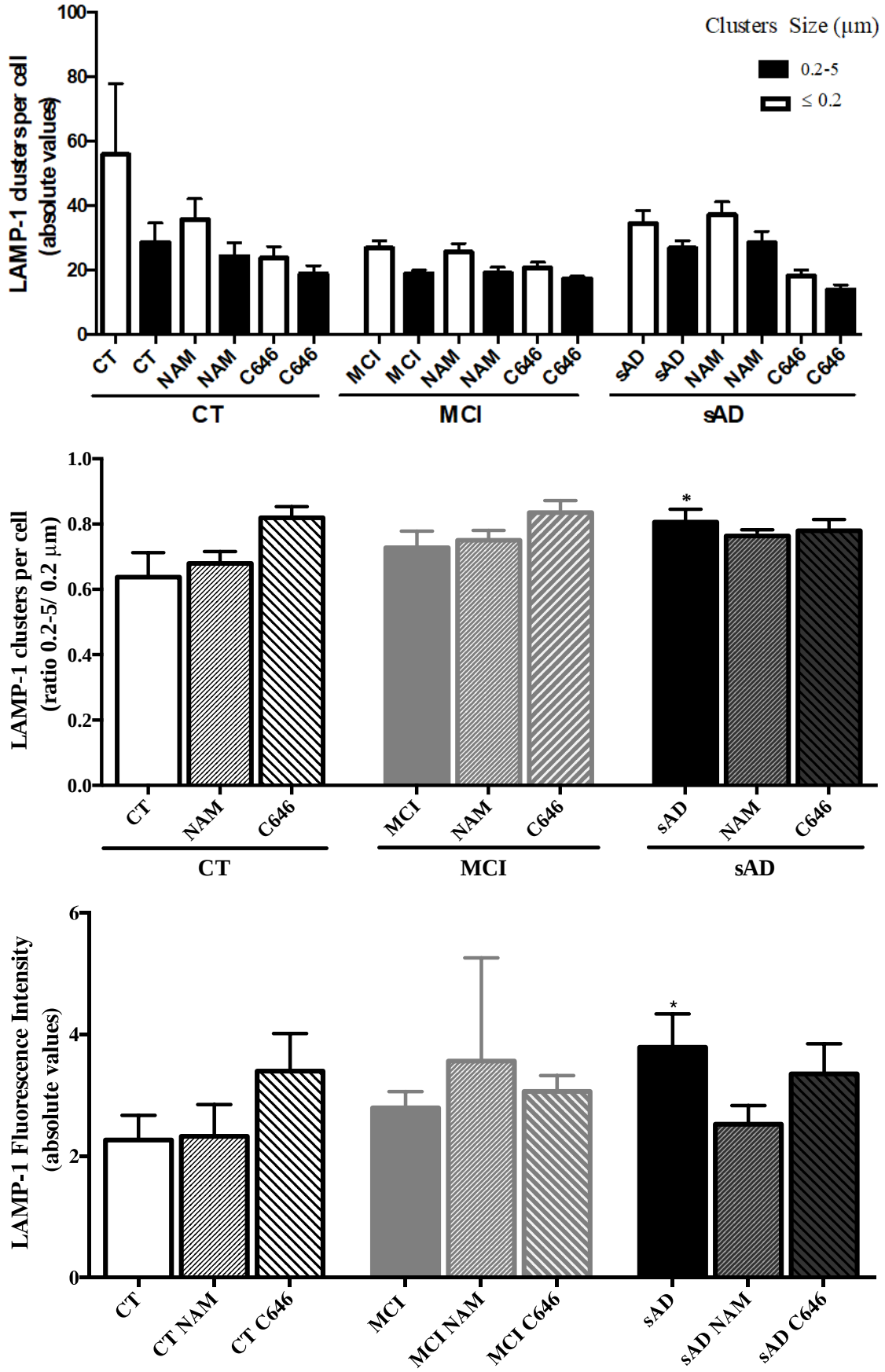


Figure 4:5 - **Endo-lysosomal fusion: an essential key on autophagic flux.** Immunoblotting for Rab7 protein levels for each separate cybrid group, treated with NAM and C646 (**A**). Immunofluorescence for LAMP-1protein levels for each separated cybrid group, treated with NAM and C646 (**B**). * indicates a $p < 0.05$ difference from the control CT cybrid cell line; *** and # indicates that the ANOVA calculation itself was not statistically significant. Data were analyzed by Student's T-test and are presented as mean ± S.E.M.

5 DISCUSSION

A growing number of evidences have indicated some irregularities at the level of autophagy induction/ autophagosomes formation, as well as at lysosomal dysfunction pathway, contributing to the AD pathogenesis (Sooyeon, Sato and Nixon, 2011; Orr and Oddo, 2013; Salminen *et al.*, 2013; Liang and Jia, 2014; Lumkwana *et al.*, 2017).

Therefore, we propose a mechanism that might be related to the impairment of autophagy pathway, described in AD. Our focus was to understand how a (de)acetylation of Beclin-1, a beginner protein involved in autophagic pathway- responsible for the protein quality control in our cells, particularly essential in neurons, can be affected, and consequently, decreases autophagy flow, leading to a lysosomal dysfunction.

In this study, the first step was to evaluate both roles of SIRT1 deacetylase and p300 acetyltransferase in our cybrid cell model from CT, MCI and sAD cybrid cell lines.

SIRT1 deacetylase is involved in regulating metabolic and aging processes in AD pathogenesis. Hence, it has received a widely attention in the last decades, representing a potential therapeutic target in neurodegenerative disorders. In addition, there is mounting evidence for a link between SIRT1 and AD malady. Also, SIRT1 protects against Aβ toxicity, inhibiting NF-κB signaling in microglia, which suggests that microglial NF-κB signalling may be critical in mediating the toxic effects of AD-related inflammatory responses (Chen *et al.*, 2005; Outeiro, Marques and Kazantsev, 2008). Data demonstrate that aging promotes the accumulation of toxic proteins and this process might be counteracted by anti-aging pathways. SIRTs may also regulate the level of these misfolded proteins by blocking their production or facilitating their removal (Gan and Mucke, 2008). For example, SIRT1 activation in mammalian neurons may promote α-secretase non-amyloidogenic pathway of APP processing, preventing Aβ production, down-regulate ROCK1 expression (Qin *et al.*, 2006).

Furthermore, on the autophagy machinery some evidences show that SIRT1 co-immunoprecipitated with Atg5, Atg7 and Atg8/LC3. All these proteins could be deacetylated by SIRT1 in a NAD^+-dependent manner *in vitro* (Ng and Tang, 2013).

According to mentioned studies, we expected a reduced basal p-SIRT1 levels in sAD cell lines, as detected, which may generate an insufficient or reduced autophagic activity, resulting in harmful proteins aggregates/ accumulation of damage organelles, such as mitochondria, lysosomes, among others. Our results clearly show that when SIRT1 deacetylase is inhibited, occurs an increase of Beclin-1 acetylation in sAD cybrid cell line, which may be responsible for an impairment of autophagic machinery, seen in AD ailment.

This matter is in synchrony with our hypothesis, proving, at the same time the neuroprotective role of SIRT1 protein in neurological disorders.

Afterwards, we noticed an increase of acetylated Beclin-1 basal levels in our sAD cybrid cell model, which may be a part of the source of a compromised autophagy-lysosomal pathway in AD. At same time, a significantly increase of acetylated Beclin-1 occurs more specifically in sAD group, when inhibited SIRT1deactylase. On the other hand, a decrease of acetylated Beclin-1 happens in sAD group, when p300 acetyltransferase is inhibited, being in accordance with our theory.

Acetylation is one of the most important post-translational protein modifications in the cell (Drazic *et al.*, 2016), with a main role in several biological processes, such as transcriptional regulation, DNA damage repair, aging, cell cycle progression and glycolysis (Yi and Yu, 2012). Protein acetylation is an important regulatory mechanism, tightly regulated itself in response to metabolism changes, contributing to control of autophagy pathway. However, the role of acetylation in autophagy remains unclear.

Another key protein in the autophagy machinery is LC3, a protein essential for autophagosome biogenesis, including formation, expansion, and cargo recruitment. In our data, we observed that in sAD cybrid cell lines untreated, a significantly decreased in autophagic flux occurs.

Nevertheless, a slight increase in autophagic flux arises, when we used C646 in sAD cybrid cell line, suggesting that a deacetyled Beclin-1 by SIRT1 may normalize autophagy pathway flow in AD disorder. Moreover, SIRT1 deacetylase is essential for LC3 translocation to shift its distribution from the nucleus to cytoplasm during cell starvation (Huang and Liu, 2015).

Simultaneously, p62 protein is used as a marker for autophagic flux, linking physically autophagic cargo to the autophagic membrane (Pugsley, 2017). p62 is localized to ubiquitin-positive inclusions bodies, especially in liver and brain, a common phenomenon that can be observed in various diseases, such as neurodegeneration; and into membrane-confined autophagosomal and lysosomal structures. In this manner, p62 relates to the formation of ubiquitin-positive inclusions bodies and binds LC3II to promote autophagic degradation (Liu *et al.*, 2016). Additionally, immunostaining shows that p62 accumulates in early stages of NFTs formation in AD patients (Kuusisto, Salminen and Alafuzoff, 2002).

Our findings show a significantly decrease of p62 flux levels in both MCI and sAD untreated cybrid cell lines. Besides, when p300 is inhibited an extreme increase of p62 flux occurs in sAD group, confirming that an acetylation of Beclin-1 may compromise autophagy pathway in AD pathology. As well, we demonstrated that accumulation of p62 protein in conjugation with a reduced autophagic flux designate an impairment of autophagic clearance in AD.

As a matter of fact, aberrant clearance of lysosomal substrates, including accumulation of AVs, has been noted in AD patients, reflecting a defect related with the progression of AD condition (Steele *et al.*, 2013). Additionally, persuasive evidences have supported that autophagy dysfunction in AD may occurs due to defective lysosomal clearance (Yang *et al.*, 2011).

It is well known that autophagy decreases with age, underlining the possibility that a compromised autophagic activity with aging process, may contribute to develop age-related disorders, such as neurodegeneration and metabolic defects (Martinez-lopez, Athonvarangkul and Singh, 2015). One common forms of autophagy pathway, CMA is responsible to ensure cellular homeostasis through the removal of damaged/malfunctioning intracellular proteins, and gradual loss of CMA functions, as well the manifestations of aging phenotypes have been observed in physiological aging process (Zhang and Cuervo, 2008; Arias and Cuervo, 2011). However, pathological aging is related with severe CMA failure, which has been described in many age-related disorders, such as neurodegenerative diseases. This CMA dysfunction may occur due to different facets of alterations in LAMP-2A level or functions (Wong, 2018).

LAMP-2A is described as a receptor for CMA substrates, where substrate proteins bind to monomers of LAMP-2A at the lysosomal membrane, creating multimeric complexes required for substrate translocation into lysosomes (Orenstein and Cuervo, 2010). It has been reported that LAMP-2A deteriorates during aging in lysosomes due to post-transcriptional changes and not because of aging process itself (Salminen and Kaarniranta, 2009).

In spite of, not all lysosomal pathways of proteolysis are equally affected by age (Cuervo and Dice, 2000). The rate of autophagy decreases with aging, which compromise both CMA and further LAMP-2A. Many neurodegenerative pathologies-linked pathogenic proteins that are substrates of CMA. Basically, these toxic proteins interact in aberrantly manner with CMA components during their degradation, leading to CMA blockage. Such CMA malfunction compromises LAMP-2A, resulting an alteration of dynamics in the trafficking and recycling of LAMP-2A from the lumen to the lysosomal membrane, as occurs in neurodegenerative diseases (Sjödin *et al.*, 2016). It is well known that the lysosomal system is altered in AD almaint. Furthermore, a broad range of lysosomal network proteins were considered in the CSF from AD patients, such as LAMP-1, LAMP-2A, Rab3 and Rab7, which found to be significantly increased, when compared with age-matched controls (Armstrong *et al.*, 2014).

Our results show increased levels of LAMP-2A in untreated sAD cybrid cell line. These findings are in accordance with a plausible CMA decrease rate and a lysosomal dysfunction, already described in AD pathology (Koga, Kaushik and Cuervo, 2011).

More interestingly, an inhibition of SIRT1 have an advantageous effect in LAMP-2A expression in treated MCI cybrids-prodromal phase, when compared with treated sAD cybrid cell

lines. Basically, this evidence emphasizes the SIRT1 deacetylase protective role in LAMP-2A expression in MCI group, which alternativetely not occurs after the condition is installed, in sAD group.

However, an inhibition of p300 decreases LAMP-2A levels in treated sAD group, comparing with treated MCI group. These findings support, that acetylation process is a critically important mechanism for metabolic network. Consequently, is quite clear that a deacetylation of Beclin-1 by SIRT1 protein, appears to have a good positive impact in LAMP-2A expression, as well in both autophagic and lysosomal pathways, specifically in AD pathology. Although, upon inhibition of acetylation the degradation of metabolic enzymes recognized by CMA into lysosome might be affected (Xiong and Guan, 2012). For this reason, more experimental studies are needed to describe in detail our hypothesis.

On the other hand, an increased and altered intracellular distributions of lysosomal hydrolases have been seen in many degenerated neurons, especially in brain areas known to become affected in AD condition (Cataldo *et al.*, 1991). CatD is a soluble lysosomal aspartic endopeptidase, essential in numerous physiological functions, including metabolic degradation of intracellular proteins, activation and degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, brain antigen processing and regulation of programmed cell death (Benes, Vetvicka and Fusek, 2008). An abnormal immunoreactivity of CatD in SPs of AD patients has been reported (Cataldo and Nixon, 1990; Cataldo, Hamilton and Nixon, 1994). CatD has been associated with important factors of AD pathogenesis, such as APP (Ladror *et al.*, 1994), APOE (Zhou *et al.*, 2006) and Tau protein (Kenessey *et al.*, 1997) . However, is described that CatD is not essential for APP processing in CatD knock-out mice (Saftig *et al.*, 1996). It is necessary to understand whether the processing of both APOE and Tau proteins by CatD in the brain must be pathologically relevant.

