UNIVERSITÀ
DEGLI STUDI
FIRENZE

Dottorato di Ricerca Internazionale in
Farmacologia, Tossicologia e Trattamenti Innovativi

CICLO XXVI

COORDINATORE: Prof.ssa Elisabetta Teodori

Beneficial effects of Oleuropein Aglycone in Alzheimer's disease models and a study of autophagy in neurodegeneration and development of the brain

Settore Scientifico Disciplinare BIO/14

Dottoranda
Dott.ssa Teresa Ed Dami

Tutore Scientifico
Prof.ssa Fiorella Casamenti

Coordinatore
Prof.ssa Elisabetta Teodori

Anni 2011/2013

This thesis reports three studies

Rat study

Supervision: Prof.ssa Fiorella Casamenti
Department of NEUROFARBA, University of Florence, Italy

Publication:
Luccarini I, Ed Dami T, Grossi C, Rigacci S, Stefani M and Casamenti F (2014) Oleuropein Aglycone counteracts Aß42 toxicity in the rat brain
Neuroscience Letters 558:67-72

Mouse study

Supervision: Prof.ssa Fiorella Casamenti
Department of NEUROFARBA, University of Florence, Italy

Publications:
Grossi C, Ed Dami T, Rigacci S, Stefani M, Luccarini I and Casamenti F (2013) Employing Alzheimer disease animal models for translational research: focus on dietary components. *Neurodegenerative Diseases* (DOI: 10.1159/000355461)

Grossi C, Rigacci S, Ambrosini S, Ed Dami T, Luccarini I, Traini C, Failli P, Berti A, Casamenti F and Stefani M (2013) The polyphenol Oleuropein Aglycone protects TgCRND8 mice against Aβ plaque pathology.
PLoS ONE 8(8): e71702. doi:10.1371/journal.pone.0071702

Zebrafish study

Supervision: Prof. David C. Rubinsztein and Dr. Angeleen Fleming
Cambridge Institute for Medical Research, Department of Medical Genetics
Department of Physiology, Development and Neuroscience
University of Cambridge, England, UK

INDEX

INTRODUCTION

ALZHEIMER'S DISEASE

1. History
2. Epidemiology
3. Etiology
   3.1 Age
   3.2 Genetics
4. Clinical presentation
5. Clinical forms and symptoms
6. Histopathological features: senile plaque and neurofibrillary tangles
   6.1 APP metabolism
   6.2 Amyloid and oligomers
7. Inflammation
   7.1 Cellular mediators
   7.2 Molecular mediators
8. Treatment strategies
   8.1 Cholinergics
   8.2 Neurotropins
   8.3 Antioxidants
   8.4 Statins
   8.5 Non steroidal anti inflammatory drugs (NSAIDs)
   8.6 Hormone replacement therapy
   8.7 Blocking of exitotoxicity
   8.8 Immunotherapy
   8.9 Secretase effectors
   8.10 Diet

AUTOPHAGY

1. History
2. Types of autophagy
3. The process of macroautophagy
   3.1 Induction of autophagy
   3.2 Cargo recognition and selectivity
   3.3 Autophagosome formation
   3.4 Expansion of the phagophore
   3.5 Vescicle fusion and autophagosome breakdown
4. Non-atg components required for autophagy
   4.1 The secretory and endocytic pathways
   4.2 Cytoskeleton
5. Signaling pathways regulating autophagy

5.1 Nutrient signaling
5.2 Insulin/growth factor pathways
5.3 Energy sensing
5.4 Stress response
5.5 Pathogen infection

6. Quality control and starvation-induced autophagy
7. Autophagy and diseases
   7.1 Autophagy and Alzheimer's disease
8. Autophagy and development

MEDITERRANEAN DIET

1. History
2. Mediterranean diet pyramid
   2.1 Plant foods
   2.2 Olive oil as the principal fat
   2.3 Dairy products
   2.4 Meat, fish and eggs
3. Beneficial roles in preventing pathologies
4. Oleuropein Aglycone
   4.1 Chemistry
   4.2 Biosynthesis
   4.3 Bioavailability
   4.4 Pharmacology
   4.5 Effects on amyloid toxicity

AIM OF THE STUDY

Aim of the study

MATERIALS AND METHODS

RAT EXPERIMENTS

1. Animals
2. Oleuropein deglycosilation
3. Aβ aggregation
4. Brain injection
5. Immunohistochemistry
6. Determination of ChAT positive cells

MOUSE EXPERIMENTS

1. TgCRND8 mice
2. Genotyping
3. Oleuropein Aglycone treatment
4. Body weight
5. Behavioral experiment

5.1 Step down inhibitory passive avoidance task
5.2 Context-dependent object recognition test

6. BrdU treatment
7. Animal tissue processing
8. Histology, histochemistry and immunohistochemistry
   8.1 Thioflavin S staining
   8.2 BrdU labeling and counting
9. Determination of Aβ plaque load
10. Western blot
11. ELISA assay
12. TBARS assay

ZEBRAFISH EXPERIMENTS

1. Zebrafish
2. TALENs technology
3. TALEN plasmids
4. Microinjection
5. PCR and enzymatic digestion

RESULTS AND DISCUSSIONS

RAT STUDY

1. Results and discussion
   1.1 Aβ aggregation
   1.2 ChAT
   1.3 Aβ peptide
2. Conclusion

MOUSE STUDY

1. Results
   1.1 Behavior
   1.2 Neurogenesis
   1.3 Aβ deposition
   1.4 Autophagy
   1.5 *Neuroinflammation*
2. Discussion

ZEBRAFISH STUDY

1. Experimental design
2. Results
3. Discussion

REFERENCES

INTRODUCTION

ALZHEIMER'S DISEASE

1. History

In 1907 Alois Alzheimer (1863-1915) described the case of a 51 year-old woman who showed rapidly deteriorating memory along with psychiatric disturbances and died four years later. At that time, a variety of progressive and fatal neurological conditions were known, including senile dementia, but two factors made this case unique: the early age at onset and the neurofibrillary tangles (NFT), a new pathological finding.

Alzheimer's disease (AD) is a progressive neurologic deterioration accompanied by hallmark pathology.

Over time, AD was split into two clinical conditions depending upon the age of onset. "Alzheimer disease" was used to indicate a type of "presenile" dementia, since its initial description in a relatively young woman, affecting individuals younger than 65 years of age, whereas a similar dementia in the elderly, in individuals over 65 years of age, was referred to as "senile dementia of the Alzheimer-type" after the pioneering studies of Tomlinson, Roth and Blessed (Roth et al., 1966). Although these age-related classifications are still sometimes used, ad has never been shown to have a bi-modal age of onset, AD was absolutely recognized as a distinct entity from "dementia senilis". AD is now generally recognized as a single entity with a prevalence that increases sharply after age of 65 (Castellani et al., 2010).

2. Epidemiology

AD constitutes approximately 70% of all dementia cases (Fratiglioni et al., 1999; Small et al., 1997) . Incidence of AD increases with age, doubling every five to ten years. For people between ages 65-69, 70-74, 75-79, 80-84, and 85 and older the incidence of AD has been estimated at 0.6%, 1.0%, 2.0%, 3.3% and 8.4%, respectively (Hebert et al., 1995). Prevalence also increases exponentially with age, rising from 3% among those 65-74, to almost 50% among those 85 or older. AD affects 25 million people worldwide. In the US, prevalence was estimated at 5 million in 2007 and, by 2050, is projected to increase to 13 million in the US alone (Zhu et al., 2006). Aside from age, other risk factors include family history of dementia, head trauma, genetic

factors (apolipoprotein E [ApoE] ε4 allele), being female, low education level, vascular disease and environmental factors.

The estimated data about incidence are in keeping with the projected demographic changes resulting from the "baby boomer" generation reaching old age, and the continued increase in life expectancy. The combination of a declining birth rate and an increased average life span is expected to increase the proportion of the population aged 65 years and older in the US from 12.4% in 2000 to 19.6% in 2030. The number of people aged 80 years old and older is also expected to double, from 9.3 million in 2000 to 19.5 million in 2030. The number of people aged 65 years and older is expected to increase from 35 million in 2000 to 71 million in 2030. The number of people aged 80 years and older is also expected to double, from 9.3 million in 2000 to 19.5 in 2030. Globally, the number of older adults (aged 65 years and older) is projected to increase even more dramatically: more than doubling from 420 million in 2000 to 973 million in 2030. Considering the fact that advanced age is the most significant risk factor for AD, one cannot underestimate the problem this disease poses in terms of its financial and human cost.

Since the current therapies could not block the progression of the pathology, AD is actually considered a clinical, social and economic problem, so it is considered the 3rd most important pathology, after cardiovascular disease and tumors (Castellani et al., 2010).

3. Etiology

3.1 Age

Age represents, by far, the single greatest risk factor in the etiology of AD. Even in genetically-predisposed individuals, the disease essentially does not occur in middle age. Therefore, regardless of whether one is genetically predisposed or not, aging is essential factor in AD, strongly suggesting that an age-related process is involved in the development of the disease. This age-related penetrance is not restricted to AD and is also a risk factor in a number of other chronic diseases including other neurodegenerative diseases, cancer, atherosclerosis, arthritis and emphysema indicating the possibility that there may be common etiologies with diverse consequences.

Nevertheless, it remains an open question whether AD is purely a manifestation of advanced age, as has been suggested, and if it still worths separating "senile dementia" as was known at the time of Alzheimer, from "Alzheimer disease."

A progressive neurological deterioration, beginning in the 6th decade with no family history, does occur. It differs dramatically from the slowly progressive memory loss in a patient in the 9th or 10th decade with concomitant atherosclerotic disease and the confounding effect of a multitude of comorbid illnesses, their complications, and a long medication list.

The difficulties in AD diagnosing in the very old has also been experienced since the original AD descriptions. Indeed, in one study, neuropathologists blinded to clinical history diagnosed AD in 76% of elderly subjects who were otherwise not clinically demented during life (Brayne et al., 2009). These data seem to emphasize again a discernible difference, clinical and pathological, between AD and senile dementia, the first is precipitated by a specific cause as yet unknown, and the latter is a manifestation of advanced age *per se*.

3.2 Genetics

Evidence for a significant genetic component has been shown in a substantial proportion of individuals with AD. First degree relatives of AD patients have a higher lifetime incidence of AD than the general population, and 15–35% of patients with AD have affected first-degree relatives. Using the traditional arbitrary distinction between early- (<65 years) and late- (>65 years) onset, it has been shown that the early familial form of the disease tends to be more aggressive, with particular familial forms having a characteristic age of onset (Bondareff et al., 1987).

A number of mutated proteins have been implicated in familial cases of AD.

Amyloid Precursor Protein . Transgenic mice overexpressing mutated APP form senile plaques (e.g., Tg2576), show age related deficiencies on cognitive-behavioral testing and show synaptic deficits, supporting a pathogenic role for mutated APP.

A small number of early-onset familial AD cases are linked to mutations in AβPP.

To date, 89 families affected by germline AβPP mutations have been described worldwide (http://www.molgen.ua.ac.be/ADMutations/default.cfm). Interestingly, such cases comprise only about 9% of familial AD families, while the most common

germline mutations linked to familial early onset disease are linked to presenilin 1 (~40%).

ApoE protein. One of the major risk factors for the development of AD is carrying the ε4 allele of APOE either in heterozygous or homozygous state (Sherrington et al., 1995). The mechanism by which ApoE influences the pathogenesis of AD is unknown; however, ApoE, a serum cholesterol transport protein, is a component of both senile plaques and NFT (Namba et al., 1991).

Interestingly, oxidative stress would also be important in the action of ApoE, since the formation of ApoE/Aβ complexes is increased in oxygenated buffer and completely abolished under reducing condition (Strittmatter et al., 1993). Moreover, oxidized ApoE ε4 forms complexes with Aβ at a significantly higher rate than in similar experiments with ApoE ε3. Therefore, the oxidation of ApoE, alone or bound with Aβ protein, might affect receptor affinity and/or other catabolic interactions by a mechanism analogous to the oxidation of low density lipoprotein in diabetic renal disease (Gupta et al., 1992).

Presenilin 1 and Presenilin 2. The majority (~70%) of early-onset familial AD cases are associated with mutations in two genes, *presenilin 1 and presenilin 2*, located on chromosomes 14 and 1, respectively (Sherrington et al., 1995). To date, over thirty different pathogenic mutations in these genes have been described from over sixty unrelated kindred (Tanzi et al., 1996). The presenilins comprise part of the γ-secretase complex, necessary for the synthesis of Aβ peptides. Mutations at these sites evidently alter Aβ processing in favor of increased synthesis and deposition. Familial early onset AD cases associated with presenilin mutations are noteworthy in the extensive deposits of Aβ throughout the brain, including white matter and extensively within blood vessels (Castellani et al., 2008). There is considerable homology between the gene products of *presenilin 1 and presenilin-2* which are transmembrane proteins of 463 and 448 amino acids, respectively, with between six and nine hydrophobic membrane spanning domains (Sherringhton et al., 1995).

CLU and PICALM. *CLU* (which encodes clusterin) and *PICALM* (phosphatidylinositol-binding clathrin assembly protein, also known as CALM, clathrin assembly lymphoid-myeloid leukemia gene) genes were identified as loci involved in the emerging disease (Harold et al., 2009).

Clusterin is a multifunctional molecule: it interacts with the soluble form of Aβ in animal models of disease and binds soluble Aβ in a specific and reversible manner,

forming complexes that have been shown to cross the blood-brain barrier. Notably, ApoE also appears to act as a molecular chaperone for Aβ and influences when Aβ aggregates and deposits; ApoE also influences Aβ conformation and toxicity. In a similar manner similar to ApoE, clusterin may regulate both the toxicity of Aβ and its conversion into insoluble forms. ApoE and clusterin have been shown to cooperate in suppressing Aβ deposition, and they may critically modify Aβ clearance at the blood-brain barrier, which could suggest a role for clusterin in the amyloidogenic pathway. Levels of ApoE protein appear to be inversely proportional to APOE ε4 allele dose levels, with protein levels reduced in ε4 homozygotes compared with heterozygotes. Conversely, clusterin levels are increased in proportion to APOE ε4 allele dose levels, suggesting an induction of clusterin in individuals with low ApoE levels (Holtzman, 2004).

PICALM is ubiquitously expressed in all tissue types with prominent expression in neurons, where it is non-selectively distributed at the pre- and postsynaptic structures. PICALM protein is involved in clathrin-mediated endocytosis, an essential step in the intracellular trafficking of proteins and lipids such as nutrients, growth factors and neurotransmitters. Of relevance to AD, PICALM appears to be involved in directing the trafficking of VAMP2. VAMP2 is a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein that has a prominent role in the fusion of synaptic vesicles to the presynaptic membrane in neurotransmitter release, crucial process to neuronal function. Genetically directed changes in PICALM function may lead to synaptic dysfunction, possibly through synaptic vesicle cycling, thereby increasing risk for AD. Alternatively, PICALM could influence AD risk through APP processing via endocytic pathways, resulting in changes in Aβ levels.

Other proteins. In 2013 a genome-wide association study of AD involving 74,046 individuals identified 11 new susceptibility loci for AD (Lambert et al., 2013).

1. The HLA-DRB5–DRB1 region (encoding major histocompatibility complex, class II, DRβ5 and DRβ1, respectively) is associated with immunocompetence and histocompatibility and, interestingly, with risk of both multiple sclerosis and Parkinson disease (Sawcer et al., 2011; Nalls et al., 2011).
2. The *SORL1* gene (encoding sortilin-related receptor, L(DLR class) 1) is associated with increased risk of both autosomal dominant and sporadic forms of AD (Pottier et al., 2012) and represents the first gene that directly connects

aberrant trafficking and metabolism of the APP to late-onset AD (Rogaeva et al., 2007).

3. The PTK2B locus (encoding protein tyrosine kinase 2β), is only approximately 130 kb away from *CLU*; the protein encoded by *PTK2B* may be an intermediate between neuropeptide-activated receptors or neurotransmitters that increase calcium flux and the downstream signals regulating neuronal activity such as mitogen-activated protein kinase (MAPK) signaling (Pandey et al., 1999); PTK2B is involved in the induction of long-term potentiation in the hippocampal CA1 (cornu ammonis 1) region, a central process in the formation of memory (Huang et al., 2001).
4. The *SLC24A4* gene [encoding solute carrier family 24 (sodium/potassium/ calcium exchanger), member 4] encodes a protein involved in iris development and hair and skin color variation in humans in addition to being associated with the risk of developing hypertension (Sulem et al., 2007; Adeyemo et al., 2009); SLC24A4 is also expressed in the brain and may be involved in neural development (Larsson et al., 2011).
5. The *ZCWPW1* gene (encoding zinc finger, CW type with PWWP domain 1) encodes for a protein that modulates epigenetic regulation (He et al., 2010).
6. Another locus was within the *CELF1* gene (encoding CUGBP, Elav-like family member 1), whose gene product is a member of the protein family that regulates pre-mRNA alternative splicing (Gallo and Spickett, 2010).
7. Another gene is *MADD* (encoding MAP kinase–activating death domain), the reduced expression of which may affect long-term neuronal viability in AD (Del Villar and Miller, 2004).
8. The *FERMT2* gene (encoding fermitin family member 2) is expressed in the brain; its corresponding protein localizes to cell matrix adhesion structures, activates integrins, is involved in the orchestration of actin assembly and cell shape modulation, and is an important mediator of angiogenesis (Pluskota et al., 2011).
9. The *CASS4* gene (encoding Cas scaffolding protein family member 4): little is known about the function of the encoded protein; however, the *Drosophila* CASS family ortholog (p130CAS) binds to CMS, the Drosophila ortholog of CD2AP

(CMS), a known AD susceptibility gene that is involved in actin dynamics (Kirsch et al., 1999).

10. The *INPP5D* gene (encoding inositol polyphosphate-5-phosphatase, 145 kDa) is expressed at low levels in the brain, but the encoded protein has been shown to interact with CD2AP, whose corresponding gene is one of the AD genes previously identified (Bao et al., 2012), and to modulate, along with GRB2, metabolism of APP (Brauer et al., 2012).
11. The *MEF2C* gene (encoding myocyte enhancer factor 2). The MEF2C protein limits excessive synapse formation during activity-dependent refinement of synaptic connectivity and thus may facilitate hippocampal-dependent learning and memory (Akhtar et al., 2012); mutations in this locus are associated with severe mental retardation, stereotypic movements, epilepsy and cerebral mal-formation (Le Meur et al., 2010).

The newly associated loci reinforce the importance of some previously suspected pathways such as APP (*SORL1* and *CASS4*) and tau (*CASS4* and *FERMT2*) in pathology. Several candidate genes at these loci are involved in pathways already shown to be enriched for association signal in AD such as immune response and inflammation (*HLA-DRB5–DRB1*, *INPP5D* and *MEF2C*), which is also supported by the described association of AD with cell migration (*PTK2B*) and lipid transport and endocytosis (*SORL1*). These recent results suggest the existence of new pathways underlying AD. These pathways could include hippocampal synaptic function (*MEF2C* and *PTK2B*), cytoskeletal function and axonal transport (*CELF1* and *CASS4*), regulation of gene expression and post-translational modification of proteins, and microglial and myeloid cell function (*INPP5D*) (Lambert et al., 2013).

4. Clinical presentation

The diagnosis of AD requires clinical evidence of memory loss and impairment of at least one other cognitive domain, with evidence of disturbance in social or occupational function. AD must be differentiated from other causes of dementia: vascular dementia, dementia with Lewy bodies, Parkinson's disease with dementia, fronto-temporal dementia (FTD) and reversible dementias. Wide variability in initial clinical presentation may be expected based on the brain regions affected: dysfunctions

concerning speech, personality and judgment, vision, sensory-motor function and memory impairment, are well known.

Braak and Braak in 1991 popularized the concept of a more or less predictable progression of pathology and clinical signs (Braak et al., 2006).

An attempt to refine the early diagnostic features of AD is reflected in the new nosologic entity, Mild Cognitive Impairment (MCI), in which patients have cognitive dysfunction that can be identified objectively, and are not demented (Peterson, 2004). While the attempt to identify early AD is strategic, if not predictable, in terms of targeting the disease for intervention, it is important to point out that MCI is not a distinct entity. Rather, MCI lacks a pathological substrate, genetic predispositions and discernible treatment benefits, whereas a given patient with this diagnosis may progress to neurodegenerative disease, remain clinically stable for many years, and even improve over time. However, in a subset of patients MCI represents a transition state between normal aging and AD.

Unfortunately, genuine AD is progressive, in spite of all available therapy thus far. Initial insidious memory impairment converts, over months and years, to disorientation, personality and judgment dysfunctions, speech abnormalities and apraxias, among other signs. The ability to care for one's self is lost over time. With aggressive management, AD patients often live out their final months and sometimes years in a vegetative state, bed bound, with sacral decubiti (Castellani et al., 2010).

The proximate cause of death in most patients is pneumonia, which comes as a merciful ending to a prolonged and tragic illness that has long since robbed the affected patient of their individuality and dignity.

5. Clinical forms and symptoms

Several clinical forms of AD have been reported, depending on the onset of age:

Early onset form (EOFAD): is a familiar form of AD (FAD) that affects people under 40, it represents 5-10% of the total cases ; it is hereditary since the main cause is mutation in three genes, coding for Amyloid Precursor Protein (APP), presenilin 1 and presenilin 2 (PSEN1, PSEN2), located respectively on chromosomes 21,14 and 1.

Late onset form (LOFAD): it affects people over 65 years, it represents the 90% of the total cases, and it is a sporadic form; have been reported some predisposing factors,

such as the ε4 allele of the ApoE gene, located on the chromosome 19 and the allele 2 of the α-2-macroglobulin, located on the chromosome 12 (Colacicco et al., 2009).

AD is characterized by a chronic and progressive impairment of memory functions. Firstly, the loss of memory involves recent events, then non-cognitive deficits appears such as mood alterations, behavioral and neurovegetative disturbs with sleep disorders. Later cognitive defects set on: short-term and long-term memory dysfunction, cognitive deficits (Làvadas and Berti, 2002), language deficits (Luttazzi, 1996) and emotion processing disturbs (Passafiume et al., 2002 and 2006).

6. Senile plaques and neurofibrillary tangles

AD is characterized by the presence of senile plaques, containing the β-amyloid peptide (Aβ) and neurofibrillary tangles (NFT), consisting of hyperphosphorylated forms of tau protein; in AD a selective death of the cholinergic system, atrophy and synaptic and neuronal loss occurs; all these features contribute to the progressive cognitive decline that characterizes AD (Selkoe, 2002, Ling et al., 2003).

The main component of the senile plaques is a fibrillar polymeric form of amyloid peptide 1-42 (Aβ42), generated from the proteolysis of Amyloid Precursor Protein (APP) (Selkoe, 1991). Currently the amyloid plaques are thought to be the main responsible of the cognitive impairments that occur in AD (Selkoe, 1991).

Moreover, a massive atrophy of the cerebral cortex (Fig.1), a wide neuronal loss in both cerebral cortex and cerebellum, basal ganglia and forebrain, encephalic trunk and bone marrow occur. The cell death involves first the cholinergic basal nuclei, then it involves amygdale and pyramidal cortex neurons (Rasool et al., 1986)

Senile plaques (Fig. 2a) are extracellular deposits consisting of the small peptide Aβ (about 4 kDa) that aggregates (Kang et al. 1987). During the preclinical stages of the pathology, amorphous aggregates of Aβ consisting mainly of non-fibrillar Aβ42 peptide, named diffuse plaques have been observed (Yamaguchi et al., 1988; Tagliavini et al., 1988). During the clinical stages, senile plaques are composed by extracellular compact deposits of Aβ40 and Aβ42 peptides, soluble oligomers and insoluble fibrils. The β-sheet structure of Aβ is kept by several proteins that are associated with α-1 antichymotrypsine (α-1 ATC), apolipoprotein E2 and E4, complement protein and proteoglycans (Selkoe, 1999). In senile plaques a central "core" of amyloid is surrounded by degenerated neurons with dystrophic dendrites and axons projected

towards the core, and two types of glial cells: microglial cells penetrating the plaque and astrocytes located around it (Selkoe, 1999).

The NFT (Fig. 2b) are filamentous inclusions consisting of hyperphosphorylated forms of tau protein. In physiologic conditions, tau protein is soluble in the cytoplasm and promotes the microtubules assembling and stabilization (Grundke-Iqbal et al., 1986); otherwise, in pathologic conditions tau protein is subjected to an abnormal phosphorylation that makes it insoluble and let it aggregate in large filaments (Hanger et al., 1991).

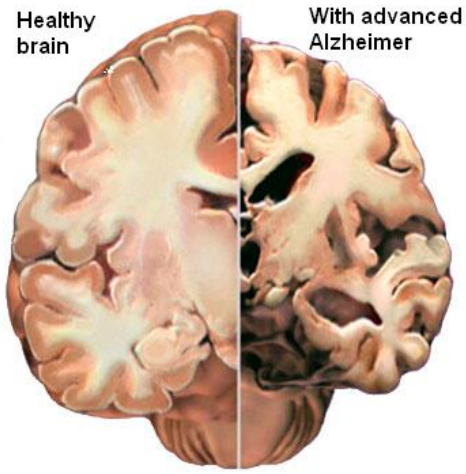


Figure 1. *Comparison between healthy and AD brain. In AD patients the volume of the cortex is decreased, lateral ventricules are wider, showing the effect of the massive neuronal loss that causes cerebral volume loss as well.*

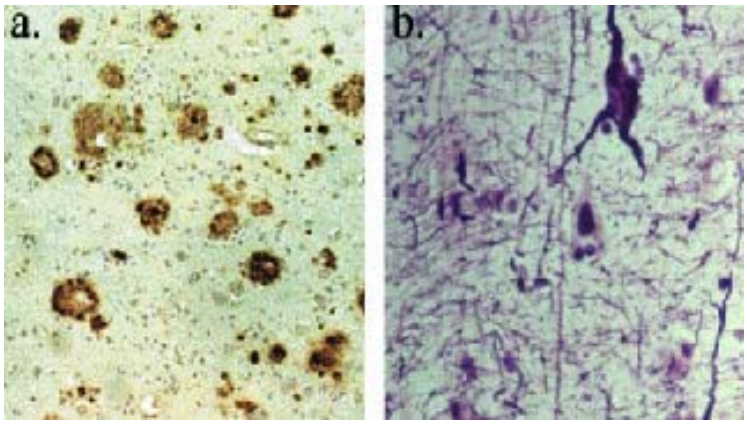


Figure 2*. a) senile plaques b) neurofibrillary tangles*

6.1 APP metabolism in Alzheimer's disease

Aβ peptide consists in 39-43 amino acids, and it is generated by an abnormal proteolysis of the APP. Human APP gene is located in the chromosome 21, it belongs to a family genes that includes APLP1 and APLP2 (human), Appl (fly) and apl-1 (worm). All these genes code for a membrane glycoprotein, but only the APP gene contains the region coding for Aβ peptide.

APP protein is a small transmembrane protein, it consists in a long extracellular N-term domain, an intracytoplasmic domain and an intramembrane portion that contains the last 14 aminoacids of the Aβ peptide sequence. APP protein is also located on endoplasmic reticule, Golgi apparatus and on endosomes (Selkoe, 1994).

APP protein has several roles in order to maintain cellular functions: cell adhesion, endocytosis and synaptogenesis.

After the alternative splicing of the APP at least three forms have been thought to be the main responsible of the pathology: APP-695, mostly expressed in central neurons, APP-751 and APP-770, ubiquitari expressed in the periphery (Selkoe, 1994).

Aβ peptide is generated by an abnormal cleavage of APP operated by specific enzymes; APP can be cleaved by three secretases: α, β and γ (Fig. 3).

APP metabolism can follow two pathways: non-amyloidogenic, when α-secretase cleaves APP, and amyloidogenic, when β and γ secretases work.

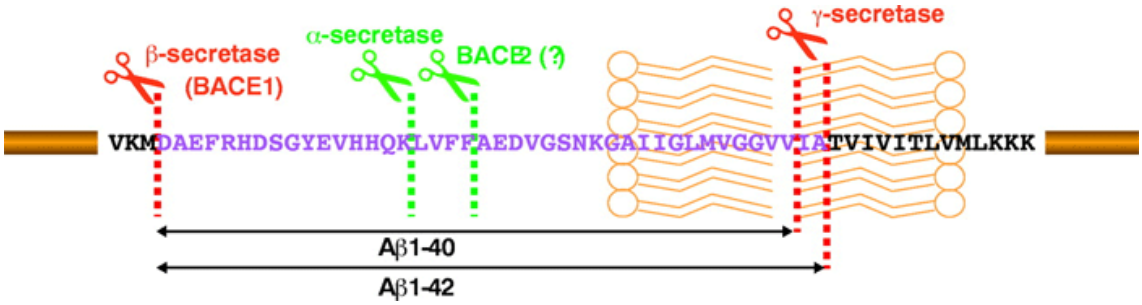


***Figure 3**. APP metabolism mediated by the secretases: α, ß e γ.*

The non-amyloidogenic pathway involves three α-secretases enzymes: ADAM 9, ADAM 10 and ADAM 17 (Lammich et al., 1999). The α-secretases cleave APP between aminoacids 16 and 17 releasing the soluble bAPPs in the extracellular space (Postina, 2008) and a 83-aa C-term fragment, named C83, that remains in the plasma membrane. Then C83 is cleaved by the γ-secretase, a multiproteic transmembrane complex composed by nicastrin, APH-1 (antheriorpharynx-defective phenotype-1),

PEN-2 (PS enhancer 2) and presenilin-1 (PS), generating a smaller fragment, called P3 (Haas et al., 1993).

The amyloidogenic pathway involves β and γ-secretases generating Aβ peptides. The β-secretase, also called BACE 1 (β-APP-cleavage-enzyme 1), is an aspartyl-protease and cuts APP generating a soluble βAPP (βAPPs) in the extracellular space and a 99-aa C-term fragment (C99), that remains in the plasma membrane. Then the γ-secretase cleaves the C99 fragment generating Aβ peptides and a small intracellular fragment called AICD (APP intracellular domain), a transcriptional activator (Pangalos et al., 2005). Depending on the cut site of the γ-secretase, different peptides are generated: if the cleavage is between 712 and 713 aa, a short Aβ peptide is produced (39-40aa), otherwise if the γ-secretase cut between 713 and 714, a longer peptide is generated (42-43 aa), the main form found in senile plaques, due to its trend to aggregate. Another enzyme with α-secretase activity has been identified, BACE 2, that has 50% identity of aa sequence of BACE1; it can cleave APP between amino acids 19 and 20, suggesting a role against BACE1 activity (Farzan et al., 2000).

Intracellular insoluble Aβ has been reported (LaFerla et al., 2007), in fact during the early stages of AD Aβ is generated inside neurons (Mora et al., 2001) and then it aggregates to form cerebral plaques (Wirths, 2001). Several studies on AD animal models correlate intracellular Aβ with the cognitive decline (Billings et al., 2005). Intracellular Aβ can result from intracellular APP proteolysis (Kinoshita et al., 2003) or it can be the result of extracellular Aβ re-uptake (Deane at al., 2003).

Endosomal-lysosomal APP cleavage is mediated by β and γ-secretases, generating potentially amyloidogenic derivates (Golde et al., 1992). The trans-Golgi APP metabolism involves the α-secretase and produces a soluble Aβ (Haass et al., 1993), that has trophic effects on neuronal growth (Selkoe, 1994).

In basal conditions, cerebral Aβ is degraded by neprilysin, a metal proteases zinc-dependent regulated by nicastrin, an enzyme of the γ-secretases complex (Pardossi-Piquard et al., 2006), and by a metal proteases endotelin-converter. Furthermore, the cerebral Aβ is regulated by systemic blood flow through the Receptor for Advanced Glycation End products (RAGE) and the Low-density Lipoprotein Receptor related Protein 1 (LRP1) (Tanzi et al., 2004). Some AD patients show RAGE upregulation and LRP1 downregulation, anomalies that contribute further to increase the amount of cerebral Aβ (Donahue et al., 2006).

6.2. Amyloid and oligomers

The aggregation and accumulation of amyloid-β plays a significant role in the pathogenesis of AD. Aβ oligomeric aggregates are believed to be the main toxic species and the causative agent underlying the pathological mechanism for AD, aggregating and accumulating within and around neurons. Excised from the APP, Aβ peptide has intrinsic property of forming aggregates with β-pleated sheet structure (Hardy and Selkoe, 2002).

The amyloid hypothesis has undergone several modification, mainly concerning the type of Aβ thought to cause AD: initially this was the amyloid plaque, followed by increased concentrations of Aβ42, then increased Aβ42:Aβ40 ratio, and finally pre-fibrillar oligomers (Pimplikar, 2009). Results from clinical trials have shown that removing plaques will not reserve the damage or stop AD (Hardy, 2009). Recent evidence suggest that this toxicity may be linked to the aggregation state of the peptide, implicating oligomers, rather than insoluble fibrils, as the primary toxic species (Baglioni et al., 2006). While both are found in the brains of post mortem AD patients, soluble Aβ oligomers are better correlated with the disease severity than are the classic amyloid plaques containing insoluble Aβ fibrillar deposits (McLean et al., 1999). Furthermore, oligomers are found both extracellularly, and are capable of moving between the interior of the cell and the extracellular space (Gaspar et al., 2010). However, Aβ oligomer structure, size, conformation and inter-relationship with other amyloid aggregates, as well as the exact mechanism of Aβ oligomer-induced neurotoxicity, remain elusive.

Monomeric Aβ undergoes conformation transition and proceeds to form low molecular oligomers (dimer/trimer) and then soluble high molecular aggregates and progress to form spherical oligomers which are composed of 12 to 24 monomers, which prolong to protofibrils and finally become insoluble fibrils (Glabe, 2008). These various structures differ not only in aggregation state, but also in their toxic effects. Recently, many have reported that fibrils, which were once thought to exhibit the highest level of toxicity, are actually second in toxicity to intermediate aggregates of Aβ (spherical oligomers and protofibrils) (Glabe, 2006).

The presence of a variety of Aβ oligomer conformations have been reported and oligomeric species differ not only in mechanism formation but also in mechanism of toxicity (Kayed and Lasagna-Reeves, 2013).

7. Neuroinflammation

Biochemical and neuropathological studies of brains from individuals with AD provide clear evidence for an activation of inflammatory pathways. Some components of this complex molecular and cellular machinery are most likely promoting pathological processes leading to AD, whereas other components serve to do the opposite.

7.1. Cellular mediators

Microglia. Microglia are generally recognized as the brain's resident macrophages, and are considered to be pivotal players in innate immune/inflammatory responses in multiple neurologic disorders, including PD (Rogers et al., 2007), HIV dementia (Garden, 2002), multiple sclerosis (Muzio et al., 2007), amyotrophic lateral sclerosis (Dewil et al., 2007), AD (Mandrekar-Colucci and Landreth, 2010) and others. There is also general consensus on mechanisms of microglial actions in both the normal and diseased central nervous system (CNS), from the remarkable ability of these cells to survey vast extents of the brain (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009) to their expression of classic pro- and anti-inflammatory mediators and receptors (Lue et al., 2001a, 2001b; Wyss-Coray, 2006; Cameron and Landreth, 2010).

Microglia in the developing brain ultimately derive from the mesenchyme, in which myeloid progenitors give rise to cells that migrate to the CNS and proliferate as microglia (Rezaie and Male, 2002). Migration of blood-derived macrophages into the CNS has also been suggested to occur later in fetal development (Chan et al., 2007). Throughout development, microglia may play an important role in remodeling of the brain by removing presumably redundant, apoptotic neurons (Bessis et al., 2007; Caldero et al., 2009).

Microglia constantly sample their immediate environment, including neighboring glia, blood vessels and neurons, by extending and retracting their processes for distances up to some 80 μm (Nimmerjahn et al., 2005). In this manner, the microglial population may survey the entire brain every few hours. Loss of synapses encountered by microglial processes has been reported using *in vivo* microscopy supporting the potential role of microglia in normal synaptic remodeling (Wake et al., 2009).

Microglia also contribute to a healthy CNS by attacking and removing potential pathogens and detritus, and by secreting tissue rebuilding factors (Wyss-Coray, 2006). Alternatively, several reports of microglial attack mechanisms in neurologic disease suggest a more complex view. Microglial cells, *in vitro*, secrete a wide range of inflammatory factors, many of which can automodulate microglial phenotype and impact bystander neurons and their processes. These include reactive oxygen species (Coraci et al., 2002); Th1 cytokines such as IL-1β, IL-6, TNF-α, and interferon γ (INF-γ) (Lue et al., 2001a); chemokines such as Macrophage Inflammatory Protein 1α (MIP1α), MIP1β, CXCL8, RANTES, and Monocyte Chemotactic Protein 1 (MCP1) (El Khoury and Luster, 2008); growth factors such as macrophage colony stimulating factor (Lue et al., 2001a); and complement components such as C1q, C3, C4, and C9 (Walker et al. 2001). They also express receptors associated with inflammatory activation, attack and phagocytosis, including cytokine receptors (John et al., 2003), chemokine receptors (Cartier et al., 2005), complement receptor 3 (Sedgwick et al., 1991), the Receptor for Advanced Glycation Endproducts (RAGE) (Walker and Lue, 2005), Fc receptors (Okun et al., 2010), CD40 (Tan et al., 1999), Formyl Peptide (FP) receptors (Brandenburg et al., 2008), various scavenger receptors (El Khoury and Luster, 2008) and Toll-Like Receptors (TLRs) (Landreth and Reed-Geaghan, 2009). Elevation of these factors in culture and animal models typically results in neurodegeneration, and all have been reported to be elevated in pathologically-vulnerable regions of the AD brain (Landreth and Reed-Geaghan, 2009).

Microglial cells are also capable of secreting anti-inflammatory mediators and growth factors such as IL-4, IL-10, IL-13, and TGF-β, just as peripheral monocytes do in the tissue-rebuilding phase that follows inflammatory attack (Wyss-Coray, 2006; Colton, 2009). Although there are multiple stimuli for the inflammatory responses of microglia in the AD brain, including simple detritus from other pathogenic reactions, aggregated Aβ deposits appear to be especially potent, as indicated by the dense

accumulations of microglia expressing MHCII and other markers of activation within and around such deposits (Rogers et al., 1988). In fact, human elderly microglia in culture have been shown to migrate to aggregated Aβ spots dried down to the well floor, and to internalize portions of the Aβ (Walker and Lue, 2005). Similarly, microglia cultured on sections of AD cortex accumulate on Aβ deposits and appear to remove them (Bard et al., 2000). Additional studies have suggested, however, that microglia may not be able to degrade the Aβ they have taken up (Majumdar et al., 2008), potentially leading to a state of "frustrated phagocytosis" and to phenotypic and functional changes in the microglia.

In transgenic mouse models of AD, recruitment of microglia to newly-formed plaques occurred within a few days and was followed by establishment of a dynamic interface between microglial processes and Aβ deposits (Meyer-Luehmann et al., 2008; Yan et al., 2009). Internalization and lysosome colocalization of Aβ was observed by Bolmont and colleagues (2008), and damage to neighboring neurons, coincident with the arrival of microglia, has also been reported (Meyer-Luehmann et al., 2008). Alternatively, Meyer-Luehmann and coworkers (2008) did not observe resolution of plaques over "days to weeks," and it remains to be shown whether or not the neurodegeneration following microglial recruitment to plaques is directly caused by the microglia.

Mechanistic studies of the inflammatory factors and receptors mediating microglial localization and responses to Aβ have also been carried on. *In vitro*, Aβ has been shown to stimulate expression of nearly all the proinflammatory mediators secreted by microglia (Lue et al., 2001a; Walker et al., 2001), and many of the innate immune response receptors found on microglia have Aβ either as a direct or indirect ligand.

Microglial cell type is neuroprotective primarily by virtue of its capacity for attack.

Microglia do not prevent apoptosis, neurodegenerative debris, bacterial invasion, or Aβ deposits; they attempt to remove such pathologies before further damage is performed. They also secrete anti-inflammatory mediators and growth factors. For these and other reasons, microglia are certainly vital to promoting a healthy CNS.

After initial pro inflammatory responses to pathogens or injury, alterations to macrophage and microglial function occur and are mediated primarily by the Th2 cytokines, IL.4, IL-10, IL-13 and TGF-β. These alterations include the frustrated phagocytosis of Aβ (Bogdan, 2008)

Some of microglial activation state nomenclatures are based on peripheral macrophage responses (M1, M2a, M2b, M2c) and reflect macrophage populations that are induced by specific Th2 cytokines and other factors (e.g., immune complexes, apoptotic cells) (Gordon and Taylor, 2005). Colton (2009), has summarized an activation nomenclature that embraces many of the findings that have been reported for macrophages but works particularly well for microglia.

Three activation states are proposed:

1. classical activation, which is stimulated by IFN-γ and characterized functionally by attack mechanisms;
2. alternative activation, which is stimulated by IL-4 and IL-13 and characterized functionally by tissue restorative, anti-inflammatory mechanisms;
3. acquired deactivation, which is stimulated by TGF-β, IL-10, apoptotic cells and is characterized functionally by immunosuppression.

A mixture of classical activation, acquired deactivation, and increasing alternative activation is observed in AD suggesting the complex roles that microglia may play in neurodegenerative diseases. (Colton, 2009; Wyss-Coray and Rogers, 2012).

Astrocytes. Astrocytes are an essential neurosupportive cell type in brain. They interact with neurons through many ways: secretion and recycling of transmitters, ion homeostasis, regulation of energy metabolism, synaptic remodeling and modulation of oxidative stress. Tiling the entire brain in contiguous, each single gray matter astrocyte has been estimated to envelope as many as 100,000 synapses (Halassa et al., 2007). Thus, perturbation of the many neurosupportive astrocyte functions can have extremely deleterious consequences for the CNS (Belanger and Magistretti, 2009).

Moreover, like microglia, astrocytes respond quickly to pathology with changes in their morphology and function and these reactive states have been increasingly recognized as a continuum with potentially beneficial and destructive consequences (Sofroniew and Vinters, 2010).

In brains of AD patients and AD transgenic mouse models reactive astrocytes occupy peri-plaque positions, encircling Aβ deposits in a manner reminiscent of glial scarring, a mechanism by which the cells may provide a barrier between healthy tissue and areas of injury or infection (Sofroniew and Vinters, 2010; Rodriguez et al., 2009).

Many mechanisms may account for the dense accumulation of reactive astrocytes observed at sites of aggregated Aβ deposition: MCP1, which is highly concentrated in Aβ plaques, is chemotactic for adult astrocytes; astrocyte cells express receptors that bind Aβ, including RAGE, low density lipoprotein receptor-like protein, membrane-associated proteoglycans and scavenger receptor-like receptors (Wyss-Coray et al., 2003).

Several studies suggest that plaque-localized, reactive astrocytes take up and may degrade Aβ (Koistinaho et al., 2004). In Tg2576 transgenic mice such effects may be linked to insulin degrading enzyme (IDE), which appears to play a key role in Aβ degradation. Astrocyte expression of IDE is increased after Aβ exposure and is pronounced within glial fibrillary acidic protein (GFAP) immunoreactive astrocytes surrounding Aβ deposits (Leal et al., 2006). Extracellular clearance of Aβ may also occur via astrocyte secretion of matrix metalloproteinases (Yin et al., 2006). Exposure to Aβ, in turn, appears to disrupt astrocyte calcium homeostasis, with subsequent increases in GFAP (Glial Fibrillar Astrocyte Protein), a typical marker of astrocyte reactivity, as well as degeneration of neurons in astrocyte-neuron cocultures (Abramov et al., 2004; Chow et al., 2010).

Reactive astrocytes may provoke neuropathology through expression or overexpression of a number of inflammation-related factors. For example, S100b, a neurotropin that induces neurite proliferation, is overexpressed in AD brain and its levels are correlated with numbers of dystrophic neurites within Aβ deposits (Mrak et al., 1996).

Exposure of cultured astrocytes to Aβ significantly increases IL-1β, TNF-α, iNOS, and NO production, with differential temporal effects depending on whether the Aβ is in fibrillar, oligomeric or non-fibrillar form (White et al., 2005).

NF-kB and CEBP transcription factor mechanisms regulate the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Astrocyte CEBPδ is upregulated in AD cortex (Li et al., 2004), and astrocyte NF-kB is activated on exposure to Aβ, leading to increased expression of IL-1β and IL-6 (Bales et al., 1998). NF-kB mechanisms in astrocytes also control the secretion of chemokines and the adhesion of molecules that might permit invasion by peripheral leukocytes, further fueling the inflammatory response (Moynagh, 2005; Wyss-Coray and Rogers, 2012).

Oligodendrocytes. Oligodendrocytes, and the myelin sheath they produce, envelope axons and are critical for neurotransmission. Studies revealed lesions and myelin abnormalities in AD white matter (Roth et al., 2005); focal demyelination of axons associated with Aβ deposits in grey matter has been shown both in familial and sporadic AD, as well as in transgenic mouse models of AD (Mitew et al., 2010). Stereotaxic injection of nM quantities of Aβ into the corpus callosum induces microglial proliferation, with damage to myelin and losses of oligodendrocytes (Jantaratnotai et al., 2003).

In vitro, oligodendrocyte toxicity has been reported for Aβ25-35 (Xu et al., 2001), Aβ40 (Xu et al., 2001; Lee et al., 2004) and Aβ42 (Roth et al., 2005). Perhaps because of their low glutathione and high iron content, oligodendroglia are also extremely vulnerable to oxidative stress, one of the main weapons of inflammatory attack (Juurlink, 1997).

7.2. Molecular mediators

The complement system. The complement system represents a key inflammatory pathway for the activation and execution of immune responses. Complement appears to be activated in neurodegenerative diseases and complement proteins are associated with plaques and tangles in AD.

The complement system is capable of recognizing molecular patterns on pathogens or molecular patterns associated with injured tissues and dying cells. Recognition may be through C1q or mannose binding proteins, which contain collagen-like receptor binding domains or through interactions with the multifunctional protein C3 (Tenner, 1999; Sahu and Lambris, 2001). Depending on the entity that triggers the complement system, two pathways could be activated: the classical pathway and the alternative one (Fig. 4). Once activated, a diverse array of almost 30 proteins in the complement pathway can attract and activate immune cells, amplify antigen-specific immune responses, promote phagocytosis, facilitate complement-mediated cytolysis by the Membrane Attack Complex (MAC) (Fig. 4) and regulate cell proliferation and differentiation (Holers, 1996).

Glial cells and neurons in the CNS can synthesize complement proteins and their production is increased in brain injury and neurodegeneration (D'Ambrosio et al., 2001; Gasque, 2004), including AD (Gasque et al., 2000). The complement proteins C1q and

MAC colocalize with amyloid plaques and tangle-bearing neurons (Fonseca et al., 2004). More recent studies showed that complement gene expression is increased in AD, suggesting the activation of complement in the disease (Katsel et al., 2009).

Aggregated Aβ activates the complement system *in vitro* through the classical pathway by binding C1q and through the alternative pathway by binding C3b (Jiang et al., 1994). Isolated tangles or tau aggregates can also activate the classical pathway by binding C1q (Shen et al., 2001). The accumulation of Aβ and tau may therefore promote the activation of complement and possibly neuroinflammation.

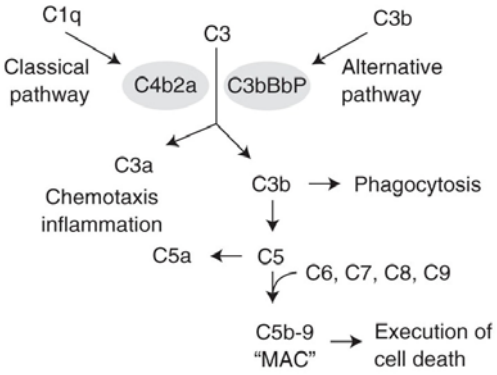


Figure 4. Scheme of the complement system. *The classical pathway of complement is triggered by antigen binding C1q and then activated C1r and C1s, followed by C4, C2, and C3. Bacteria can activate the alternative pathway by binding C3b and Factor B. Both the pathways result in the formation of multiprotein catalytic activities, the C3 convertases, generating two proteolytic C3 fragments: C3a, which is released in the fluid phase and involved in the chemotaxis of phagocytes, and C3b, which can bind covalently to acceptor molecules in solution or on cellular surfaces. C3b binding can mediate phagocytosis or may activate the lytic pathway involving C5, C6, C7,C8 and C9, which then form the MAC, C5b-9, and may lead to cytotoxicity. This pathway also leads to the formation of C5a, another small proinflammatory peptide (Wyss-Coray and Rogers, 2012).*

The complement can recognize degenerating or dying cells and has an important role in the clearance of dead or degenerating cells in various tissues (Taylor et al., 2000). Although there is no direct support for this mechanism in AD, C1q has been shown to tag apoptotic neurons or neuronal blebs and promote their uptake by microglia in cell culture (Fraser et al., 2010; Wyss-Coray and Rogers, 2012).

Thus, complement is activated in AD, where it may exert regulatory functions and aid in the clearance of degenerating cells and protein aggregates. However, it is likely also to promote unwanted inflammation.

Cytokines and chemokines. The soluble network of communication factors includes cytokines, chemokines and relates immune proteins; these proteins are potent regulators

of inflammation and immune function, have pleiotropic effects in many tissues, including CNS, and regulate diverse cellular processes such as proliferation, survival and differentiation.

In AD many of these proteins have altered levels (Akiyama et al., 2000) cytokine or chemokine changes in the brain parenchyma are often accompanied by changes in protein levels in the CSF, and some of these proteins appear to be dysregulated in the periphery as well (Hu et al., 2010). Thus far, the consequences of cytokine and chemokine changes on brain function and neurodegeneration relevant to AD are unknown.

Genetic studies in mice show that these proteins have potent effects on amyloidosis, neurodegeneration and cognition (Wyss-Coray, 2006)

Tumor necrosis factor (TNF)-α is able to promote Parkinson's disease (PD) progression (McCoy et al., 2006), whereas TNF receptor 1 knockout protects against AD- and PD-like disease in mice (Sriram et al., 2002; He et al., 2007). The cytokine transforming growth factor (TGF)-β1 is increased in human AD brains at the transcript and protein level, whereas TGF receptor expression is decreased (Tesseur et al., 2006). TGF-β1 promotes cerebrovascular amyloidosis but delays parenchymal Aβ accumulation in APP mice that also overproduce this cytokine from astrocytes (Wyss-Coray et al., 2001).

Several other cytokine mRNAs or proteins are expressed at increased or decreased levels in AD brain or periphery (Wyss-Coray, 2006). A number of these have also been studied in AD mouse models, including IL-1β and IL-6, which surprisingly both appear to have beneficial effects on amyloidosis.

Short-term expression of IL-1β in adult APP mice resulted in strong activation of glial cells, induction of various inflammatory factors and reduced amyloid pathology (Shaftel et al., 2007). Similarly, viral overexpression of IL-6 in the hippocampus led to massive gliosis and neuroinflammation, reduced amyloidosis and improvement of cognitive deficits in APP mice (Chakrabarty et al., 2010). Interestingly, lifelong stable overexpression of IL-1 receptor antagonist or IL-6 had no effect on pathogenesis in another APP mouse model consistent with the concept that acute but not chronic activation of certain types of immune responses in the brain may be beneficial (Wyss-Coray 2006 and 2012).

8. Treatment strategies

8.1. Cholinergics

Reduction in the activity of the cholinergic neurons is a well-known feature of AD. Acetylcholinesterase inhibitors are employed to reduce the rate at which acetylcholine (ACh) is broken down, thereby increasing the concentration of ACh in the brain and combating the loss of ACh caused by the death of cholinergic neurons. (Stahl et al., 2000). The nucleus basalis of Meynert, a distinct population of basal forebrain neurons, is a major source of cholinergic innervation to the cerebral cortex. In 1982, Whitehouse and colleagues found that these neurons are selectively (>75%) degenerated in patients with AD (Whitehouse, 1982 and 2006). The loss of cholinergic function has been found to be closely related to cognitive dysfunction (Farlow et al., 1988). Over the last two decades, a number of therapeutics targeting cholinesterase inhibition, choline precursors, post-synaptic cholinergic stimulation with muscarinic agonists, and presynaptic cholinergic stimulation with nicotinic agonists has been investigated (Hebert et al., 2003). These drugs stabilize cognitive decline for up to 3–6 months, although no modification of disease duration or general disease progression has been accomplished (Giacobini, 2000 and 2001).

Acetylcholinesterase inhibitors (ChEIs) are the best developed therapy and are used for mild to moderate disease. The mechanism by which ChEIs slow progression of disease is thought to be by decreasing levels of APP and production of Aβ and amyloidogenic compounds (Giacobini et al., 1996).

Tacrine was the first widely used ChEI. A 30-week randomized control clinical trial showed a significant dose-related improvement in cognitive function (Knapp et al., 1994). However, subsequent studies were less impressive and a short half-life, hepatotoxicity, and cholinergic side effects have limited the use of this drug. Second generation cholinergics, including donepezil (trade name Aricept®, Eisai Company and Pfizer Inc.), galantamine (Hoechst Marion Roussel Inc., Shire Pharmaceutical Group, and Janssen Pharmaceutical, trade names Reminyl and Nivalin, U.S. trade name Razadyne) and rivastigmine (trade name Exelon, Novartis Pharmaceuticals) have been developed. These drugs have fewer side effects, longer half-lives and greater efficacy.
Typically, ChEIs are started at low doses to minimize side effects such as facial flushing, dyspepsia, nausea, vomiting, and diarrhea, all linked to cholinergic excess. These side effects arise in approximately 10-20% of users and are mild to moderate

severity. Less common secondary effects include muscle cramps, decreased heart rate (bradycardia), decreased appetite and weight, and increased gastric acid production (Birks et al., 2006). The dose is then titrated up to the maximum tolerated dose (Mulugeta et al., 2003).

Precursor (lecithin and choline) and nicotine supplementation have not been found to improve overall cognition (Farlow et al., 1998).

Direct activation of postsynaptic cholinergic receptors by muscarinic agonists may decrease secretion of Aβ and increase secretion of nerve growth factor (Mulugeta et al., 2003). A Phase 2 clinical study is going on testing EVP-6124, a partial selective agonist of the α-7 nicotin acetylcholine receptor that improves memory performance by potentiating the acetylcholine response (Prickaerts et al., 2012).

8.2. Neurotropins

Beginning in 1986, the infusion of Nerve Growth Factor (NGF) into the adult rat brain was found to completely prevent the death of basal forebrain cholinergic neurons both spontaneously and after injury (Fischer et al., 1987). Over the next decade, studies in rodent models went further to show improvement in cognitive deficits (Markowska et al., 1994). Primate studies in the 1990s confirmed the ability of NGF to prevent degeneration of basal forebrain cholinergic neurons in monkeys (Koliatsos et al., 1991; Kordower et al., 1994). However, the inability of the NGF protein to cross the blood brain barrier, its short half-life, and its broad ranging effect of biological signals has made efficacious delivery of these neurotropins difficult to attain in the clinical setting.

Administration of mouse NGF in AD patients and clinical trials of growth factors in patients with either amyotrophic lateral sclerosis or PD failed secondary to adverse side effects and lack of efficacy (Eriksdotter et al., 1998; Kordower et al., 1999). In response to this dilemma, multiple CNS gene delivery studies were initiated.

Ex vivo preclinical studies demonstrated targeted, efficacious, sustained responses to NGF with no associated toxicity (Smith et al., 1999). In 2001, human clinical trials were initiated. A Phase 1 clinical trial with eight early stage AD patients was performed where in autologous NGF secreting cells were injected into the nucleus basalis of three dose cohorts. In the patients who received injection without complications, there were no adverse events observed over 2 years and no signs of non-targeted NGF spread

(Tuszynski et al., 2005), and the phase 2 study is going on using CERE-110 (Ceregene Inc) (Mandel 2010).

8.3.Antioxidants

Oxidative stress is an important pathogenic process associated with aging and AD, while markers of oxidative stress have been shown to precede pathological lesions in AD, including senile plaques and NFT (Nunomura et al., 1999). Antioxidants may thus blunt the cognitive decline in AD or slow disease progression (Perrig et al., 1997). The Alzheimer's Disease Cooperative Study compared selegiline, α-tocopherol, or both with placebo (Sano et al., 1997). Given the delay in progression to adverse outcome in the treatment groups, an American Academy of Neurology practice parameter states that vitamin E likely delays time to clinical worsening. Vitamin E in combination with vitamin C is also associated with a decrease in the prevalence and incidence of AD (Zandi et al., 2004).

Since several beneficial effects have been reported, dietary supplementation with some of the main antioxidants (resveratrole, epigallocatechin-gallate, docosahexanoic acid [DHA] and α-tocopherol) represent one of the several phase 3 clinical trials for AD in 2013 (*www.alzhforum.org*).

8.4. Statins

Cerebral Aβ levels have been shown to be decreased *in vivo* with simvastatin and results in the specific neuropathologic change in statin use of decreased NFT burden at autopsy (Fassbender et al., 2001; Li et al., 2007).

The Adult Changes in Thought (ACT) Study also showed a significant protective effect of statins against dementia; however, a later analysis of a larger sample from ACT suggested a protective effect in subjects who began statin use before age 80 (Li et al., 2004). In line with these results, numerous early epidemiologic studies indicated that the use of statins significantly reduces the risk of AD (Jick et al., 2000). The pattern of results obtained thus far suggests that statins may slow progression of the neurodegenerative process, but may not be able to reverse neuronal degeneration once it has occurred, so both were not approved for the treatment of AD by the FDA.

8.5. Non steroidal anti inflammatory drugs (NSAIDs)

Aβ deposition and plaque formation are associated with an innate immune response that includes activation of complement, secretion of pro-inflammatory cytokines, expression of chemokines, and excretion of nitric oxide which mediates apoptosis (Dickson et al., 1993). NSAIDs downregulate pro-inflammatory signals, microglia, and astrocytes and may reduce risk of AD by lowering Aβ1–42 production (Breitneir et al., 1994). Case control studies of individuals taking NSAIDs for arthritis, a small clinical trial of indomethacin, and a number of familial studies also indicated protection from development of AD or progression of disease (Andersen et al., 1995). NSAIDs may help prevent cognitive decline if started in midlife.

8.6. Hormone replacement therapy

Estrogen enhances cerebral blood flow, prevents atrophy of cholinergic neurons, reduces oxidative stress, and modulates the effects of nerve growth factors (Goutte et al., 2002). It may also reduce neuronal injury by decreasing formation of Aβ. Three prospective, population-based epidemiologic studies suggested that post-menopausal estrogen replacement therapy (HRT) may delay the onset of AD and showed some improvement in cognitive function (Bard et al., 2000). However, other randomized trials actually showed an increase in the development of dementia in healthy individuals and no improvement in cognitive or functional outcome (Becher et al., 2000). As such, further work is clearly needed to explore the use of HRT in AD and whether any effects are direct or indirect (Casadeus et al., 2007).

8.7. Blocking of exitotoxicity

Glutamate is a useful excitatory neurotransmitter of the nervous system, although excessive amounts in the brain can lead to cell death through a process called excitotoxicity, which consists in the overstimulation of the glutamate receptor NMDA. Excessive activation of NMDA receptors by glutamate increases the vulnerability of CNS neurons leading to neuronal degeneration. Memantine (trade name Namanda, Forest) is a non-competitive NMDA receptor antagonist, it blocks glutamate gated NMDA channels, thereby blocking pathological activation, inhibiting their overstimulation by glutamate and preserving physiological activation (Kornhuber et al., 1989). Memantine has been shown to be moderately efficacious in the treatment of moderate to severe AD. In contrast with ChEIs, which are approved for patients with

mild to moderate AD, memantine is approved for patients with moderate to severe AD. Reported adverse events with memantine are infrequent and mild, including hallucinations, confusion, headache and fatigue. There is evidence that combination therapy with donepezil may provide better outcomes with regards to cognition, global outcome and behavior (Tariot et al., 2004).

8.8. Immunotherapy

In the attempt to avoid adverse T cell mediated immune response, many vaccination modalities under current investigation are directed towards the humoral response. Nasal immunization of an AD mouse model with AdPEDI-(Aβ1–6) demonstrated a predominantly IgG1 response and reduced Aβ load in the brain (Kim et al., 2007). Transcutaneous immunization has also been studied in mice with aggregated Aβ 1–42 plus the adjuvant cholera toxin. This animal study showed significant decreases in cerebral Aβ1–40/42 levels in the setting of increased circulating levels of Aβ1–40/42 without the side effects of brain T cell infiltration or microhemorrhage (Nikolic et al., 2007; Asuni et al., 2006). Aβ derivatives in alum adjuvant also promote humoral immunity and decrease the Aβ burden (Asuni et al., 2006). Preliminary clinical results indicate that intravenous immunoglobulin (IVIg) has the potential to deliver a controlled immune attack on the Aβ peptide (Solomon et al., 2007). Clinical trials testing the safety and tolerability of the monoclonal antibody Bapineuzumab (AAB-001), an Aβ peptide antibody, have been conducted by Wyeth and Elan in patients with mild to moderate AD. Phase 3 trials were terminated on August 6, 2012 because two large Phase 3 studies showed no clinical benefit. Biomarker analyses indicated that bapineuzumab engaged its target but had no benefit.

The Aβ vaccine trials. Removal of Aβ excess from the brain occurs by a reactive T cell response. In 1999, active immunization of transgenic mice producing human Aβ was shown to produce a significant reduction of Aβ plaques (Schenk et al., 1999). For the immunization of the PDAPP transgenic mouse model Aβ was administered prior to the onset of AD-type neuropathologies or once these changes were already established. Immunization of the young animals prevented the development of Aβ plaque formation, neuritic dystrophy and astrogliosis. Treatment of the older animals reduced the extent and progression of these neuropathologies (Schenk et al., 1999).

There is evidence for a number of mechanisms of plaque removal:

1) solubilization by binding of antibody to Aβ;

2) phagocytosis of opsonized Aβ by microglial cells;

3) the "sink" hypothesis in which Aβ antibodies remain in the plasma and extract Aβ from the brain by altering equilibrium across the blood brain barrier to retain functional effects following immunization (Janus et al., 2000; Morgan et al., 2000).

Following these animals, clinical trials of the immunogen AN-1792 [Aβ1–42 with adjuvant (QS-21)] (Janseen, Pfizer) were performed by Elan Pharmaceuticals/Wyeth. Phase 2a clinical trials were suspended when 6% of patients in the active treatment group developed meningoencephalitis characterized by subacute neurologic deterioration, lymphocytic pleocytosis, and white matter abnormalities on imaging (Orgogozo et al., 2003). Five patients receiving the trial drug died, with one death (secondary to cerebral infarction) considered to be related to study treatment (Gilman et al., 2005).

Gantenerumab is a fully human IgG1 antibody designed to bind with subnanomolar affinity to a conformational epitope on Aβ fibrils. It encompasses both N-terminal and central amino acids of Aβ. The therapeutic rationale for this antibody is that it acts centrally to disassemble and degrade amyloid plaques by recruiting microglia and activating phagocytosis. Gantenerumab preferentially interacts with aggregated brain Aβ, both parenchymal and vascular. In APP/PS-1 transgenic mice, gantenerumab binds to cerebral Aβ, reduces small plaques by recruiting microglia, and prevents new plaque formation. Gantenerumab does not alter plasma Aβ (Bohrmann et al., 2012). It is being studied as a potential combination therapy with the Roche BACE inhibitor R7129 in mouse models of Aβ amyloidosis.

Phase 1 BACE inhibitor and its Phase 3 antibody reduced Aβ levels and plaque burden more strongly in a transgenic mouse overexpressing mutant human APP than did either treatment alone (Novakovic et al., 2013).

8.9. Secretase effectors

Memapsin 2 (β-secretase, BACE1) is the protease that initiates cleavage of APP leading to the production of Aβ. Immunization of transgenic AD mice (Tg2576) with BACE has also resulted in Aβ reduction and cognitive improvement in the absence of an inflammatory response (Chang et al., 2007). A nonselective γ-secretase inhibitor, LY450139 (Eli Lilly) was evaluated in PDAPP transgenic mice and found to result in a 60% reduction of Aβ40 in plasma, CSF and the hippocampus. There was also a reduction in the median value for Aβ plaque burden in the cortex and hippocampus.

Two clinical trials showed dose dependent reduction of plasma Aβ, however, following the inhibitory phase, plasma Aβ levels exceeded baseline levels. Gastrointestinal side effects and eosinophilia were also properties of concern (LY450139 Dihydrate or semagacestat). Phase 3 study showed that the drug increased the risk of skin cancer and infections and had no efficacy. Both cognition and function were not improved but rather worsened in all treatment groups (Doody et al., 2013). Lilly terminated development of semagacestat because disruption of hippocampal network function was found (Hajos et al., 2013).

The Phase 2 trial of another BACE inhibitor by Eli Lilly & co. (LY2886721) was discontinued in 2013 due to liver abnormalities showed up in four out of 45 patients during routine testing (*www.alzforum.org*).

Tarenflurbil (MPC-7869; Myriad Pharmaceuticals) is a γ-secretase modulator in the class of drugs referred to as Selective Amyloid Lowering Agents (SALAs). As these drugs do not interfere with γ-secretase substrates, they are well tolerated. A phase 2 study in 207 patients with mild to moderate AD showed improvement on ADLs, global function, and cognition in patients with mild AD, with a significant plasma concentration to response relationship. There was no benefit observed in patients with moderate AD. The phase 3 study of Tarenflurbil showed that it does not slow cognitive decline or the loss of activities of daily living in patients with mild AD, it was discontinued (Green et al., 2009).

8.10. Diet

The Mediterranean diet (MD) was recently demonstrated to be associated with lower AD risk (Singh et al., 2014). A recent study has now shown that the Mediterranean diet is also associated with lower mortality in AD. This diet is characterized by high intake of vegetables, fruits, and cereals; a low-to-moderate intake of saturated fatty acids, moderately high intake of fish, low-to-moderate intake of dairy products, low intake of meat and poultry, and a moderate amount of ethanol.

This thesis will provide an insight in the benefits that MD has against AD onset.

AUTOPHAGY

1. History

The term "autophagy" was chosen to distinguish the lysosomal degradation, or "eating" (phagy), of part of the cell's self (auto) from the breakdown of extracellular material (heterophagy).