Some studies have been described that CatD polymorphisms (Touitou *et al.*, 1994), have impact in intracellular routing and maturation of the proenzyme, being associated with AD pathogenesis (Ntais, Polycarpou and Ioannidis, 2004). Moreover, an impairment of lysosomal pathway has been demonstrated to occur in early stage of AD pathology, before the robust accumulation of NFTs and SPs. Our data shows that CatD protein levels in untreated sAD cells are significantly increased comparing with both CT and MCI cells. Although, when p300 is inhibited CatD levels decrease in sAD treated cybrid cell line. These results suggesting that acetylated Beclin-1 may compromise the lysosome organelle, affecting CatD enzyme proper function, leading to an extreme increase of levels expression.

In addition, our theory reveals that a deacetylation of Beclin-1 plays a cytoprotective role in lysosomal pathway. Therefore, an upregulation of CatD protein expression in AD condition, may

represent a compensatory protective mechanism to counteract the autophagic-lysosomal deficit of the neurons in eliminating toxic protein aggregates.

Abnormalities and progressive dysfunction of the endosomal-lysosomal network have been described as a signature feature of AD malady. Lysosomes become dysfunctional as a reflection by their enlargement as they accumulate autophagic and endocytic substrates (Nixon, 2017) .

Rab7 is commonly viewed as a late endosome maker protein and involved in multiple processes, including early-to-late endosome transition, biogenesis of lysosomes, transport of autophagosomes to endosomes/ lysosomes, and vacuole fusion (Wen *et al.*, 2017).

Gutierrez *et al.* (Gutierrez *et al.*, 2004) have described the function of Rab7 protein for the progression of autophagy in mammalian cells (Gutierrez *et al.*, 2004). Although, an expression of a constuctively active form of Rab7 results in the formation of large perinuclear lysosome clusters, while dominant- negative Rab7 disperses lysosomes throughout the cytosol (Bucci *et al.*, 2000). Interestingly, in basal forebrain, frontal cortex and hippocampus of MCI and AD patients, *Rab7* gene expression and proteins levels have been reported to be upregulated (Ginsberg, Mufson, *et al.*, 2010). Lately, Rab7 protein levels were described to be increased in CSF of AD patients. Also, a new study reports that an increase of Rab7 in AD condition can enhance secretion of Tau protein (Rodriguez *et al.*, 2017) . During our study, we observed an increase of Rab7 basal levels in sAD cybrid cell line, as expected. Additionally, further inhibition of SIRT1 over-increases Rab7 protein levels in sAD group. However, when we used C646, a decrease of Rab7 protein levels occurs, especially in sAD cells. These results are in accordance with our theory, that a deacetylated Beclin-1 by SIRT1 deacetylase influences in a positive manner, regulating autophagy-lysosomal and endocytic pathways.

Another, not less important protein is LAMP-1, a marker of lysosomes. It was shown that LAMP-1 protein levels are increased in AD brains, but its expression is inversely correlated with hyperphosphorylated Tau deposition in individual neurons with NFTs, one of the hallmarks of AD ailment (Wang *et al.*, 2016).

Additionally, we observed an increase of LAMP-1 basal expression in sAD cybrid cell line, as expected indicating a feedback mechanism to facilitate autophagosome clearance. Curiously, when we inhibited p300, a decrease of LAMP-1 clusters size in sAD group occurs, suggesting that acetylation process may be implicate in autophagy-lysosomal dysfunction.

Furthermore, is clear that a deacetylation of Beclin-1 influences the improvement of both autophagy and lysosomal pathways in AD pathology. SIRT1 has protective role in different pathological conditions, especially in neurodegenerative diseases and its potential therapeutic effect in different pathways, may be a future focus on neurosciences.

6 CONCLUSION AND FUTURE PERSPECTIVES

For decades, autophagy has been considered as an active death pathway, but only recently its cell survival functions have been underlined. Autophagy plays a crucial role in AD pathology. It is conceived that activation of autophagy may promote neuronal survival in physiological and pathological situations under a strictly regulated process, suggesting that increasing levels of Beclin-1 by gene therapy or by a drug acting at this level, could be a promising strategy. Our results clearly indicate that SIRT1 deacetylase mediated deacetylation of Beclin-1 may be a feasible strategy to overcame the defective autophagy-lysosomal pathway observed in AD condition.

During these years, several studies have indicated that pharmacological induction of autophagy can be beneficial for treatment of neurodegenerative diseases, and increased autophagy has been shown to ameliorate pathology in various disease models by enhancing the clearance of intracytoplasmic protein aggregates, including hyperphosphorylated Tau protein.

As a complex group of diseases, scientists are focus to understand how the nervous system works at the molecular and genetic levels, helping to develop more effective treatments for these maladies. However, medicines treat the symptoms of these pathologies, but cannot slow, prevent or reverse the progressive deterioration.

We identify in this study, that a deacetylation of Beclin-1 in sAD cybrid cell line can improve the autophagy pathway flux, as well as the maturation of autophagosomes and finally lysosomal network. We show for the first time, in sAD cells, that acetylation-dependent regulatory mechanism can compromise autophagosome maturation and later lysosome compartment.

Further work is needed to clarify in detail these mechanisms responsible for an autophagy-lysosomal pathway dysfunction described in AD context.

In summary, our current study on Beclin-1, indicates that acetylation, a post-translational modification, can occur at different stages of autophagy pathway. However, under a variety of conditions, autophagic response may be induced, inhibited or fine-tuning.

Our research found a new therapeutic target, Beclin-1 since acetylation process is responsible for perturbing autophagy-lysosomal pathway, leading to an accumulation of autophagosomes, as well, inhibiting its maturation and promoting autophagic dysfunction in AD ailment. This approach allows for an intervention up-stream in the events leading to AD pathogenesis.

REFERENCES

Alers, S., Loffler, A. S., Wesselborg, S. and Stork, B. (2012) 'Role of AMPK-mTOR-Ulk1 / 2 in the Regulation of Autophagy : Cross Talk, Shortcuts, and Feedbacks', *Molecular and Cellular Biology*, 32(1), pp. 2–11. doi: 10.1128/MCB.06159-11.

Alzheimer, A. (1991) 'Uber eigenartige Krankheitsfälle des späteren Alters (On certain peculiar diseases of old age)', *History of Psychiatry*, 2(5), pp. 74–101. doi: 10.1177/0957154X9100200506.

Anastasiou, D. and Krek, W. (2006) 'SIRT1: Linking Adaptive Cellular Responses to Aging-Associated Changes in Organismal Physiology', *Physiology*, 21(6), pp. 404–410. doi: 10.1152/physiol.00031.2006.

Anderson, K. A., Huynh, F. K., Fisher-Wellman, K., Stuart, J. D., Peterson, B. S., Douros, J. D., Wagner, G. R., Thompson, J. W., Madsen, A. S., Green, M. F., Sivley, R. M., Ilkayeva, O. R., Stevens, R. D., Backos, D. S., Capra, J. A., Olsen, C. A., Campbell, J. E., Muoio, D. M., Grimsrud, P. A. and Hirschey, M. D. (2017) 'SIRT4 Is a Lysine Deacylase that Controls Leucine Metabolism and Insulin Secretion', *Cell Metabolism*. Elsevier Inc., 25(4), pp. 838–855. doi: 10.1016/j.cmet.2017.03.003.

Ansari, A., Rahman, M. S., Saha, S. K., Saikot, F. K., Deep, A. and Kim, K. H. (2017) 'Function of the SIRT3 mitochondrial deacetylase in cellular physiology, cancer, and neurodegenerative disease', *Aging Cell*, 16(1), pp. 4–16. doi: 10.1111/acel.12538.

Appelqvist, H., Wa, P. W., Kagedal, K. and Ollinger, K. (2013) 'The lysosome : from waste bag to potential therapeutic target', *Journal of Molecular Cell Biology*, 5, pp. 214–226.

Arduino, D. M. (2012) *Mitochondria and Protein Homeostasis in Parkinson's Disease: From quality control pathways to an integrated network*. Universidade de Coimbra.

Arias, E. and Cuervo, A. M. (2011) 'Chaperone-mediated autophagy in protein quality control', *Current Opinion in Cell Biology*. Elsevier Ltd, 23(2), pp. 184–189. doi: 10.1016/j.ceb.2010.10.009.

Armstrong, A., Mattsson, N., Appelqvist, H., Janefjord, C., Sandin, L., Agholme, L., Olsson, B., Svensson, S., Blennow, K., Zetterberg, H. and Kågedal, K. (2014) 'Lysosomal network proteins as potential novel CSF biomarkers for Alzheimer's disease', *NeuroMolecular Medicine*, 16(1), pp. 150–160. doi: 10.1007/s12017-013-8269-3.

Armstrong, R. A. (2013) 'What causes alzheimer's disease?', *Folia Neuropathologica*, 51(3), pp. 169–188. doi: 10.5114/fn.2013.37702.

Avila, J., Lucas, J. J., Perez, M. and Hernandez, F. (2004) 'Role of Tau Protein in Both Physiological and Pathological Conditions', *Physiological Reviews*, 84(2), pp. 361–384. doi: 10.1152/physrev.00024.2003.

Axe, E. L., Walker, S. A., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., Griffi, G. and Ktistakis, N. T. (2008) 'Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum',

The Journal of Cell Biology, 182(4), pp. 685–701. doi: 10.1083/jcb.200803137.

Ballabio, A. (2016) 'The awesome lysosome', *EMBO Molecular Medicine*, 8(2), pp. 73–76. doi: 10.15252/emmm.201505966.

Ballatore, C., Lee, V. M. Y. and Trojanowski, J. Q. (2007) 'Tau-mediated neurodegeneration in Alzheimer's disease and related disorders', *Nature Reviews Neuroscience*, 8(9), pp. 663–672. doi: 10.1038/nrn2194.

Bandyopadhyay, D., Cyphersmith, A., Zapata, J. A., Kim, Y. J. and Payne, C. K. (2014) 'Lysosome transport as a function of lysosome diameter', *PLoS ONE*, 9(1), pp. 18–23. doi: 10.1371/journal.pone.0086847.

Bánréti, Á., Sass, M. and Graba, Y. (2013) 'The emerging role of acetylation in the regulation of autophagy', *Autophagy*, 9(6), pp. 819–829. doi: 10.4161/auto.23908.

Barrachina, M., Maes, T., Buesa, C. and Ferrer, I. (2006) 'Lysosome-associated membrane protein 1 (LAMP-1) in Alzheimer's disease', *Neuropathology and Applied Neurobiology*, 32(5), pp. 505–516. doi: 10.1111/j.1365-2990.2006.00756.x.

Beau, I., Mehrpour, M. and Codogno, P. (2011) 'Autophagosomes and human diseases', *The International Journal of Biochemistry & Cell Biologynternational Journal of Biochemistry and Cell Biology*. Elsevier Ltd, 43(4), pp. 460–464. doi: 10.1016/j.biocel.2011.01.006.

Benes, P., Vetvicka, V. and Fusek, M. (2008) 'Cathepsin D - Many Functions of One Aspartic Protease', *Crit Review Oncol Hematol.*, 68(1), pp. 12–28. doi: 10.1016/j.critrevonc.2008.02.008.CATHEPSIN.

Bergamini, E., Cavallini, G., Donati, A. and Gori, Z. (2007) 'The Role of Autophagy in Aging Its Essential Part in the Anti-Aging Mechanism of Caloric Restriction', *Annals of the New York Academy of Sciences*, 1114, pp. 69–78. doi: 10.1196/annals.1396.020.

Bertram, L., Lill, C. M. and Tanzi, R. E. (2010) 'The genetics of alzheimer disease: Back to the future', *Neuron*. Elsevier Inc., 68(2), pp. 270–281. doi: 10.1016/j.neuron.2010.10.013.

Binder, L. I., Guillozet-Bongaarts, A. L., Garcia-Sierra, F. and Berry, R. W. (2005) 'Tau, tangles, and Alzheimer's disease', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1739(2), pp. 216–223. doi: 10.1016/j.bbadis.2004.08.014.

Blander, G. and Guarente, L. (2004) 'The Sir2 family of protein deacetylases', *Annual Review of Biochemistry*, 73, pp. 417–435.

Bolanda, B., Kumara, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H. and Nixon, R. A. (2008) 'Autophagy Induction and Autophagosome Clearance in Neurons: Relationship to Autophagic Pathology in Alzheimer's Disease', *Journal of Neuroscience*, 28(27), pp. 6926–6937. doi: 10.1523/JNEUROSCI.0800-08.2008.

Boya, P., Gonzalez-Polo, R.-A., Casares, N., Perfettini, J.-L., Dessen, P., Larochette, N., Métivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P. and Kroemer, G. (2005) 'Inhibition of Macroautophagy Triggers Apoptosis', *Molecular and Cellular Biology*, 25(3), pp. 1025–1040. doi: 10.1128/MCB.25.3.1025.

Braidy, N., Jayasena, T., Poljak, A. and Sachdev, P. S. (2012) 'Sirtuins in cognitive ageing and Alzheimer's disease', *Current Opinion in Psychiatry*, 25(3), pp. 226–230. doi: 10.1097/YCO.0b013e32835112c1.

Bucci, C., Thomsen, P., Nicoziani, P., Mccarthy, J. and Deurs, B. Van (2000) 'Rab7: A Key to Lysosome Biogenesis.pdf', *Molecular biology of the cell*, 11, pp. 467–480.

Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A. and Hof, P. R. (2000) 'Tau protein isoforms, phosphorylation and role in neurodegenerative disorders', *Brain Research Reviews*, 33(1), pp. 95–130. doi: 10.1016/S0165-0173(00)00019-9.

Caballero, B. and Coto-Montes, A. (2012) 'An insight into the role of autophagy in cell responses in the aging and neurodegenerative brain Histology and', *Histology and Histopathology*, 27(3), pp. 263–275. doi: 10.14670/HH-27.263.

Caccamo, A., Ferreira, E., Branca, C. and Oddo, S. (2017) 'p62 improves AD-like pathology by increasing autophagy', *Mol Psychiatry*, 22(6), pp. 865–873. doi: 10.1016/j.ejps.2014.09.005.

Camandola, S. and Mattson, M. P. (2017) 'Brain metabolism in health, aging, and neurodegeneration', *The EMBO Journal*, 36(11), pp. 1474–1492. doi: 10.15252/embj.201695810.

Candeias, E., Duarte, A. I., Sebastião, I., Fernandes, M. A., Plácido, A. I., Carvalho, C., Correia, S., Santos, R. X., Seiça, R., Santos, M. S., Oliveira, C. R. and Moreira, P. I. (2017) 'Middle-Aged Diabetic Females and Males Present Distinct Susceptibility to Alzheimer Disease-like Pathology', *Molecular Neurobiology*. Molecular Neurobiology, 54(8), pp. 6471–6489. doi: 10.1007/s12035-016-0155-1.

Canto, C. and Auwerx, J. (2009) 'PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure', *Current Opinion in Lipidology*, 20(2), pp. 98–105. doi: 10.1097/MOL.0b013e328328d0a4.

Cantó, C. and Auwerx, J. (2012) 'Targeting SIRT1 to improve metabolism : all you need is NAD+ ?', *Pharmacol Reviews*, 64(1), pp. 166–187. doi: 10.1124/pr.110.003905.

Cao, Y. and Klionsky, D. J. (2007) 'Physiological functions of Atg6 / Beclin 1 : a unique autophagy-related protein', *Cell Research*, 17, pp. 839–849. doi: 10.1038/cr.2007.78.

Cardoso, S. M. (2015) 'Special Focus on Autophagy', *DNA and Cell Biology*, 34(4), p. 2015. doi: 10.1089/dna.2015.1501.

Carmona-Gutierrez, D., Hughes, A. L., Madeo, F. and Ruckenstuhl, C. (2016) 'The crucial impact of lysosomes in aging and longevity', *Ageing Research Reviews*, 32, pp. 2–12. doi: 10.1016/j.arr.2016.04.009.

Castellani, R. J., Rolston, R. K. and Smith, M. A. (2010) 'Alzheimer Disease', *Disease a Month*, 56(9), pp. 484–546. doi: 10.1016/j.disamonth.2010.06.001.

Castino, R. and Isidoro, C. (2008) 'The Transport of soluble lysosomal hydrolases from the Golgi complex to lysosomes', in Mironov, A. and Pavelka, M. (eds) *The Golgi Apparatus State of the art 110 years after Camillo Golgi's discovery*. 2008th edn. Springer, p. 716.

Cataldo, A. M., Barnett, J. L., Berman, S. A., Li, J., Quarless, S., Bursztajn, S., Lippa, C. and

Nixon, R. A. (1995) ‘Gene Expression and Cellular Content of Cathepsin D in Alzheimer’s Disease Brain: Evidence for Early Up-Regulation of the EndosomaI-Lysosomal System’, *Neuron*, 14, pp. 671–680. doi: 10.1016/0896-6273(95)90324-0.

Cataldo, A. M., Hamilton, D. J. and Nixon, R. A. (1994) ‘Lysosomal abnormalities in degenerating neurons link neuronal compromise to senile plaque development in Alzheimer disease’, *Brain Research*, 640(1–2), pp. 68–80. doi: 10.1016/0006-8993(94)91858-9.

Cataldo, A. M. and Nixon, R. A. (1990) ‘Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain’, *Proceedings of the National Academy of Sciences of the United States of America*, 87, pp. 3861–3865. doi: 10.1073/pnas.87.10.3861.

Cataldo, A. M., Paskevich, P. A., Kominami, E. and Nixon, R. A. (1991) ‘Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease.’, *Proceedings of the National Academy of Sciences of the United States of America*, 88, pp. 10998–1002. doi: 10.1073/pnas.89.6.2509a.

Cataldo, A. M., Petanceska, S., Terio, N. B., Peterhoff, C. M., Durham, R., Mercken, M., Mehta, P. D., Buxbaum, J., Haroutunian, V. and Nixon, R. A. (2004) ‘Aβ localization in abnormal endosomes: Association with earliest Aβ elevations in AD and Down syndrome’, *Neurobiology of Aging*, 25(10), pp. 1263–1272. doi: 10.1016/j.neurobiolaging.2004.02.027.

Caughey, B. and Lansbury, P. T. (2003) ‘PROTOFIBRILS,PORES,FIBRILS, AND NEURODEGENERATION:Separating the Responsible Protein Aggregates from The Innocent Bystanders’, *Annual Review of Neuroscience*, 26(1), pp. 267–298. doi: 10.1146/annurev.neuro.26.010302.081142.

Van Cauwenberghe, C., Van Broeckhoven, C. and Sleegers, K. (2016) ‘The genetic landscape of Alzheimer disease: Clinical implications and perspectives’, *Genetics in Medicine*, 18(5), pp. 421–430. doi: 10.1038/gim.2015.117.

Chang, H. C. and Guarente, L. (2014) ‘SIRT1 and other sirtuins in metabolism’, *Trends in Endocrinology and Metabolism*. Elsevier Ltd, 25(3), pp. 138–145. doi: 10.1016/j.tem.2013.12.001.

Chang, J. R., Ghafouri, M., Mukerjee, R., Bagashev, A., Chabrashvili, T. and Sawaya, B. E. (2011) ‘Role of p53 in neurodegenerative diseases’, *Neurodegenerative Diseases*, 9(2), pp. 68–80. doi: 10.1159/000329999.

Chen, B., Retzlaff, M., Roos, T. and Frydman, J. (2011) ‘Cellular Strategies of Protein Quality Control’, *Cold Spring Harbor perspectives in biology*, 3(8), pp. 1–14. doi: 10.1101/cshperspect.a004374.

Chen, G.-F., Xu, T.-H., Yan, Y., Zhou, Y.-R., Jiang, Y., Melcher, K. and Xu, H. E. (2017) ‘Amyloid beta: Structure, biology and structure-based therapeutic development’, *Acta Pharmacologica Sinica*. Nature Publishing Group, 38(9), pp. 1205–1235. doi: 10.1038/aps.2017.28.

Chen, J., Zhou, Y., Mueller-Steiner, S., Chen, L.-F., Kwon, H., Yi, S., Mucke, L. and Gan, L. (2005) ‘SIRT1 protects against microglia-dependent amyloid-β toxicity through inhibiting NF-κB signaling’, *Journal of Biological Chemistry*, 280(48), pp. 40364–40374. doi:

10.1074/jbc.M509329200.

Chen, L.-F., Fischle, W., Verdin, E. and Greene, W. C. (2001) 'Duration of nuclear NF-κB action regulated by reversible acetylation', *Science*, 293(5535), pp. 1653–1657. doi: 10.1126/science.1062374.

Ciechanover, A. and Kwon, Y. T. (2015) 'Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies', *Experimental & Molecular Medicine*. Nature Publishing Group, 47, p. e147. doi: 10.1038/emm.2014.117.

Citron, M. (2010) 'Alzheimer's disease: strategies for disease modification', *Nature Reviews Drug Discovery*. Nature Publishing Group, 9, pp. 387–98. doi: 10.1038/nrd2896.

Codogno, P. and Meijer, A. J. (2005) 'Autophagy and signaling : their role in cell survival and cell death', *Cell Death and Differentiation*, 12, pp. 1509–1518. doi: 10.1038/sj.cdd.4401751.

Cook, C., Stankowski, J. N., Carlomagno, Y., Stetler, C. and Petrucelli, L. (2014) 'Acetylation: A new key to unlock tau's role in neurodegeneration', *Alzheimer's Research and Therapy*, 6(29), pp. 1–8. doi: 10.1186/alzrt259.

Coutts, A. S. and La Thangue, N. B. (2016) 'Regulation of actin nucleation and autophagosome formation', *Cellular and Molecular Life Sciences*. Springer International Publishing, 73(17), pp. 3249–3263. doi: 10.1007/s00018-016-2224-z.

Cuervo, A. M. and Dice, J. F. (2000) 'Age-related decline in chaperone-mediated autophagy', *Journal of Biological Chemistry*, 275(40), pp. 31505–31513. doi: 10.1074/jbc.M002102200.

Cuervo, A. M. and Wong, E. (2014) 'Chaperone-mediated autophagy : roles in disease and aging', *Nature Publishing Group*. Nature Publishing Group, 24(1), pp. 92–104. doi: 10.1038/cr.2013.153.

Della-Morte, D., Dave, K. R., DeFazio, R. A., Bao, Y. C., Raval, A. P. and Perez-Pinzon, M. A. (2009) 'Resveratrol Pretreatment Protects Rat Brain from Cerebral Ischemic Damage via a SIRT1-UCP2 Pathway', *Neuroscience*, 159(3), pp. 993–1002. doi: 10.1016/j.neuroscience.2009.01.017.

Denu, J. M. (2003) 'Linking chromatin function with metabolic networks: Sir2 family of NAD+-dependent deacetylases', *Trends in Biochemical Sciences*, 28(1), pp. 41–48. doi: 10.1016/S0968-0004(02)00005-1.

Deretic, V. (2008) *Autophagosome and Phagosome*. Edited by J. M. Walker.

Domenico, F. Di, Tramutola, A. and Perluigi, M. (2016) 'Cathepsin D as a therapeutic target in Alzheimer's disease', *Expert Opinion on Therapeutic Targets*. Taylor & Francis, 20(12), pp. 1393–1395. doi: 10.1080/14728222.2016.1252334.

Drazic, A., Myklebust, L. M., Ree, R. and Arnesen, T. (2016) 'The world of protein acetylation', *Biochimica et Biophysica Acta - Proteins and Proteomics*. The Authors, 1864(10), pp. 1372–1401. doi: 10.1016/j.bbapap.2016.06.007.

Du, Y., Wooten, M. C., Gearing, M. and Wooten, M. W. (2009) 'Age-Associated Oxidative Damage to the p62 Prmoter: Implications for Alzheimer's Disease', *Free Radic Biol Med*, 46(4), pp. 492–501. doi: 10.1016/j.freeradbiomed.2008.11.003.

Du, Y., Wooten, M. C. and Wooten, M. W. (2009) 'Oxidative damage to the promoter region of SQSTM1/p62 is common to neurodegenerative disease', *Neurobiology of Disease*, 35(2), pp. 302–310. doi: 10.1016/j.nbd.2009.05.015.

Dubnovitsky, A., Sandberg, A., Rahman, M. M., Benilova, I., Lendel, C. and Härd, T. (2013) 'Amyloid-β Protofibrils: Size, Morphology and Synaptotoxicity of an Engineered Mimic', *PLoS ONE*, 8(7), pp. 1–8. doi: 10.1371/journal.pone.0066101.

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) 'Tissue fractionation studies. Intracellular distribution patterns of enzymes in rat-liver tissue', *Biochemical Journal*, 60(4), pp. 604–617. doi: 10.1042/bj0600604.

Eitan, E., Suire, C., Zhang, S. and Mattson, M. P. (2016) 'Impact of Lysosome Status on Extracellular Vesicle Content and Release', *Ageing Research Reviews*, 32, pp. 65–74. doi: 10.1016/j.arr.2016.05.001.

Erlich, S., Shohami, E. and Pinkas-Kramarski, R. (2006) 'Neurodegeneration induces upregulation of Beclin 1', *Autophagy*, 2(1), pp. 49–51. doi: 10.4161/auto.2156.

Eskelinen, E. L. (2006) 'Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy', *Molecular Aspects of Medicine*, 27(5–6), pp. 495–502. doi: 10.1016/j.mam.2006.08.005.

Esteves, A. R., Arduíno, D. M., Swerdlow, R. H., Oliveira, C. R. and Cardoso, S. M. (2010) 'Dysfunctional mitochondria uphold calpain activation: Contribution to Parkinson's disease pathology', *Neurobiology of Disease*, 37(3), pp. 723–730. doi: 10.1016/j.nbd.2009.12.011.

Esteves, A. R., Oliveira, C. R. and Cardoso, S. M. (2013) 'Autophagy, the "Master" Regulator of Cellular Quality Control: What Happens when Autophagy Fails?', in *Autophagy- A double-edged sword- Cell survival or death?*, pp. 81–120. doi: 110.5772/intechopen.68549.

Feldman, J. L., Dittenhafer-Reed, K. E. and Denu, J. M. (2012) 'Sirtuin catalysis and regulation', *Journal of Biological Chemistry*, 287(51), pp. 42419–42427. doi: 10.1074/jbc.R112.378877.

Forgac, M. (2007) 'Vacuolar ATPases: Rotary proton pumps in physiology and pathophysiology', *Nature Reviews Molecular Cell Biology*, 8(11), pp. 917–929. doi: 10.1038/nrm2272.

Frade, J. M. and Ovejero-Benito, M. C. (2015) 'Neuronal cell cycle: The neuron itself and its circumstances', *Cell Cycle*, 14(5), pp. 712–720. doi: 10.1080/15384101.2015.1004937.

Frake, R. A., Ricketts, T., Menzies, F. M. and Rubinsztein, D. C. (2015) 'Autophagy and neurodegeneration', *The Journal of Clinical Investigation*, 125(1), pp. 65–74. doi: 10.1172/JCI73944.