Autophagy is a ubiquitous process in eukaryotic cells that results in the breakdown of cytoplasm within the lysosome in response to stress conditions and that allows the cell to adapt to environmental and/or developmental changes.

Christian de Duve is considered to be the founding father of autophagy research area: autophagy is a degradative mechanism that is part of the lysosomal system an de Duve carried out the pioneering biochemical work that helped lead to the discovery of the lysosome as a distinct entity in 1955. He conied the term "lysosome" and received the Nobel Prize in Physiology and Medicine in 1974, mostly for his work on this organelle. De Duve also came up with the term "autophagy", which he introduced at the CIBA Foundation Symposium on Lysosomes in 1963.

The descriptive name "autophagy" was intended to illustrate observations from electron microscopy studies, which showed novel single- or double-membrane vesicles that contained parts of the cytoplasm, including organelles, in various degrees of disintegration (Ashford et al., 1962; Clark et al., 1957; de Duve et al., 1966; Novikoff, 1959). At the time, de Duve and others suggested that the sequestering organelle, or "autophagosome" was derived from a preformed membrane such as smooth endoplasmic reticulum, but the current view is that the autophagosome forms in a largely *de novo* process, starting with a core membrane that expands through vesicular addition. Whereas autophagy was, and still is, considered to be primarily non-specific (Kopitz et al., 1990), de Duve suggested the possibility of selective types of autophagy that might allow the targeted degradation of abnormal cellular constituents, an aspect of autophagic function that has gained considerable prominence in recent years (Klionsky, 2007).

Whereas the initial studies of de Duve and others primarily examined the terminal stages of the process, and focused on steps just before or after fusion with the lysosome, Seglen's laboratory began to study the early and intermediate steps using electroinjected

radioactive probes (Seglen et al., 1987); these analyses identified the phagophore, the initial sequestering organelle that develops into an autophagosome, as well as the amphisome (Gordon et al., 1988), which marks the convergence of the autophagic and endocytic pathways.

The identification of autophagy genes in higher eukaryotes made it possible to analyse mammalian cells that express autophagy proteins tagged with fluorescent markers. Time-lapse studies provide images which suggest that autophagosome formation proceeds in a step-wise manner, marked by the expansion of the sequestering membrane (Mizushima et al., 2001).

Analysis of autophagy in the yeast system lagged behind considerably until 1992, when a study by Yoshinori Ohsumi's laboratory demonstrated that the autophagy morphology in yeast was similar to that documented in mammals (Takeshige et al., 1992). This result was crucial as a foundation for further studies in this genetically tractable organism. In yeast, the autophagic machinery is concentrated at a perivacuolar site (the vacuole is the yeast equivalent to the lysosome), termed the "pre-autophagosomal structure" (PAS), which acts as a phagophore assembly site (Kim et al., 2002; Suzuki et al., 2001).

Autophagy research clearly has its roots in the mammalian system, however, significant breakthroughs in our understanding of the molecular basis of autophagy only occurred following analyses in the yeast system. Ohsumi's group carried out the first genetic screen for autophagy mutants (Tsukada and Ohsumi, 1993), and this was rapidly followed by various similar screens (Klionsky, 2003): the first gene, Atg1 (autophagy-related gene 1) has been published in 1997 (Matsuura et al., 1997), ten years on, the thirty-first gene (Atg31, according to the unified nomenclature, Klionsky 2003) was recently identified (Kabeya et al., 2007).

The identification of the Atg genes in yeast allowed the subsequent explosion of research into the molecular analysis of autophagy in higher eukaryotes. Mizushima and colleagues identified the first mammalian autophagy genes, Atg5 and Atg12, and demonstrated that the Atg12-Atg5 conjugation system, necessary to trigger autophagy, is conserved from yeast to human (Mizushima et al., 1998). Another important step for autophagy analysis in higher eukaryotes was the identification of the mammalian Atg8 homologue MAP1LC3 (also known as LC3) by Yoshimori and Mizushima (Kabeya et al., 2000) and the subsequent development of LC3-based assay for monitoring

autophagy in mammals and other higher eukaryotic systems (Mizushima, 2004). It is important to note, however, that steady state LC3 levels alone are not sufficient for evaluating autophagy, as it is crucial to follow flux through the entire pathway (Klionsky et al., 2007; Klionsky, 2007).

2. Types of autophagy

At least three forms of autophagy have been identified: chaperone-mediated autophagy, microautophagy and macroautophagy (Fig. 5).

The main degradation pathway is *macroautophagy*, it involves the sequestration of cytoplasmic components (long-lived proteins, damaged organelles and even invasive pathogens) into a double-membrane cytosolic vescicle, termed autophagosome, that transports the cargo to the lysosome; there, the outer-membrane and the inner vescicle, together with the cargo, is degraded; the resulting macromolecules can be recycled back to the cytosol for reuse during starvation (Yorimitsu and Klionsky, 2005). This sequestration process is controlled by the mammalian target of Rapamycin (mTOR) kinase pathway, the major negative regulator of autophagy, which in turn, is regulated by insulin via the phosphoinositol 3 kinase/serine threonine protein kinase (PI3K/AKT) pathway by specific amino acids via AMP kinases (Petiot et al., 2000).

Microautophagy involves the direct engulfment of cytoplasm at the surface of the lysosome by protrusion and/or invagination of the limiting membrane. It is an active process in basal conditions, and it is responsible of the intracellular components turnover, it is not activated by stress or starvation.

Although initially described in the liver, most of recent molecular characterization of this pathway has been done in yeast (Ahlberg and Glaumann, 1985). This type of autophagy involves the direct engulfment of cytoplasm at the surface of the lysosome by protrusion and/or invagination of the limiting membrane (Fig. 5). It is an active process in basal conditions, and it is responsible of the intracellular components turnover (Ahlberg and Glaumann, 1985). In addition, studies have shown that microautophagy is responsible for the selective removal of organelles when they are no longer needed, for example after treatment with drugs that induce proliferation of peroxisomes, normal numbers of this organelle are attained through their degradation by microautophagy (Farré and Subramani, 2004, Martinez-Vicente and Cuervo, 2007).

The *chaperone-mediated autophagy* is a lysosomal pathway of proteolysis that is responsible for the selective degradation of 30% of cytosolic proteins under conditions of prolonged nutrient deprivation; CMA can mediate the degradation of soluble proteins containing a particular pentapeptide consensus motif (KERFQ) that can be detected by the cytosolic chaperone HSC70 (heat-shock protein 70) and its co-chaperones, that selectively bring the protein to the lysosome for the degradation. The complex substrate-chaperone moves toward lysosomes, where the lysosome-associated membrane protein 2 (LAMP-2A) receptor recognizes the protein, that then is traslocated through the lysosomal membrane (Kon and Cuervo, 2010). CMA activity is present in most tissues, but maximum activation occurs under condition of stress (Massey et al., 2006). The first response to nutritional starvation is activation of macroautophagy as a source of amino acids; however, if starvation persists, this random degradation of intracellular components cannot be maintained, and it is then that CMA becomes the new supplier of amino acids for the synthesis of new proteins (Massey et al., 2006). The selectivity of this pathway is also particularly useful under conditions that result in protein damage (oxidative stress, exposure to different toxic compounds, etc.) because it allows removal of the altered proteins without affecting neighbouring healthy ones (Kiffin et al., 2004). CMA can be reduced by inhibitors of glucose-6-phosphate dehydrogenase and of the heat shock protein of 90 kDa; reduction of levels of LAMP-2A using RNAi strategies reduces CMA activity, but macroautophagy is activated as result (Dice, 2007). The decrease in CMA causes cells to be more susceptible to oxidative and other stresses.

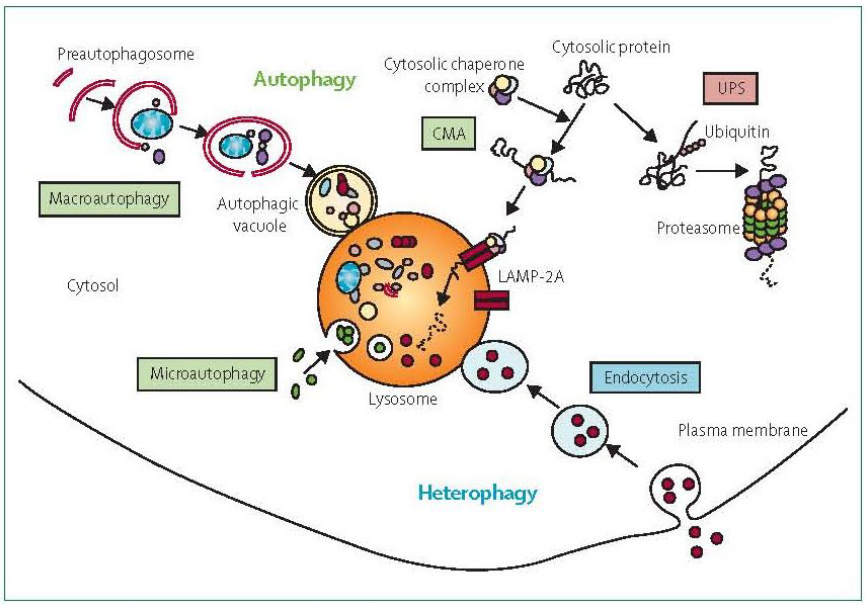


Figure 5. Types of autophagy*. In chaperone mediated autophagy, proteins marked with the KFERQ motif are detected by chaperones, brought to the lysosome and translocated into the lumen through LAMP2 for degradation. In microautophagy small cytosolic components are carried through invagination of the lysosomal membrane. In macroautophagy Atg proteins generate the preautophagosomal structure which engulfs cytoplasmic components and organelles as it matures into a full double-membraned autophagosome then it fuses with the lysosome forming an autophagolysosome, degrading the components trapped within (Martinez-Vicente and Cuervo, 2007).*

3. The process of macroautophagy

Macroautophagy is a general term for the degradation of cytoplasmic components within lysosomes (Cuervo, 2004; Levine and Klionsky, 2004; Shintani and Klionsky, 2004; Klionsky, 2005; Mizushima and Klionsky, 2007). This process is quite distinct from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins.

The term “autophagy” usually indicates macroautophagy. Autophagy is mediated by a unique organelle called the autophagosome. As autophagosomes engulf a portion of cytoplasm, autophagy is generally thought to be a nonselective degradation system. This feature is in contrast to the ubiquitin-proteasome system, which specifically recognizes only ubiquitinated proteins for proteasomal degradation. It is therefore reasonable to assume that the ubiquitin-proteasome system has numerous specific functions because it can selectively degrade thousands of substrates. However, recent studies have clearly demonstrated that autophagy has a greater variety of physiological and pathophysiological roles than expected, such as starvation adaptation, intracellular protein and organelle clearance, development, antiaging, elimination of

microorganisms, cell death, tumor suppression, and antigen presentation (Mizushima, 2005).

To understand the various roles of autophagy, it may be useful to subclassify macroautophagy into "induced autophagy" and "basal autophagy" (Mizushima, 2005). The former is used to produce amino acids following starvation, while the latter is important for costitutive turnover of cytosolic components.

The autophagy process is divided into mechanistically distinct steps, including induction, cargo recognition and selection, vesicle formation, autophagsome-vacuole fusion, and breakdown of the cargo followed by release of the degradation products back into the cytosol.

Different sets of Atg proteins are involved in these steps and consist of the core autophagic machinery.

3.1. Induction of autophagy

Basal-level autophagy is very low under normal conditions; therefore, an efficient mechanism to induce autophagy is crucial for organisms to adapt to stress and extracellular cues. A central inhibitor of autophagy is the serine/threonine protein kinase TOR (target of rapamycin). In yeast and *Drosophila*, Tor/dTOR integrates input information from multiple upstream signal transduction pathways and negatively regulates another serine/threonine kinase, Atg1, in nutrient-rich conditions (Chang et al., 2009). In yeast, upon Tor inhibition by starvation or rapamycin treatment, the kinase activity of Atg1 is activated and Atg1 binding affinity to Atg13 and Atg17 may also increase, which promotes the formation of an Atg1-Atg13-Atg17 scaffold and the recruitment of multiple Atg proteins to the PAS to initiate autophagosome formation (Suzuki et al., 2007). Thus, a role of the Atg1 kinase complex in protein recruitment is indispensable for autophagy induction. Moreover, in *Drosophila*, Atg1 is able to inhibit the phosphorylation and activation of a downstream TOR effector, S6K, during nutrient starvation, yet it is not clear how S6K signaling modulates other autophagy proteins and/or autophagy activity (Lee et al., 2007).

There are two mammalian homologs of Atg1 that appear to function in autophagy, the Unc-51-like kinase 1 (ULK1) and -2 (ULK2), and one homolog of yeast Atg17, FIP200 (the focal adhesion kinase family-interacting protein of 200 kD), which forms a complex with ULKs and mammalian Atg13 and localizes to the phagophore upon

starvation (Hara et al., 2008). Regarding the substrates of the Atg1 kinase during autophagy, it is suggested that mammalian Atg13 and FIP200 are phosphorylated by ULKs, and ULKs also undergo autophosphorylation, which is conducive to a conformational change and autophagy induction (Chan et al., 2009). Different from what has been reported in yeast, ULKs-Atg13-FIP200 seem to form a stable complex regardless of nutritional conditions in mammalian cells. mTOR interacts with, phosphorylates, and inactivates ULKs and Atg13 under nutrient-rich conditions. Upon mTOR inhibition by starvation or rapamycin, ULK1 and ULK2 are activated and phosphorylate Atg13 and FIP200, which are essential for autophagy activity (Jung et al., 2009). These studies raise an interesting hypothesis that Atg13 is phosphorylated by TOR or Atg1/ULKs on different residues, which may exert opposite effects on autophagy induction dependent on nutrient status. Indeed, yeast Atg13 is rapidly dephosphorylated during starvation, whereas Atg13 phosphorylation is enhanced during autophagic conditions in *Drosophila* (Kamada et al., 2000; Chang Y-Y et al., 2009); it is likely that phosphorylation of Atg13 is dependent more on Tor in yeast, and to a greater extent on Atg1 in *Drosophila*. Atg101, a newly identified protein component in the ULKs-Atg13- FIP200 complex, binds and stabilizes Atg13, and is required for autophagy in mammals (Mercer et al., 2009).

3.2. Cargo recognition and selectivity

In selective autophagy, cargos are recognized through interactions with specific receptor proteins. The yeast Cvt pathway delivers prApe1 into the vacuole and generates the mature enzyme Ape1. The cargo prApe1 contains a vacuolar targeting signal, which can be recognized and bound by the receptor protein Atg19. An adaptor protein, Atg11, binds Atg19 and recruits the Atg19-prApe1 complex to the PAS, where Atg19 interacts with one of the key components in the vesicle-forming machinery, Atg8, for packaging the receptor-cargo complex into Cvt vesicles (analogous to autophagosomes) (Shintani et al., 2002).

In multicellular organisms, an important function of autophagy is the clearance of cytosolic ubiquitinated substrates or aggregateprone proteins. Recent studies suggest that this degradative process is also selective and mediated through the mammalian protein p62/sequestosome 1 (SQSTM1), the *Drosophila* homolog of p62 (Bjørkøy G et al., 2005); p62 directly binds both poly- or monoubiquitin via its ubiquitin-associated

(UBA) domain and the mammalian Atg8 homolog, LC3 (microtubule-associated protein 1 light chain 3), and links the ubiquitinated cargos to the autophagy machinery for autophagic degradation (Pankiv et al., 2007). The structural and functional similarity between a C-terminal motif in mammalian p62 and yeast Atg19 suggests that p62 is an Atg19 analog in higher eukaryotes and acts as a receptor for ubiquitinated proteins or organelles in selective autophagy (Noda et al., 2008).

3.3. Autophagosome formation

In the first step of autophagosome formation, cytoplasmic constituents, including organelles, are sequestered by a unique membrane called the phagophore or isolation membrane, which is a very flat organelle. Complete sequestration by the elongating phagophore results in formation of the autophagosome, which is typically a double-membraned organelle. This step is a simple sequestration, and no degradation occurs (Suzuki et al., 2001).

In yeast, 31 Atg (autophagy-related) proteins have been identified, and many of them gather at a site that can be identified by fluorescence microscopy as a punctate spot very close to the vacuolar membrane. Since autophagosomes are generated from this site, it is called the "preautophagosomal structure" (PAS) (Kim et al., 2001; Suzuki and Ohsumi, 2007). Equivalent structures have not been observed in mammalian cells. Yeast cells may have a stable PAS because it is shared with the cytoplasm-to-vacuole targeting (Cvt) pathway, a costitutive transport pathway for amino peptidase 1 (Ape1) and α-mannosidase (Ams1) from the cytosol to the vacuole (Klionsky and Ohsumi, 1999).

Among the 31 Atg proteins, 18 Atg proteins (Atg1-10, Atg12-14, Atg16-18, Atg29, and Atg31) are involved in autophagosome formation and are called "AP-Atg proteins". A recent systematic analysis showed that the AP-Atg proteins depend on each other for recruitment to the PAS (Suzuki et al., 2007); in particular, Atg17 was found to be a scaffold for PAS organization. The recently identified Atg29 (Kawamata et al., 2005) and Atg31 (Kabeya et al., 2007) appear to function together with Atg17. Atg11 is also important for PAS organization but is essential only for the Cvt pathway (Suzuki et al., 2007).

Multiple Atg proteins are recruited to the phagophore to participate in autophagosome formation, and this step requires the highly regulated coordination of all

of these proteins. The nucleation and assembly of the initial phagophore membrane require the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which is composed of the PtdIns3K Vps34 (vacuolar protein sorting 34), a myristoylated serine/threonine kinase Vps15 (p150 in mammalian cells), Atg14 (Barkor or mAtg14 in mammalian cells) and Atg6/Vps30 (Beclin 1 in mammalian cells) (Italkura et al., 2008). The function of Beclin 1 in autophagy is regulated by Bcl-2 (B-cell lymphoma/leukemia-2), an antiapoptotic protein that inhibits autophagy by binding and sequestering Beclin 1 under nutrient-rich conditions; dissociation of Beclin 1 from Bcl-2 is required for autophagy induction. The PtdIns3K complex produces PtdIns3P (phosphatidylinositol 3-phosphate) and is involved in PAS targeting of a number of yeast Atg proteins that bind PtdIns3P, such as Atg18, Atg20, Atg21, and Atg24 (Obara et al., 2008). In yeast, Atg20 and Atg24 interact with the Atg1-Atg13-Atg17 complex, and the latter mediates autophagy induction; however, the mammalian homologs of Atg20 and Atg24 are either not identified (Atg20) or not well characterized in autophagy (Atg24). The PtdIns3K complex, in part together with the above Atg proteins, further recruits two interrelated ubiquitin-like (Ubl) conjugation systems, Atg12-Atg5-Atg16 and Atg8-PE (phosphatidylethanolamine), to the phagophore, which play an essential role in regulating the membrane elongation and expansion of the forming autophagosome. The two Ubl proteins, Atg12 and Atg8, undergo conjugation in a similar manner as ubiquitin. Atg12 is activated by Atg7 (E1 activating enzyme), transferred to Atg10 (E2 conjugating enzyme) and attached to an internal lysine of the substrate protein Atg5 covalently. In contrast to ubiquitination, Atg12-Atg5 conjugation is constitutive and irreversible, and apparently no substrate-specific E3 ligase counterpart is required in this process (Geng and Klionsky, 2008). The Atg12-Atg5 conjugate further interacts with a coiled-coil protein Atg16, which links the Atg12-Atg5-Atg16 complex into a tetramer by self-oligomerization and attaches it to the phagophore (Mizushima et al., 2003). In the Atg8 conjugation system, Atg8 is first processed by a cysteine protease, Atg4, exposing a C-terminal glycine residue. The same E1 enzyme Atg7 activates Atg8 and transfers it to Atg3 (E2). Atg8 is finally conjugated to the target lipid PE via an amide bond, facilitated by the E3-like Atg12–Atg5 conjugate (Fujita et al., 2008) . In nutrient rich conditions, the majority of Atg8 is cytosolic; upon autophagy induction, Atg8 largely exists as the lipid-conjugated form and is localized to both sides of the phagophore. Atg8 controls the size of the

autophagosome, which may result from its ability to determine membrane curvature (Xie et al., 2008). The lipidation of Atg8 and its mammalian homolog LC3 is widely used to monitor autophagy induction. Various sources, including mitochondria, the Golgi complex, the ER and the plasma membrane, are proposed to be the origins of the autophagosomal membrane (Hailey et al., 2010; Hayashi-Nishino et al., 2009; Ravikumar et al., 2010; van der Vaart et al., 2010; Yen et al., 2010; Yla-Anttila et al., 2009). Clathrin-mediated endocytosis provides membrane that contributes to the formation of preautophagosomal structures and the subsequent generation of phagophores and mature autophagosomes (Ravikumar et al., 2010).

SNAREs (*N*-ethylmaleimidesensitive factor attachment protein receptors) have recently been shown to regulate autophagosome formation in both mammalian cells and in yeast (Moreau et al., 2011; Nair et al., 2011) In mammalian cells, the SNAREs vesicle-associated membrane protein (VAMP)7, syntaxin-7, syntaxin-8, and VTI1B regulate the homotypic fusion of phagophore precursors (Moreau et al., 2011). These fusion events enable the growth of these structures into a tubular network leading to the formation of phagophores and autophagosomes (Moreau et al., 2011). Indeed, the fusion of small, apparently single-membrane phagophore precursor vesicles to form tubulovesicular structures may be a mechanism contributing to the genesis of double-membraned phagophores or autophagosomes (Moreau et al., 2013).

Atg9 is the only identified integral membrane protein required for autophagosome formation. In yeast, Atg9 localizes to the PAS and peripheral structures and is suggested to cycle between the two sites. Atg11, Atg23, and Atg27 are essential in the anterograde transport of Atg9 to the PAS, and the Atg1- Atg13 complex, Atg2, Atg18, and the PtdIns3K complex are involved in its retrograde transport (Legakis et al., 2007; Reggiori et al., 2004). Thus, Atg9 may function as a carrier in supplying membrane and may also play a role during phagophore expansion through its dynamic self-interaction. Similarly, mammalian Atg9 (mAtg9) transports from the *trans*-Golgi network (TGN) to late endosomes, which are co-labeled with LC3, when autophagy is induced. In mammalian cells, it is the redistribution of mAtg9 from the TGN to late endosomes that is dependent on ULK1 and human Atg13 (Chan et al., 2009).

3.4. Expansion of the phagophore

Canonical macroautophagy is controlled by two central enzymatic pathways that drive the formation and expansion of the phagophore through the recruitment of various autophagy proteins. The first involves the formation of the Atg5-12-16 complex, Atg12 is first covalently conjugated to Atg5 by the catalytic activities of Atg7 and Atg10. Atg16 then interacts with the Atg5-12 complex and homo oligomerizes to form a tetrameric Atg5-12-16 complex. This complex is then recruited to the phagophore assembly site, where it helps to promote the second pathway, namely, the processing of LC3. LC3 is cleaved to LC3-I by Atg4, activated by Atg3 and Atg7, and then conjugated to phosphatidylethanolamine (PE), forming LC3-II. This reaction is thought to be targeted to the developing phagophore by the Atg5-12-16 complex (rew. Levine and Kroemer, 2008). Since the full Atg5-12-16 complex is required for LC3-II formation and autophagic function, components of the Atg5-12-16 complex make attractive targets for impairing autophagy (Fig. 6).

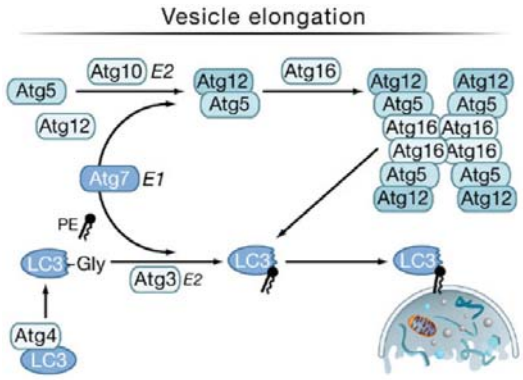


***Figure 6. The two major conjugation systems that drive autophagic vescicle elongation**. The enzymatic activities of Atg7 and Atg10 act for the formation of the Atg5-12-16 complex which then homo-oligomerizes to form a tetrameric structure; LC3 is cleaved by Atg4 and activated by Atg7 before being covalently conjugated to PE by Atg3 and the Atg5-12-16 complex, forming LC3-II. The Atg5-12-16 complex also correctly localizes LC3-II to the developing phagophore (Levine and Kroemer, 2008).*

As the phagophore expands the Atg5-12-16 complex continues to recruit LC3-II. The Atg5-12-16 complex primarily localizes the outer surface of the phagophore, while LC3-II is present throughout the membrane surface (Levine and Kroemer, 2008). LC3-II is believed to function as a scaffolding protein, helping to maintain the structure of the autophagosome and recruit membrane to the developing phagophore. LC3-II drives the maturation of the phagophore and expansion of the membrane. As the

autophagosome continues maturing and approaches closure, the Atg5-12-16 complex dissociates from the outer membrane. Since LC3-II only forms on autophagosomes, it has traditionally been used as a marker for autophagy, in western blot it runs at lower molecular weight than LC3-I (Tanida et al., 2005).

As the phagophore matures, it expands in size and eventually closes, forming a mature autophagosome. In this process LC3-II may help bring the two membrane surfaces close enough to fuse. Following closure, autophagosomes are trafficked by dynein motors along microtubules to the lysosomes in the perinuclear region. There they fuse with the lysosomes through a process mediated by the SNARE proteins Vamp3, Vamp7 and Vti1, that are involved in fusing the two membranes together (Levin and Kroemer, 2008).

3.5. Vesicle fusion and autophagosome breakdown

When autophagosome formation is completed, Atg8 attached to the outer membrane is cleaved from PE by Atg4 and released back to the cytosol. Autophagosome-lysosome fusion is mediated by the same machinery that is involved in homotypic vacuole membrane fusion. In mammalian cells, the fusion event requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7, although the mechanism is less characterized (Jaeger et al., 2004). In yeast, the machinery consists of the Rab family GTPase Ypt7 (the homolog of Rab7), the NSF(N-ethylmalemide-sensitive fusion protein) homolog Sec18, the SNARE proteins Vam3, Vam7, Vti1, and Ykt6, the class C Vps/HOPS complex proteins, and two other proteins, Ccz1 and Mon1 (Klionsky, 2005). After fusion, degradation of the inner vesicle is dependent on a series of lysosomal/vacuolar acid hydrolases, including proteinases A and B (encoded by *PEP4* and *PRB1*, respectively) and the lipase Atg15 in yeast and cathepsin B, D (a homolog of proteinase A), and L in mammalian cells (Tanida et al., 2005; Teter et al., 2001). The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions. The identification of Atg22, together with other vacuolar permeases (such as Avt3 and Avt4) as vacuolar amino acid effluxers during yeast autophagy, has helped in the understanding of the mechanisms of nutrient recycling; these permeases represent the last step in the degradation and recycling process (Yang et al., 2006).

4. Non-Atg components required for autophagy

Besides the Atg proteins, certain subcellular systems, including the secretory pathway, the endocytic pathway, and the cytoskeletal network, may also carry out essential functions during autophagy, such as providing membrane, facilitating autophagosome transport, and enabling clearance of autophagic substrates.

4.1. The secretory and endocytic pathways

Autophagy involves dramatic subcellular membrane remodeling. The role of the secretory pathway in autophagy is largely studied in yeast, where a functional ER and Golgi complex are required for autophagy and the Cvt pathway (Reggiori et al., 2004). A subset of GTP exchange factors, including Sec12 and Sec16, and two coatomer subunits of the COPII coat, Sec23 and Sec24, are needed for the biogenesis of autophagosomes but not Cvt vesicles, although it is possible that a defect in the Cvt pathway is more subtle compared with nonselective autophagy because of the relatively reduced need for membrane (Ishihara et al., 2001).

Although completed autophagosomes can directly fuse with lysosomes, when cells are overloaded with aggregate-prone proteins, fusion of autophagosomes with the endocytic compartments is essential to facilitate efficient autophagic removal of these proteins. In *Drosophila* and mammalian cells, the ESCRT (endosomal sorting complexes required for transport) machinery and multivesicular body (MVB)-localized Rab11 play an important role in the fusion of MVBs with completed autophagosomes, and the *Drosophila* endosomal PtdIns3P 5-kinase Fab1 is involved in fusion of the resulting amphisomes with lysosomes (Rusten at al., 2007). Both steps are required for promoting degradation of aggregate-prone substrate proteins sequestered by autophagosomes.

4.2. Cytoskeleton

Efficient protein trafficking during autophagosome formation is presumably mediated by cytoskeletal networks. A functional actin cytoskeleton and the Arp (actin-related protein) 2/3 complex, which nucleates branching of actin filaments, are required for Atg9 anterograde transport to the PAS and selective autophagy activity in yeast (Reggiori et al., 2005). Microtubules are involved in autophagy in higher eukaryotes. In primary rat hepatocytes, the microtubule depolymerising drug nocodazole inhibits

autophagosome formation (Kochl et al., 2007). In addition, microtubules, the tubulin deacetylase HDAC6, and the microtubule motor protein dynein are required for the autophagic clearance of various aggregate prone proteins in flies and mammalian cells (Iwata et al., 2005). In mammalian cells, it appears that autophagosomes are formed at random locations in the cell, but transported directionally toward the nucleus after completion (Jahreiss et al., 2008). Several lines of evidence suggest that autophagosomes associate with microtubule tracks, move to the microtubule-organizing center, and fuse with endosomes or lysosomes, and the dynamic process is driven by the dynein motor (Jahreiss et al., 2008).

5. Signalling pathways regulating autophagy

5.1. Nutrient signalling

During nutrient deprivation, autophagosome formation is dramatically induced. In both yeast and mammalian cells, two well-characterized signaling cascades that sense nutrient status, activate cell division and growth, and negatively regulate autophagy are the TOR and Ras-cAMP-PKA pathways.

TOR complex 1. TOR complex 1 (TORC1) is sensitive to inhibition by rapamycin. Inactivation of TORC1 by rapamycin stimulates autophagy in the presence of nutrients, suggesting that TOR downregulates autophagy (Noda and Ohsumi, 1998). Extracellular amino acids enter mammalian cells through transporters such as SLC1A5 (solute carrier family 1 member 5) and SLC7A5, and it is proposed that mTORC1 directly senses and is phosphorylated in response to nutrient signals (Long et al., 2005); however, recent observations in *Drosophila* and mammals suggest that Rag proteins, the Ras-related small GTPases, activate TORC1 in response to amino acids (Kim et al., 2008) through mediating translocation of TORC1 to a specific subcellular compartment that contains the TORC1 activator Rheb (Ras homolog enriched in brain) (Rusten et al., 2007). Other studies indicate that amino acids also activate mTOR via class III PtdIns3K (hVps34) (Nobukuni et al., 2005). The presence of amino acids stimulates hVps34, which leads to mTOR activation and autophagy inhibition. However, this creates a discrepancy with the role of the class III PtdIns3K in promoting nucleation and assembly of Atg proteins at early steps of autophagosome formation (Juhasz et al., 2008). A possible explanation is that the PtdIns3K exists in distinct subpopulations or protein complexes in the cell, which carry out different functions, or function at different times. Besides regulating the

Atg1/ULK complex, in yeast TORC1 also suppresses autophagy via phosphorylation of Tap42, which activates the catalytic subunits of PP2A (the serine/threonine protein phosphatase 2A), a negative regulator of autophagy (Yorimitsu et al., 2009).

The Ras/PKA pathway. The Ras/cAMPdependent protein kinase A (PKA) signalling pathway plays an important role in glucose sensing from yeast to mammals. Yeast PKA contains a heterotetramer composed of the regulatory subunit Bcy1 and three apparently redundant catalytic subunits, Tpk1, Tpk2, and Tpk3. In nutrient-rich conditions, the small GTPases Ras1 and Ras2 are active and enhance cAMP generation by the adenylyl cyclase. Elevated cAMP binds to Bcy1 and releases its inhibitory effect on PKA. Constitutive activation of the Ras/PKA pathway suppresses autophagy induced by TOR inhibition in yeast (Schmelzle et al., 2004), suggesting that the Ras-PKA pathway downregulates autophagy in parallel with the TOR-Tap42 pathway. Autophagy inhibition by Ras/PKA may be mediated through regulation of Atg1, which is identified as a phosphorylation substrate of PKA. In the presence of nutrients, PKA phosphorylation causes Atg1 to be largely cytosolic and dissociated from the PAS, whereas during starvation, Atg1 is dephosphorylated and localized to the PAS.

In addition to PKA, the protein kinase Sch9, the closest yeast homolog to the mammalian protein kinase B (PKB)/Akt as well as to the TOR target S6 kinase (S6K), is involved in nutrient sensing (Zhang et al., 2008). Simultaneous inactivation of PKA and Sch9 induces autophagy, which can be further increased by inactivation of TORC1, suggesting that autophagy is negatively regulated by at least three parallel pathways in yeast, TORC1, Ras/PKA, and Sch9. Ras/PKA and Sch9 may regulate autophagy at the transcriptional level, as the transcription factors Msn2/4 and the Rim15 kinase are required for autophagic flux induced by inactivation of both PKA and Sch9, but not for that induced by inactivation of TOR (Yorimitsu et al., 2007).