Fraldi, A., Klein, A. D., Medina, D. L. and Settembre, C. (2016) 'Brain Disorders Due to Lysosomal Dysfunction', *Annual Review of Neuroscience*, 39, pp. 277–295. doi: 10.1146/annurev-neuro-070815-014031.

Fryer, J. D. and Holtzman, D. M. (2005) 'The bad seed in Alzheimer's disease', *Neuron*, 47(2), pp. 167–168. doi: 10.1016/j.neuron.2005.07.002.

Fu, L., Cheng, Y. and Liu, B. (2013) 'Beclin-1: Autophagic regulator and therapeutic target in cancer', *International Journal of Biochemistry and Cell Biology*. Elsevier Ltd, 45(5), pp. 921–

924. doi: 10.1016/j.biocel.2013.02.007.

Fukuda, M., Yoshizawa, T., Karim, M. F., Sobuz, S. U., Korogi, W., Kobayasi, D., Okanishi, H., Tasaki, M., Ono, K., Sawa, T., Sato, Y., Chirifu, M., Masuda, T., Nakamura, T., Tanoue, H., Nakashima, K., Kobashigawa, Y., Morioka, H., Bober, E., Ohtsuki, S., Yamagata, Y., Ando, Y., Oike, Y., Araki, N., Takeda, S., Mizuta, H. and Yamagata, K. (2018) 'SIRT7 has a critical role in bone formation by regulating lysine acylation of SP7/Osterix', *Nature Communications*. Springer US, 9(2833), pp. 1–14. doi: 10.1038/s41467-018-05187-4.

Funderburk, S. F., Marcellino, B. K. and Yue, Z. (2010) 'Cell "Self Eating" (Autophagy) Mechanism in Alzheimer's Disease', *Mount Sinai Journal of Medicine*, 77(1), pp. 59–68. doi: 10.1002/msj.20161.

Funderburk, S. F., Wang, Q. J. and Yue, Z. (2010) 'The Beclin 1-VPS34 complex - at the crossroads of autophagy and beyond', *Trends in Cell Biology*. Elsevier Ltd, 20(6), pp. 355–362. doi: 10.1016/j.tcb.2010.03.002.

Gan, L. and Mucke, L. (2008) 'Paths of Convergence: Sirtuins in Aging and Neurodegeneration', *Neuron*, 58(1), pp. 10–14. doi: 10.1016/j.neuron.2008.03.015.

Ghosh, A. K. and Varga, J. (2007) 'The Transcriptional Coactivator and Acetyltransferase p300 in Fibroblast Biology and Fibrosis', *Journal of cellular physiology*, 213, pp. 663–671. doi: 10.1002/JCP.

Giannakou, M. E. and Partridge, L. (2004) 'The interaction between FOXO and SIRT1: tipping the balance towards survival', *Trends in Cell Biology*, 14(8), pp. 404–408. doi: 10.1016/j.tcb.2004.07.001.

Ginsberg, S. D., Alldred, M. J., Counts, S. E., Cataldo, A. M., Neve, R. L., Jiang, Y., Wuu, J., Chao, M. V., Mufson, E. J., Nixon, R. A. and Che, S. (2010) 'Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression', *Biol Psychiatry*, 68(10), pp. 885–893. doi: 10.1016/j.biopsych.2010.05.030.

Ginsberg, S. D., Mufson, E. J., Counts, S. E., Wuu, J., Alldred, M. J., Nixon, R. A. and Shaoli, C. (2010) 'Regional selectivity of rab5 and rab7 protein up regulation in mild cognitive impairment and Alzheimer's disease', *Journal of Alzheimer 's Disease*, 22(2), pp. 631–639. doi: 10.3233/JAD-2010-101080.

Gomes, P., Outeiro, T. F. and Cavadas, C. (2015) 'Emerging Role of Sirtuin 2 in the Regulation of Mammalian Metabolism.', *Trends in Pharmacological Sciences*. Elsevier Ltd, 36(11), pp. 756–768. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26538315.

Götz, M. and Huttner, W. B. (2005) 'The cell biology of neurogenesis', *Nature Reviews Molecular Cell Biology*, 6(10), pp. 777–788. doi: 10.1038/nrm1739.

Guarente, L. (2007) 'Sirtuins in aging and disease.', *Cold Spring Harbor symposia on quantitative biology*, 72, pp. 483–488. doi: 10.1101/sqb.2007.72.024.

Guedouari, H., Daigle, T., Scorrano, L. and Hebert-Chatelain, E. (2017) 'Sirtuin 5 protects mitochondria from fragmentation and degradation during starvation', *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier B.V., 1864(1), pp. 169–176. doi:

10.1016/j.bbamcr.2016.10.015.

Guo, F., Xinyao, L., Cai, H. and Le, W. (2017) 'Autophagy in Neurodegenerative Diseases: Pathogenesis and Therapy', *Brain Pathology*, 28(1), pp. 3–13. doi: 10.1111/bpa.12545.

Gutierrez, M. G., Munafó, D. B., Berón, W. and Colombo, M. I. (2004) 'Rab7 is required for the normal progression of the autophagic pathway in mammalian cells', *Journal of Cell Science*, 117(13), pp. 2687–2697. doi: 10.1242/jcs.01114.

Haass, C. and Selkoe, D. J. (1993) 'Cellular Processing of beta-Amyloid Precursor Protein and the Genesis of Amyloid beta-Peptide', *Cell Press*, 75, pp. 1039–1042.

Haass, C. and Selkoe, D. J. (2007) 'Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid β-peptide', *Nature Reviews Molecular Cell Biology*, 8(2), pp. 101–112. doi: 10.1038/nrm2101.

Hanada, T., Noda, N. N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F. and Ohsumi, Y. (2007) 'The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy', *Journal of Biological Chemistry*, 282(52), pp. 37298–37302. doi: 10.1074/jbc.C700195200.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H. and Mizushim, N. (2006) 'Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice', *Nature*, 441(4), pp. 885–889. doi: 10.1038/nature04724.

Hardy, J. A. and Higgins, G. A. (1992) 'Alzheimer's Disease: The Amyloid Cascade Hypothesis', *Science*, 26, pp. 184–185. doi: 10.1126/science.1566067.

Haung Yu, W., Cuervo, A. M., Kumar, A., Peterhoff, C. M., Schmidt, S. D., Lee, J. H., Mohan, P. S., Mercken, M., Farmery, M. R., Tjernberg, L. O., Jiang, Y., Duff, K., Uchiyama, Y., Näslund, J., Mathews, P. M., Cataldo, A. M. and Nixon, R. A. (2005) 'Macroautophagy - a novel β-amyloid peptide-generating pathway activated in Alzheimer's disease', *Journal of Cell Biology*, 171(1), pp. 87–98. doi: 10.1083/jcb.200505082.

Hayat, M. A. (2013) 'Introduction to Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection and Aging, Volumes 1-4', in *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging*. 1st edn. Elsevier Inc., pp. 1–32. doi: 10.1016/B978-0-12-405530-8.00001-7.

Hayat, M. A. (2016) 'Overview of Autophagy', in Hayat, M. A. (ed.) *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging*. 1st edn. Elsevier Inc., pp. 3–84. doi: 10.1016/B978-0-12-805420-8.00001-9.

Head, E., Powell, D., Gold, B. T. and Schmitt, F. A. (2012) 'Alzheimer's Disease in Down Syndrome', *European Journal Neurodegenerative Diseases*, 1(3), pp. 353–364.

Herskovits, A. Z. and Guarente, L. (2014) 'SIRT1 in neurodevelopment and brain senescence', *Neuron*, 81(3), pp. 471–483. doi: 10.1016/j.neuron.2014.01.028.

Hippius, H. and Neundorfer, G. (2003) 'The discovery of Alzheimer's disease', *Dialogues in Clinical Neuroscience*, 5(1), pp. 101–108.

Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., Iemura, S., Natsume, T., Takehana, K., Yamada, N., Guan, J., Oshiro, N. and Mizushima, N. (2009) 'Nutrient-dependent mTORC1 Association with the ULK1 – Atg13 – FIP200 Complex Required for Autophagy', *Molecular Biology of the cell*, 20, pp. 1981–1991. doi: 10.1091/mbc.

Houtkooper, R. H., Pirinen, E. and Auwerx, J. (2012) 'Sirtuins as regulators of metabolism and healthspan', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 13(4), pp. 225–238.

Huang, R. and Liu, W. (2015) 'Identifying an essential role of nuclear LC3 for autophagy', *Autophagy*, 11(5), pp. 852–853. doi: 10.1080/15548627.2015.1038016.

Huang, Y. and Mucke, L. (2012) 'Alzheimer mechanisms and therapeutic strategies', *Cell*. Elsevier Inc., 148(6), pp. 1204–1222. doi: 10.1016/j.cell.2012.02.040.

Iqbal, K., Liu, F. and Gong, C.-X. (2016) 'Tau and neurodegenerative disease: The story so far', *Nature Reviews Neurology*, 12(1), pp. 15–27. doi: 10.1038/nrneurol.2015.225.

Irwin, D. J., Cohen, T. J., Grossman, M., Arnold, S. E., Xie, S. X., Lee, V. M. Y. and Trojanowski, J. Q. (2012) 'Acetylated tau, a novel pathological signature in Alzheimer's disease and other tauopathies', *Brain*, 135(3), pp. 807–818. doi: 10.1093/brain/aws013.

Itakura, E. and Mizushima, N. (2011) 'p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding', *Journal of Cell Biology*, 192(1), pp. 17–27. doi: 10.1083/jcb.201009067.

Jack, C. R. and Holtzman, D. M. (2013) 'Biomarker modeling of alzheimer's disease', *Neuron*. Elsevier Inc., 80(6), pp. 1347–1358. doi: 10.1016/j.neuron.2013.12.003.

Jaeger, P. A., Pickford, F., Sun, C.-H., Lucin, K. M., Masliah, E. and Wyss-Coray, T. (2010) 'Regulation of Amyloid Precursor Protein Processing by the Beclin 1 Complex', *PLoS ONE*, 5(6), p. e11102. doi: 10.1371/journal.pone.0011102.

Jaeger, P. A. and Wyss-Coray, T. (2010) 'Beclin 1 complex in autophagy and Alzheimer disease', *Archives of Neurology*, 67(10), pp. 1181–1184. doi: 10.1001/archneurol.2010.258.

Jazwinski, S. M., Belancio, V. P. and Hill, S. M. (2017) *Circadian Rhythms and Their Impact on Aging*. Edited by S. I. S. Rattan. Aarhrus, Denmark. doi: 10.1007/978-3-319-64543-8.

Jouanne, M., Rault, S. and Voisin-Chiret, A. S. (2017) 'Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents', *European Journal of Medicinal Chemistry*. Elsevier Masson SAS, 139, pp. 153–167. doi: 10.1016/j.ejmech.2017.07.070.

Julien, C., Tremblay, C., Émond, V., Lebbadi, M., Salem, N., Bennett, D. A. and Calon, F. (2009) 'SIRT1 Decrease Parallels the Accumulation of tau in Alzheimer Disease', *J Neuropathol Exp Neurol*, 68(1), pp. 1–26. doi: 10.1097/NEN.0b013e3181922348.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000) 'LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing', *The EMBO Journal*, 19(21), pp. 5720–5728.

Kang, R., Zeh, H. J., Lotze, M. . T. and Tang, D. (2011) 'The Beclin 1 network regulates autophagy and apoptosis', *Cell Death and Differentiation*. Nature Publishing Group, 18(4), pp. 571–580. doi: 10.1038/cdd.2010.191.

Kaur, J. and Debnath, J. (2015) 'Autophagy at the crossroads of catabolism and anabolism', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 16(8), pp. 461–472. doi: 10.1038/nrm4024.

Kelly, G. S. (2010) 'A review of the sirtuin system, its clinical implications, and the potential role of dietary activators like resveratrol: part 2.', *Alternative Medicine Review*, 15(4), pp. 313–328.

Kenessey, A., Nacharaju, P., Ko, L. and Yen, S.-H. (1997) 'Degradation of Tau by Lysosomal Enzyme Cathepsin D: Implication for Alzheimer Neurofibrillary Degeneration', *Journal of Neurochemistry*, 69(5), pp. 2026–2038. doi: 10.1046/j.1471-4159.1997.69052026.x.

Kim, J., Onstead, L., Randle, S., Price, R., Smithson, L., Zwizinski, C., Dickson, D. W., Golde, T. and Mcgowan, E. (2007) 'Neurobiology of Disease Abeta 40 Inhibits Amyloid Deposition In Vivo', 27(3), pp. 627–633. doi: 10.1523/JNEUROSCI.4849-06.2007.

Kim, K. H. and Lee, M.-S. (2014) 'Autophagy - A key player in cellular and body metabolism', *Nature Reviews Endocrinology*. Nature Publishing Group, 10(6), pp. 322–337. doi: 10.1038/nrendo.2014.35.

King, M. and Attardi, G. (1989) 'Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation', *Science*, 246(4929), pp. 500–503. doi: 10.1126/science.2814477.

Koga, H., Kaushik, S. and Cuervo, A. M. (2011) 'Protein Homeostasis and Aging: the importance of exquisite quality control Hiroshi', *Ageing Research Reviews*, 10(2), pp. 205–215. doi: 10.1016/j.arr.2010.02.001.

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J. and Ripova, D. (2012) 'Structure and pathology of tau protein in Alzheimer disease', *International Journal of Alzheimer's Disease*, 2012, pp. 1–13. doi: 10.1155/2012/731526.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J. I., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E. and Tanaka, K. (2006) 'Loss of autophagy in the central nervous system causes neurodegeneration in mice', *Nature*, 441(7095), pp. 880–884. doi: 10.1038/nature04723.