5.2. Insulin/Growth Factor Pathways

When growth factors are withdrawn from the extracellular milieu, in spite of sufficient nutrients, autophagy is induced to maintain cellular functions and energy production (Lum et al., 2005). In higher eukaryotes such as *Drosophila* and mammalian cells, the pathways through which hormones regulate autophagy are different from those of nutrients, but both converge on TOR (Fig. 7). Insulin and insulin-like growth factors regulate mTOR through the class I PtdIns3K. Upon insulin binding,

autophosphorylation of the insulin receptor on tyrosine residues results in the recruitment and phosphorylation of IRS1 and IRS2 (insulin receptor substrate 1 and 2), which creates a docking scaffold that allows binding of adaptor proteins, including subunits of the class I PtdIns3K such as p85. Generation of PIP3 [phosphatidylinositol (3,4,5)-trisphosphate; Fig. 7 , red circles in the membrane] by the class I PtdIns3K increases membrane recruitment of both protein kinase B (PKB)/Akt and its activator PDK1 (phosphoinositide-dependent protein kinase 1), leading to phosphorylation and activation of PKB/Akt by PDK1 (Stokoe et al., 1997). The 3-phosphoinositide phosphatase PTEN reverses PIP3 production, decreasing the downstream PKB/Akt signalling, positively regulates autophagy (Arico et al., 2001), but has been recently observed that this effect in promoting autophagy could be independely of its lipidic phosphatase activity (Errafiy et al., 2013).

Recently Renna et al. (2013) reported that IGF-1 (insulin- growth factor) inhibition attenuates autophagosome formation; the reduced amount of autophagosomes present in IGF-1R depleted cells has beeen, at least in part, explained by a reduced formation of autophagosomal precursors at the plasma membrane. In particular, IGF-1R depletion inhibits mTORC2, which, in turn, reduces the activity of protein kinase C (PKCα/β). This perturbs the actin cytoskeleton dynamics and decreases the rate of clathrin-dependent endocytosis, which impacts autophagosome precursor formation.

Basically, when hormones are absent, mTOR is inactivated, which releases the inhibitory effect on autophagy. Besides TOR, Ras signaling also plays a role in autophagy regulation by growth factors (Fig. 7). Ras transduces signals from growth factor receptor tyrosine kinases to intracellular effectors, such as Raf-1/MAP (mitogen-activated protein) kinases and the class I PtdIns3K (Furuta et al., 2004).

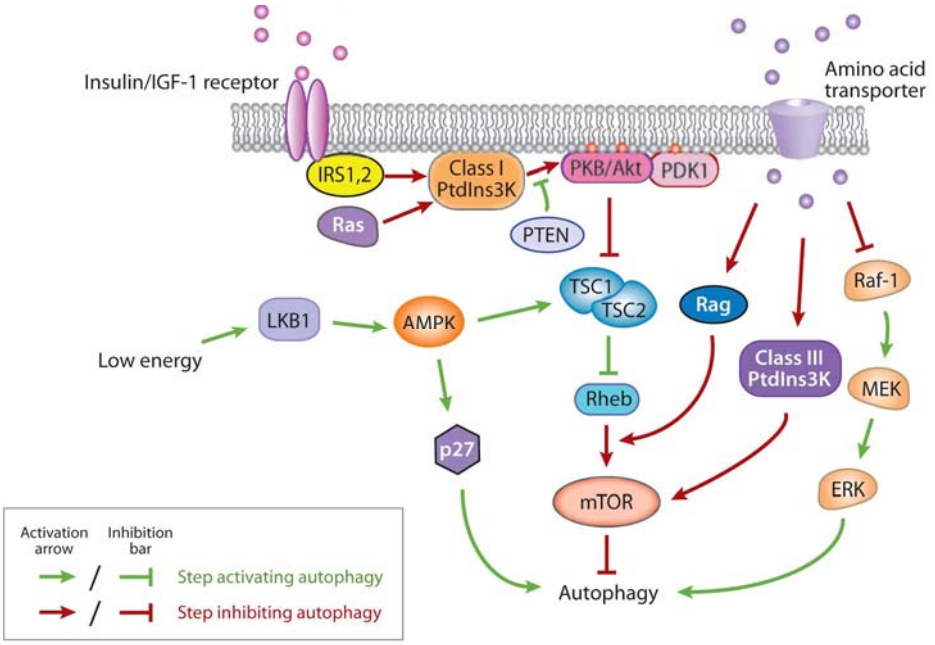


Figure 7. Autophagy is induced by deprivation of nutrients, hormones, and energy*. Regulatory pathways of autophagy by amino acids, hormones, and energy in mammals (He and Klionsky, 2009)*

5.3. Energy Sensing

During periods of intracellular metabolic stress, activation of autophagy is essential for cell viability. In mammalian cells, a reduced cellular energy (ATP) level is sensed by AMPK (5'-AMP-activated protein kinase) (Fig. 7). AMPK is activated by a decreased ATP/AMP ratio through the upstream LKB1 kinase (encoded by the Peutz-Jeghers syndrome gene). Active AMPK leads to phosphorylation and activation of the TSC1/2 complex, which inhibits mTOR activity through Rheb (Inoki et al., 2003). Autophagy stimulated by mTOR downregulation results in elevated ATP production via recycling of nutrients. In addition, the LKB1-AMPK pathway phosphorylates and activates p27kip1, a cyclin-dependent kinase inhibitor leading to cell cycle arrest, which is essential to prevent cells from undergoing apoptotic death and to induce autophagy for survival in response to bioenergetic stress during growth factor withdrawal and nutrient deprivation (Liang et al., 2007). Similarly, Snf1, the yeast homolog of mammalian AMPK, also positively modulates autophagy, possibly through independent mechanisms involving regulation of Atg1 (Wang et al., 2001).

5.4. Stress Response

Various extra- and intra-cellular stresses potently induce autophagy, which is important for organisms to adapt to, or overcome, unfavourable conditions. Recent studies have provided insight into the molecular mechanisms that regulate autophagy in response to different stresses.

ER stress. The ER is the key compartment in the cell to facilitate folding of newly synthesized proteins and initiate the pathway of vesicular movement of membrane and proteins to various organelles and the cell surface. In mammalian cells, the ER also serves as the major intracellular Ca^{2+} reservoir. A number of ER stress stimuli, for example, expression of aggregate prone proteins, glucose deprivation (resulting in reduced glycosylation and decreased energy for chaperone activity), hypoxia and oxidative stress (causing decreased disulfide bond formation), and Ca^{2+} efflux from the ER, lead to the accumulation of unfolded proteins in the ER, which exceeds its folding capacity. Autophagy is induced by ER stress in organisms from yeast to mammals. However, the signalling mechanisms linking ER stress to autophagy vary, dependent on the specific stress conditions and the organisms is still studied (Fig. 8). In yeast, ER chemical stressors blocking formation of disulfide bonds or protein glycosylation, such as DTT and tunicamycin, effectively trigger autophagy, which requires Atg1 kinase activity (Yorimitsu et al., 2006). ER stress-induced autophagy is required for cell survival in the presence of tunicamycin, likely through compensatory removal of expanded and disorganized ER (which results from the unfolded protein response; UPR), along with the misfolded proteins within (Bernales et al., 2006). The UPR signaling pathway in yeast is mediated by Ire1 (inositol-requiring kinase 1), an ER transmembrane protein with a lumenal stress sensing domain and a cytosolic endoribonuclease domain. In response to the accumulation of unfolded proteins, an ER-specific member of the heat shock protein 70 family, Grp78/BiP, dissociates from its ER-sensing domain and activates the cytosolic endonuclease domain of Ire1, which triggers the splicing of the Ire1 substrate Hac1. The latter encodes a transcription factor (the yeast homolog of mammalian XBP1) that activates transcription of target genes involved in protein modification/folding, vesicle transport, phospholipid biosynthesis, and ERAD (ER-associated degradation) (Mammucari et al., 2007). Although the Ire1-Hac1 pathway is required for autophagy induction by ER stress, it is dispensable for the transcriptional upregulation of *ATG* genes (Bernales et al., 2006). In mammalian cells,

knockdown of the upstream UPR regulator Grp78/BiP by siRNA inhibits autophagosome formation induced by both ER stress and nutrient deprivation, but does not affect the conversion of LC3-I to LC3-II, suggesting that Grp78/BiP is necessary for autophagy and may function at the phagophore expansion rather than induction step (Li et al., 2008). But the knockdown of Grp78/BiP is an artificial condition that spontaneously activates UPR pathways and induces LC3 conversion, and this situation makes it difficult to differentiate between the roles of Grp78/BiP and UPR signaling in autophagy induction.

Mammalian UPR signaling is more complex than in yeast and involves three distinct downstream pathways, IRE1 (similar to yeast Ire1), ATF6 (activating transcription factor 6), and PERK (RNA-dependent protein kinase-like ER kinase). These factors signal misfolded protein levels in the ER and activate transcription of different target genes. One downstream target of IRE1 is c-Jun N-terminal kinase (JNK), which is essential for lipid conjugation of LC3 induced by tunicamycin or by accumulation of cytosolic misfolded proteins due to proteasome inhibition in MEFs and cancer cells (Ogata et al., 2006).

Data using murine cells suggest that in response to ER stress induced by expression of misfolded polyQ72 or mutant dysferlin proteins, phosphorylation of eIF2α (eukaryotic initiation factor 2α) by the eIF2α kinase PERK is required for mediating LC3 conversion and autophagic degradation of the mutant proteins in the ER (Kouroku et al., 2007). Thus, both the IRE1-JNK and the PERK-eIF2α pathways seem to play an important role in UPR-induced autophagy.

In addition to UPR signaling, ER stress also induces release of lumenal Ca^{2+} to the cytosol. Calcium-activated calmodulin-dependent kinase kinase-β (CaMKKβ) is stimulated by the increase in the intracellular Ca^{2+} level and further activates AMPK, the latter potently inducing autophagy (Høyer-Hansen et al., 2007). Elevated Ca^{2+} levels also trigger phosphorylation of protein kinase Cθ (PKCθ), which induces LC3 conversion and autophagy in immortalized hepatocytes in response to the ER stressors thapsigargin and tunicamycin (Sakaki and Kaufman, 2008). Although some studies show that ER stress-induced autophagy has a pro-survival role in mammalian cells, others suggest that ER stressors may cause autophagic cell death. Injection of tunicamycin into renal tubules in mice induces kidney tubular cell death via both apoptosis and autophagy, which are mediated by the catalytic activity of the tumor

suppressor DAPk (calmodulin-regulated serine/threonine kinase death-associated protein kinase). DAPk is activated by PP2A-like phosphatase-dependent dephosphorylation of an inhibitory serine that is derived from autophosphorylation and activated DAPk induces autophagy likely through its ability to phosphorylate Beclin 1 and promote Beclin 1 dissociation from Bcl-2 (Gozuacik et al., 2008).

It is possible that autophagy plays dual roles in determining cell fate, depending on specific cell types and stimuli (Ding et al., 2007).

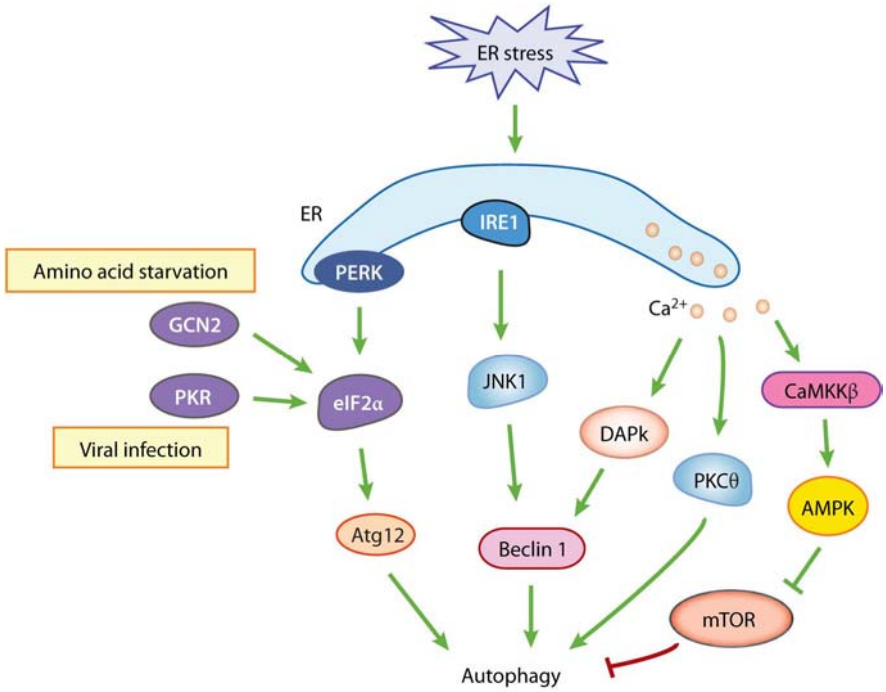


Figure 8. Autophagy regulation in response to stress. *ER stress stimulates autophagy through the PERK-eIF2α pathway, the IRE1-JNK1 pathway and Ca2+ release. Activation of eIF2α by PERK may upregulate transcription of certain autophagy genes, such as ATG12. Phosphorylation and activation of eIF2α by two other eIF2α kinases, GCN2 and PKR, upon amino acid starvation and viral infection, respectively, are also depicted. (He and Klionsky, 2009)*

Hypoxia. Low levels of oxygen at or below 1% (hypoxic stress) versus 2–9% (normoxia for most mammalian cell types) exist in physiologically developing embryos as well as many pathological conditions, such as solid tumors, cardiovascular ischemia, and brain injuries.

Hypoxia can induce autophagy in mammalian cells, and the signalling pathways responsible for autophagy induction and its cellular consequences seem to be different, contingent on the types of cells and autophagic pathways (Fig. 9). Enhanced mitochondrial autophagy (mitophagy) during hypoxia is suggested to be an adaptive

response, reducing the levels of reactive oxygen species (ROS) and protecting cell integrity, although in several glioma and breast cancer cell lines, prolonged hypoxia mediates autophagic cell death (Azad et al., 2008).

Hypoxia-inducible factor-1 (HIF-1) is the primary transcription factor acutely induced by hypoxic conditions, and it drives transcription of hundreds of genes that promote erythropoiesis and angiogenesis and decrease mitochondrial biogenesis and respiration, counterbalancing deleterious effects caused by O2 deficiency. In MEFs, mitochondria are removed by mitophagy in response to hypoxia, which is dependent on HIF-1 and the induction of its downstream target BNIP3 (Bcl-2 adenovirus E1a Nineteen kDa Interacting Protein 3; a pro-death Bcl-2 family member) (Zhang et al., 2008). In addition, during reticulocyte maturation in mice, another HIF-1-induced target, BNIP3L (BNIP3-like protein or NIX), is required for programmed mitochondrial clearance by autophagy (Sandoval et al., 2008). BNIP3 competes with Beclin 1 for binding Bcl-2 and thus releases Beclin 1 for participating in mitophagy.

Besides being under the control of HIF-1, BNIP3 is also a target gene of the E2F transcription factors, which are inhibited by the RB tumor suppressor during RB-induced cell cycle arrest (Tracy et al., 2007). Therefore, hypoxia induces BNIP3 transcription via binding of HIF-1 and/or E2F to the BNIP3 promoter, and the RB-E2F-BNIP3 signalling is also involved in hypoxia-induced autophagy.

Upregulation of bulk autophagy by hypoxia in tumor cells seems independent of the HIF-1 pathway: the AMPK-mTOR andPKCδ (protein kinaseCδ)-JNK1 cascades are responsible for signaling autophagy induction (Chen JL et al., 2008; Papandreou et al., 2008).

In addition, hypoxia inhibits TOR and blocks eIF4F (eukaryotic initiation factor 4F) complex formation and mRNA translation (Brugarolas et al., 2004). Thus, autophagy induced by hypoxia can be at least partially TORdependent, and hypoxia-stimulated ER stress may also play a role in autophagy induction.

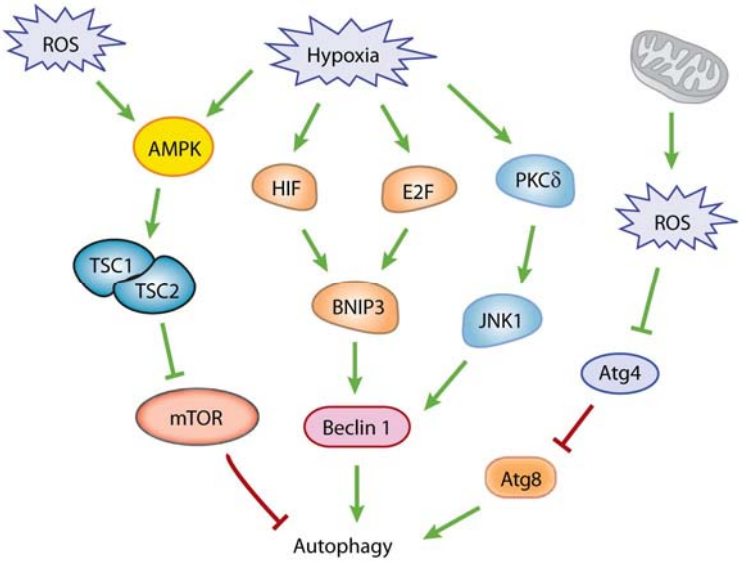


Figure 9. Autophagy regulation in response to stress*.Autophagy induction by mechanisms sensing hypoxia or oxidative stress. JNK1, DAPk and BNIP3 induce autophagy by disrupting the Bcl-2-Beclin 1 interaction and activating Beclin 1.*

Oxidative stress. A common intracellular stress that effectively leads to induction of autophagy is the formation of Reactive Oxygen Species (ROS). Mitochondria are the major source generating ROS, which will in turn damage these organelles. ROS generating agents (such as hydrogen peroxide and 2-methoxyestradiol), or chemicals inhibiting the mitochondrial electron transport chain, induce ROS production and autophagic cell death in transformed and cancer cell lines (ChenY et al., 2008). These drugs induce a much lower level of ROS in non-transformed primary mouse astrocytes compared with cancer cells and fail to stimulate autophagy, suggesting that normal cells effectively maintain ROS at a tolerable level and are able to protect themselves from mitochondrial damage, likely through antioxidant mechanisms such as superoxide dismutase (SOD), catalase, and the redox system, because applying chemical ROS scavengers or overexpressing SOD2 reduces autophagy (Chen Y et al., 2007).

Impaired mitochondria may be selectively degraded through mitophagy in yeast and mammalian cells, which may constitute another mechanism to reduce ROS levels and maintain cell survival (Priault et al., 2005). The link between ROS and autophagy induction may be the cysteine protease Atg4 (Fig. 9), which cleaves Atg8/LC3 from the autophagosome outer-membrane before, or soon after, autophagosome-lysosome fusion. ROS targets a conserved Cys81 on Atg4, which is in the vicinity of the catalytic Cys77 residue; oxidation of cysteines inhibits Atg4 protease activity and promotes lipidation of

Atg8/LC3, an essential step for autophagy (Scherz-Shouval et al., 2007). It is not known, however, how ROS levels might be temporally and spatially controlled inside the cell, so that Atg4 can be locally activated to allow de-lipidation and recycling of Atg8/LC3, or whether another mechanism is involved.

In addition, Atg4 seems to not be the only molecule that underlies the oxidative regulation of autophagy. A recent study suggests that hydrogen peroxide activates poly(ADP-ribose) polymerase-1 (PARP-1), which stimulates the LKB1-AMPK pathway and leads to autophagy induction (Huang et al., 2009). It is likely that DNA damage induced by oxidative stress is involved in the activation of PARP-1 and autophagy (Munoz-Gamez et al., 2009).

5.5. Pathogen infection

Autophagy has an important role in eliminating invading pathogens, and pathogen-induced autophagy appears to be TOR-independent (Fig. 10).

The innate immune system is an evolutionarily ancient and conserved defense mechanism and is found in almost all types of multicellular organisms, including insects, plants, and mammals. In *Drosophila*, which depend nearly entirely on innate immunity to fight against infection, the peptidoglycan-recognition protein (PGRP) family members play crucial roles in surveillance and microbe sensing. The PGRP receptors are present in immune cells, recognize bacteria-derived peptidoglycans, and activate the production of anti-microbial peptides.

A PGRP family member, PGRP-LE, is the first identified cytoplasmic sensor in insects that recognizes the presence of intracellular bacteria and triggers autophagy in host cells, which is required for suppressing the growth of *Listeria monocytogenes* in hemocytes and enhancing host viability (Yano et al., 2008).The pathway that transduces signals from the PGRP-LE receptor to the autophagy machinery, and whether it cross-talks to the classical signaling pathways downstream of PGRP receptors that activate genes encoding antimicrobial peptides, remain to be investigated.

Signalling of Toll-like receptors (TLRs) triggers autophagy during mammalian innate and adaptive immunity. TLRs are membrane receptors localized at the cell surface and endosomes, and TLR signalling activates transcription of genes responsible for T-cell stimulation, inflammation, and antiviral immune responses. Different TLRs are involved in autophagy induction upon binding their specific pathogen-derived

ligands. For example, viral ssRNA (single-stranded RNA) induces autophagy via TLR7, zymosan stimulates LC3 translocation to phagosomes by activating TLR2 and lipopolysaccharide (LPS), which is derived from cell walls of Gram negative bacteria, triggers autophagy through TLR4. TLRs recruit adaptor proteins to transduce signals to downstream effectors (Del Gado et al., 2008; Sanjuan et al., 2007).
In human and murine macrophages, the TLR4 adaptor protein TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-β) is suggested to mediate LPS-induced autophagy, and the same downstream signalling pathway is shared by both innate immunity and autophagy upregulation (Xu et al., 2007).

Moreover, interferon (IFN)-γ and its effector Irgm1 (the IFN-inducible immunity-related GTPase family M member 1; also known as LRG-47) are also required to stimulate autophagy in macrophages and inhibit the survival of intracellular mycobacteria (Singh et al., 2006).

In response to viral infection, the antiviral eIF2α kinase signaling pathway, including eIF2α and the IFN-inducible double-stranded RNA-dependent protein kinase R (PKR), is activated and upregulates autophagy (Tallòczy et al., 2002). Some viral proteins may antagonize autophagy induction by directly modulating Atg proteins: the HSV-1 (herpes simplex virus type 1)-encoded neurovirulence protein ICP34.5 inhibits autophagy via interaction and sequestration of Beclin 1 (Orvedhal et al., 2007). It is also reported that viral infection, such as infection with hepatitis C virus, induces ER stress, and autophagy is triggered through the unfolded protein response, including the downstream IRE1, ATF6, and PERK signalling pathways (Sir et al., 2008).

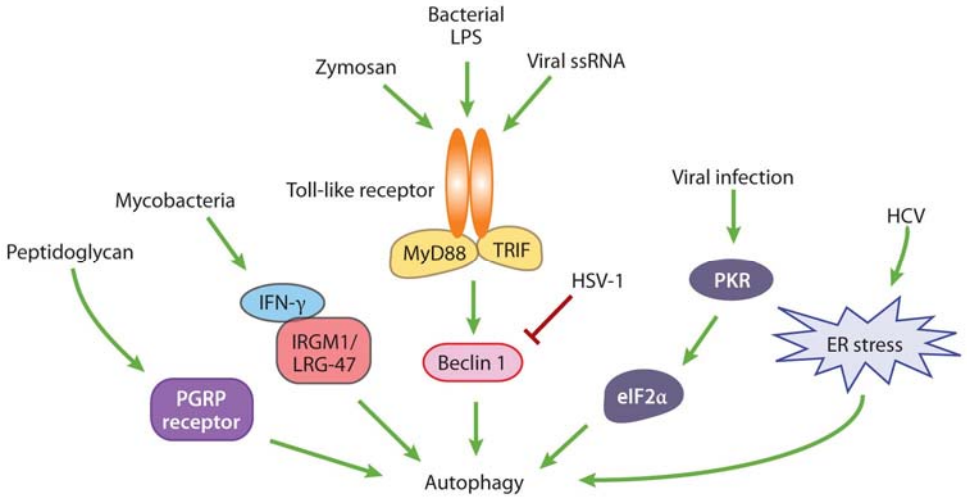


Figure 10. *Different mechanisms that regulate autophagy by pathogen invasion (he and Klionsky, 2009)*

6. Quality control and starvation-induced autophagy

Autophagy can be distinguished into two forms: quality control (QC) autophagy and starvation-induced autophagy.

QC autophagy is present in normal cell function to degrade proteins and organelles that become mutated, misfolded or oxidized and recycle the remaining component lipids, nucleic acids, amino acids and micronutrients back to the cytosol as nutrients. Damaged cytosolic components are selected for degradation by QC autophagy through ubiquitin tags. Then Histone deacetylase 6 (HDAC6) binds ubquinated proteins and organelles, selecting them for degradation (Lee et al., 2010).

Starvation-induced autophagy does not require ubiquitination of substrates for degradation. Starvation-induced autophagy is regulated through mTOR inhibition to compensate for limited nutrients in the environment (Hosokawa et al., 2009). Macroautophagy is also regulated through mTOR by nutrient supply and environmental toxins that cause intracellular damage; mTOR is itself the target of multiple signalling pathways. To ensure that autophagy does not become overactive and cytotoxic, certain amino acids released from protein degradation act as negative feedback regulators (Tassa et al., 2003). Activation of the metabolic state sensor AMPK by AMP at low energy levels inhibits mTOR to upregulate autophagy (Fig. 11). When AMP levels rise from consumption of ATP, AMPK phosphorylated mTOR, a protein kinase that phosphorylates ULK1/2 and Atg13. mTOR has two subunits: mTORC1 and mTORC2, buy only mTORC1 directly inhibits autophagy along with protein synthesis and mitosis

(Raught et al., 2001). mTORC1 inhibits the ULK1/2-Atg13 complex through phosphorylation. When mTORC1 is inactive, dephosphorylation of ULK1/2 activates its kinase activity, phosphorylating Atg13, FIP200 and ULK1/2 itself (Jung et al., 2010). Active ULK1/2-Atg13 complex is localized to the isolation membrane, aiding in vesicle nucleation (Hara et al., 2008). Rapamycin is a useful drug that directly inhibits mTORC1 to activate autophagy. Intracellular signals that inhibit mTOR activity include inadequate energy supply and protein. When the amount of available energy reaches a certain threshold, AMPK is activated. The kinase activity of AMPK phosphorylates TSC1/2, a point of convergence for multiple autophagy regulators (Inoki et al., 2005).
One intracellular protein that regulates mTOR activity is the tumor suppressor p53; depending on where it resides in the cell, p53 can either stimulate or inhibit the process of autophagy (Tasdemir et al., 2008).

Litium and amino acids regulate autophagy through mTOR independent pathways. Litium inhibits the activity of inositol monophosphate (IMPase), so the levels of myo-1.4.5-triphosphate (IP3) decrease in the cell. Low IP3 levels induce autophagy independently of mTOR activity. The specific mechanism for IP3 regulation of autophagy is still unknown (Sarkar et al., 2005).

Certain amino acids that are released when autophagy digests proteins (leucine, phenylalanine, alanine, methionine and glutamine) inhibit autophagy, since they are negative feedback regulators of autophagy through the PI3 K-III complex (Fig. 11). Amino acid inhibition of the PI3 K-III complex stops vesicle nucleation, downstream of mTOR. Conversely, low levels of these amino acids induce autophagy through PI3 K-III activation (Tassa et al., 2003).

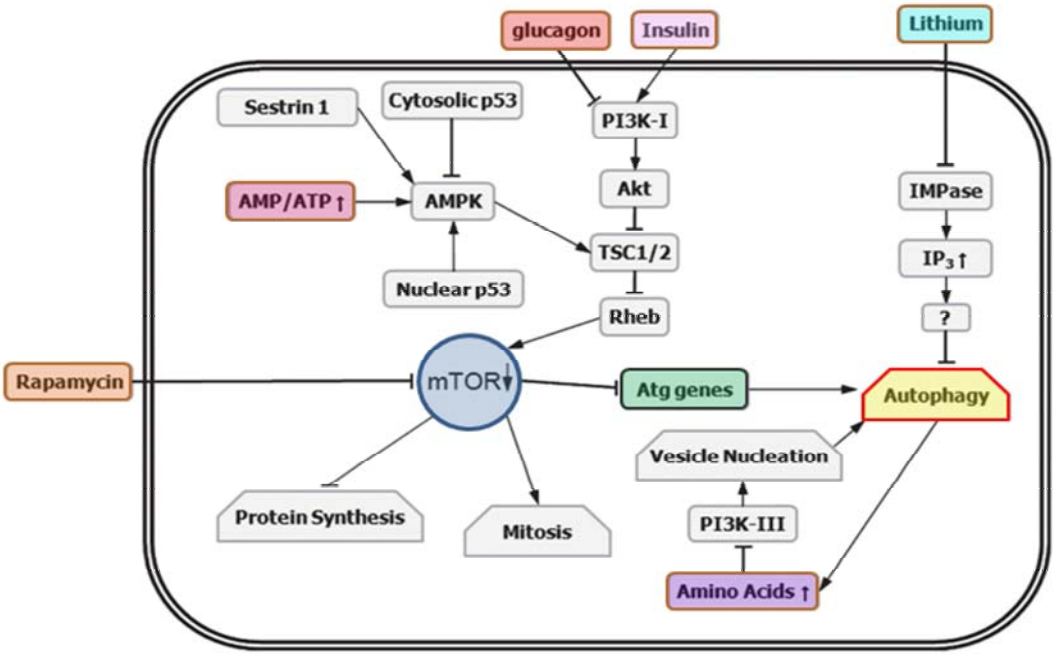


Figure 11. *Regulation of autophagy is regulated by both extracellular and intracellular signals. Low AMP/ATP, growth factor deprivation or nutrient restriction inhibit mTOR activity to promote autophagy, although amino acids and lithium act independently of mTOR. The inhibition of mTOR induces autophagy, protein synthesis and mitosis. Hexagon = cellular processes regulated by mTOR. (Barnett and Brewer, 2011)*

As an important catabolic pathway, autophagy maintains crosstalk with several other regulatory pathways including the ubiquitin proteasome system (UPS) and ER associated degradation. For example, impairment of the proteasome results in upregulation of autophagy, and autophagy cooperates with ER associated degradations to degrade certain disease proteins (Kroeger 2009).

In mammals the importance of autophagy in maintaining homeostasis and modulating cytoplasmic contents also makes it very important in cell death and proliferation. As a pathway controlling cellular stress such as oxidative damage and protein misfolding, activation of autophagy can promote cell survival (Eskelinen 2005). Conversely, loss of these pro-survival activities by inhibition of autophagy can result in apoptosis. During cell proliferation autophagy can act as a sensor of stress, helping determine if the cell is at an appropriate stage to divide.

8. Autophagy and diseases

A basal and constitutive level of autophagy is necessary for intracellular homeostasis and quality control for healthy individuals. Mounting evidence has demonstrated that disruption of its physiological function is strongly related to human diseases, including neurodegeneration, cancer, liver and heart diseases, ageing, myopathies, and infections (Mizushima et al., 2008).

Neurodegenerative diseases such as AD, PD, amyotrophic lateral sclerosis, prion diseases, Huntington's disease (HD) and spinocerebellar ataxias have the common characteristic of protein aggregation (Sarkar and Rubinsztein, 2008). For example, β-amyloid and tau aggregates accumulate in AD. Expanded polyglutamine (PolyQ) stretch caused by the expansion of CAG trinucleotide repeat within several gene exons seen in HD and several types of spinocerebellar ataxias. α-synuclein fibrils linked to familial PD self-aggregate and deposit in the brain (Honson et al., 2007; Williams et al., 2006). These protein aggregates, the neurotoxic components, are substrates of the autophagy degradation system. Boland et al (2008) suggested that the basal expression of autophagy is crucial in primary cortical neurons and there is no large amount of autophagosome accumulation in healthy brains because of efficient lysosomal hydrolysis of newly formed autolysosomes.

It is known that autophagy plays a role in the protection against neurodegeneration but in patients with diverse neurodegenerative diseases the autophagic clearance system may reach saturation and thereby become unable to eliminate excess aggregate-prone mutant proteins, or dysregulation or defection may occur in the autophagy pathway.

Impaired autophagic vacuoles (AVs) clearance during autophagosome transport to lysosomes or during substrate degradation within the lysosome may lead to AVs accumulation with morphologies that are observed in AD (Boland et al., 2008).

Contrary to the beneficial effect of basal autophagy, an excess accumulation of autophagosomes promotes neuronal cell loss under certain circumstances, such as dysfunctional endosomal sorting complex required for transport III (ESCRT-III), which is associated with FTD (Lee and Gao 2009). This implies that either excessive autophagosome synthesis or reduced autophagosome-lysosome fusion may contribute to the pathogenesis of neurodegeneration by the dysfunctional autophagic process. Owing to the protective role of autophagy in neuron systems, it may be a therapeutic target against neurodegenerative diseases. Upregulation of autophagy may improve the

clearance of neurotoxic aggregate-prone proteins. The inhibition of TOR, a negative regulator of autophagy, by rapamycin or its analogue CCI-779 is protective in *Drosophila* and mouse models of HD (Ravikumar et al., 2004). mTOR is required for various cellular events, including protein synthesis (e.g. the initiation of mRNA translation, ribosome biogenesis, transcription), cell growth and proliferation, and cytoskeletal reorganization (Sarbassov et al., 2005), but its inhibition by rapamycin causes some adverse effects such as cell cycle arrest, poor wound healing, and mouth ulcers (Bissler et al., 2008). Small molecule enhancers of rapamycin (SMERs), however, induce autophagy in an mTOR independent mechanism. They enhance the elimination of mutant huntingtin and α-synuclein, and protect against neurodegeneration in the HD model of *Drosophila* (Sarkar et al., 2007). Recently, autophagy induction by a group of new drugs, L-type Ca^{2+} channel antagonists, K^{+} ATP channel opener minoxidil, and Gi signaling activator clonidine in an mTOR independent pathway were reported in HD mammalian cell, fly and zebrafish models (Williams et al., 2008). Nevertheless, it is important to understand the pathology underlined by autophagy in different neurodegenerative diseases. Pharmacologically enhancing autophagosome formation in AD where the autophagosome clearance system is defective may exacerbate the accumulation of the unmatured autophagosomes, some of which may generate toxic proteolytic product Aβ and/or others (Yu et al., 2005; Boland et al., 2008). Therefore, improvement in the late stages of the autophagy process may be the best therapeutic modulation of autophagy in AD. In addition, the inhibition of autophagy by small molecules might be promising therapeutic strategies for the early stages of some forms of FTD or other neurodegenerative diseases where excess produces autophagosomes (Lee and Gao, 2009).

The impairment of autophagy clearance for intracellular proteins and organelles is associated with the pathogenesis of several diseases, such as neurodegeneration, PD and ageing. Therefore, targeting autophagy may be a potential strategy for the treatment of these diseases (Bao et al., 2010; Rubinsztein et al., 2012).

8.1. Autophagy and Alzheimer's disease

Cytoplasmic, nuclear and extracellular inclusions composed of aggregated and ubiquitinated proteins are key pathological hallmarks of numerous neurodegenerative diseases. It is believed that protein aggregation contributes to organelle damage, synaptic dysfunction and neuronal degeneration, so clearance pathways for intracellular protein aggregates have been suggested as a potential therapeutic approach for such disorders. It is now apparent that aggregate-prone proteins and damaged organelles observed in a variety of neurodegenerative disorders are eliminated more efficiently via the autophagy–lysosome pathways than via the ubiquitin–proteasome machinery. Efficient autophagosome trafficking along microtubules is essential for autophagic degradation in neurons given the distance between neuronal processes, where endosomes and autophagosomes are formed, and the neuronal soma, where lysosomes are concentrated.

In AD there is massive accumulation of autophagosomes within large swellings along dystrophic and degenerating neurites, primarily due to deficits in the maturation of autophagosomes and their retrograde transport towards the neuronal cell body (Nixon, 2007).

The general consensus is that autophagy is activated in AD primarily because of impaired clearance of autophagosomes that contain both APP and its processing enzymes, thereby increasing the propensity to generate toxic Aβ peptides (Yu et al., 2005). Although most Aβ formed during autophagy is normally degraded within lysosomes via macroautophagy, Aβ also accumulates within the large pool of autophagosomes in dystrophic neurites and becomes a major intracellular reservoir of toxic peptides in AD brains. It is well established that Aβ can disrupt autophagosome membranes and trigger the release of hydrolytic enzymes into the cytoplasm. It has been suggested that local accumulation of autophagosomes in dystrophic neurons can contribute to Aβ generation within plaques and that increased autophagy in the neuropil could be a major source of Aβ (Nixon et al., 2005). This is achieved via increased turnover of APP and enrichment of the γ-secretase complex inside autophagosomes that cleaves APP to Aβ. In fact, autophagosome membranes are rich in presenilin 1, a component of the APP-cleaving γ-secretase complex, which is encoded by the presenilin gene, mutations of which lead to early-onset forms of autosomal dominant AD. Human AD brains with mutant presenilin 1 show accumulation of extensive

lysosomal pathology, together with amyloid pathology and neurodegeneration (Cataldo et al., 2004).

It was recently demonstrated that neurons and blastocysts from presenilin1 hypomorphic mice and fibroblasts from presenilin 1 mutant AD patients show loss of macroautophagy, resulting in increased Aβ accumulation due to impaired maturation of the V0a1 subunit of the bimodular v-type H+-ATPase proton pump that acidifies the lysosome (Lee et al., 2010).

Furthermore, levels of beclin 1 (or Atg6), which is required for autophagosome formation, are consistently reduced in postmortem AD brains (Pickford et al., 2008). In a transgenic mouse model of AD that expresses human APP, genetic reduction of beclin 1 expression stimulated Aβ accumulation and neurodegeneration due to a decrease in autophagy. Conversely, increased expression of beclin 1 in APP transgenic mice significantly reduced amyloid pathology and neurodegeneration. If reduced induction of autophagy is the underlying mechanism for the higher levels of Aβ peptides and aggregate observed, then these results are consistent with findings that autophagosome maturation can be impaired in AD (Pickford et al., 2008).

However, Aβ levels are affected by both production and clearance (Selkoe 2001). Therefore, defects in autophagosome maturation could enhance Aβ production, whereas reduced autophagy through beclin 1 deficiency might inhibit Aβ clearance by altering APP metabolism (Jaeger et al., 2010). Studies indicate ultrastructural alterations in mitochondrial morphology, such as reduced size, broken cristae and abnormalities in mitochondrial dynamics, in AD (Santos et al., 2010).

In patients with sporadic AD, fibroblasts and cells overexpressing the Swedish variant of APP demonstrate an imbalance in mitochondrial fission–fusion proteins, either via post-translational modification such as S-nitrosylation or by alterations in their expression leading to an increase in mitochondrial fission (Wang et al., 2008, Cho et al., 2009). It has been suggested that increased mitochondrial fission is probably an attempt to segregate and eliminate damaged mitochondria via a macroautophagy process known as mitophagy, which is consistent with reductions in the level and size of mitochondria observed in human post-mortem tissue (Hirai et al., 2001).

It has been suggested that alterations in mTOR pathway, which plays a central role in signaling induced by nutrients and growth factors, support autophagic failure in AD. Accumulation of Aβ peptides increases signaling of the mTOR pathway, whereas a

decrease in mTOR signaling reduces Aβ levels: in a mouse model of AD, pharmacological inhibition of mTOR signaling by rapamycin (an FDA-approved drug) rescues cognitive deficits and ameliorates amyloid and tau pathology by increasing autophagy (Spilman et al., 2010). Rapamycin-induced inhibition of mTOR led to increased neuronal autophagy in mutant AD mice, suggesting that reductions in Aβ levels and improvements in cognitive function are due in part to increased autophagy via mTOR inhibition.

Considering that age is an important risk factor for the development of AD and many other neurodegenerative disorders, rapamycin administration in animals extended lifespan in multiple recent studies (Anisimov et al., 2010). This effect of rapamycin on lifespan due to increased autophagy reinforces the benefits of promoting autophagy in age-related neurodegenerative disorders

Thus, autophagy induction might serve as a potential therapeutic target for blocking of AD pathogenesis.

9. Autophagy and development

During embryonic development, cells undergo several and various processes like proliferation, differentiation and death. Autophagy plays a role in the development of all organisms, from unvertebrates like Drosophila (Tracy and Baehrecke, 2013) to vertebrates as zebrafish and mice (Di Bartolomeo, 2010).

During critical mammalian developmental stages in which nutrients are restricted, autophagy is important; one such stage is pre-implantation development of embryos (Tsukamoto et al., 2008a). Autophagic activity is low in unfertilized oocytes and increases shortly after fertilization (Fig.12); it is transiently suppressed between the late one-cell and middle two-cell stages and is then activated again after the late two-cell stage.

Atg5-deficient oocytes fertilized by Atg5-null sperm failed to develop beyond the four- and eight-cell stages, but could develop if fertilized by wild-type sperm. It is not known whether the main role of autophagy in pre-implantation development is to provide nutrients to the growing embryo or to clear maternal proteins (Tsukamoto et al., 2008a, 2008b). Other possible consequences of autophagy deficiency are also possible, including secondary effects on the ubiquitin-proteasome system, which may have an impact on the coordination of the levels of critical proteins required for regulating cell

division. It is possible that some of these secondary consequences of autophagy compromise may contribute to the necessity for autophagy during development (Wu et al., 2013). Sudden termination of the fetal nutrient supply from the mother presents a stressful situation for the newborn infant before it establishes breastfeeding. During this transition period, autophagy provides necessary nutrients to the infant through increased turnover of proteins. This has been shown experimentally in a transgenic mouse model expressing GFP-tagged LC3 to visualize autophagosomes *in vivo* (Ravikumar et al., 2004). In these mice, autophagy in various tissues increases soon after birth, peaks at ~6 h after birth, and declines back to basal levels within 24-48 h (Ravikumar et al., 2004). To assess the specific role of autophagy, the effect of starvation during this critical period in Atg5- and Atg7-knockout mice was studied. Whereas mice generated from animals with conditional deletion of Atg5 in oocytes and Atg5-null sperm cannot develop beyond the four- and eight-cell stages, conventional $Atg5^{-/-}$ mice generated by mating $Atg5^{+/-}$ mice survive until after birth, since the maternally derived Atg5 proteins stored in oocytes is sufficient to rescue the autophagy-deficient phenotype of early embryogenesis (Tsukamoto et al., 2008).

Atg5- and Atg7-knockout mice die within 1 day of birth (Ravikumar et al., 2004; Komatsu et al., 2005). Under forced starvation conditions, the survival time of the Atg5-knockout mice was nearly half (12–13 h) that of their $Atg5^{+/-}$ or wild-type littermates (21–24 h) (Ravikumar et al., 2004). These experiments support the role of autophagy in normal developmental processes that occur in the context of diminished nutrient supply, lending importance to the need for the functional turnover of amino acids. However, it is possible that other mechanisms may also be relevant.

Autophagy is also important for neuronal development: inactivation of Ulk1 (Atg1) in immature granule cells impairs axon outgrowth and differentiation of neurons; Ulk1 activity at the growth cone is thus crucial for the formation of fibres, and allows the progression of cerebellar development (Di Bartolomeo et al., 2010; Wu et al., 2013).
It is necessary to consider also that embryogenesis and neuronal development is controlled by central signalling pathways, such as Shh (Sonic Hedgehog), TGFβ (transforming growth factor β) and Wnt. In embryogenesis, these pathways are essential for patterning, formation of the body axis, limb development and formation of the neural tube (Copp et al., 2003; Tabata 2001; Harvey 2002; Raya and Izpisua Belmonte,2006). Furthermore, each pathway regulates individual cell fate in embryonic

and adult tissue. It has been reported that autophagy interacts with these developmental pathways. The interaction is a likely cause for the defects in development observed in the knockout mice, since the pathways are important for formation of embryonic structures (Wu et al., 2013).

The Shh signalling pathway plays an important role in patterning of the central nervous system, neural tube development and limb development. Previously, we reported that the Hedgehog signalling pathway inhibits autophagosome synthesis, both in basal and in autophagy-induced conditions (Jimenez-Sanchez et al., 2012; Han et al., 2008). On the other hand, Shh induces autophagy in hippocampal neurons (Petralia et al., 2013). This might be due to dual functions of Shh serving as mitogen in NSCs, but as an autophagy enhancer in differentiated neurons (Lai et al., 2003; Breunig et al., 2008).

TGFβ signalling is one of the most important players that drives developmental programmes that control cell behavior, and plays crucial roles in pluripotency and differentiation of embryonic stem cells *in vitro* (Massague, 2012). Recent evidence demonstrates that TGFβ activates autophagy through up-regulation of several autophagy-related genes, such as Beclin-1, Atg5, Atg7 and DAPK (death-associated protein kinase) (Suzuki et al., 2010).

The Wnt signalling pathway is crucial for embryogenesis, planar cell polarity and maintenance of stem cell fate (MacDonald et al., 2009). Autophagy was found to negatively regulate Wnt signaling (Gao et al., 2010). On the other hand, GSK3β (an inhibitor of Wnt signalling) was reported to stimulate autophagy through an mTOR-dependent mechanism (Zhai and Sadoshima, 2012).There may be a feedback mechanism through the master sensor GSK3β, which regulates Wnt signalling by multiple mechanisms.

Since embryogenesis is mainly governed by developmental pathways, such as Shh, TGFβ, Wnt and FGF, and there is an intensive cross-talk of autophagic proteins with these pathways (with implications in differentiation and proliferation of a variety of cell types), autophagy might regulate embryogenesis through these routes. However, it is likely that there are further mechanisms and regulatory loops to be discovered (Wu et al., 2013).

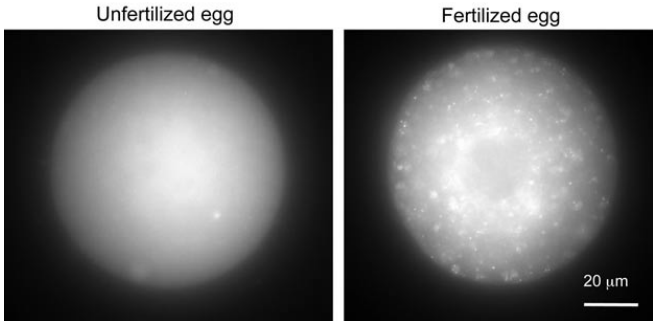


Figure 12. Autophagy is activated in fertilized mouse embryos. *Small bright dots represent LC3-positive autophagosomes and are shown in oocytes only after fertilization. Autophagy may be important to generate amino acids through degradation of maternal proteins, which is important for the synthesis of embryonic proteins.*

Also in zebrafish autophagy plays an important role, but unlike mammals, the fish are not dependent on the implantation in uterus, but develop from a fertilized egg to an adult outside the female in a transparent egg and the yolk provides nutrients to the embryo during all the early stages of development. In zebrafish Atg12 is ubiquitously expressed and the formation of the Atg12-Atg5 complex is dependent on Atg5; Atg5 is required in zebrafish for normal morphogenesis of brain regionalization and body plan. Atg5 also regulates expression of neural gene markers and is involved in regulation of LC3-II (Hu et al., 2011): that makes it an interesting target to study autophagy in development.

MEDITERRANEAN DIET

The Mediterranean diet is a modern nutritional recommendation inspired by the traditional dietary patterns of countries of the Mediterranean area: Greece, Italy, Spain and Morocco.

On November 17th 2010, UNESCO recognized this diet pattern as an Intangible Cultural Heritage of Italy, Greece, Spain and Morocco.

The most commonly understood version of the MD was presented, among others, by Dr. Willet of Harvard University's School of Public Health from the mid-1990s (Kushi et al., 1995) based on the food patterns typical of Greece and Southern Italy in the early 1960s. This diet, in addition to a regular physical activity emphasizes abundant plant foods (vegetables, legumes, fruits and cereals), fresh fruits as daily dessert, olive oil as the principal source of fat, dairy products (principally cheese and yogurth), fish and poultry consumed in moderate amount, from zero to four eggs consumed weekly, low intake of red meat and wine (Trichopoulou et al., 2003).

1. History

In the 1975 the american scientist Ancel Keys of the University of Minnesota School of Power pointed out the correlation between cardiovascular disease and MD for the first time (Keys and Keys, 1975). In the fifties he discovered its healthy benefits by observing a phenomenon which could not, at first, provide a full explanation. The poor population of small towns of southern Italy was, against all predictions, much healthier than the wealthy citizen of New York, either of their own relatives who emigrated in earlier decades in the United States. Keys suggested that this dependon food, and focused his attention on foods that made up the diet of these populations. Thus, he led the famous "Seven Countries Study", conducted in Finland, Holland, Italy, Greece, Jugoslavia, United States and Japan, in order to find out the relationship between lifestyles, nutrition and cardiovascular disease between different population, including through cross-sectional studies, being able to prove scientifically the nutrition value of the Mediterranean diet and its contribution to the health of the population that adopted it (Keys 1980). From this study emerged clearly that the populations that had adopted a diet based on the MD presented a very low rate of cholesterol in the blood and, consequently, a minimum percentage of coronary heart disease. This was mainly due to

the plentiful use of olive oil, bread, pasta, vegetables, herbs, garlic, red onions and other foods of vegetable origin compared to a rather moderate use of meat (Menotti et al., 1996).

Starting from Key's studies, many other scientific researchers have analyzed the association between dietary habits and chronic diseases. There is a convergence of assessments agreed in the direction of full recognition of the beneficial qualities of the Mediterranean way of eating (World Cancer Research Fund, 1997; Altomare et al., 2013). Many studies and clinical trials have shown that the Mediterranean diet reduces the risk of cardiovascular disease and metabolic syndrome. In particular has been put into evidence a remarkable decrease of abdominal circumference, an increase in high density lipoprotein (HDL), a decrease in triglycerides, a lowering of blood pressure and a decrease in the concentration of glucose in the blood (Estruch et al., 2006; Fitò et al., 2007).

The MD may contribute to produce the benefits listed above, but other risk factors should be considered. In fact, ischemic heart disease depends not only on errors in the composition of the diet, to which attaches a dominant role, but also by other factors, such as a reduced or absent physical activity, caloric intake in excess of the energy needs of the organism, the presence of metabolic diseases such as diabetes and obesity, stress, cigarette smoking, high levels of homocysteine in the blood, high levels of triglycerides. Therefore, it is not surprising that about half of all cases of stroke occur in individuals with a normal level of cholesterol in the blood. To prevent a heart attack is therefore imperative to take not only a balanced diet, as is indeed the Mediterranean diet, but also a healthy lifestyle, as Ancel Keys had already pointed out (Altomare et al., 2013).

2. Mediterranean diet pyramid

The Mediterranean Diet is characterized by the balanced use of foods rich in fiber, antioxidants and unsaturated fats, a healthy approach designed to reduce the consumption of animal fats and cholesterol in a diet with an appropriate balance between energy intake and expenditure. The relationships between the macronutrient energy answer to those recognized as adequate, ie 55–60% of carbohydrates of which 80% complex carbohydrates (bread, pasta, rice), 10–15% of proteins about 60% of

animal origin (especially white meat, fish), 25–30% fat (mostly olive oil) (Arienti and Fidanza, 1998) .

The guidelines developed by nutritionists to improve the eating habits of consumers can be represented by an effective image, the "Food Pyramid" designed for the first time in 1992 by the U.S. Department of Agriculture, which simply represents a fair and balanced way of eating, displaying the proportions and the frequencies with which foods should be consumed, style that coincides with the Mediterranean Model identified by the physiologist Ancel Keys.

The pyramid is meant to provide an overall impression of healthy food choices rather than to define recommended weights of certain foods or proportions of energy obtained from them. Where the pyramid indicates relative frequencies, they are intentionally nonspecific because good health has been associated with considerable variation within the overall pattern. The Food pyramid is designed as a dietary guide for the general adult population and may need to be modified to meet the needs of children, pregnant women and other special population groups.

The main concepts of the Food Pyramid are the "proportionality", that is the right amount of foods to choose from for each group, the "portion" standard quantity of food in grams, which is assumed as the unit of measurement to be a balanced feeding, the "variety", i.e., the importance of changing the choices within a food group, and "moderation" in the consumption of certain foods, such as fat or sweets.

At the base of the pyramid are grains, followed by fruits and vegetables, legumes, olive oil, low-fat cheese and yogurt, which should be eaten daily. Meat is not excluded, but is given the preference to that of chicken, rabbit and turkey than beef. Along with fish and eggs should be eaten a few times a week, for the supply of high quality protein. Beef or red meat should be eaten a few times a month (Fig. 13).

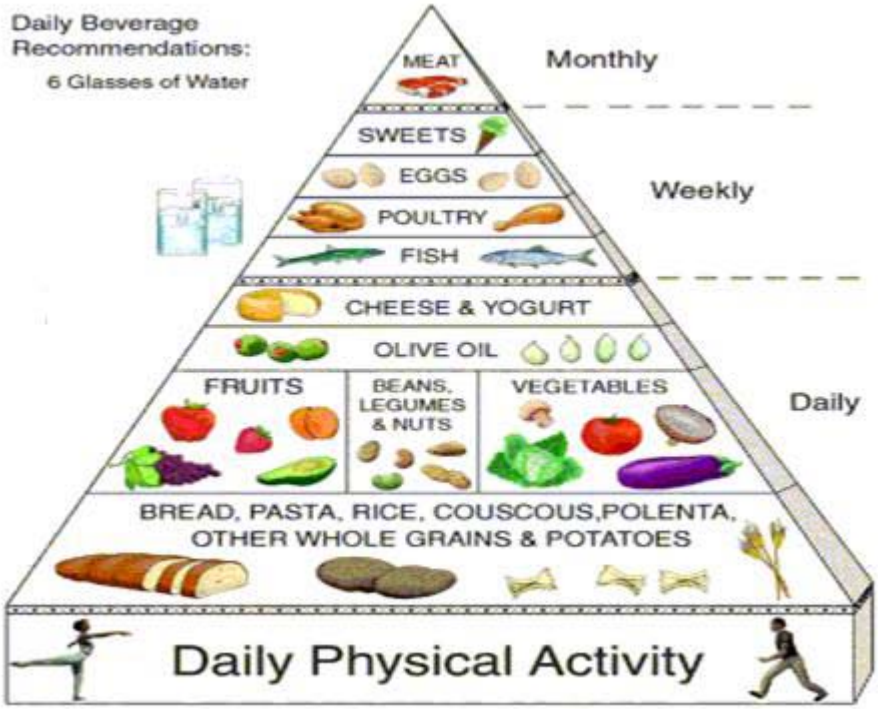


Figure 13. *Food pyramid in the Mediterranean diet (Altomare et al., 2013)*

Each group includes foods that provide nearly the same type of nutrients. Within the same group, foods can have small differences in terms of quality and quantity of patrimony in nutrients. However, this does not affect the concept of "interchangeability" of foods. The latter in fact, if they belong to the same group, being nutritionally equivalent, may be substitutes for each other, without, however, affecting the adequacy of the diet, provided you comply with the variety. It is necessary to vary as much as possible food choices and properly combine foods from the different groups to balance the diet. A very varied diet avoids the risk of nutritional imbalances and possible consequent metabolic imbalances, and it also satisfies the taste of fighting the monotony of flavors (Altomare et al., 2013)

Each group expected is represented by at least a portion of the foods that constitute it, to vary the choices within the same group (Mariani Costantini et al., 2006).

2.1. Plant foods

Plant foods constituted the core of the daily intake, whereas foods from animals were more peripheral. Examples of traditional Mediterranean dietary patterns with foods from plants at the center of the plate include the use of couscous, vegetables and legumes in North Africa; pasta, polenta, rice or potatoes along with vegetables and legumes in southern Europe; bulgur and rice, along with vegetables, chickpeas and other beans in eastern Mediterranean regions. Foods from plants also accompanied meals. Bread for example was a fundamental component of all meals. Fresh vegetables, salads, fruit, nuts, seeds and olives were consumed frequently and garlic, onions and herbs were used as condiments.

Cereals and tubers. This group includes bread, pasta, rice, corn, oats, barley and potatoes. These foods are the most important source of starch, easily usable energy from our body; cereals should be consumed in proportion to their needs. Some of these foods contain vitamins of the B group and a fair amount of protein that, associated with legumes, constitute a meal with high protein intake and high biological value. This combination occurs frequently in the Mediterranean diet since the cereals and their derivatives are the basic ingredients for the preparation of many dishes.

Fruits and vegetables are a source of fiber, vitamin A (found mainly in tomatoes, peppers, carrots, cantaloupe, apricots, etc), vitamin C (primarily in tomatoes, strawberries, citrus fruits, kiwi, etc), other vitamins and many minerals like potassium. In addition, plant foods contain minor components (antioxidant and others) which play an important protective action for the body and water that can reach even 95% of the weight (watermelon). Moreover, they contain relevant quantities of dietary fiber (cellulose, hemicellulose and pectin) that has a role in facilitating the intestinal transit and in moderating the levels of blood cholesterol and glucose. More consistent is, instead, the contribution in sugar (sucrose and fructose) made from fruit. The role of fruits and vegetables in the diet is also linked to its physiological regulator of water balance for their considerable supply of water. Finally, these foods has a role in the prevention of obesity, due to their high content of fiber and water and low in calories that provide compared to the volume ingested and the high satiating power (INRAN 2003; Altomare et al., 2013).

This diet, when consumed in sufficient amounts, provided all of the known essential vitamins, minerals, fiber and other plant food substances believed to promote health (Allbaugh, 1953). Minimal processing, seasonal use and freshness of foods would be expected to maximize contents of dietary fiber, antioxidants, vitamins and minerals found in foods from plants (Willet et al., 1995).

2.2. Olive oil as the principal fat

Olive oil was the Mediterranean regions' principal source of fat and was used instead of animal fats typical of northern European diets. Olive oil contains a large proportion of monounsaturated fat, is relatively low in saturated fat and is a source of antioxidants. All these features make oil preferable to animal fats. Olive oil contains a very high level of monounsaturated fats, most notably oleic acid, which is considered to be antithrombotic compared with saturated fatty acids (Ulbricht and Southgate, 1991); in fact epidemiological studies suggest it may be linked to a reduction in coronary heart disease risk (Keys et al., 1986).

Diets high in polyunsaturated fat are thought to be involved in the oxidation of low-density lipoproteins (LDLs), a process that increases the risk of atherogenesis and coronary heart disease (Reaven et al., 1991); on the other hand, substitution of olive oil for carbohydrates in certain short-term clinical studies has been shown to increase concentrations of high-density lipoproteins (HDLs) without increasing LDLs and should be expected, therefore, to reduce coronary risk (Mattson and Grundy, 1985; Mensink and Katan, 1987).

There is also evidence that the antioxidants in olive oil improve cholesterol regulation and LDL cholesterol reduction, and that it has anti-inflammatory and anti-hypertensive effects (Covas 2007).

Olive oil facilitates the typical consumption of large amounts of vegetables and legumes throughout much of the Mediterranean region by enhancing taste and energy density. The proportion of energy from total fat in Mediterranean diet ranged from 28% in southern Italy to 40% in Greece (Keys, 1980). Proportions of energy from fat across this range appear to have been compatible with excellent adult health with diets in which most of the fat was derived from olive oil (Willet et al., 1995).

2.3. Dairy products

Mediterranean diet typically included small amounts of dairy products from a variety of animals: goat, sheep, buffalo, cow and camel (Kromhout et al., 1989). Dairy products were consumed in low to moderate amounts. Since refrigeration was lacking and the climate was often hot, milk in the Mediterranean region was frequently preserved and consumed as yogurt and cheese. These foods represented a greater proportion of dairy food intake than is typical of modern diets in the U.S or in northern Europe (Willet et al., 1995).

This food group provides calcium in a highly bioavailable form, that is easily assimilated by the body. Moreover, these foods contain high biological quality proteins, vitamins B2 and A. Within the group are preferred low-fat milk and low-fat cheese. The calcium in milk (either be partially or completely skimmed) and its derivates is the most significant nutrient and it is easily adsorbed and used in the body. The milk contains more than 1 gram of calcium per litre, while cheese, according to the technology of preparation, contains several more percent, so that in the hard cheeses and seasoned ones the quantity of calcium can be 10 times higher than that present in milk to equal weight.

In addition to calcium, dairy products provide significant amounts of proteins of high biological value, among these casein is the most represented (the part that coagulates when the milk sours and which forms the basis for the preparation of cheese) and lactalbumin which together constitute 3.5%.

The consumption of milk is therefore the most immediate way to intake these nutrients, but an equally valid alternative is represented by yogurt and cheeses, for people that have lactose intolerance, due to the lack or absence of the lactase, an intestinal enzyme responsible for the breakdown of lactose in the two compounds: galactose and glucose (INRAN 2003; Altomare et al., 2013).

2.4. Meat, fish and eggs

Traditional Mediterranean diet included foods from animals in limited amounts (Kromhout et al., 1989). Evidence increasingly support associations between western diets that include high intakes of meats such as beef, pork and lamb, and the incidence of chronic diseases such as coronary heart disease and cancer of the colon and other sites. Whether the fat in meat is the sole contributor to these relations is uncertain. Other

factors may also be involved: research has suggested the possible involvement of the carcinogens formed during frying and cooking of meat, and the protein, cholesterol or iron content of the meat (Willet et al., 1995).

The consumption of low amounts of red meat by traditional Mediterranean populations posed no evident risk to adult health and was consistent with better health profiles than those observed in industrialized countries where much higher amounts are consumed (Willet et al., 1994).

Food in this group must be present in our diet a few times a week, with the exception of red meat, which should be eaten a few times a month.

The meat has high protein content (from 15% to 25%) of high biological value, able to provide all the amino acids necessary for protein synthesis (essential amino acids) in optimal amounts. It also provides B-complex vitamins, iron, zinc and copper. However, not all foods in this group are the same: it is better to prefer lean bovine, lean pork, white meat and fish, and to moderate the consumption of fatter meat and sausages.
The amount of fish consumed varied widely within the Mediterranean countries, but together with the data from japan and clinical evidence, has been observed that weekly consumption of low to moderate amounts of fish would be compatible with the excellent health (NDHSRG 1968; TJDA 1992).

The consumption of zero to four whole eggs per week was typical of the Mediterranean diets of the early 1960s (Kromhout et al., 1989), this figure includes eggs used in baking and preparing foods (Willet et al., 1995).

Any food of animal origin belonging to this group, whether fresh, chilled and frozen, provides protein of high biological value, trace elements and vitamins of the B complex, including in particular thiamine (vitamin B1), niacin (vitamin PP) and vitamin B12; some foods also provide other minerals, such as iodine in fish and vitamin A and D, contained primarily in the liver.

The amount of protein contained in the food group is equal to 18-20% of the total weight, with higher values for preserved meats like salami, where it may reach up to 37% due to the loss of water consequent to drying (Altomare et al., 2013).

3. Beneficial roles in preventing pathologies

The Mediterranean diet has been widely reported to be a model of healthy eating for its contribution to a favourable health status and a better quality of life (USDA 2005). Since the first data from the seven countries study (Keys, 1980), several studies in different populations have established a beneficial role for the main components of the Mediterranean diet on the occurrence of cardiovascular diseases and chronic degenerative diseases (Serra-Majern et al., 2006).

The meta-analysis by Sofi and colleagues showed, in an overall analysis comprising more than 2 million healthy subjects and 50,000 deaths or incident cases from any causes and/or from any cardiovascular, neoplastic, and neurodegenerative diseases, that the greater adherence to a Mediterranean diet is significantly associated with a reduced risk of overall mortality, cardiovascular mortality, cancer incidence and mortality, and incidence of PD and AD (Sofi et al., 2010).

Adherence to the Mediterranean diet significantly determined a 8% reduction of death from any causes, a 10% reduction from death and/or incidence of cardio- and cerebrovascular diseases, a 6% reduction from death and/or the incidence of neoplastic diseases, and a 13% reduction of the incidence of neurodegenerative diseases.

A diet rich in fruits, vegetables, legumes, and cereals, with olive oil as the only source of fat, moderate consumption of red wine especially during meals, and low consumption of red meat has been shown to be beneficial for all cause and cardiovascular mortality, lipid metabolism, blood pressure, and several different disease states such as endothelial dysfunction and overweight (Willet, 2006).

Only recently, higher adherence to a Mediterranean-type diet was associated with decreased cognitive decline, although the MD combines several foods, micro- and macro-nutrients already separately proposed as potential protective factors against dementia and pre-dementia syndromes. In fact, recent prospective studies provided evidence that higher adherence to a Mediterranean-typ diet could be associated with slower cognitive decline, reduced risk of progression from MCI to AD, reduced risk of AD and a decreased all-cause mortality in AD patients (Scarmeas et al., 2006). These findings suggested that adherence to the MD may affect not only the risk of AD, but also of pre-dementia syndromes and their progression to overt dementia.

Studies in rodents suggest that supplementation of the diet with polyphenols found in red wine and extra virgin olive oil are associated with better cognitive performances

(Pitozzi et al., 2010; Valls-Pedret et al., 2012). In fact polyphenols are highly bioactive molecules with beneficial effects that go beyond the modulation of oxidative stress to improve brain function, at least in experimental models of aging (Joseph et al., 2005). Furthermore, animals fed antioxidant and polyphenol-rich foods showed improved cellular signaling and neuronal communication that translated into better cognitive and motor performance (Joseph et al., 2009).

However, following dietary advice for lowering the risk of cardiovascular and metabolic disorders, high levels of consumption of fats from fish, vegetable oils, vegetables, low glycemic index fruits and a diet low in foods with added sugars and with moderate wine intake should be encouraged. Hopefully this will open new opportunities for the prevention and management of dementia and AD (Solfrizzi et al., 2011).

4. Oleuropein Aglycone

Cumulative evidence suggests that extra virgin olive oil (EVOO) may have a role in the protection against cognitive decline, in addition to protection other than against coronary disease and several types of cancer, because of its high levels of monounsaturated fatty acids and polyphenolic compounds (Solfrizzi et al., 1999).