Kovacs, G. G. (2014) 'Introduction Classification of neurodegenerative diseases', in Kovacs, G. G. (ed.) *Neuropathology of Neurodegenerative Diseases: A Pratical Guide*. Cambridge University Press, pp. 1–7. doi: 10.1017/CBO9781107588660.001.

Kramer, L., Turk, D. and Turk, B. (2017) 'The Future of Cysteine Cathepsins in Disease Management', *Trends in Pharmacological Sciences*, 38(10), pp. 873–898. doi: 10.1016/j.tips.2017.06.003.

Kumar, R., Chaterjee, P., Sharma, P. K., Singh, A. K., Gupta, A., Gill, K., Tripathi, M., Dey, A. B. and Dey, S. (2013) 'Sirtuin1: A Promising Serum Protein Marker for Early Detection of Alzheimer's Disease', *PLoS ONE*, 8(4), pp. 1–6. doi: 10.1371/journal.pone.0061560.

Kümmel, D. and Ungermann, C. (2014) ‘Principles of membrane tethering and fusion in endosome and lysosome biogenesis’, *Current Opinion in Cell Biology*, 29(1), pp. 61–66. doi: 10.1016/j.ceb.2014.04.007.

Kundu, M. and Thompson, C. B. (2008) ‘Autophagy: Basic Principles and Relevance to Disease’, *Annual Review of Pathology: Mechanisms of Disease*, 3, pp. 427–455. doi: 10.1146/annurev.pathmechdis.

Kuusisto, E., Salminen, A. and Alafuzoff, I. (2002) ‘Early accumulation of p62 in neurofibrillary tangles in alzheimer’s disease: Possible role in tangle formation’, *Neuropathology and Applied Neurobiology*, 28(3), pp. 228–237. doi: 10.1046/j.1365-2990.2002.00394.x.

Ladror, U. S., Snyder, S. W., Wang, G. T., Holzman, T. F. and Krafft, G. A. (1994) ‘Cleavage at the amino and carboxyl termini of Alzheimer’s amyloid-β by cathepsin D’, *Journal of Biological Chemistry*, 269(28), pp. 18422–18428.

Lee, I. H., Cao, L., Mostoslavsky, R., Lombard, D. B., Liu, J., Bruns, N. E., Tsokos, M., Alt, F. W. and Finkel, T. (2008) ‘A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy’, *Proceedings of the National Academy of Sciences*, 105(9), pp. 3374–3379. doi: 10.1073/pnas.0712145105.

Lee, J.-A. (2012) ‘Neuronal Autophagy: A Housekeeper or a Fighter in Neuronal Cell Survival?’, *Experimental Neurobiology*, 21(1), pp. 1–8. doi: 10.5607/en.2012.21.1.1.

Lee, J. A. and Gao, F. B. (2008) ‘Regulation of Abeta pathology by beclin 1: A protective role for autophagy?’, *Journal of Clinical Investigation*, 118(6), pp. 2015–2018. doi: 10.1172/JCI35662.

Lee, Y.-K. and Lee, J.-A. (2016) ‘Role of the mammalian ATG8/LC3 family in autophagy: Differential and compensatory roles in the spatiotemporal regulation of autophagy’, *BMB Reports*, 49(8), pp. 424–430. doi: 10.5483/BMBRep.2016.49.8.081.

Lenk, S. E., Dunn, W. A., Trausch, J. S., Ciechanover, A. and Schwartz, A. L. (1992) ‘Ubiquitin-activating enzyme, E1, is associated with maturation of autophagic vacuoles’, *The Journal of Cell Biology*, 118(2), pp. 301–308. doi: 10.1083/jcb.118.2.301.

Levine, B. and Kroemer, G. (2008) ‘Autophagy in the Pathogenesis of Disease’, *Cell*, 132(1), pp. 27–42. doi: 10.1016/j.cell.2007.12.018.

Levine, B., Liu, R., Dong, X. and Zhong, Q. (2015) ‘Beclin orthologs: integrative hubs of cell signaling, membrane trafficking, and physiology’, 25(9), pp. 533–544. doi: 10.1158/1940-6207.CAPR-14-0359.Nrf2-dependent.

Levites, Y., Das, P., Price, R. W., Rochette, M. J., Kostura, L. A., Mcgowan, E. M., Murphy, M. P. and Golde, T. E. (2006) ‘Anti-Aβ42– and anti-Aβ40–specific mAbs attenuate amyloid deposition in an Alzheimer disease mouse model’, *The Journal of Clinical Investigation*, 116(1), pp. 193–201. doi: 10.1172/JCI25410.

Li, L., Shi, L., Yang, S., Yan, R., Zhang, D., Yang, J., He, L., Li, W., Yi, X., Sun, L., Liang, J., Cheng, Z., Shi, L., Shang, Y. and Yu, W. (2016) ‘SIRT7 is a histone desuccinylase that functionally links to chromatin compaction and genome stability’, *Nature Communications*. Nature Publishing Group, 7(12235), pp. 1–17.

Liang, C., Lee, J.-S., Inn, K.-S., Gack, M. U., Li, Q., Roberts, E. A., Vergne, I., Deretic, V., Feng, P., Akazawa, C. and Jung, J. U. (2008) 'Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking', *Nature Cell Biology*, 10(7), pp. 776–787. doi: 10.1038/ncb1740.

Liang, J. H. and Jia, J. P. (2014) 'Dysfunctional autophagy in Alzheimer's disease: Pathogenic roles and therapeutic implications', *Neuroscience Bulletin*, 30(2), pp. 308–316. doi: 10.1007/s12264-013-1418-8.

Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B. and Levine, B. (1998) 'Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein.', *Journal of virology*, 72(11), pp. 8586–8596. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=110269&tool=pmcentrez&rendertype=abstract.

Libert, S., Cohen, D. and Guarente, L. (2008) 'Neurogenesis directed by Sirt1', *Nature Cell Biology*, 10(4), pp. 373–374.

Lilienbaum, A. (2013) 'Relationship between the proteasomal system and autophagy', *Int J Biochem Mol Biol*, 4(1), pp. 1–26.

Lim, C. Y. and Zoncu, R. (2016) 'The lysosome as a command-and-control center for cellular metabolism', *Journal of Cell Biology*, 214(6), pp. 653–664. doi: 10.1083/jcb.201607005.

Liu, C.-C., Kanekiyo, T., Xu, H. and Bu, G. (2013) 'Apolipoprotein e and Alzheimer disease: Risk, mechanisms and therapy', *Nature Reviews Neurology*. Nature Publishing Group, 9(2), pp. 106–118. doi: 10.1038/nrneurol.2012.263.

Liu, W. J., Ye, L., Huang, W. F., Guo, L. J., Xu, Z. G., Wu, H. L., Yang, C. and Liu, H. F. (2016) 'p62 Links the Autophagy Pathway and the Ubiqutin-Proteasome System Upon Ubiquitinated Protein Degradation', *Cellular and Molecular Biology Letters*. Cellular & Molecular Biology Letters, 21(29), pp. 1–14. doi: 10.1186/s11658-016-0031-z.

Longstaff, A. (2005) *Neuroscience*. 2nd edn. Edited by E. Owen. New York: Taylor & Francis Group.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. and Kroemer, G. (2013) 'The Hallmarks of aging', *Cell*, 153(6), pp. 1194–1217. doi: 10.1016/j.cell.2013.05.039.

Lu, X., Wang, L., Yu, C., Yu, D. and Yu, G. (2015) 'Histone Acetylation Modifiers in the Pathogenesis of Alzheimer's Disease', *Frontiers in Cellular Neuroscience*, 9(226), pp. 1–9. doi: 10.3389/fncel.2015.00226.

Lumkwana, D., du Toit, A., Kinnear, C. and Loos, B. (2017) 'Autophagic flux control in neurodegeneration: Progress and precision targeting—Where do we stand?', *Progress in Neurobiology*. Elsevier Ltd, 153, pp. 64–85. doi: 10.1016/j.pneurobio.2017.03.006.

Ma, T., Tan, M.-S., Yu, J.-T. and Tan, L. (2014) 'Resveratrol as a therapeutic agent for alzheimer's disease', *BioMed Research International*. Hindawi Publishing Corporation, 2014, pp. 1–13. doi: 10.1155/2014/350516.

Mandelkow, E.-M. and Mandelkow, E. (2012) 'Biochemistry and Cell Biology of Tau Protein in

Neurofibrillary Degeneration', *Cold Spring Harbor Perspectives Medicine*, 2(7), p. a006247. doi: 10.1101/cshperspect.a006247.

Mandelkow, E. and Mandelkow, E. (1998) 'Tau in Alzheimer's disease', *Trends Cell Biology*, 8(11), pp. 425–427.

Martinez-lopez, N., Athonvarangkul, D. and Singh, R. (2015) 'Autophagy and Aging', *Adv Exp Med Biol.*, 847, pp. 73–87. doi: 10.1007/978-1-4939-2404-2_3.

Martinez-Vicente, M. (2015) 'Autophagy in neurodegenerative diseases: From pathogenic dysfunction to therapeutic modulation', *Seminars in Cell and Developmental Biology*. Elsevier Ltd, 40, pp. 115–126. doi: 10.1016/j.semcdb.2015.03.005.

Mathews, P. M., Guerra, C. B., Jiang, Y., Grbovic, O. M., Kao, B. H., Schmidt, S. D., Dinakar, R., Mercken, M., Hille-Rehfeld, A., Rohrer, J., Mehta, P., Cataldo, A. M. and Nixon, R. A. (2002) 'Alzheimer's disease-related overexpression of the cation-dependent mannose 6-phosphate receptor increases Aβ secretion. Role for altered lysosomal hydrolase distribution in β-amyloidogenesis', *Journal of Biological Chemistry*, 277(7), pp. 5299–5307. doi: 10.1074/jbc.M108161200.

Mattison, J. A., Colman, R. J., Beasley, T. M., Allison, D. B., Kemnitz, J. W., Roth, G. S., Ingram, D. K., Weindruch, R., De Cabo, R. and Anderson, R. M. (2017) 'Caloric restriction improves health and survival of rhesus monkeys', *Nature Communications*. Nature Publishing Group, 8(14063), pp. 1–12. doi: 10.1038/ncomms14063.

Mattson, M. P. (2004) 'Pathway towards and away from Alzheimer's disease', *Nature*, 430(7000), pp. 631–639. doi: 10.1038/nature02621.

Mattson, M. P. (2012) 'Energy intake and exercise as determinants of brain health and vulnerability to injury and disease', *Cell Metabolism*. Elsevier Inc., 16(6), pp. 706–722. doi: 10.1016/j.cmet.2012.08.012.

Maurer, K., Volk, S. and Gerbaldo, H. (1997) 'Auguste D and Alzheimer ' s disease', *The lancet*, 349, pp. 1546–49.

Mazon, J. N., de Mello, A. H., Ferreira, G. K. and Rezin, G. T. (2017) 'The impact of obesity on neurodegenerative diseases', *Life Sciences*. Elsevier Inc., 182, pp. 22–28. doi: 10.1016/j.lfs.2017.06.002.

McClellan, A. J., Tam, S., Kaganovich, D. and Frydman, J. (2005) 'Protein quality control: Chaperones culling corrupt conformations', *Nature Cell Biology*, 7(8), pp. 736–741. doi: 10.1038/ncb0805-736.

Mcknight, N. C., Mizushima, N. and Yue, Z. (2012) 'THE CELLULAR PROCESS OF AUTOPHAGY AND CONTROL OF AUTOPHAGY IN NEURONS', in Yue, Z. and Chu, C. T. (eds) *Autophagy of the Nervous System*, p. 440.

McKnight, N. C. and Zhenyu, Y. (2013) 'Beclin 1, an Essential Component and Master Regulator of PI3K-III in Health and Disease', *Curr Pathobiol Rep.*, 1(4), pp. 231–238. doi: 10.1007/s40139-013-0028-5.

Michán, S., Li, Y., Chou, M. M., Parrella, E., Long, J. M., Allard, J. S., Lewis, K., Miller, M., Xu,

W., Mervis, R. F., Chen, J., Guerin, K. I., Smith, L. E. H., Mcburney, M. W., Sinclair, D. A., Baudry, M., de Cabo, R. and Longo, V. D. (2010) 'SIRT1 is essential for normal cognitive function and synaptic plasticity', *Journal of Neuroscience*, 30(29), pp. 9695–9707. doi: 10.1523/JNEUROSCI.0027-10.2010.

Michan, S. and Sinclair, D. (2007) 'Sirtuins in mammals: insights into their biological function', *Biochemical Journal*, 404(1), pp. 1–13. doi: 10.1042/BJ20070140.

Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C. and Horikawa, I. (2005) 'Evolutionarily Conserved and Nonconserved Cellular Localizations and Functions of Human SIRT Proteins', *Molecular biology of the cell*, 16, pp. 4623–4635.

Mietelska-Porowska, A., Wasik, U., Goras, M., Filipek, A. and Niewiadomska, G. (2014) 'Tau Protein Modifications and Interactions:Their Role in Function and Dysfunction', *International Journal of Molecular Sciences*, 15(3), pp. 4671–4713. doi: 10.3390/ijms15034671.

Miller, S. W., Trimmer, P. A., Parker, W. D. and Davis, R. E. (1996) 'Creation and Characterization of Mitochondrial DNA-Depleted Cell Lines with "Neuronal-Like" Properties', *Journal of Neurochemistry*, 67(5), pp. 1897–1907. doi: 10.1046/j.1471-4159.1996.67051897.x.

Ming, G. and Song, H. (2011) 'Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions', *Neuron*, 70(4), pp. 687–702. doi: 10.1016/j.neuron.2011.05.001.