EVOO is a staple of the Mediterranean diet and is rich in antioxidants. Antioxidants are considered to be the primary reason for these benefits. EVOO is a natural product particularly rich in monounsaturated fatty acids, mainly oleic acid, a constituent of the biological membranes that may progressively substitute polyunsaturated fatty acids. Cellular membranes rich in monounsaturated fatty acids have optimal fluidity and are less prone to lipid peroxidation, a process that requires two or more double links to take place (Scislowski et al., 2005). The antioxidants in EVOO include carotenes, alpha-tocopherol, and hydrophilic and lipophilic phenolic substances. It is worth noting that while tocopherols and carotenes can be bound in other plant and animal fats, the simple polyphenols, such as phenyl acids and phenyl alcohols, like several oleuropein derivatives, are compounds found exclusively in olives and the oils extracted mechanically from them (Servili et al., 2004; Farr et al., 2012). Olive oil is rich in more than 30 phenolic compounds such as oleuropein, tyrosol, and hydroxytyrosol.

Oleuropein, a secoiridoid glucoside characteristic of *Oleaceae*, is the main phenolic compound in leaves and fruit oil, and it is always present in extra virgin olive oil (Soler-

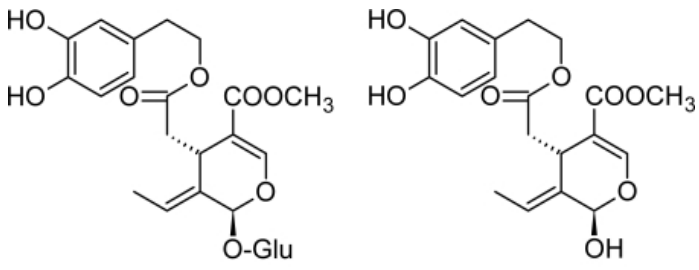
Rivas et al., 2000) and can reach concentrations of up to 140 mg/g on a dry matter basis in young olives and 60-90 mg/g of dry matter in the leaves (Amiot et al., 1986; Le Tutor and Guedon, 1992).

The amount of this polyphenol in olive fruit and oil varies depending on the maturation time of the olive fruit or on the oil tree subtype (Perri et al., 1999). In Mediterranean countries, it is estimated that the intake of olive oil varies between 30 and 50 g/person/day; if we use the average of this amount of oil, one person consumes 1.16 mg of oleuropein per day (Vissers et al., 2004).

4.1. Chemistry

Oleuropein belongs to the secoiridoids, compounds that are usually glycosilically bound and are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids. The secoiridoids in *Oleaceae* are usually derived from the oleoside type of glucosides (oleosides), which are characterized by an exocyclic 8,9-olefinic functionality, a combination of elenolic acid and a glucosidic residue. Oleuropein is an ester of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of *Oleaceae* (Soler-Rivas et al., 2000), mainly in its aglycone form, which makes the sugar moiety insoluble in oil (Fig. 14).

Oleuropein hydrolysis, carried out by an endogenous β-glycosidase during olive ripening and in the technological process of olive oil production, releases the aglycon moiety of the molecule in the olive oil (Soler-Rivas et al., 2000). Oleuropein aglycone (OLE) originates from deglycosylation of the polyphenol oleuropein during olive crushing (Brenes et al., 1999); the increased hydrophobicity of the aglycon allows it to cross the water/oil barrier and to be recovered in the oil, where it is far more abundant than its glycoside precursor (Cicerale et al., 2009).



Oleuropein Oleuropein aglycon

Figure 14. *Structures of oleuropein and oleuropein aglycone*

4.2. Biosynthesis

The biosynthesis of oleuropein in *Oleaceae* proceeds via a branching in the mevalonic acid pathway from the secondary metabolism, resulting in the formation of oleosides (Damtoft et al., 1992). From these compounds, secoiridoids are derived (Damtroft et al., 1993). The sequences of the intermediate steps that lead to oleuropein biosynthesis may differ between plant species and times of the year (Damtoft et al., 1995). A plausible biosynthetic route from deoxyloganic acid, 7-epiloganic acid, 7-ketologanic acid, 8-epikingisidic acid, oleoside 11-methyl ester, 7-β-1-D-glucopyranosyl 11-methyl oleoside and ligustroside to oleuropein was proposed by Damtoft et al. (1993) for *Oleaceae* (Fig. 15).

In the development of the olive fruit, three phases are usually distinguished: a growth phase, during which accumulation of oleuropein occurs; a green maturation phase that coincides with a reduction in the levels of chlorophyll and oleuropein; and a black maturation phase that is characterized by the appearance of anthocyanins and during which the oleuropein levels continue to fall (Amiot et al., 1989). Therefore, oleuropein is very abundant in the early stages: in young fruits, it can reach 14% of dry matter.

4.3. Bioavailability

Phenolic compounds from virgin olive oil have been demonstrated to be highly bioavailable. Vissers et al. (2002) found that absorption of administered hydroxytyrosol, tyrosol and oleuropein aglycone was 55-60% in human subjects .

Oleuropein is rapidly adsorbed after oral administration with a maximum plasma concentration occurring 2h after administration, and hydroxytyrosol is its most important metabolite. Both compounds are rapidly distributed and excreted in urine mainly as glusoronides or in very low concentrations as free forms (Tan et al., 2003; Boccio et al., 2003). Furthermore, the mechanism of absorption of olive oil compounds remains unclear.

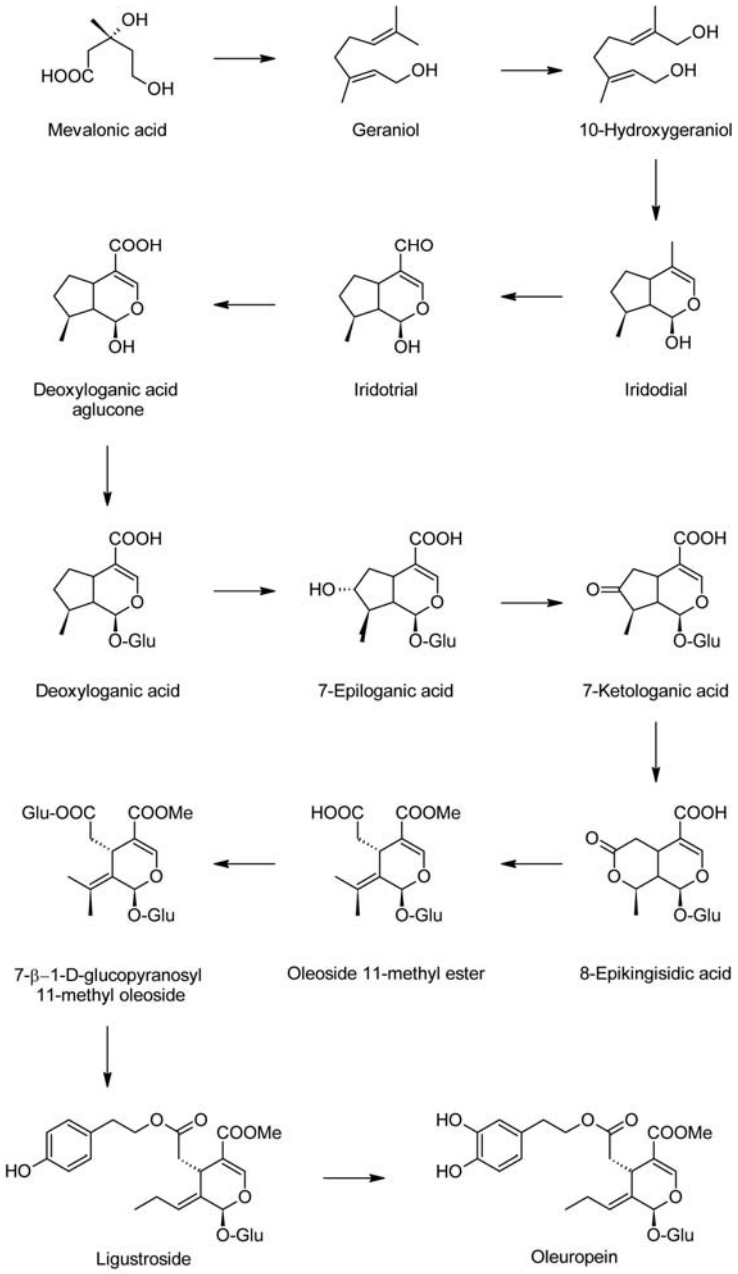


Figure 15. *Proposed biosynthesis pathway for oleuropein in Oleaceae (Damtoft et al., 1993)*

4.4. Pharmacology

Oleuropein has been shown to possess several pharmacological properties, including antioxidant (Visioli et al., 2002), anti-inflammatory (Visioli et al., 1998), anti-atherogenic (Carluccio et al., 2003) and anti-cancer (Owen et al., 2000) activities, and for these reasons, it is commercially available as food integrator in many countries. In addition, oleuropein has been shown to enhance ostoblastogenesis, to inhibit adipogenesis (Santiago-Mora et al., 2011), to be cardioprotective against acute

adriamycin cardiotoxicity (Andreadou et al., 2007) and to exhibit anti-ischemic and hypolipidemic activities (Andreadou et al., 2006).

Further pharmacological activity of oleuropein includes diverse healing properties due to its vasodilatatory (Petkov and MAnolov 1978), anti-platelet aggregation (Petroni et al., 1995), hypotensive (Ribeiro et al., 1986), anti-rehumatic (Visioli et al., 1998), diuretic (Ribeiro et al., 1986) and antipyretic (Visioli et al., 1995) effects.

Prevention of free radical formation by oleuropein occurs through its ability to chelate metal ions, such as Cu and Fe, which catalyze free radical generation reactions (Andrikopoulos et al., 2002) and through its inhibitory effect on several inflammatory enzymes like lipoxygenases (De la Puerta et al., 1999). Also, oleuropein has an anti-hyperglycemic effect in diabetic rats (Gonzalez et al., 1992); oleuropein inhibits hypeglycemia and oxidative stress induced by diabetes, which suggests that administration of oleuropein is helpful in the prevention of diabetic complications associated with oxidative stress (Al-Azzawie and Alhamdani, 2006).

4.5. Effects on amyloid toxicity

The protective effect of several polyphenols against amyloid cytotoxicity comes from their physical association with aggregation-prone proteins, rather than from their antioxidant activity (Porat et al., 2006). OLE must be present during amyloid aggregation to prevent the formation of toxic species; it modifies the aggregation path of amylin inhibiting the formation of toxic amyloid species. In particular, OLE appears capable of interfering with the early steps of amylin aggregation hindering the proper reorganization of the polypeptide chain and the appearance of the most cytotoxic species, and delaying, but not suppressing, the growth of harmless amyloid fibrils likely structurally different from those grown in the absence of the polyphenol (Rigacci et al., 2010).

Recently, Oleuropein (the glycated precursor of OLE) has been shown to form a noncovalent complex with the Aβ peptide (Bazoti et al., 2006 and 2008), and *in vitro* studies showed that the best protection against Aβ42 cytotoxicity was achieved when OLE was present during peptide aggregation. In fact, when OLE was given to cells together with Aβ42 aggregates grown in its absence, cell viability was still significantly reduced, although slightly less, respect to control cells (Rigacci et al., 2011). The relief of amyloid toxicity could, in part, result from the anti-oxidant activity of OLE;

however, the direct, early interaction of OLE with the amyloidogenic peptide, inhibiting the growth of toxic species, appears to provide the best protection. This behavior resembles that of other polyphenols and has led to hypothesize that the inhibitory activity of this class of substances results from the interference either in aromatic-aromatic stacking or in the formation of hydrophobic clusters, or in both (Yang et al., 2005; Mishra et al., 2009; Rivière et al., 2007; Ono et al., 2008).

The interaction of oleuropein with Aβ40 was shown using ESI-MS and NMR (Bazoti et al., 2006, 2008; Benaki et al., 2009). Three peptide regions (4-11, 12-22 and 17-28) were shown to be involved in the interaction. It was proposed that while the sugar unit would be preferably accommodated into the polar N-terminal fragment (4-11) of the peptide, the hydrophobic (17-21) region of the latter would interact with the non-polar moiety of oleuropein (Bazoti et al., 2008). It is conceivable that OLE interacts with the same 17-21 hydrophobic region, which is also critical for Aβ fibrillization (Kallberg et al., 2001).

The *in vitro* studies by Rigacci and colleagues showed that OLE abrogates the formation of toxic oligomeric species, both pre-fibrillar and fibrillar, not only during the whole path of Aβ42 aggregation but also once added to pre-formed amyloid fibrils (Rigacci et al., 2011).

OLE would represent a very good candidate for amyloid cytotoxicity prevention, being able to interfere with the aggregation of amyloidogenic peptides by inhibiting the formation of early toxic oligomeric species while allowing deposition of stable fibrillar material (Rigacci et al., 2011). Oleuropein has also anti-amyloidogenic effects, since it promotes the α-secretase cleavage of the APP *in vitro*, causing a substantial decrease in the formation of Aβ oligomers; the potential anti-amyloidogenic activity of oleuropein adds to the multiple health benefits of olive oil and of the Mediterranean diet (Kostomoiri et al., 2013).

AIM OF THE STUDY

Aim of the study

Mounting evidence supports the beneficial effects of the Mediterranean diet in preventing age-related dysfunctions, neurodegenerative diseases and in attenuating AD-like pathology and cognitive deterioration.

Rat study

OLE counteracts *in vitro* aggregation of the Aβ42 peptide and protects cultured cells and model organisms against aggregates toxicity.

We investigated the relative tissue toxicity of Aβ42 aggregated *in vitro* in the presence or in the absence of OLE by injecting the nucleus basalis magnocellularis (NBM) of adult rats with solutions containing Aß42 aggregated in the absence or in the presence of OLE.

Mouse study

Adherence to the Mediterranean diet is associated with a reduced risk of developing MCI and AD, and reduced risk of MCI conversion to AD.

We aimed to detect *in vivo* the effects of dietary supplementation with OLE on AD physiopathology using the CRND8 transgenic mouse model of AD. Young and adult mice, which represent the early and intermediate stage of cerebral Aβ deposition, were fed with OLE diet and its effects in preventing/slowing down the neuropathology were investigated on memory functions, brain amyloid load and on autophagy.

Zebrafish study

Autophagy represents a potential therapeutic target in pathologies characterized by aggregates, and further studies will provide an insight into the role of this process in the brain; atg5-knockdown studies in zebrafish showed that Atg5 is required for normal morphogenesis of brain regionalization.

In order to study the role of the autophagy in the embryonic zebrafish neurodevelopment, we aimed to generate a Atg5-knockout zebrafish stable line using TALEN technology.

MATERIALS AND METHODS

RAT EXPERIMENTS

1. Animals

Three-month old, 230–250 g male Wistar rats (Harlan, Milan, Italy) were used. The rats were housed in macrolon cages with ad libitum food and water and maintained on a 12-h light/dark cycle at 23 °C. All experiments were carried out according to the EC Directive 86/609/EEC for animal experiments and National guidelines for animal care (Permit Number: 283/2012-B)

2. Oleuropein deglycosylation

Oleuropein (Extrasynthase) deglycosilation was performed according to Konno et al. (1999) with minor modifications (Rigacci et al., 2010). Briefly, a 10 mM solution of oleuropein in 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 28.7 I.U./mL of β-glycosidase overnight at room temperature (RT) in the dark. The reaction mixture was centrifuged at 10,000×g for 15 min to precipitate the aglycone. The complete oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma). GC-MS analysis showed the absence of any oleuropein in the precipitate and the substantially total recover of OLE in the same precipitate. A 100 mM Oleuropein Aglycone (OLE) stock solution in DMSO was stored protected from light until use.

3. Aβ aggregation

The Aβ42 peptide (Bachem) was dissolved and aggregated following the procedure described by Rigacci et al. (2011)

An alkaline 500 μM monomeric peptide solution was obtained after sonication for 5 min in a mixture of CH_3CN/Na_2CO_3 (300 μM)/NaOH (250 mM) (48.3/48.3/3.4, v/v/v), this was diluted to 50 μM in 10 mM phosphate buffer, pH 7.7, containing 11 mM NaCl (PBS), in the presence (Aβ-OLE) or in the absence (Aβ) of 450 μM OLE (obtained by enzymatic digestion of glycated oleuropein (Rigacci et al., 2010) and incubated at 25 °C for 24 h. The structural and morphological features of the aggregates grown at these conditions, as well as their toxicity to cultured cells (which was maximal after 24 h of aggregation in the absence of OLE), were previously characterized (Rigacci et al., 2011).

4. Brain injections

The rats were anesthetised with chloral hydrate (400 mg/kg i.p.) and 1.5 µl of a solution containing Aβ42 (50 µM) aggregated in the presence or in the absence of OLE (450 µM), or OLE (450 µM) alone, was injected by means of a Hamilton microsyringe into the right NBM at the stereotaxic coordinates: AP=-0.2, L=-2.8, from Bregma and H= 6.8 from the dura (Paxinos and Watson, 1997).Control rats were injected with 1.5 µl of PBS.

Thirty days after injection, the rats were killed by decapitation, the brains were rapidly removed and postfixed in phosphate-buffered 4.0% paraformaldehyde, pH 7.4, at 4 °C for 48 h, rinsed in PBS and embedded in paraffin for immunohistochemistry.

5. Immunohistochemistry

Histochemical and immunohistochemical analyses were performed on 5.0 µm coronal paraffin-embedded sections obtained by microtome sectioning (Leica Microsystem, UK). Brain sections were placed on slides and incubated for 30 min in xylene at RT to allow the removal of the paraffin; then section were rehydrated using decreasing ethanol concentrations solutions (100%, 95%, 90% and 70%) and distilled water. At the time of the experiment, sections were incubated overnight at 4 °C with the primary antibodies, at the optimized working dilution, made up in PBS 0.1 M (pH 7.4) with Triton X-100 (0.3%) and BSA (5 mg/ml). On the second day, sections were incubated for 1 h with the secondary antibody at 1:1000 dilution made up in PBS 0.1 mM plus BSA (1 mg/ml) and the immunostaining was visualized using the avidin-biotin system (Vectastain: Vector Laboratories, Burlingame, CA) and 3,39-diaminobenzidine plus Nickel (DAB Kit: Vector Laboratories, Burlingame, CA) as the chromogen.

Fluorescent staining

Fluorescent labeling followed previously described protocols (Fiorentini et al. 2010). Coronal brain sections were rinsed 3 times then placed in blocking solution (PBS, pH 7.4+0.3% Triton X-100, 2g/l BSA and 5% NGS for polyclonal antibodies or 5% NHS for monoclonal antibodies), for 30 min at RT. Primary antibodies were diluted in fresh blocking solution and applied overnight at 4°C. Next, sections were washed in PBS (3X10 min) at RT and subsequently incubated for 2 h in the dark with the appropriate fluorescent secondary antibody (monoclonal antimouse Alexa Fluor 594 red conjugated and polyclonal anti-rabbit or anti-goat Alexa Fluor 488 green conjugated, Invitrogen,

Eugene, OR) diluted 1:200 in blocking solution. Following 3 additional rinses, sections were coverslipped using Vectashield water-based mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The analysis of negative controls (omission of primary antibody) was simultaneously performed in order to exclude the presence of non-specific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through. An Olympus BX63 microscope coupled to CellSense Dimension Software (Olympus, MI, Italy) was used to acquire representative images.

6. Determination of ChAT positive cells

The number of ChAT-positive cells in the nucleus basalis magnocellularis was determined by two analysts blinded for the administration, using a 20X objective with a calibrated eyepiece grid. Eight sections (5.0 μm each) spaced about 60 μm from each other per animal were analyzed and the total number of ChAT-positive cells in the NBM was averaged. An Olympus BX63 microscope coupled to *CellSens Dimension* Software (Olympus, MI, Italy) was used to acquire representative images. GraphPad Prism version 5.0 for Windows was used for ChAT positive cell calculations and statistically significant differences were evaluated by One-way ANOVA followed by Bonferroni's post-hoc test. A P-value of less than 0.05 was considered significant. Data were expressed as mean ± standard error of the mean (S.E.M).

MOUSE EXPERIMENTS

1. TgCRND8 mice

The TgCRND8 mice express two mutated human APP genes implicated in AD (Swedish, KM670/672NL and Indiana, V717F) under the regulation of Syrian hamster prion promoter gene (Chishti et al., 2001). The mice were maintained on a hybrid (C57)/(C57/CH3) background by crossing transgenic heterozygous TgCRND8 males with wild-type female mice.

The main feature of this model is a very rapid development of amyloid pathology in the brain: amyloid β deposits start quite early since the two mutations involve both the β and γ secretase APP cleavage sites; in fact, all mice display amyloid plaques in the cortex and hippocampus by three months of age (Chishti et al., 2001). These neuropathologic manifestations are accompanied by impaired acquisition and learning deficits.

The transgenic mice were generated and supplied by the group of Dr. P StGeorge Hyslop (Center for research in neurodegenerative Diseases, Toronto, Canada) and then the colony was bred in our animal house, Ce.S.A.L. (Centro Stabulazione Animali da Laboratorio), University of Florence.

2. Genotyping

Transgenic TgCRND8 mice were identified by DNA extraction from tail samples and PCR analysis using specific primers (5'-aggactgaccactcgacca-3' and 5'-tggattttcgtagccgttc-3') for the human mutated (KM670/672NL+V717F) cDNA insert (Bellucci et al., 2006).

The primers directed against the insert used the following program: lid heating to 105°C, five minutes at 99°C in order to degrade any DNAses, then five minutes at 95°C followed by 35 cycles: each cycle involved 1 min at 95°C, 1 min at 52°C and 1 min at 72°C. After cycles were completed, samples were held at 72°C for an additional 5 minutes and then were stored at 8°C.

In order to check the presence of the transgene, the PCR product was run in a gel electrophoresis using 0.5X Tris/borate/EDTA buffer and 0.8% agarose gel with etidium bromide (Sigma). DNA was visualized on a UV lightbox imaged with a camera. Only in transgenic samples a 300 bp band is detected.

3. Oleuropein Aglycone treatment

Since several beneficial effects of OLE have been reported (Rigacci et al., 2010; Diomede et al., 2013), we decided to test its effect on transgenic mouse model of AD by oral administration of a diet containing OLE.

After deglycosylation, the 100 mM OLE stock solution was protected from light, stored at -20°C and diluted in oil immediately before diet supplementation.

The experimental diet was prepared using components purchased from Piccioni (Gessate, Mi, Italy). Fresh diet was put in the trough every two days. We used a modified low-fat American Institute of Nutrition (AIN)-76 diet (5.0% fat) composed of: 50.0% sucrose, 5.0% fat, 20.0% casein, 15.0% corn starch, 5.0% powdered cellulose, 3.5% AIN-76 mineral mix, 1.0% AIN-76A vitamin mix, 0.3% DL-methionine and 0.2% choline bitartrate.

Different diet treatments were equally given to each age group of wt and Tg mice: for 8 weeks the 5.0% fat diet (10 g/day per mouse) was given either alone (untreated) or containing OLE (50 mg/kg of diet) (OLE-fed).

The following 2 age groups of mice (equally divided for sex) were used: 1.5 month-old wt (n=16) and pre-plaque Tg (n=16) mice and 4 month-old wt (n=16) and Tg (n=16) mice. These two ages correspond to different stages of the pathologies in terms of cerebral β-amyloid deposition.

We decided to treat young mice, 1.5 month-old, that don't show cerebral amyloid deposition yet since we would like to test if OLE could interfere in early stages of amyloid deposition or at least slow down the aggregation of cerebral Aβ peptide and we chose adult mice in order to evaluate any effect on an intermediate stage of the pathology.

4. Body weight

The animals were weighted once a week during the treatment; body weight was slightly, not significantly, increased in all mice. The treatment was not toxic and animals were healthy.

5. Behavioral experiments

At the end of the diet treatment, 3.5 month-old and 6 month-old OLE-fed and untreated Tg and wt mice were pooled according to the treatment and genotype and memory performance was investigated by two widely used behavioural tests.

TgCRND8 mice, as previously demonstrated in our laboratories by performing other behavioural tests as rotarod, hole platform and grip strength, don't show any motor deficits or exploration impairment (Bellucci et al., 2006).

5.1. Step down inhibitory passive avoidance task

In this test, the animals learn to associate exploration of a compartment with a foot shock delivered through the floor grid. On subsequent exposure to the same environment, the mice will avoid stepping down or will increase the latency before ''stepping down'' onto the floor grid.

The term "passive avoidance" (Netto and Izquierdo, 1985) is used to indicate in vivo experiments in which the animal learns to avoid a specific event, usually concerned an innate behavior, that is dangerous due to a linked punishment.

A mouse in an open field naturally spends most of the time in dark places, as near walls and corners; when the mouse is placed on an elevated-platform in the center of the field, it will try to get off the platform, to explore the environment and go near the walls. Based on this natural behavior, several different variants of the step down inhibitory avoidance task have been used (Abdel-Hafez et al., 1998; Chorover and Schiller, 1981; Kubanis and Zornetzer, 1981; Zoernetzer et al., 1982)

In this study, the following procedure was used:

- the apparatus was an open field plexiglas box (40 × 40 cm) with a steel rod floor and a plexiglas platform (4 × 4 × 4 cm) set in the centre of the grid floor to which intermittent electric shocks (20 mA, 50 Hz) were delivered (Fig. 16).
- on day 1 (training test, TT), each mouse was placed on the platform and received an electric shock (1 Hz, 0.5 s, 40 V DC) for 3 s when it stepped down placing all paws on the grid floor (Hiramatsu et al., 1995) considering 180 s as the upper cut-off, then the mouse was put back into the cage.

Responsiveness to the punishment in the TT was assessed by animal vocalization; only those mice that vocalized touching the grid (about 70% of mice) were used for retention test (RT).

- 24 h after TT, each mouse was placed on the platform again (RT). The latencies were measured, considering 180 s as the upper cut-off. The test ended when the mouse stepped down or when the cut-off time was over. During retention test no electric shock was released.
- latencies recorded during TT and RT were compared: a latency longer in the RT than in TT meant the mouse learnt and could remember the punishment received the day before, otherwise the mouse showed memory impairment.

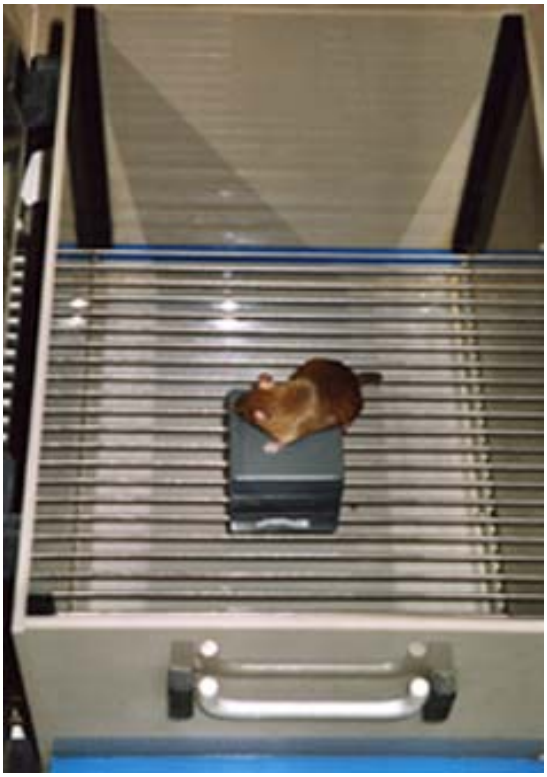


Figure 16. *A mouse performing the step down inhibitory avoidance task*

5.2. Context-dependent object recognition test

The object recognition paradigm utilized the innate tendency of mice to explore a novel object over a familiar one. The test measures spontaneous behavior, and thus requires no length training or preparation (Ennaceur and Delacour, 1988).

Pre-training: To avoid neophobic interference a habituation phase preceded the testing. Habituation consisted in placing the mice for 5 min into a square open-field arena (60 x 50x 25 cm). During this phase the mice could explore freely the empty arena with one object inside (Fig. 17).

Testing: The object recognition task was conducted 1 day after the habituation phase and comprised two trials (T1 and T2). During the T1 the animals could explore two identical objects while in the T2 one object was replaced by a novel one. Each trial lasted 5 min, with an intertrial interval of about 60 min. All objects were made of a biologically neutral material such as plastic or metal, and animals could not move them around in the arena. Objects were not known to have any ethological significance for the mice and they never had been associated with a reinforcer (Ennaceur et al., 2005).

Fecal boli were removed after each tested animal.

Frequency and duration of object exploration was recorded by a video-tracking/computer-digitizing system (HVS Image, UK).

Exploration of an object was defined as directing the nose towards an object at a distance of less than a half head length and/or touching the object with the paws. Sitting on an object was not considered as exploratory behaviour. Mice without any exploration behavior towards the objects were excluded from the analysis of learning behaviour.

To analyze the object recognition test, a discrimination score was calculated: discrimination score=exploration time of the novel object (s)/total exploration time of both objects (s). A discrimination score above 0.5 indicates the ability of mice to discriminate between the familiar and novel objects while a score below or equal to 0.5, reflecting a novel object exploration time less or equal to the half of the total time spent between the two objects, indicates memory impairment in this task Mice with a total exploration time of less than 5 s in either the T1 or the T2 were excluded from the analysis of learning behaviour on this test day.

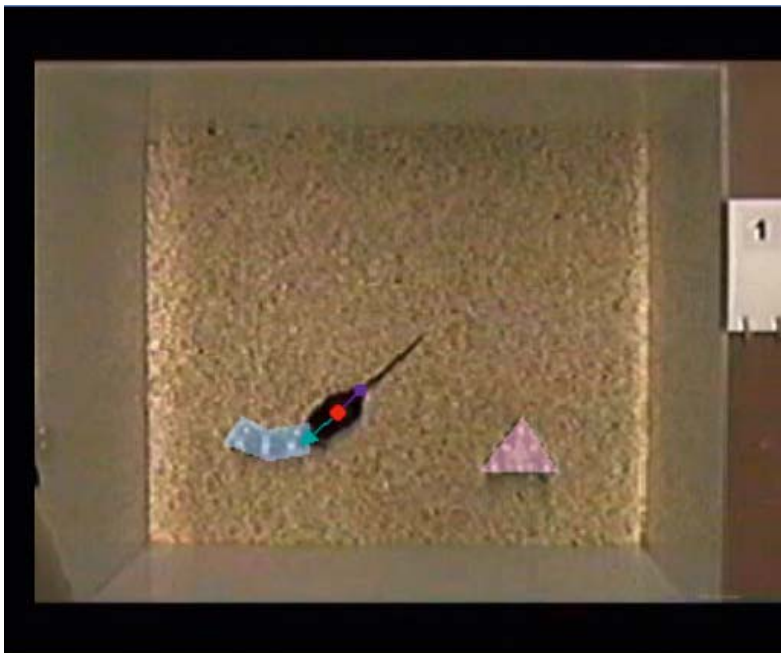


Figure 17. *A view from above of the arena during a mouse is performing the object recognition test.*

6. BrdU treatment

BrdU is a synthetic nucleoside that is an analogue of thymidine; BrdU was used in the detection of proliferating cells since it can be incorporated into the newly synthetized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication.

In order to evaluate proliferation of neural progenitor cells, 5-bromo-2'-deoxyuridine (BdrU, Sigma-Aldrich, MI, Italy) (50 mg/kg of body weight) was administrated to the mice twice a day for three days. 24h after the last BrdU injection, mice were sacrificed. Cells marked by BrdU incorporation were detected using an anti-BrdU antibody.

7. Animal tissue processing

After completing the behavioral tests, mice were sacrificed by cervical dislocation and the brains were rapidly removed and divided sagittally. For protein analysis, cortical and hippocampal samples from one hemibrain were immediately sectioned, snap-frozen in dry ice or liquid nitrogen and stored at -80 °C. The other hemibrain was postfixed in phosphate-buffered 4.0% paraformaldehyde, pH 7.4, at 4 °C for 48 h, rinsed in PBS and paraffin embedded for immunohistochemistry and Thioflavin S staining.

8. Histology, histochemistry and immunohistochemistry

Histochemical and immunohistochemical analyses were performed on 5.0 µm coronal paraffin-embedded sections obtained by microtome sectioning (Leica Microsystem, UK). Immunohistochemistry and immunofluorescence experiments were performed as previously described in "rat experiments", using the antibodies reported in Table 1.

8.1. Thioflavin S staining

Thioflavin S staining was used to detect deposit containing β-sheet structures in mouse brains. We performed Thioflavin S staining following the protocol described by Yamamoto and Hirano (1986).

The rehydrated sections were incubated with 0.25% $KMnO_4$ for 4.0 min, washed twice with distilled water, incubated with a 0.1% $NaBH_4$ solution for 5.0 min, washed twice with distilled water and placed in phosphate buffer (411 mM NaCl, 8.1 mM KCl, 30 mM Na_2HPO_4, 5.2 mM KH_2PO_4), pH 7.2 for 30 min at 4 °C. The sections were washed, incubated in 0.05% Thioflavin S (in 50% ethanol) for 8.0 min in the dark, washed twice in 80% ethanol for 10 s and incubated in phosphate buffer for 30 min at 4 °C. The sections were cover slipped using Vectashield mounting medium with or without DAPI (Vector Laboratories, USA). Representative images were acquired by an Olympus BX63 fluorescence microscope coupled to *CellSens Dimension* Imaging Software (Olympus, Italy).