Mizushima, N. and Klionsky, D. J. (2007) 'Protein Turnover Via Autophagy: Implications for Metabolism', *Annual Review of Nutrition*, 27(1), pp. 19–40. doi: 10.1146/annurev.nutr.27.061406.093749.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M. and Ohsumi, Y. (1998) 'A protein conjugation system essential for autophagy', *Nature*, 395(6700), pp. 395–398. doi: 10.1038/26506.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T. and Ohsumi, Y. (2004) 'In Vivo Analysis of Autophagy in Response to Nutrient Starvation Using Transgenic Mice Expressing a Fluorescent Autophagosome Marker', *Molecular biology of the cell*, 15, pp. 1101–1111. doi: 10.1091/mbc.E03.

Mizushima, N., Yoshimori, T. and Ohsumi, Y. (2011) 'The Role of Atg Proteins in Autophagosome Formation', *Annual Review of Cell and Developmental Biology*, 27(1), pp. 107–132. doi: 10.1146/annurev-cellbio-092910-154005.

Mizushima, N., Yoshimorim, T. and Levine, B. (2010) 'Methods in Mammalian Autophagy Research', *Cell*, 140(3), pp. 313–326. doi: 10.1016/j.cell.2010.01.028.

Moreira, P. I., Santos, R. X., Zhu, X., Lee, H. and Smith, M. A. (2010) 'Autophagy in Alzheimer's disease', *Expert Review of Neurotherapeutics*, 10(7), pp. 1209–1218.

Morris, D. H., Yip, C. K., Shi, Y., Chait, B. T. and Wang, Q. J. (2015) 'BECLIN 1-VPS34 COMPLEX ARCHITECTURE: UNDERSTANDING THE NUTS AND BOLTS OF THERAPEUTIC TARGETS', *Front Biol (Beijing)*, 10(5), pp. 398–426. doi: 10.1007/s11515-015-1374-y.

Mosmann, T. (1983) 'Rapid Colorimetric Assay for Cellular Growth and Survival : Application

to Proliferation and Cytotoxicity Assays', *Jounal of Imunological Methods*, 65, pp. 55–63.

Murphy, M. P. and Levine III, H. (2010) 'Alzheimer's Disease and the β-Amyloid Peptide', *Journal of Alzheimer's Disease*, 19(1), pp. 1–17. doi: 10.3233/JAD-2010-1221.Alzheimer.

Murrow, L. and Debnath, J. (2013) 'Autophagy as a Stress-Response and Quality-Control Mechanism: Implications for Cell Injury and Human Disease', *Annual Review of Pathology: Mechanisms of Disease*, 8(1), pp. 105–137. doi: 10.1146/annurev-pathol-020712-163918.

Nah, J., Yuan, J. and Jung, Y.-K. (2015) 'Autophagy in Neurodegenerative Diseases: From Mechanism to Therapeutic Approach.', *Molecules and Cells*, 38(5), pp. 381–389. doi: 10.14348/molcells.2015.0034.

Nakatogawa, H., Ichimura, Y. and Ohsumi, Y. (2007) 'Atg8, a Ubiquitin-like Protein Required for Autophagosome Formation, Mediates Membrane Tethering and Hemifusion', *Cell*, 130(1), pp. 165–178. doi: 10.1016/j.cell.2007.05.021.

Nascimento-Ferreira, I., Santos-Ferreira, T., Sousa-Ferreira, L., Auregan, G., Onofre, I., Alves, S., Dufour, N., Colomer Gould, V. F., Koeppen, A., Déglon, N. and Pereira De Almeida, L. (2011) 'Overexpression of the autophagic beclin-1 protein clears mutant ataxin-3 and alleviates Machado-Joseph disease', *Brain*, 134(5), pp. 1400–1415. doi: 10.1093/brain/awr047.

Nemoto, S., Fergusson, M. M. and Finkel, T. (2005) 'SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1α', *Journal of Biological Chemistry*, 280(16), pp. 16456–16460. doi: 10.1074/jbc.M501485200.

Ng, F. and Tang, B. L. (2013) 'Sirtuins' modulation of autophagy', *Journal of Cellular Physiology*, 228(12), pp. 2262–2270. doi: 10.1002/jcp.24399.

Ng, F., Wijaya, L. and Tang, B. L. (2015) 'SIRT1 in the brain—connections with aging-associated disorders and lifespan', *Frontiers in Cellular Neuroscience*, 9(64), pp. 1–13. doi: 10.3389/fncel.2015.00064.

Nikoletopoulou, V., Papandreou, M.-E. and Tavernarakis, N. (2015) 'Autophagy in the physiology and pathology of the central nervous system', *Cell Death and Differentiation*. Nature Publishing Group, 22(3), pp. 398–407. doi: 10.1038/cdd.2014.204.

Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., Tanaka, M., Iwata, N., Saito, T. and Saido, T. C. (2013) 'Aβ Secretion and Plaque Formation Depend on Autophagy', *Cell Reports*. The Authors, 5(1), pp. 61–69. doi: 10.1016/j.celrep.2013.08.042.

Nixon, R. A. (2007) 'Autophagy, amyloidogenesis and Alzheimer disease', *Journal of Cell Science*, 120(23), pp. 4081–4091. doi: 10.1242/jcs.019265.

Nixon, R. A. (2013) 'The role of autophagy in neurodegenerative disease', *Nature Medicine*. Nature Publishing Group, 19(8), pp. 983–997. doi: 10.1038/nm.3232.

Nixon, R. A. (2016) 'The Lysosome in Aging-Related Neurodegenerative Diseases', in Frederick R. Maxfield, James M.Willard, and Lu., S. (eds) *Lysosomes: Biology, Diseases, and Therapeutics*. First edit. John Wiley & Sons, Inc., pp. 137–179. doi: 10.1002/9781118978320.ch9.

Nixon, R. A. (2017) 'Amyloid precursor protein & endosomal-lysosomal dysfunction in

Alzheimer's disease: Inseparable partners in a multifactorial disease', *The FASEB Journal*, 31(7), pp. 2729–2743. doi: 10.1096/fj.201700359.

Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A. and Cuervo, A. M. (2005) 'Extensive involvement of autophagy in Alzheimer disease: An immuno-electron microscopy study', *Journal of Neuropathology and Experimental Neurology*, 64(2), pp. 113–122. doi: 10.1093/jnen/64.2.113.

Nixon, R. A. and Yang, D.-S. (2011) 'Autophagy Failure in Alzheimer's Disease-Locating the Primary Defect', *Neurobiology of Disease*, 43(1), pp. 38–45. doi: 10.1016/j.nbd.2011.01.021.

Nogueiras, R., Habegger, K. M., Chaudhary, N., Finan, B., Banks, A. S., Dietrich, M. O., Horvath, T. L., Sinclair, D. A., Pfluger, P. T. and Matthias, T. H. (2012) 'SIRTUIN 1 AND SIRTUIN 3: PHYSIOLOGICAL MODULATORS OF METABOLISM', *Physiological Reviews*, 92(3), pp. 1479–1514. doi: 10.1152/physrev.00022.2011.SIRTUIN.

Ntais, C., Polycarpou, A. and Ioannidis, J. P. A. (2004) 'Meta-Analysis of the Association of the Cathepsin D Ala224Val Gene Polymorphism with the Risk of Alzheimer's Disease: A HuGE Gene-Disease Association Review', *American Journal of Epidemiology*, 159(6), pp. 527–536. doi: 10.1093/aje/kwh069.

de Oliveira, R. M., Sarkander, J., Kazantsev, A. G. and Outeiro, T. F. (2012) 'SIRT2 as a therapeutic target for age-related disorders', *Frontiers in Pharmacology*, 3(82), pp. 1–9. doi: 10.3389/fphar.2012.00082.

Orenstein, S. J. and Cuervo, A. M. (2010) 'Chaperone-mediated autophagy: Molecular mechanisms and physiological relevance', *Seminars in Cell and Developmental Biology*, 21(7), pp. 719–726. doi: 10.1016/j.semcdb.2010.02.005.

Orr, M. E. and Oddo, S. (2013) 'Autophagic/lysosomal dysfunction in Alzheimer's disease.', *Alzheimer's research & therapy*, 5(5), p. 53. doi: 10.1186/alzrt217.

Outeiro, T. F., Marques, O. and Kazantsev, A. (2008) 'Therapeutic role of sirtuins in neurodegenerative disease', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1782(6), pp. 363–369. doi: 10.1016/j.bbadis.2008.02.010.

Pan, P. W., Feldman, J. L., Devries, M. K., Dong, A., Edwards, A. M. and Denu, J. M. (2011) 'Structure and biochemical functions of SIRT6', *Journal of Biological Chemistry*, 286(16), pp. 14575–14587. doi: 10.1074/jbc.M111.218990.

Paraíso, A. F., Mendes, K. L. and Santos, S. H. S. (2013) 'Brain activation of SIRT1: Role in neuropathology', *Molecular Neurobiology*, 48(3), pp. 681–689. doi: 10.1007/s12035-013-8459-x.

Patel, B. and Cuervo, A. M. (2015) 'Methods to study chaperone-mediated autophagy.', *Methods*, 75, pp. 133–140. doi: 10.1016/j.ymeth.2015.01.003.

Pawlowski, M., Meuth, S. G. and Duning, T. (2017) 'Cerebrospinal Fluid Biomarkers in Alzheimer's Disease—From Brain Starch to Bench and Bedside', *Diagnostics*, 7(42), pp. 1–21. doi: 10.3390/diagnostics7030042.

Peden, A. H. and Ironside, J. W. (2012) 'Molecular pathology in neurodegenerative diseases.',

Current drug targets, 13(12), pp. 1548–59. doi: doi:10.2174/138945012803530134.

Pereira, H., Oliveira, C. S. F., Castro, L., Preto, A., Chaves, S. R. and Corte-Real, M. (2015) 'Yeast as a tool to explore cathepsin D function', *Microbial Cell*, 2(7), pp. 225–234. doi: 10.15698/mic2015.07.212.

Perera, R. M. and Zoncu, R. (2016) 'The Lysosome as a Regulatory Hub', *Annual Review of Cell and Developmental Biology*, 32, pp. 223–253. doi: 10.1146/annurev-cellbio-111315-125125.

Perez, M., Santa-Maria, I., De Barreda, E. G., Zhu, X., Cuadros, R., Cabrero, J. R., Sanchez-Madrid, F., Dawson, H. N., Vitek, M. P., Perry, G., Smith, M. A. and Avila, J. (2009) 'Tau - An inhibitor of deacetylase HDAC6 function', *Journal of Neurochemistry*, 109(6), pp. 1756–1766. doi: 10.1111/j.1471-4159.2009.06102.x.

Perez, S. E., He, B., Nadeem, M., Wuu, J., Ginsberg, S. D., Ikonomovic, M. D. and Mufson, E. J. (2015) 'Hippocampal Endosomal, Lysosomal and Autophagic Dysregulation in Mild Cognitive Impairment: Correlation with Aβ and Tau Pathology', *J Neuropathol Exp Neurol.*, 74(4), pp. 345–358. doi: 10.1097/NEN.0000000000000179.

Peters, R. (2006) 'Ageing and the brain', *Postgrad Med J*, 82, pp. 84–88. doi: 10.1136/pgmj.2005.036665.

Pickford, F., Masliah, E., Britschgi, M., Lucin, K., Narasimhan, R., Jaeger, P. A., Small, S., Spencer, B., Rockenstein, E., Levine, B. and Wyss-coray, T. (2008) 'The autophagy-related protein beclin 1 shows reduced expression in eary Alzheimer disease and regulates amyloid beta accumulation in mice', *The Journal of Clinical Investigation*, 118(6), pp. 2190–2199. doi: 10.1172/JCI33585.

Polito, L., Biella, G. and Albani, D. (2017) 'Sirtuin Modulation as Novel Neuroprotective Strategy for Alzheimer's Disease', in Gozes, I. (ed.) *Neuroprotection in Alzheimer's Disease*. Tel Aviv: Elsevier Inc., pp. 149–173. doi: 10.1016/B978-0-12-803690-7.00008-9.

Posse de Chaves, E. and Narayanaswami, V. (2008) 'Apolipoprotein E and cholesterol in aging and disease in the brain', *Future Lipidol.*, 3(5), pp. 505–530.

Prozorovski, T., Schulze-Topphoff, U., Glumm, R., Baumgart, J., Schröter, F., Ninnemann, O., Siegert, E., Bendix, I., Brüstle, O., Nitsch, R., Zipp, F. and Aktas, O. (2008) 'Sirt1 contributes critically to the redox-dependent fate of neural progenitors', *Nature Cell Biology*, 10(4), pp. 385–394. doi: 10.1038/ncb1700.

Przedborski, S., Pahan, K., Kabanov, A. V., Kipnis, J. and Taylor, R. (2008) *Neuroimmune Pharmacology*. Edited by T. Ikezu and H. E. Gendelman.

Pu, J., Guardia, C. M., Keren-Kaplan, T. and Bonifacino, J. S. (2016) 'Mechanisms and functions of lysosome positioning', *Journal of Cell Science*, pp. 4329–4339. doi: 10.1242/jcs.196287.

Pugsley, H. R. (2017) 'Assessing Autophagic Flux by Measuring LC3, p62, and LAMP1 Co-localization Using Multispectral Imaging Flow Cytometry', *Journal of Visualized Experiments*, (125), pp. 1–13. doi: 10.3791/55637.

Purushotham, A., Schug, T. T., Xu, Q., Surapureddi, S., Guo, X. and Li, X. (2009) 'Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and

inflammation', *Cell Metabolism*, 9(4), pp. 327–338.

Pyo, J.-O., Nah, J. and Jung, Y.-K. (2012) 'Molecules and their functions in autophagy', *Experimental and Molecular Medicine*, 44(2), pp. 73–80. doi: 10.3858/emm.2012.44.2.029.

Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., Zhao, W., Thiyagarajan, M., MacGrogan, D., Rodgers, J. T., Puigserver, P., Sadoshima, J., Deng, H., Pedrini, S., Gandy, S., Sauve, A. A. and Pasinetti, G. M. (2006) 'Neuronal SIRT1 activation as a novel mechanism underlying the prevention of alzheimer disease amyloid neuropathology by calorie restriction', *Journal of Biological Chemistry*, 281(31), pp. 21745–21754. doi: 10.1074/jbc.M602909200.

Rahman, S. and Islam, R. (2011) 'Mammalian Sirt1: Insights on its biological functions', *Cell Communication and Signaling*. BioMed Central Ltd, 9(1), p. 11. doi: 10.1186/1478-811X-9-11.

Ramadori, G. and Coppari, R. (2011) 'Does hypothalamic SIRT1 regulate aging?', *Aging*, 3(3), pp. 325–328. doi: 100311 [pii].

Ramadori, G., Fujikawa, T., Fukuda, M., Anderson, J., Morgan, D. A., Mostoslavsky, R., Stuart, R. C., Perello, M., Vianna, C. R., Nillni, E. A., Rahmouni, K. and Coppari, R. (2010) 'SIRT1 deacetylase in POMC neurons is required for homeostatic defenses against diet-induced obesity', *Cell Metabolism*, 12(1), pp. 78–87. doi: 10.1016/j.cmet.2010.05.010.

Raval, A. P., Dave, K. R. and Pérez-Pinzón, M. A. (2006) 'Resveratrol mimics ischemic preconditioning in the brain', *Journal of Cerebral Blood Flow and Metabolism*, 26(9), pp. 1141–1147. doi: 10.1038/sj.jcbfm.9600262.

Ravikumar, B., Acevedo-Arozena, A., Imarisio, S., Berger, Z., Vacher, C., O'Kane, C. J., Brown, S. D. M. and Rubinsztein, D. C. (2005) 'Dynein mutations impair autophagic clearance of aggregate-prone proteins', *Nature Genetics*, 37(7), pp. 771–776. doi: 10.1038/ng1591.

Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-arencibia, M., Green-thompson, Z. W., Jimenez-sanchez, M., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C. O., Menzies, F. M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F. H., Underwood, B. R., Winslow, A. R. and Rubinsztein, D. C. (2010) 'Regulation of Mammalian Autophagy in Physiology and Pathophysiology', *Physiological Reviews*, 90, pp. 1383–1435. doi: 10.1152/physrev.00030.2009.

Reggiori, F. and Ungermann, C. (2017) 'Autophagosome Maturation and Fusion', *Journal of Molecular Biology*. The Authors, 429(4), pp. 486–496. doi: 10.1016/j.jmb.2017.01.002.

Roberts, R. and Ktistakis, N. T. (2013) 'Omegasomes: PI3P platforms that manufacture autophagosomes', *Essays In Biochemistry*, 55(2), pp. 17–27. doi: 10.1042/bse0550017.

Rodriguez, L., Mohamed, N.-V., Desjardins, A., Lippé, R., Fon, E. A. and Leclerc, N. (2017) 'Rab7A regulates tau secretion', *Journal of Neurochemistry*, 141(4), pp. 592–605. doi: 10.1111/jnc.13994.

Rohn, T. T., Wirawan, E., Brown, R. J., Harris, J. R., Masliah, E. and Vandenabeele, P. (2011) 'Depletion of Beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain', *Neurobiology of Disease*, 43(1), pp. 68–78. doi: 10.1016/j.nbd.2010.11.003.

Rothgiesser, K. M., Erener, S., Waibel, S., Luscher, B. and Hottiger, M. O. (2010) 'SIRT2 regulates NF-kB-dependent gene expression through deacetylation of p65 Lys310', *Journal of*

Cell Science, 123(24), pp. 4251–4258. doi: 10.1242/jcs.073783.

Rubinsztein, D. C., Bento, C. F. and Deretic, V. (2015) 'Therapeutic targeting of autophagy in neurodegenerative and infectious diseases', *The Journal of Experimental Medicine*, 212(7), pp. 979–990. doi: 10.1084/jem.20150956.

Rubinsztein, D. C., Mariño, G. and Kroemer, G. (2011) 'Autophagy and aging', *Cell*, 146(5), pp. 682–695. doi: 10.1016/j.cell.2011.07.030.

Russell, R. C., Yuan, H.-X. and Guan, K.-L. (2014) 'Autophagy regulation by nutrient signaling', *Cell Research*. Nature Publishing Group, 24, pp. 42–57. doi: 10.1038/cr.2013.166.

Saftig, P. and Klumperman, J. (2009) 'Lysosome biogenesis and lysosomal membrane proteins : trafficking meets function', *Molecular Cell Biology*. Nature Publishing Group, 10, pp. 623–635. doi: 10.1038/nrm2745.

Saftig, P., Peters, C., Figura, K. von, Craessaerts, K., Leuven, F. Van and Strooper, B. De (1996) 'Amyloidogenic processing of the human amyloid precursor protein in hippocampal neurons devoid of Cathepsin D', *The Journal of Biological Chemistry*, 271(4), pp. 27241–27244.

Saftig, P., Schröder, B. and Blanz, J. (2010) 'Lysosomal membrane proteins: life between acid and neutral conditions', *Biochemical Society Transactions*, 38(6), pp. 1420–1423. doi: 10.1042/BST0381420.

Saharan, S., Jhaveri, D. J. and Bartlett, P. F. (2013) 'SIRT1 regulates the neurogenic potential of neural precursors in the adult subventricular zone and hippocampus', *Journal of Neuroscience Research*, 91(5), pp. 642–659. doi: 10.1002/jnr.23199.

Salminen, A. and Kaarniranta, K. (2009) 'Regulation of the aging process by autophagy', *Trends in Molecular Medicine*, 15(5), pp. 217–224. doi: 10.1016/j.molmed.2009.03.004.

Salminen, A., Kaarniranta, K., Kauppinen, A., Ojala, J., Haapasalo, A., Soininen, H. and Hiltunen, M. (2013) 'Impaired autophagy and APP processing in Alzheimer's disease: The potential role of Beclin 1 interactome', *Progress in Neurobiology*. Elsevier Ltd, 106–107, pp. 33–54. doi: 10.1016/j.pneurobio.2013.06.002.

Sampaio-Marques, B. and Ludovico, P. (2015) 'Sirtuins and Proteolytic Systems: Implications for Pathogenesis of Synucleinopathies', *Biomolecules*, 5, pp. 735–757. doi: 10.3390/biom5020735.

Satoh, A., Brace, C. S., Ben-josef, G., West, T., Wozniak, D. F., Holtzman, M., Herzog, E. D. and Imai, S. (2010) 'SIRT1 promotes the central adaptive response to diet restriction through activation of the dorsomedial and lateral nuclei of the hypothalamus', *Journal of Neuroscience*, 30(30), pp. 10220–10232. doi: 10.1523/JNEUROSCI.1385-10.2010.

Satoh, A. and Imai, S. (2014) 'Systemic regulation of mammalian ageing and longevity by brain sirtuins', *Nature Communications*. Nature Publishing Group, 5(4211), pp. 1–11. doi: 10.1038/ncomms5211.

Satoh, A., Imai, S. I. and Guarente, L. (2017) 'The brain, sirtuins, and ageing', *Nature Reviews Neuroscience*. Nature Publishing Group, 18(6), pp. 362–374. doi: 10.1038/nrn.2017.42.

Satoh, A., Stein, L. and Imai, S. (2011) 'The Role of Mammalian Sirtuins in the Regulation of Metabolism, Aging, and Longevity', *Handbook Exp Pharmacol*, 206, pp. 125–162. doi: 10.1007/978-3-642-21631-2_7.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L. and Elazar, Z. (2007) 'Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4', *EMBO Journal*, 26(7), pp. 1749–1760. doi: 10.1038/sj.emboj.7601623.

Selkoe, D. J. (2000) 'The Origins of Alzheimer Disease A Is for Amyloid', *American Medical Association*, 283(12), pp. 1615–1617.

Serrano-Pozo, A., Frosch, M. P., Masliah, E. and Hyman, B. T. (2011) 'Neuropathological Alterations in Alzheimer Disease', *Cold Spring Harbor perspectives in medicine*, 1(1), p. a006189. doi: 10.1101/cshperspect.a006189.

Settembre, C., Fraldi, A., Medina, D. L. and Ballabio, A. (2013) 'Signals from the lysosome : a control centre for cellular clearance and energy metabolism', *Nature Publishing Group*. Nature Publishing Group, 14(5), pp. 283–296. doi: 10.1038/nrm3565.

Shibata, M., Lu, T., Furuya, T., Degterev, A., Mizushima, N., Yoshimori, T., MacDonald, M., Yankner, B. and Yuan, J. (2006) 'Regulation of intracellular accumulation of mutant huntingtin by beclin 1', *The Journal of Biological Chemistry*, 281(20), pp. 14474–14485. doi: 10.1074/jbc.M600364200.

Shrimp, J. H., Sorum, A. W., Garlick, J. M., Guasch, L., Nicklaus, M. C. and Meier, J. L. (2015) 'Characterizing the Covalent Targets of a Small Molecule Inhibitor of the Lysine Acetyltransferase P300', *ACS Medicinal Chemistry Letters*, 7(2), pp. 151–155. doi: 10.1021/acsmedchemlett.5b00385.

Silva, D. F., Esteves, A. R., Oliveira, C. R. and Cardoso, S. M. (2016) 'Mitochondrial Metabolism Power SIRT2-Dependent Deficient Traffic Causing Alzheimer's-Disease Related Pathology', *Molecular Neurobiology*. Molecular Neurobiology, 54(6), pp. 4021–4040. doi: 10.1007/s12035-016-9951-x.

Sinha, S. and Levine, B. (2008) 'The autophagy effector Beclin 1:a novel BH3-only protein', *Oncogene*, 27(Suppl 1), pp. S127–S148. doi: 10.1038/onc.2009.51.

Sjödin, S., Öhrfelt, A., Brinkmalm, G., Zetterberg, H., Blennow, K. and Brinkmalm, A. (2016) 'Targeting LAMP2 in human cerebrospinal fluid with a combination of immunopurification and high resolution parallel reaction monitoring mass spectrometry', *Clinical Proteomics*. BioMed Central, 13(4), pp. 1–14. doi: 10.1186/s12014-016-9104-2.

Skibinski, G., Parkinson, N. J., Brown, J. M., Chakrabarti, L., Lloyd, S. L., Hummerich, H., Nielsen, J. E., Hodges, J. R., Spillantini, M. G., Thusgaard, T., Brandner, S., Brun, A., Rossor, M. N., Gade, A., Johannsen, P., Sørensen, S. A., Gydesen, S., Fisher, E. M. C. and Collinge, J. (2005) 'Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia', *Nature Genetics*, 37(8), pp. 806–808. doi: 10.1038/ng1609.

Skovronsky, D. M., Lee, V. M.-Y. and Trojanowski, J. Q. (2006) 'Neurodegenerative Diseases: New Concepts of Pathogenesis and Their Therapeutic Implications', *Annual Review of Pathology: Mechanisms of Disease*, 1, pp. 151–170. doi: 10.1146/annurev.pathol.1.110304.100113.

Son, J. H., Kim, K., Son, J. H., Shim, J. H. and Kim, K. (2012) 'Neuronal Autophagy and Neurodegenerative Diseases', *Experimental & molecular medicine*, 44(2), pp. 89–98. doi: 10.3858/emm.2012.44.2.031.

Sooyeon, L., Sato, Y. and Nixon, R. A. (2011) 'Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's- like axonal dystrophy', *Journal Neuroscience*, 31(21), pp. 7817–7830. doi: 10.1523/JNEUROSCI.6412-10.2011.

Steele, J. W., Emily Fan, Kelahmetoglu, Y., Tian, Y. and Bustos, V. (2013) 'Modulation of Autophagy as a Therapeutic Target for Alzheimer's Disease', *Posdoc J.*, 1(2), pp. 21–34.

Swerdlow, R. H., Parks, J. K., Miller, S. W., Tuttle, J. B., Trimmer, P. A., Sheehan, J. P., Bennett, J. P., Davis, R. E. and Parker, W. D. (1996) 'Origin and functional consequences of the complex I defect in Parkinson's disease', *Annals of Neurology*, 40(4), pp. 663–671. doi: 10.1002/ana.410400417.

Takahashi, R. H., Milner, T. A., Li, F., Nam, E. E., Edgar, M. A., Yamaguchi, H., Beal, M. F., Xu, H., Greengard, P. and Gouras, G. K. (2002) 'Intraneuronal Alzheimer AB42 Accumulates in Multivesicular Bodies and Is Associated with Synaptic Pathology', *American Journal of Pathology*, 161(5), pp. 1869–1879.

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H. D., Sun, M., Sato, Y., Chengyu, L., Jung, J. U., Cheng, J. Q., Mulé, J. J., Pledger, W. J. and Wang, H.-G. (2007) 'Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis', *Nature Cell Biology*, 9(10), pp. 1142–1151. doi: 10.1016/j.coi.2010.04.008.Monocytes.

Tammineni, P., Ye, X., Feng, T., Aikal, D. and Cai, Q. (2017) 'Impaired retrograde transport of axonal autophagosomes contributes to autophagic stress in Alzheimer's disease neurons', *eLife*, 6(e21776), pp. 1–26. doi: 10.7554/eLife.21776.

Tanji, K., Miki, Y., Ozaki, T., Maruyama, A., Yoshida, H., Mimura, J., Matsumiya, T., Mori, F., Imaizumi, T., Itoh, K., Kakita, A., Takahashi, H. and Wakabayashi, K. (2014) 'Phosphorylation of serine 349 of p62 in Alzheimer's disease brain', *Acta Neuropathologica Communications*, 2(50), pp. 1–14. doi: 10.1186/2051-5960-2-50.

Tarawneh, R. and Holtzman, D. M. (2012) 'The clinical problem of symptomatic Alzheimer disease and mild cognitive impairment.', *Cold Spring Harbor Perspectives in Medicine*, 2(5), p. a006148. doi: 10.1101/cshperspect.a006148.