8.2. BrdU labelling and counting

For BrdU labelling, sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS 1X, pH 7.5) to block endogenous peroxidises. DNA was denaturated by exposing sections to 2N HCl for 15 min at 37°C followed by 0.1M borate buffer (pH 8.5) for 10 min at RT. Sections were then incubated for 1h in TBS with 0.1% Tween-20, 1% BSA and 3% Normal Horse Serum (NHS), and then overnight at 4°C with the primary antibody anti-BrdU (1:100 dilution) in the same buffer. On the second day, sections were washed (3x10min) in TBS-Tween20 at RT and subsequently incubated for 2h in the dark with the appropriate fluorescent secondary antibody (polyclonal anti-rat AlexaFluor 488 green conjugated, Invitrogen, Eugene, OR) diluted 1:500 in blocking solution. After washing with TBS-Tween20 (3x10 min) and briefly with water, sections

were finally cover-slipped using Vectashield water-based mounting medium (Vector Laboratories, Burlingame, CA). An Olympus BX63 microscope coupled to Cell Sens Dimension software was used to acquire representative images from the examined specimens.

Quantification of BrdU positive (BrdU+) cells was performed in the subgranular zone (SGZ) of the hippocampus in 6 coronal sections per mouse spaced at 80nm. The counts were conducted at 40X magnification with an Olympus BX63 microscope. The corresponding surface area of SGZ sampled was measured using the Cell Sens Dimension Software. The density of BrdU+ cells was calculated by dividing the number of BrdU+ cells by SGZ sectional area (mm^2).

9. Determination of Aβ plaque-load

To quantify Aβ plaque burden, cortices and hippocampi of the sections stained with anti-Aβ (1–42) antibody were digitized (Olympus BX63, Olympus, Germany) under constant light and filter settings. Six to seven coronal brain sections, each separated by 60 µm interval, from each mouse (4-5 animals per group) were analyzed.

Morphometry was conducted by using CellSens Dimension software (Olympus, Germany). Colour images were converted to greyscale by extracting blue to grey values to obtain best contrast between positive immunoreactivity and background. A constant threshold was chosen for all images to detect immunoreactive staining. Plaque number, size (maximum area, minimum area) and total area were determined automatically in the cortices and hippocampus. Brain regions were based on the Paxinos and Franklin mouse brain atlas. Data from the six-seven sections were summed to derive representative values for each animal for the total plaque area. Data were expressed as mean ± S.E.M.

10. Western blot

The western blot is an analytical technique used to detect specific proteins in the given sample of tissue homogenate. A gel electrophoresis is used to separate denaturated proteins by the length of the polypeptide; proteins are then transferred to a membrane, where they are stained with antibodies specific to the target protein. Proteins were then visualized using specific antibodies and a chemiluminescence detection system.

For western blotting analysis, TgCND8 and wt tissue brain samples were homogenized in ice-cold RIPA lysis buffer composed by:

-2X lysis buffer (50 mM Tris-HCl pH 7.5; 50 mM NaCl; 10 mM EGTA; 5 mM EDTA);

- Phosphatase inhibitors (2 mM NaPP; 4 mM PNFF; 1 mM Na_3VO_4)

- Proteases inhibitor (1 mM PMSF, 20 µg/ml leupeptin; 30 µg/ml aprotinin)

The samples were homogenized in 100 µl of RIPA buffer, then centrifuged for 15 min at 12,000 rpm and surnatant was recovered and used for the next steps.

In order to detect the amount of proteins in each sample, a protein assay was performed. Dilutions ranging from 1µg/ml to 0.075 µg/ml of Bovine Serum Albumine (BSA) were prepared to outline the standard curve of reference. Into a 96-multi well plate 4µl of each sample (blank, BSA dilutions and surnatant of the homogenized tissues) and 200 µl of Bradford reagent (Bio Rad, Hercules, CA) were put in each well: the Bradford colouring depending on the amount of proteins, became from deep to light blue. After 10 min incubation at RT, samples were assayed with a spectrophotometer associated a computer (E1800, Universal Microplate Reader, Bio-Tex Instruments, Inc). Samples absorbance was detected and the standard samples were useful to correlate absorbance measurement detected to known proteins amount; comparing the absorbance values obtained with the standard curve, the amount of protein in unknown samples could be extracted.

Forty µg of proteins were loaded on a gel and the final volume was adjusted by adding lysis buffer. Before loading on a gel, loading buffer (300 mM Tris-HCl pH 6.8; 12% SDS; 60% glycerol; 0.6% bromophenol blue; 600 mM DTT; H_2O) was added to unfold proteins and charge negatively: the samples were boiled for 5 min at 100°C in order to denature the proteins, then they were placed on ice, briefly centrifuged to spin down the samples to the bottom of the tubes, and finally loaded on a gel.

Meanwhile, 10% running gel SDS-PAGE was prepared (10% acrylamide; 1.5 M Tris-HCl pH 8.8; 10% SDS, 10% $(NH_4)_2S_2O_8$; TEMED; H_2O), poured into the gel mould and let solidified for at least 30 min, adding temporary some buthanol to avoid it could dry up. Once the gel was solidified, the buthanol was removed and the 5% stacking gel (5% acrylamide, 1mM Tris-HCl pH 6.8, 10% SDS, 10% ammonium persolphatum, TEMED, H2O) was poured on and a plastic comb was inserted to design the wells. After about 30 min the gel was solidified, the samples and a molecular weight standard solution (Biorad dual color standard) were loaded in the wells. The samples

were run in the gel at 4°C using Running buffer (100 ml 10X buffer; 5 ml 10% SDS), applying 95 V, constant voltage.

When the run was over, the stacking gel is cut out, and the resolving gel containing the proteins was placed on a 0.45 μm nitrocellulose/PVDF membrane (Hybond-C, Amersham Life Science); in order to make proteins transfer from the gel to the membrane, a tight "sandwich" with sponges and filter paper was made to keep the gel against the membrane; the sandwich is then placed into a chamber containing cold Blotting buffer (100ml 10X Buffer; 10% SDS; 10% glycine; 100ml methanol) and current was applied to allow protein transfer on the membrane; the current could move the proteins from the gel since they had negative charges due to SDS, and stick them on the membrane. The transfer at 4°C could be performed overnight at 12 mA constant voltage or for 2 h at 80 V.

Then the membrane was removed from the sandwich, washed with distilled H_2O and the effective run and transfer was checked with the unspecific Ponceau staining (Bio Rad, Hercules, CA).

Ponceau solution was washed out with H_2O and the membrane was incubated overnight at 4°C in Blocking solution containing TBST (1X TBS, 0.05% Tween 20) and 5% milk for antigen retrieval.

The membrane was incubated with the primary antibody diluted in Blocking solution for 2 h at RT or overnight at 4°C, all primary antibody concentrations were titrated to provide optimal staining (Table 1). The membrane was washed three times for 15 minutes with TBST and incubated for 1 h RT with the HRP-conjugated secondary antibody (Bio Rad) diluted 1:7500 in TBST. The immunocomplexes were visualized using enhanced chemiluminescence (ECL chemiluminescence kit, Pierce, Rockford, USA) in the dark room.

After washing out the secondary antibody with TBST for 10 min, the membrane was incubated 1 min RT with 1 ml of reagent A and 1 ml of reagent B from ECL kit. The peroxidase conjugated with the secondary antibody triggered a chemical reaction producing a chemiluminescent signal that could be detected and acquired using ImageQuant 350 system (GE Healthcare, UK).

The membrane could then reused to perform further experiments: it could be put in 10 ml of stripping buffer (Bio rad, Hercules, CA) shacking thoroughly for 15 min and after a 10 min wash with H_2O it's ready to be used again to determine house-keeping

proteins levels, like β-actin or GAPDH, necessary to normalize the samples levels detected and to check the amount of proteins loaded.

The normalized data were collected and the statistical analysis was performed using Graph Pad (Graph Pad Prism Software version 4.0, San Diego, CA, USA).

Table 1. *Antibodies employed in the rat and mouse studies*

Name	Target	Dilution	Experiment	Study
6E10	Aβ peptide, aa 1-16	1:400	IHC	Rat
A11	Soluble amyloid oligomers	1:500	IHC	Rat
Aβ42	Aβ peptide, aa 1-42	1:200	IHC	Mouse
β-actin	C-terminal β-actin fragment	1:5000	WB	Mouse
Beclin 1	Beclin 1 protein	1:200	IHC	Mouse
		1:1000	WB	Mouse
BrdU	BrdU	1:100	IHC	Mouse
Cathepsin B	Procathepsin B and mature cathepsin B	1:100	IHC	Mouse
		1:1000	WB	Mouse
ChAT	Cholinergic neurons	1:200	IHC	Rat
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase	1:1000	WB	Mouse
GFAP	Glial fibrillar acidic protein	1:500	IHC	Rat and mouse
Iba1	Ionizied calcium adaptor molecule	1:250	IHC	Rat and mouse
LC3	Microtubule-associated protein light chain 3	1:200	IHC	Mouse
		1:1000	WB	Mouse
OC	Amyloid fibrils and fibrillar oligomers	1:1000	IHC	Mouse
P-p70S6K	P-p70S6 kinase (Thr389)	1:1000	WB	Mouse
p70S6K	p70 S6 kinase	1:1000	WB	Mouse
SQSTM1/p62	SQSTM1/p62 protein	1:200	IHC	Mouse
		1:1000	WB	Mouse

11. ELISA assay

The Enzyme-Linked ImmunoSorbent Assay (ELISA) is a test that uses antibodies and color change to identify and quantify a specific substance.

Soluble and insoluble Aβ fractions were isolated from cortex homogenates using a four step extraction protocol (Rasool et al., 2012). At each step, sonication in an appropriate buffer was followed by centrifugation at 100,000 x g for 1 hr at 4°C. The supernatant was then removed, and the pellet was sonicated in the next solution used in the sequential extraction process. For four-step extraction, sonication of the frozen tissue began in Tris-buffered saline (TBS) (20 mM Tris and 137 mM NaCl, pH 7.6), which contained protease inhibitors (protease inhibitor cocktail from Sigma St. Louis USA). The next three sequential extraction steps used 1% Triton X-100 in TBS with protease inhibitors, 2% SDS in water with the same protease inhibitors, and 70% formic acid (FA) in water. SDS-soluble fractions were loaded directly onto ELISA plates, whereas FA-soluble fractions were diluted 1:20 in a neutralization buffer (1 mol/L Tris base, 0.5 mol/L NaH_2PO4) before loading. Levels of Aβ40 and Aβ42 were analyzed using an ELISA kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions. The plates were read at 450 nm using a plate reader (Molecular Dynamics, Sunnyvale, CA). All values were calculated as pmol per g based on the wet weight of brain cortical tissues.

12. Thiobarbituric acid reactive substances (TBARS) assay

ThioBarbituric Acid Reactive Substances (TBARS) are naturally present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress (Armstrong and Browne, 1994). TBARS assay values are usually reported in malonaldehyde (malondialdehyde, MDA) equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. The TBARS assay is a well-recognized, established method for quantifying these lipid peroxides (Ohkawa et al., 1979).

This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 100°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm, as shown below in figure 18.

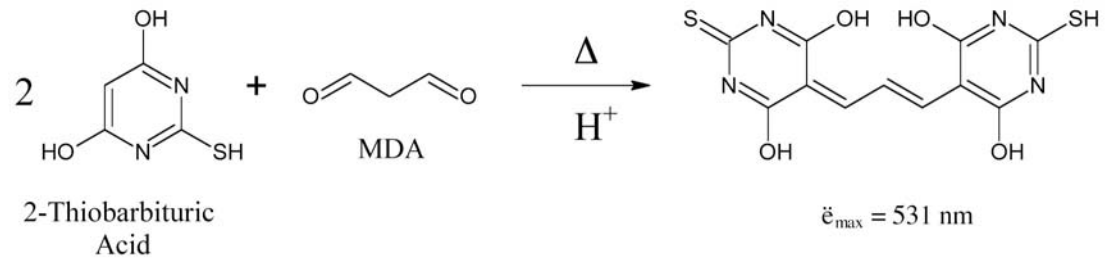


Figure 18. *Reaction between 2-thiobarbituric acid and MDA under acidic conditions ends up with a product which adsorbance is 531 nm*

The mouse cerebral cortex was homogenized in RIPA buffer (10% w/v) containing the chelating agent diethylenetriamine pentaacetic acid (500 μM); 0.2 mL of suspension were added to 4.0 mL of 36 mM TBA (thiobarbituric acid) solubilised in 10% CH_3COOH, pH 4 (LaMotta et al., 2007). After heating for 60 min at 100 °C, the reaction was stopped by cooling the test tubes in ice for at least 2 h. 1.5 ml of n-butanol was added and each test tube was vigorously mixed for 30 s and centrifuged at 250 ×g for 10 min. The organic layer was taken and the resulting pink colour was determined in a spectrophotometer at 532 nm. The acid did not produce colour when tested without the addition of the homogenate, demonstrating the absence of a direct reaction with TBA. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the homogenate. The results were reported as nmol of malondialdehyde per mg protein. All samples were run in twice.

ZEBRAFISH EXPERIMENT

1. Zebrafish

All zebrafish husbandry and experiments were performed in accordance with UK legislation under a license granted by the Home Office and with local ethical approval. Zebrafish (*Danio rerio*) were reared under standard conditions on a 14 h light:10 h dark cycle in the fish facility (Department of Physiology, Development and Neuroscience, University of Cambridge, UK).

Embryos were collected from natural spawning, staged according to established criteria (Kimmel, 1995) and reared in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 5 mM HEPES) at 28.5°C. *Tupfel long fin* (TL) wildtype strain were used.

Zebrafish are commonly used as *in vivo* model of development: they have several advantages like transparency, external development with large number of embryos, the development of zebrafish is very fast and all stages are well described (Fig. 19) . Homologs of many autophagy related genes are found in zebrafish, so it is an excellent model for studying autophagy.

Autophagy can be studied in zebrafish by knocking down, knocking out or overexpressing autophagy related genes in different ways, and the most advanced technologies thus far to knock out genes in zebrafish are TALENs and CRISPR.

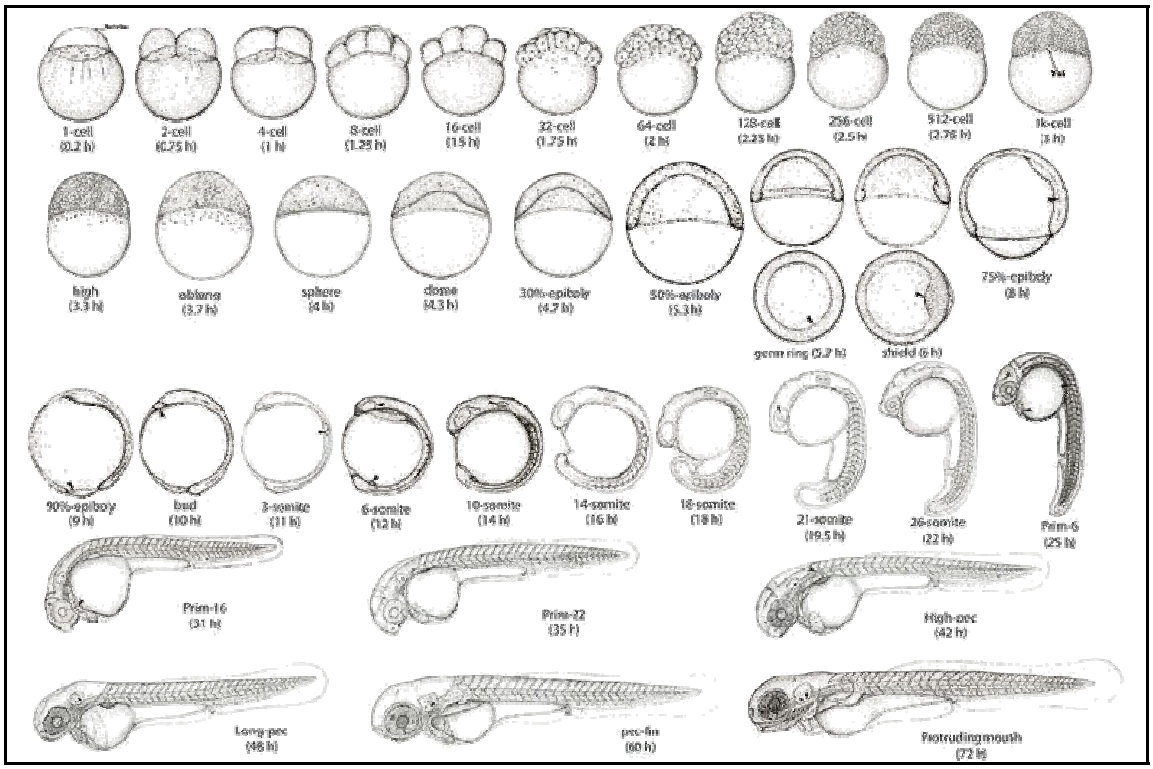


Figure 19. Stages of the embryonic development of the zebrafish. *Zebrafish develop from one cell to the entire organism in 72 hours. All the stages are well described and recognizable (source:http://www.uoneuro.uoregon.edu/k12/zfk12.html)*

2. TALENs technology

TALEN (Transcription Activator-Like Effector Nucleases) is an innovative technique to editing the genome with engineered nucleases. This technique enables researchers to target the genome exactly where they want with high precision. TALEs are transcriptional activators that specifically bind and regulate plant genes during pathogenesis. TALENs are made of two parts: one part, TALE domain, binds the DNA: within the TALE structure, a central repeat domain mediates DNA recognition, and each repeat unit of 33-35 amino acids can bind a specific base. The base preference of each units is determined by two critical adjacent amino acid referred to as the "repeat variable di-residues" (RVD); this DNA binding part consist of 18-24 repeat modules that are not thought to interfere each other. To the DNA binding part, a nuclease domain is fused so TALENs are used to generate double-stranded breaks by using two TAL effectors to each cleave a single strand of DNA (Fig. 20).

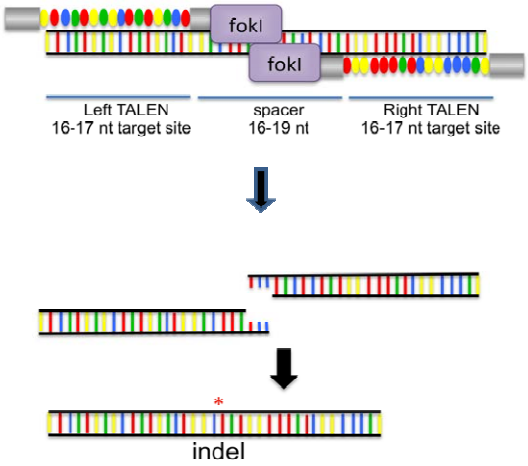


Figure 20. Transcription Activator-Like Effector Nucleases *(TALEN) bind target DNA sequences to direct a FokI nuclease domain to cut the DNA. TALENs have multiple DNA binding domains linked in series to create an array. The arrays each bind a separate sequence and a dimeric FokI nuclease domain cleaves the DNA within what is referred to as a space region. These engineered nucleases frequently induce breaks that are repaired via Non Homologous End Joining (NHEJ), which is error-prone and can result in insertion or deletion of a few bases, creating an indel (Moore et al., 2012).*

The space between the two individual TALENs DNA binding site is an important parameter for effective cleavage, usually a spacer of about 15 bp works well.
The DNA ends formed by the cleavage are repaired by the cell, often leaving deletion or small insertions. So most of the repair events would alter the reading frame of a gene and create a knock-out.

3. TALEN plasmids

The TALENs plasmids were made by an academic consortium (Sander et al., 2011), purchased from Addgene and supplied as bacterial stabs. The bacteria was streaked on agar plates (with ampicillin) and grown overnight at 37°C. A single colony was picked and grown in LB (Lysogeny Broth) medium containing ampicillin overnight, in shaking incubator at 37°C. Then the DNA was purified using Macherey Nagel plasmid midi prep kit according to manufactures instructions.

The plasmids were then linearized through a restriction enzyme and TALEN Atg5 encoding RNA was transcribed *in vitro* from TALEN expression vectors using the mMESSAGE mMACHINE® T7 Ultra (Ambion, Life techonologies) and then introduced into early zebrafish embryos by microinjection

4. Microinjection

Approximately 2nl of TALEN Atg5-encoding RNA (300 pg/nl) was injected in one-cell stage wild type embryos using pulled glass capillaries; the embryos were lined up on a glass slide and injected through the yolk into the cell using a microinjector. Injection volume was calibrated by measuring the size of injection volume in mineral oil using a microscope with eyepiece reticule. For every injection a portion of wt uninjected embryos were saved and examined to ensure that those were a good quality embryos, otherwise the batch was discarded and not analysed further.

After injection, embryos were stored at 28.5°C in the dark in an incubator, at 6 hours post fertilization (h.p.f.) they were cleared of dead and unfertilized eggs, at 24 h.p.f. and 48 h.p.f. they were cleared of the dead and the abnormal ones; 10 single embryos from each batch of injection were collected in order to check the effectiveness of injection and once successful mutation was confirmed, all the remaining injected embryos were raised to adulthood.

5. PCR and enzymatic digestion

DNA extraction from single embryos was performed using the Extract-N-Amp Tissue PCR kit (Sigma), and genomic DNA was used to perform standard PCR. The primers directed against *atg5* used the following program: lid heating to 110°C, 5 min at 95°C followed by 40 cycles: each cycle involved 30 sec at 95°C, 30 sec at 60.3°C-60.8°C and 40 sec at 72°C. After cycles were completed, samples were held at 72°C for an additional 10 min and then were stored at 8°C.

In order to check the presence of the amplificated gene, a portion of the PCR product was run in a gel electrophoresis using 0.5X Tris/borate /EDTA buffer and 0.8% agarose gel with SafeView DNA staining agent. DNA was visualized on a UV lightbox imaged with a camera and then printed.

Then the remaining PCR product was added to a mix with the restriction enzyme BspHI (10000 units/ml, BioLabs), 1µl of enzyme was used to digest 1µg of DNA, incubated at 37°C overnight with no star activity. The digestion products were run on a 0.8% agarose gel with Gel Green Nucleic Acid Gel Stain (Biotium) to check the products after restriction enzyme digestion. In wild type DNA the BspHI-mediated digestion produces two fragments: 500 bp and 200 bp, in mutated DNA an additional undigested product band, 700bp, is shown.

The undigested band was cut out of the gel and DNA and extracted and sequenced to check the mutation site.

RESULTS AND DISCUSSION

RAT STUDY

In this study we investigated the tissue toxicity of Aβ42 aggregated *in vitro* in the presence or in the absence of OLE by injecting the NBM of adult male Wistar rats with a solution containing OLE or Aβ42 aggregated in the absence (oligomers) or in the presence of OLE.

1. Results and discussion

1.1. Aβ aggregation

The structural features of the amyloid aggregates grown in the presence or in the absence of OLE were analyzed by TEM prior to injection. The Aβ42 sample aggregated in the absence of OLE consisted of oligomeric species and short protofibrils (see arrows and arrow-heads respectively, figure 21), while in the Aβ42 sample aggregated with OLE essentially amorphous material was present (Fig. 21), as previously reported (Rigacci et al., 2011). The use of a mixture of variously aggregated material resembles the condition that develops in the brain during the disease, since the aggregation of the Aβ peptide is not a synchronous process. Moreover, in the presence of OLE the Aβ42 aggregation path did not exhibit a clear sequence of steps with well defined conformationally and morphologically distinct species; rather, at the Aβ:OLE ratio used in our experiments, the aggregating material displayed a delay time of over 5 h, and subsequently poor Thioflavin T positivity and no toxicity. At 24 h amorphous aggregates were present that do not change remarkably upon further aging and only at long incubation times beaded curvy fibrillar assemblies emerge from the amorphous material (Rigacci et al., 2011).

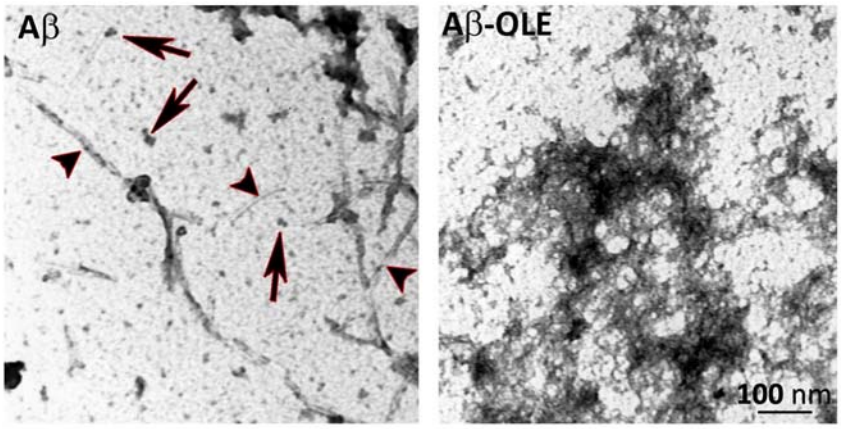


Figure 21. TEM analysis of Aβ aggregates. *50 μM. Aβ was incubated in aggregation conditions for 24 h in the absence (left) or in the presence (right) of 450 μM OLE. Aggregates were loaded onto a formvar- and carbon-coated nickel grid, negatively stained with 1.0% (w/v) uranyl acetate and examined using a JEM 1010 transmission electron microscope at 80 kV excitation voltage. Oligomers (arrows) and short protofibrils (arrowheads) are indicated on the left panel*

1.2. ChAT

Thirty days after injection into the NBM of variously aggregated Aβ, the number of ChAT-positive magnocellular neurons detected in the injected area was significantly reduced (≈−33%) as compared with those found in all other groups (Fig. 22 A and B). Notably, the number of ChAT-positive neurons was substantially unchanged in the NBM injected with Aβ aggregated for 24 h with OLE, respect to control rats. Beside confirming Aβ aggregate toxicity to cholinergic neurons in the NBM, these data indicated that OLE is, by itself, innocuous to the neuronal cells and, more interestingly, that Aβ aggregation in the presence of OLE results in the growth of aggregates completely non-toxic not only to cultured cells, as previously reported (Rigacci et al., 2011), but also in tissue.

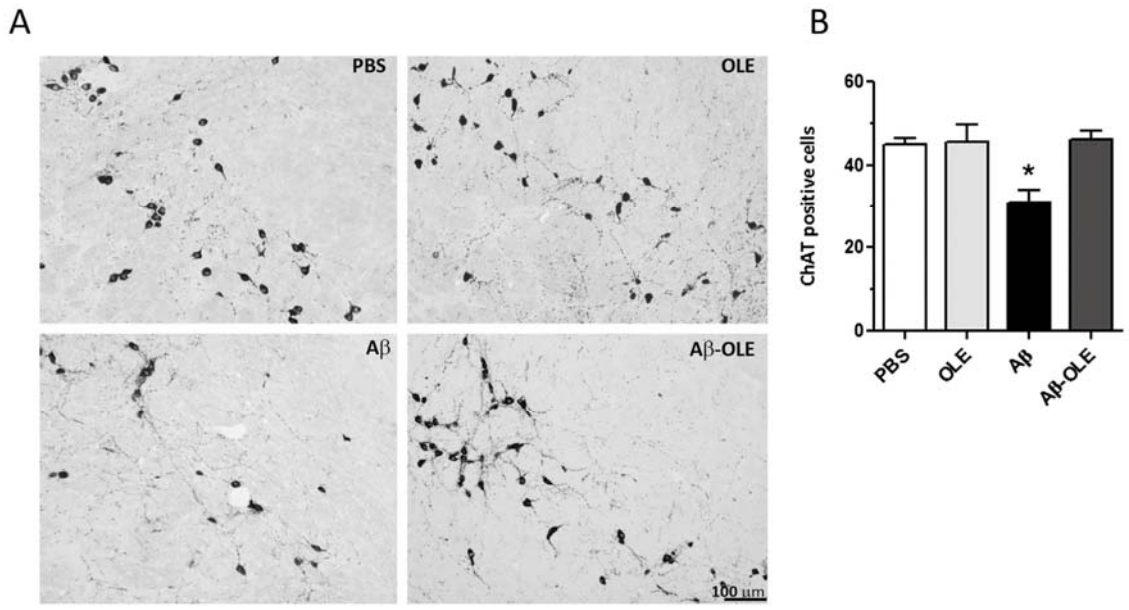


Figure 22. ChAT positive neurons in the injected NBM of rats.
(A) Representative photomicrographs of ChAT positive magnocellular neurons in the NBM injected with PBS, OLE, Aβ and Aβ-OLE.
*(B) Quantitative analysis of ChAT-positive neurons revealed a statistically significant reduction of ≈33% (*P < 0.05) in the number of ChAT-positive cells in the Aβ-injected NBM respect to all other groups. (One-way ANOVA plus Bonferroni post-comparison test). Scale bar 100 µm applies to all images .*

1.3. Aβ peptide

To confirm that the different toxicity to the cholinergic neurons of the injected material resulted from the presence, in the injected area, of the differently aggregated peptide (Aβ or Aβ-OLE) we performed an immunohistochemical analysis with two different antibodies: 6E10, which recognizes Aβ in its non-aggregated and aggregated form, or A11, which is specific for Aβ oligomers (Kayed et al., 2003). Aβ detection was carried out in double labeling immunofluorescence with GFAP which recognizes astrocytes and the so-revealed astrogliosis drove us to the site of injection, a reference that is useful to detect the small amount of injected Aβ. We found an intense astrocyte reaction, revealed as GFAP-positive hypertrophic astrocytes with long and thick branches, in a large area of the Aβ-injected NBM (Fig. 23 A) . Astrocyte reaction was also detected in the Aβ-OLE injected NBM, however it was confined to the needle trace, as expected, such that the area displaying astrogliosis was reduced as compared to that imaged in the Aβ-injected NBM (Fig. 23 b). Double labeling immunohistochemistry with the 6E10 antibody and the GFAP antibody plus DAPI showed a roughly equal amount of 6E10-positive Aβ peptide in the NBM injected with

Aβ or Aβ-OLE (Fig. 23 c and d). By contrast, double labeling immunohistochemistry with the A11 antibody, which recognizes Aβ soluble amyloid oligomers, and GFAP antibody plus DAPI, showed that the amount of A11-positive oligomers, entangled by astrocytes, was much higher in the Aβ-injected NBM than in the Aβ-OLE-injected one (Fig. 23 e and f).

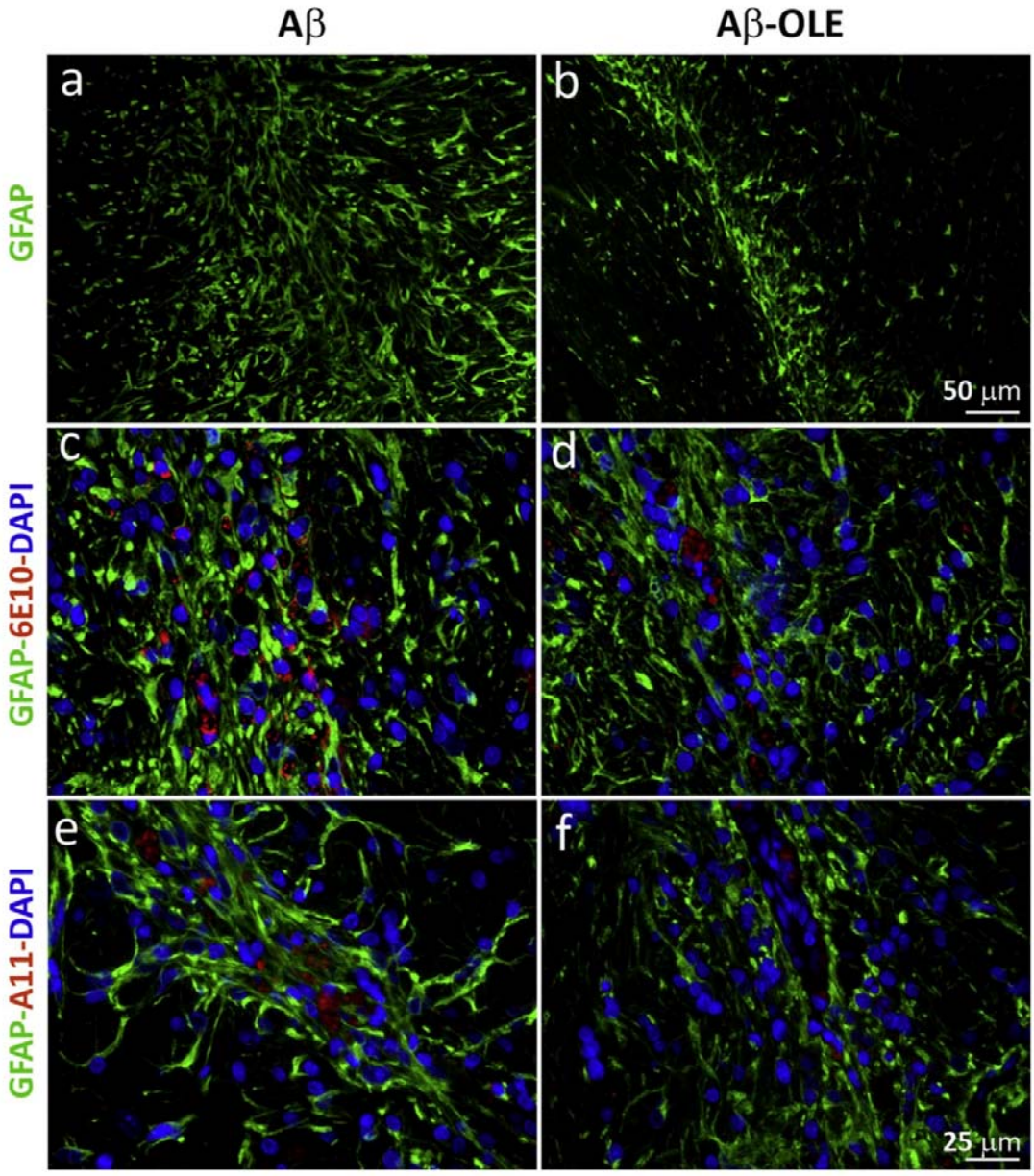


Figure 23. Fluorescent staining for astrocytes, Aβ peptide and soluble amyloid oligomers in the Aβ and Aβ-OLE injected NBM of rats.
(a–b) Single fluorescent immunohistochemistry for GFAP (green) antibody in the NBM injected with Aβ and Aβ-OLE.
(c–f) Double labeling immunohistochemistry with GFAP (green) and the anti-Aβ 6E10 (red) antibodies (c–d) and with GFAP (green) and the anti-amyloid oligomers A11 (red) antibodies (e–f) plus DAPI (blue). Scale bars: 50 µm applies to a–b images; 25 µm applies to c–f images).