Tasselli, L., Zheng, W. and Chua, K. F. (2017) 'SIRT6: Novel Mechanisms and Links to Aging and Disease', *Trends in Endocrinology and Metabolism*. Elsevier Ltd, 28(3), pp. 168–185. doi: 10.1016/j.tem.2016.10.002.

Teng, Y., Jing, H., Aramsangtienchai, P., He, B., Khan, S., Hu, J., Lin, H. and Hao, Q. (2015) 'Efficient Demyristoylase Activity of SIRT2 Revealed by Kinetic and Structural Studies', *Scientific Reports*, 5(8529), pp. 1–8. doi: 10.1038/srep08529.

Toh, W. H., Tan, J. Z. A., Zulkefli, K. L., Houghton, F. J. and Gleeson, P. A. (2017) 'Amyloid precursor protein traffics from the Golgi directly to early endosomes in an Arl5b- and AP4-dependent pathway', *Traffic*, 18(3), pp. 159–175. doi: 10.1111/tra.12465.

Toorie, A. M. and Nillni, E. A. (2014) ‘Minireview : Central Sirt1 Regulates Energy Balance via the Melanocortin System and Alternate Pathways’, *Molecular Endocrinology*, 28(9), pp. 1423–1434. doi: 10.1210/me.2014-1115.

Touitou, I., Capony, F., Brouillet, J. P. and Rochefort, H. (1994) ‘Missense polymorphism (C/T224) in the human cathepsin D pro-fragment determined by polymerase chain reaction-Single strand conformational polymorphism analysis and possible consequences in cancer cells’, *European Journal of Cancer*, 30A(3), pp. 390–394. doi: 10.1016/0959-8049(94)90261-5.

Uddin, M. S., Stachowiak, A., Mamun, A. Al, Tzvetkov, N. T., Takeda, S., Atanasov, A. G., Bergantin, L. B., Abdel-Daim, M. M. and Stankiewicz, A. M. (2018) ‘Autophagy and Alzheimer’s Disease: From Molecular Mechanisms to Therapeutic Implications’, *Frontiers in Aging Neuroscience*, 10(4), pp. 1–18. doi: 10.3389/fnagi.2018.00004.

Vachharajani, V. T., Liu, T., Wang, X., Hoth, J. J., Yoza, B. K. and Mccall, C. E. (2016) ‘Sirtuins Link Inflammation and Metabolism’, *Journal of Immunology Research*, 2016, pp. 1–11.

Vanlandingham, P. A. and Ceresa, B. P. (2009) ‘Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration’, *Journal of Biological Chemistry*, 284(18), pp. 12110–12124. doi: 10.1074/jbc.M809277200.

van de Ven, R. A. H., Santos, D. and Haigis, M. C. (2017) ‘Mitochondrial Sirtuins and Molecular Mechanisms of Aging’, *Trends in Molecular Medicine*. Elsevier Ltd, 23(4), pp. 320–331. doi: 10.1016/j.molmed.2017.02.005.

Vinters, H. V. (2015) ‘Emerging Concepts in Alzheimer’s Disease’, *Annual Review of Pathology: Mechanisms of Disease*, 10, pp. 291–319. doi: https://doi.org/10.1146/annurev-pathol-020712-163927.

Viscomi, M. T., D’Amelio, M., Nobili, A., Cavallucci, V., Latini, L., Bisicchia, E., Sasso, V. and Molinari, M. (2017) ‘Autophagy Mechanismsfor Brain Recovery. Keep It Clean, Keep It Alive’, in Petrosini, L. (ed.) *Neurobiological and Psychological Aspects of Brain Recovery*, p. 302. doi: 10.1007/978-3-319-52067-4.

Walker, L. C. and Jucker, M. (2015) ‘Neurodegenerative Diseases: Expanding the Prion Concept’, *Annual Review of Neuroscience*, 38, pp. 87–103. doi: 10.1146/annurev-neuro-071714-033828.

Wan, W., You, Z., Xu, Y., Zhou, L., Guan, Z., Peng, C., Wong, C. C. L., Su, H., Zhou, T., Xia, H. and Liu, W. (2017) ‘mTORC1 Phosphorylates Acetyltransferase p300 to Regulate Autophagy and Lipogenesis’, *Molecular Cell*. Elsevier Inc., 68, pp. 1–13. doi: 10.1016/j.molcel.2017.09.020.

Wang, C., Telpoukhovskaia, M. A., Bahr, B. A., Chen, X. and Gan, L. (2018) ‘Endo-lysosomal dysfunction: a converging mechanism in neurodegenerative diseases’, *Current Opinion in Neurobiology*. Elsevier Ltd, 48, pp. 52–58. doi: 10.1016/j.conb.2017.09.005.

Wang, H., Wang, R., Xu, S. and Lakshmana, M. K. (2016) ‘Transcription Factor EB Is Selectively Reduced in the Nuclear Fractions of Alzheimer’s and Amyotrophic Lateral Sclerosis Brains’, *Neuroscience Journal*, 2016, pp. 1–8. doi: 10.1155/2016/4732837.

Wang, J., Gu, B. J., Masters, C. L. and Wang, Y.-J. (2017) ‘A systemic view of Alzheimer disease - insights from amyloid-β metabolism beyond the brain’, *Nature Reviews Neurology*, 13(10), pp.

612–623. doi: 10.1038/nrneurol.2017.111.

Wang, T., Cui, H., Ma, N. and Jiang, Y. (2013) 'Nicotinamide-mediated inhibition of SIRT1 deacetylase is associated with the viability of cancer cells exposed to antitumor agents and apoptosis', *Oncology Letters*, 6(2), pp. 600–604. doi: 10.3892/ol.2013.1400.

Wang, Y. (2014) 'Molecular Links between Caloric Restriction and Sir2 / SIRT1 Activation', *Diabetes & Metabolism Journal*, 38, pp. 321–329. doi: 10.4093/dmj.2014.38.5.321.

Wang, Y., Martinez-Vicente, M., Krüger, U., Kaushik, S., Wong, E., Mandelkow, E.-M., Cuervo, A. M. and Mandelkow, E. (2009) 'Tau fragmentation, aggregation and clearance: The dual role of lysosomal processing', *Human Molecular Genetics*, 18(21), pp. 4153–4170. doi: 10.1093/hmg/ddp367.

Wartosch, L., Bright, N. A. and Luzio, J. P. (2015) 'Lysosomes', *Current Biology*, pp. R315–R316. doi: 10.1016/j.cub.2015.02.027.

Wen, H., Zhan, L., Chen, S., Long, L. and Xu, E. (2017) 'Rab7 may be a novel therapeutic target for neurologic diseases as a key regulator in autophagy', *Journal of Neuroscience Research*, 95(10), pp. 1993–2004. doi: 10.1002/jnr.24034.

Wilhelmus, M. M. M., De Waal, R. M. W. and Verbeek, M. M. (2007) 'Heat shock proteins and amateur chaperones in amyloid-beta accumulation and clearance in Alzheimer's disease', *Molecular Neurobiology*, 35(3), pp. 203–216. doi: 10.1007/s12035-007-0029-7.

Willcox, B. J., Willcox, D. C., Todoriki, H., Fujiyoshi, A., Yano, K., He, Q., Curb, J. D. and Suzuki, M. (2007) 'Caloric restriction, the traditional okinawan diet, and healthy aging: The diet of the world's longest-lived people and its potential impact on morbidity and life span', *Annals of the New York Academy of Sciences*, 1114, pp. 434–455. doi: 10.1196/annals.1396.037.

Wirawan, E., Lippens, S., Berghe, T. Vanden, Romagnoli, A., Fimia, G. M., Piacentini, M. and Vandenabeele, P. (2012) 'Beclin 1: A role in membrane dynamics and beyond', *Autophagy*, 8(1), pp. 6–17. doi: 10.4161/auto.8.1.16645.

Witte, A. V., Fobker, M., Gellner, R., Knecht, S. and Floel, A. (2009) 'Caloric restriction improves memory in elderly humans', *PNAS Proceedings of the National Academy of Sciences of the United States of America*, 106(4), pp. 1255–1260. doi: 10.1073/pnas.0808587106.

Wong, E. (2018) 'Chaperone-Mediated Autophagy', in *Conn's Handbook of Models for Human Aging*. Second Edi. Singapore: Elsevier Inc., pp. 1117–1132. doi: 10.1016/B978-0-12-811353-0.00078-6.

Wong, E. and Cuervo, A. M. (2010) 'Autophagy gone awry in neurodegenerative diseases', *Nature neuroscience*, 13(7), pp. 805–811. doi: 10.1038/nn.2575.

Wong, P. M., Puente, C., Ganley, I. G. and Jiang, X. (2013) 'The ULK1 complex sensing nutrient signals for autophagy activation', *Autophagy*, 9(2), pp. 124–137. doi: 10.4161/auto.23323.

Wong, S. Y. and Tang, B. L. (2016) 'SIRT1 as a therapeutic target for Alzheimer's disease', *Reviews in the Neurosciences*, 27(8), pp. 813–825. doi: 10.1515/revneuro-2016-0023.

Woulfe, J. (2008) 'Nuclear bodies in neurodegenerative disease', *Biochimica et Biophysica Acta*,

1783, pp. 2195–2206. doi: 10.1016/j.bbamcr.2008.05.005.

Wyss-Coray, T. (2016) ‘Ageing, neurodegeneration and brain rejuvenation’, *Nature*, 539, pp. 180–186. doi: 10.1038/nature20411.

Xiong, Y. and Guan, K.-L. (2012) ‘Mechanistic insights into the regulation of metabolic enzymes by acetylation’, *The Journal of Cell Biology*, 198(2), pp. 155–164. doi: 10.1083/jcb.201202056.

Yamamoto, A. and Yue, Z. (2014) ‘Autophagy and Its Normal and Pathogenic States in the Brain’, *Annual Review of Neuroscience*, 37(1), pp. 55–78. doi: 10.1146/annurev-neuro-071013-014149.

Yamamoto, H., Schoonjans, K. and Auwerx, J. (2007) ‘Sirtuin Functions in Health and Disease’, *Molecular Endocrinology*, 21(8), pp. 1745–1755. doi: 10.1210/me.2007-0079.

Yang, D.-S., Stavrides, P., Mohan, P. S., Kaushik, S., Kumar, A., Ohno, M., Schmidt, S. D., Wesson, D., Bandyopadhyay, U., Jiang, Y., Pawlik, M., Peterhoff, C. M., Yang, A. J., Wilson, D. A., St George-Hyslop, P., Westaway, D., Mathews, P. M., Levy, E., Cuervo, A. M. and Nixon, R. A. (2011) ‘Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer’s disease ameliorates amyloid pathologies and memory deficits’, *Brain*, 134(1), pp. 258–277. doi: 10.1093/brain/awq341.

Yang, X. J. and Seto, E. (2007) ‘HATs and HDACs: From structure, function and regulation to novel strategies for therapy and prevention’, *Oncogene*, 26, pp. 5310–5318. doi: 10.1038/sj.onc.1210599.

Yi, C. and Yu, L. (2012) ‘How does acetylation regulate autophagy?’, *Autophagy*, 8(10), pp. 1529–1530. doi: 10.4161/auto.21156.

Younkin, S. G. (1998) ‘The role of Aβ42 in Alzheimer’s disease’, *J Physiology (Paris)*, 92, pp. 289–292. doi: 10.1016/S0928-4257(98)80035-1.

Yu, W. H., Kumar, A., Peterhoff, C., Shapiro Kulnane, L., Uchiyama, Y., Lamb, B. T., Cuervo, A. M. and Nixon, R. A. (2004) ‘Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: Implications for β-amyloid peptide over-production and localization in Alzheimer’s disease’, *International Journal of Biochemistry and Cell Biology*, 36(12), pp. 2531–2540. doi: 10.1016/j.biocel.2004.05.010.

Zhang, C. and Cuervo, A. M. (2008) ‘Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function’, *Nature Medicine*, 14(9), pp. 959–965. doi: 10.1038/nm.1851.

Zhang, L., Sheng, R. and Qin, Z. (2009) ‘The lysosome and neurodegenerative diseases’, *Acta Biochim Biophys Sin*, 41(6), pp. 437–445. doi: 10.1093/abbs/gmp031.Review.

Zhang, M., Chen, L., Wang, S. and Wang, T. (2009) ‘Rab7: roles in membrane trafficking and disease’, *Bioscience Reports*, 29(3), pp. 193–209. doi: 10.1042/BSR20090032.

Zhou, W., Scott, S. A., Shelton, S. B. and Crutcher, K. A. (2006) ‘Cathepsin D-mediated proteolysis of apolipoprotein E: Possible role in Alzheimer’s disease’, *Neuroscience*, 143(3), pp. 689–701. doi: 10.1016/j.neuroscience.2006.08.019.

Zhu, H. and He, L. (2015) ‘Beclin 1 biology and its role in heart disease’, *Current Cardiology*

Reviews, 11(3), pp. 229–237. Available at: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L604796654.

Zhu, H., Zhao, L., Wang, E., Dimova, N., Liu, G., Feng, Y. and Cambi, F. (2012) 'The QKI-PLP pathway controls SIRT2 abundance in CNS myelin', *Glia*, 60(1), pp. 69–82.

Zilfou, J. T. and Lowe, S. W. (2009) 'Tumor suppressive functions of p53.', *Cold Spring Harbor perspectives in biology*, 1(5), pp. 1–12. doi: 10.1101/cshperspect.a001883.

Zschoernig, B. and Mahlknecht, U. (2008) 'SIRTUIN 1: Regulating the regulator', *Biochemical and Biophysical Research Communications*, 376(2), pp. 251–255. doi: 10.1016/j.bbrc.2008.08.137.