Taken together, the reported data confirm those obtained by *in vitro* aggregation experiments showing that the different structural features of Aβ aggregates grown in the presence of OLE respect to those grown in the absence of OLE (Rigacci et al., 2011) persist even in the complex environment found in tissue, with a significantly reduced toxic impact to cholinergic neurons. Our findings also suggest that the apparently amorphous Aβ aggregates grown in the presence of OLE do not display any tendency to undergo disassembly with leakage of toxic oligomers or monomeric Aβ subsequently undergoing oligomerization in tissue, a behavior that has been shown for aggregates of other peptides/proteins (Giorgetti et al., 2011). Moreover, it appears that the injected aggregates persist in tissue without any major modification over the investigated time period skipping the action of proteases or other tools aimed at clearing the material.

Finally, the Aβ-OLE injected NBM displayed a reduced area of microglia activation. In fact, in this case activated microglial cells with swollen cell body and short processes were detected along the needle trace, as expected, but not elsewhere (Fig. 24 A and B). By contrast, in the Aβ-injected NBM many activated microglial cells were detected both in the injected area and in the internal capsula/lateral globus pallidus and deep layers of the cortex (Fig. 24 A and B). In the NBM injected with PBS or OLE none, or only a few activated microglial cells were detected, respectively (Fig. 24 A and B), confirming the substantial lack of neurotoxicity of the phenol.

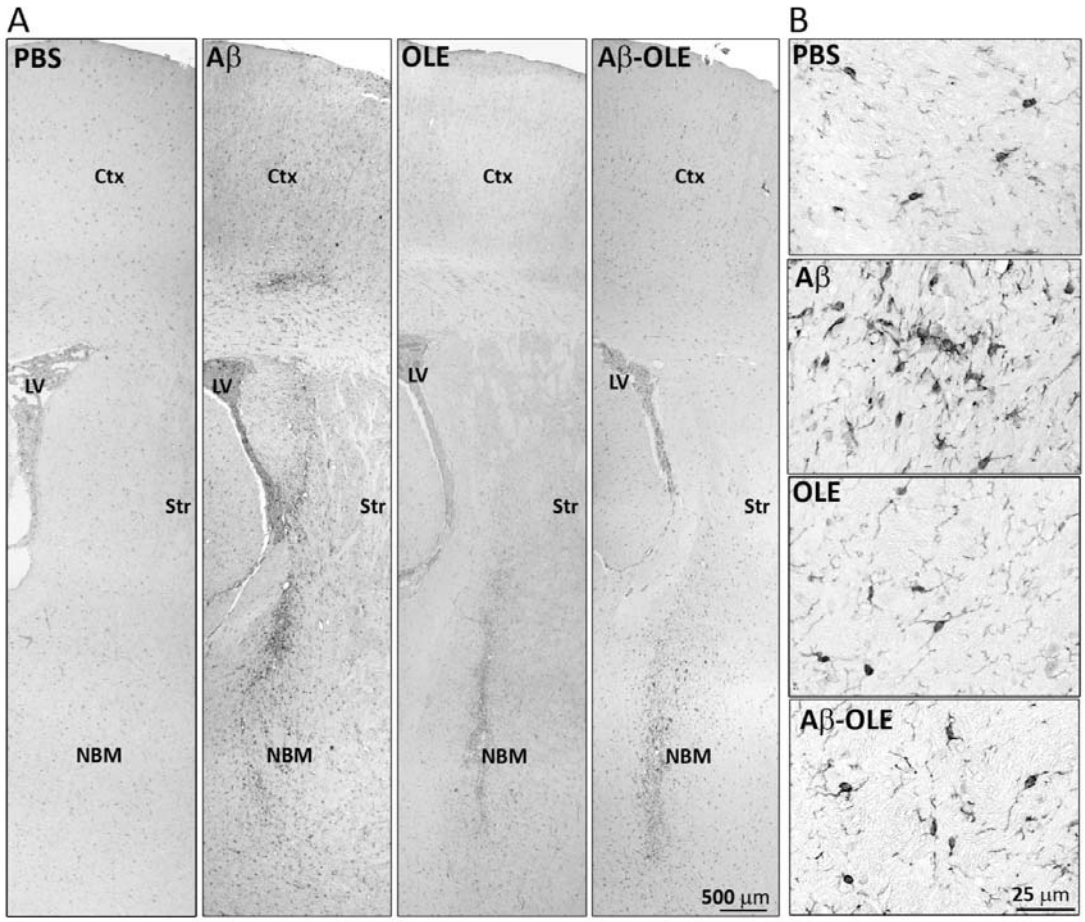


Figure 24. Microglia reactivity in the injected NBM of rats.
(A) Representative photomicrographs of Iba 1 immunopositive glial cells in the NBM and along the needle trace of PBS, Aβ, OLE and Aβ-OLE injected rats. Note in the Aβ-injected rats the presence of activated microglial cells in the NBM as well as along the needle trace.
(B) Representative high magnification images of each treatment. Ctx, cortex; LV, lateral ventricle; Str, striatum; NBM, nucleus basalis magnocellularis.

These data suggest that the aggregates grown in the absence of OLE possess conformational features (possibly increased hydrophobic exposure) that promote glia involvement in the attempt to clear them from tissue, at variance, the aggregates grown in the presence of the phenol can be considered much more inert and unable to activate glia, further explaining the lack of any evident impairment of tissue/cell viability and/or functionality of the NBM injected with this material respect to that injected with Aβ aggregated in the presence of OLE.

The path of Aβ aggregation is quite different from that occurring in the absence of the phenol. In the presence of OLE, the exposure of hydrophobic regions of the peptide (possibly favoring the interaction with lipid membranes) is hindered and the appearance of toxic oligomers is skipped; at short incubation times the peptide already precipitates

into a disordered mesh of apparently amorphous and harmless aggregated material from which at later times some ordered fibrillar organization arises, yet lacking the morphological features of typical mature fibrils.

2. Conclusion

The present study demonstrates that Aβ aggregated in the presence of OLE, the main phenolic component of extra virgin olive oil, is not toxic to cholinergic neurons in the rat NBM and unable to raise an evident inflammatory reaction as it is the case of Aβ aggregates grown under the same conditions but in the absence of OLE. The amount of soluble A11-positive oligomers was much lower in the NBM-injected with Aβ-OLE than in the NBM injected with Aβ alone, confirming that OLE stably hinders the formation of toxic species.

A marked reduction of astrocytes and microglia reaction was revealed thirty days after injection of Aβ-OLE into the NBM respect to the NBM injected with Aβ aggregates. In conclusion, these findings indicate that OLE interferes with Aβ aggregation, reduces aggregate cytotoxicity and counteracts the associated neuroinflammation.

MOUSE STUDY

Recently has been reported that OLE hinders amyloid aggregation of Aβ42 and its cytotoxicity *in vitro* (Rigacci et al., 2011). In this study we investigated the *in vivo* consequences of dietary supplementation of OLE on transgenic mouse model of AD by detecting the effects of the treatment on memory functions, brain amyloid deposition and autophagic machinery.

1. Results

1.1 Behavior

TgCRND8 mice are cognitively impaired since the age of 3 months (Chishti et al., 2001) and, as already reported, no behavioral differences between 3- and 7-month-old TgCRND8 mice have been observed (Fiorentini et al., 2010).

Therefore, transgenic mice of 3.5 and 6 months of age were grouped to increase the number of animals evaluated for cognitive function after 8 weeks of administration of food supplemented or not with OLE (n= 12/group for all groups).

OLE treatment was well tolerated and no treated animals died.

During the last week of treatment, memory performance was investigated by two widely used tests, the step down inhibitory avoidance (SD) and the object recognition tests (ORT). No significant differences were observed between wt and OLE-fed or untreated Tg mice during the training test of the SD test. However, in the retention test step-down latencies recorded for Tg mice were significantly reduced compared to wt mice and not significantly different from training latency, indicating that Tg mice were unable to memorize the punishment and to perform the inhibitory avoidance. OLE administration to Tg mice significantly improved their performance, that reached the level displayed by wt mice (Fig. 25).

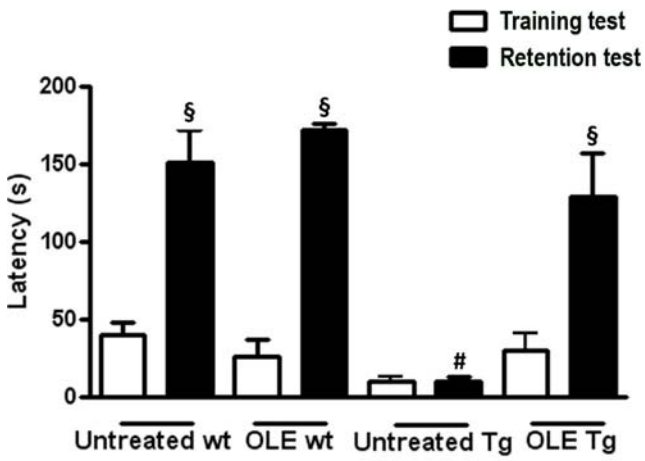


Figure 25. Step down inhibitory avoidance test. *One-way ANOVA plus Bonferroni's post comparison test shows a statistically significant increase in the mean retention latencies in untreated and OLE-treated wt and in OLE-treated Tg mice, as compared to their respective training latencies (§P<0.001). Untreated Tg mice do not show significant differences between training and retention latencies. The retention latencies of untreated Tg mice significantly differ from the retention latencies of all the other groups (#P<0.001); data are reported as mean values ± S.E.M. Number of animals: n = 12/group.*

Then the same mice were tested for ORT with a retention interval of 60 min. In the T1 trial the exploration time of the familiar object was comparable in the four groups, where OLE-fed and untreated animals showed no deficiencies in exploratory activity, directional movement towards the objects and locomotor activity. In the T2 trial, untreated Tg mice exhibited impairments in novel object preference compared to wt mice, as shown by the significant reduction in the discrimination score (untreated Tg: 0.3767±0.051; untreated wt: 0.5909±0.037) (Fig. 26). The ability of OLE-fed Tg mice to discriminate between the familiar and novel object was significantly improved (0.60±0.073) compared to that of untreated Tg mice and undistinguishable from that of wt mice (OLE-fed: 0.6525±0.044; untreated: 0.5909±0.037). Altogether, the results of the memory performance tests indicate that in our mouse model cognitive impairment is completely prevented/rescued by OLE administration to young/middle-aged Tg mice.

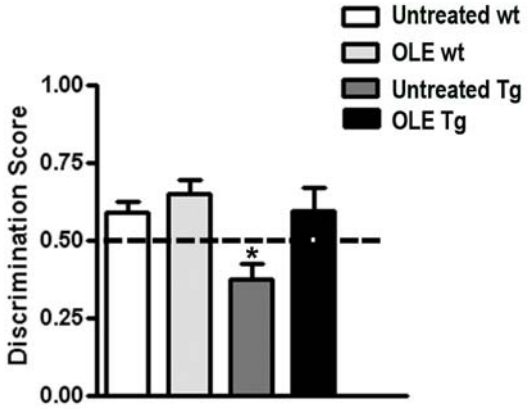


Figure 26. Object recognition test. *The discrimination score recorded in the T2 trial during untreated Tg mice performance significantly differs from the discrimination index of all other groups (*P<0.05). The dotted line indicates the chance level performance; data are reported as mean values ± S.E.M. Number of animals: n = 12/group .*

1.2. Neurogenesis

In the attempt to examine the neuronal progenitor cell proliferation, wild type control and Tg mice were administered BrdU during the last 3 days of treatment and were then sacrificed 24 h after the final BrdU injection.

Mice of both genotypes displayed newly generated cells mostly in the sub granular zone of dentate gyrus of the hippocampus, as shown by the fluorescent BrdU immunoreactivity which labels the nuclei of replicating cells irrespective of cell lineage (Fig. 27 A). Notably, quantitative analysis of BrdU+ cells revealed a significant reduction in the number of proliferating cells in the untreated Tg compared to untreated wt mice, thus indicating that the neurodegenerative process associated with the transgene expression dramatically dampens the proliferation, as previously described (Fiorentini et al., 2010). Following OLE treatment, the proliferation of neural progenitor cells in the sub granular zone of dentate gyrus appeared to be increased in Tg mice (Fig. 27 A). Quantitative analysis of the fluorescent BrdU+ cells demonstrated a significantly higher number of proliferating cells in the OLE-treated Tg than in untreated Tg mice (Fig. 27 B). Altogether these findings indicate that OLE administration increases the proliferation of newborn cells in the subgranular zone of the hippocampus in 3.5-month-old Tg mice.

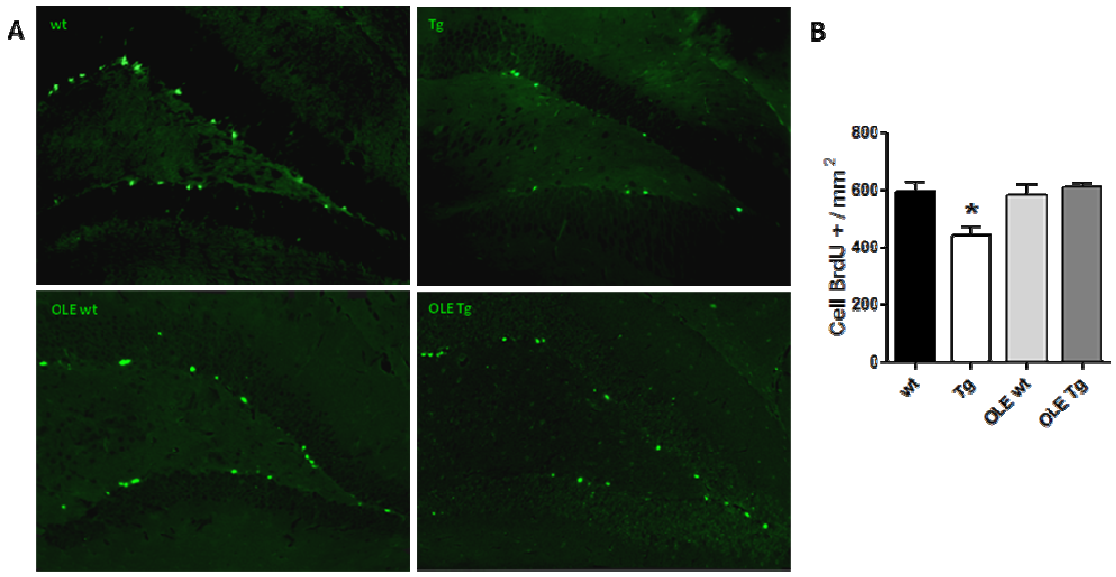


Figure 27. Cell proliferation in the subgranular zone and effect of OLE diet.
(A) BrdU-positive cells of the neurogenic dentate gyrus area in 3.5-month-old OLE-fed and untreated tg and wt mice.
*(B) The number of BrdU+ cells is significantly lower in the untreated tg than in the OLE-fed tg mice (* $p < 0.01$, one-way ANOVA plus Newman-Keuls post hoc test; $n = 5$/group), which showed a number of new born cells similar to wild type mice .*

1.3. Aβ deposition

Next, we checked whether the improved cognitive performance of OLE-fed young/middle-aged Tg mice resulted from any altered amyloid load respect to untreated Tg mice. Few, round-shaped, small to medium size plaques were detected by an anti-Aβ42 antibody in the cortex and hippocampus of untreated 3.5-month-old Tg mice (Fig. 28 A). In 6-month-old Tg mice the Aβ load became heavier with a calculated total plaque area in the cortex and hippocampus of about 1600 μm^2 and 1400 μm^2, respectively (Fig. 28 B), which was markedly reduced in the brains of age-matched Tg mice fed with OLE (Fig. 28 A). Quantitative analysis of total Aβ plaque area and number in the cortex and the hippocampus revealed that the effect of OLE treatment was significant both in 3.5- and 6-month-old Tg mice, supporting a remarkable protective effect of OLE against early and middle stage of Aβ deposition.

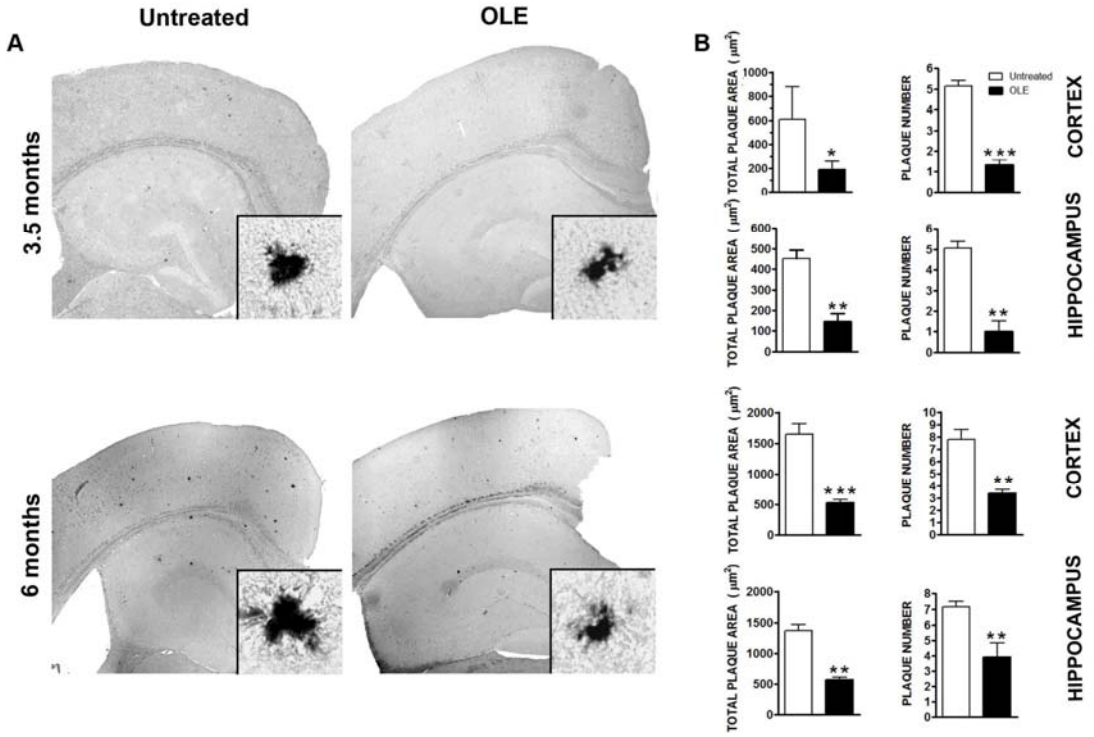


Figure 28. Analysis of Aβ plaque burden in the cortex and hippocampus of TgCRND8 mice.
(A) Representative photomicrographs of Aβ42 immunopositive deposits. (n = 5/group, six sections from each mouse). Insets: high magnification images of representative plaques. Scale bars = 500 µm applies to all reconstructed images and 20 µm to all magnified images.
*(B) Quantitative analysis of total plaque area and plaque number in untreated and OLE-fed Tg mice (n = 6 for 3.5 and 6 months Tg mice). *P<0.05, **P<0.01 and ***P<0.001. Data are reported as mean values ± S.E.M.)*

ELISA assay results showed that Aβ40 and Aβ42 SDS and formic-acid soluble fractions measured in the cortex of OLE-fed Tg mice of both ages were significantly reduced as compared to those measured in age-matched untreated Tg mice (Fig. 29). This data confirmed the one obtained by quantitative analysis of Aβ42 previously described and showed an effect of OLE also in Aβ40 peptide formation .

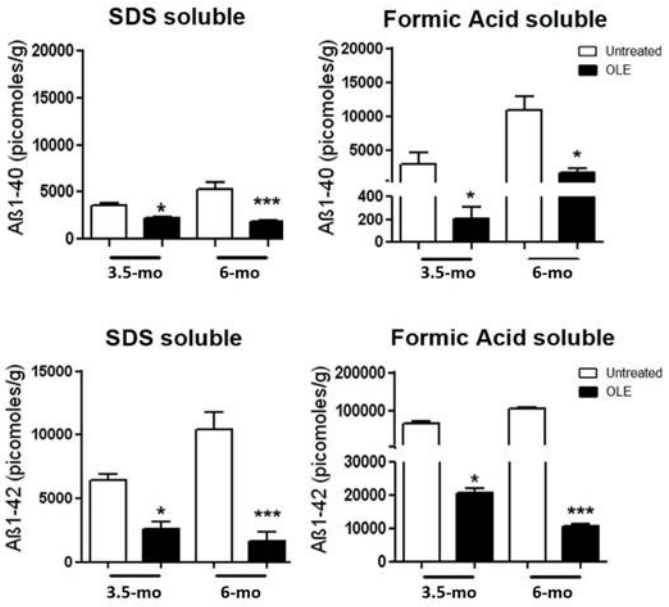


Figure 29. ELISA assay for Aβ40 and Aβ42 in the cortex of TgCRND8 mice. *Cortical levels of SDS- and formic acid-soluble Aβ40 and Aβ42 peptides in OLE-fed and untreated Tg mice were compared. Both Aβ40 and Aβ42 levels were significantly decreased in OLE-fed Tg versus age-matched untreated Tg (n = 5/group) mice. *P<0.05, **P<0.01 and ***P<0.001. Data are reported as mean values ± S.E.M. mo= month-old*

Plaque morphology was investigated through Thioflavin S staining, which detects β-sheet structures, and immunofluorescence with OC antibody, which specifically recognizes fibrillar oligomers and amyloid fibrils (Kayed et al., 2007). These reactions showed that in both the cortex (Fig. 30) and hippocampus of OLE-fed Tg mice of both ages the radiating plaques displayed a less dense core surrounded by fewer and smaller round-shaped deposits than in untreated Tg mice. Moreover, in the brain of 6-month-old Tg mice, displaying an intermediate stage of plaque deposition, feeding with OLE resulted in the presence of several radiating plaques with ribbon-like/diffuse core and in a remarkable presence of fluffy deposits (arrow in figure 30), whereas the plaques found in untreated Tg mice typically displayed a very dense core (arrowhead in figure 30).

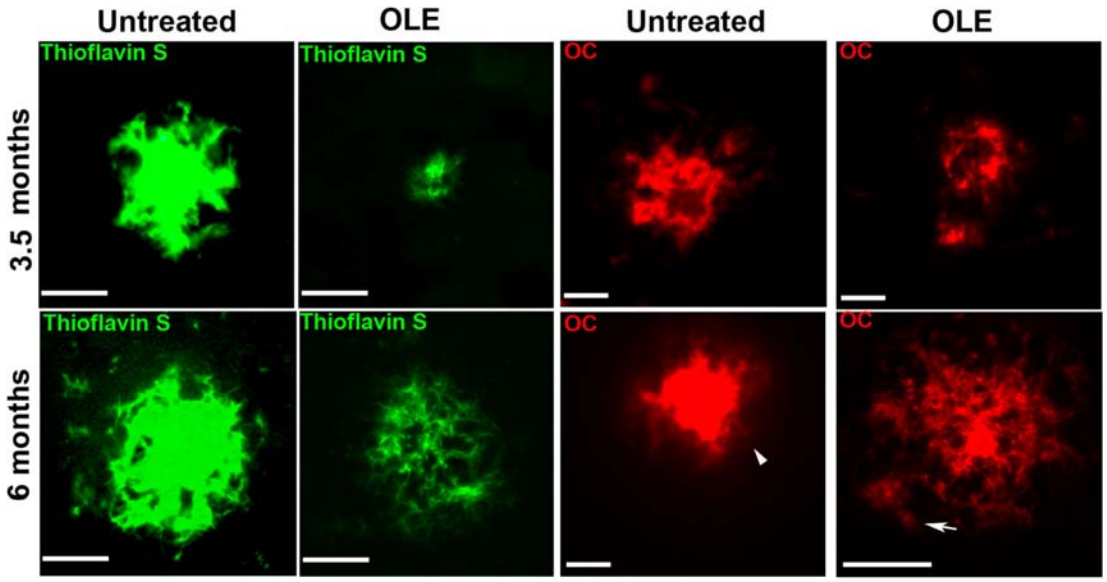


Figure 30. Plaque morphology: Thioflavin S and OC immunofluorescence. *Representative photomicrographs of Thioflavin S histochemistry (green) (n = 4/group) and OC immunolabeling (red) (n = 5/group) of amyloid plaques in the cortex of untreated and OLE-fed Tg mice. In the brains of 6-month-old OLE-fed Tg mice several radiating plaques with ribbon-like/diffuse core and fluffy deposits (arrow) are present. Arrowhead indicates dense core amyloid plaques. Scale bars = 25µm*

Altogether, these findings suggest that OLE, besides interfering with *de novo* amyloid deposition, favours preformed plaque disassembly.

1.4. Autophagy

It is widely recognized that autophagy protects neurons from Aβ-induced cytotoxicity, thus we investigated whether autophagy was involved in the interference of OLE with the *de novo* deposition and disassembly of Aβ. Actually, an intense bright and punctate immunoreactivity for Beclin 1, one of the proteins involved in the initiation and execution of autophagy, was detected in the soma, perikarya and dendrites of neurons in different layers of somatosensory/parietal and entorhinal/piriform cortices of Tg mice fed with OLE as compared to age-matched untreated Tg mice with no apparent age-related differences (Fig. 31 A). Although to a lesser extent than in OLE-fed Tg mice, Beclin 1 immunoreactivity was stronger also in OLE-fed wt mice than in untreated animals,as exemplified for 3.5-month-old mice in figure 31 A, suggesting a general effect of OLE as inducer of Beclin 1 expression. In the cortex of OLE-fed

animals of both ages, Beclin 1 levels showed a trend towards an increase in wt mice and a significant increase in the Tg mice compared to the respective age-matched untreated animals, as exemplified in figure 31 B for 3.5-month-old mice.

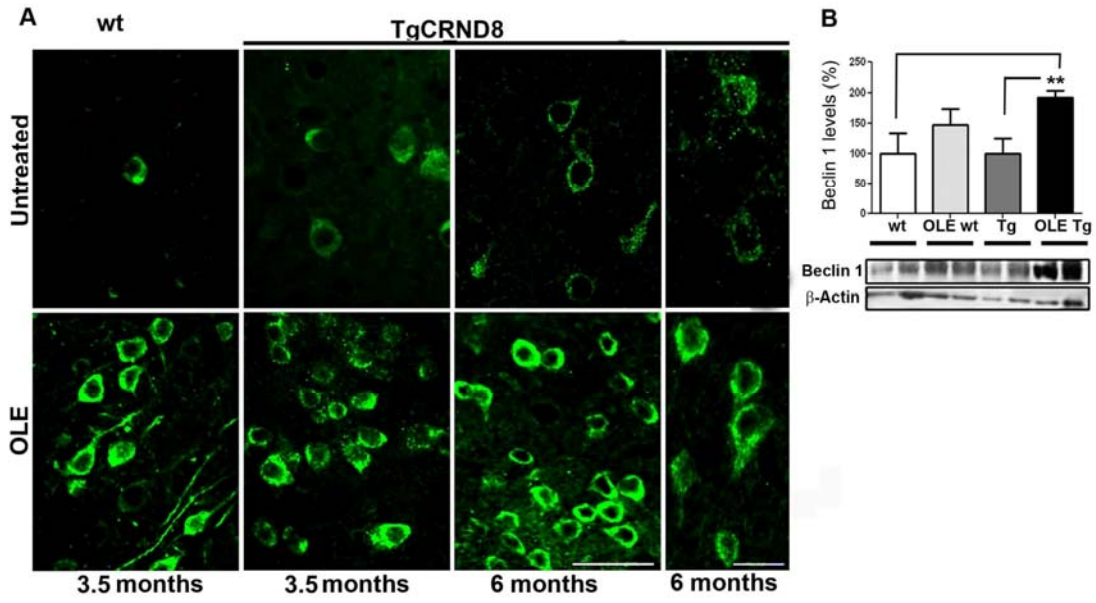


Figure 31. Autophagic protein Beclin 1 expression in the cortex of wt and TgCRND8 mice.
(A) Representative images of Beclin 1 immunoreactivity showed an intense bright and punctuate Beclin 1 staining in the soma, perikarya and dendrites of neurons in the somatosensory/ parietal cortex of Tg mice and, to a lesser extent, in the wt mice fed with OLE, as compared to age-matched untreated Tg and wt mice (n=5/group). Scale bars=50µm for the low magnification images and 20 µm for the high magnification images of untreated and OLE-fed 6-month-old Tg mice.
*(B) Western blotting analysis of Beclin 1 levels in cortical tissue, exemplified for 3.5 month-old mice, normalized for β-actin (n=6-7/group). In the cortex of OLE-fed animals Beclin 1 levels show a trend towards an increase in the wt mice and in the OLE-fed Tg mice Beclin 1 levels were significantly increased respect to age-matched untreated wt and Tg mice (**p<0.01)*

OLE-induced autophagy was further confirmed by immunohistochemical analysis of LC3-II, the membrane-associated lipidated LC3 form that appears in newly formed autophagosomes (Hansen and Johansen, 2011). Stronger and brighter LC3 puncta were detected in the neuronal cell bodies and processes in the somatosensory/parietal (Fig. 32 A) and entorhinal/piriform cortices of Tg mice fed with OLE, as compared to age-matched untreated Tg mice. As for Beclin 1, LC3 immunoreactivity was stronger also in all OLE-fed than in untreated (Fig. 32 A) wt mice. As for Beclin 1, in the cortex of OLE-fed Tg and wt mice of both ages LC3-II levels showed a trend towards an increase

in the wt mice and a significant increase in the Tg mice respect to age-matched untreated wt and Tg mice, as exemplified for 3.5-month-old mice (Fig. 32 B).

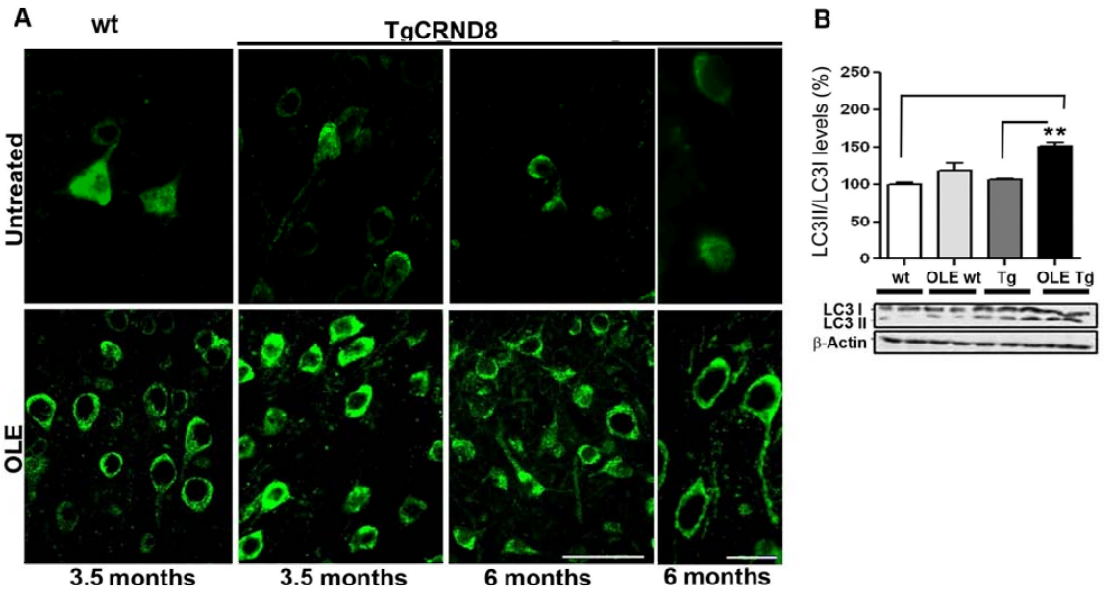


Figure 32. Autophagic protein LC3 expression in the cortex of wt and TgCRND8 mice.
(A) Representative images f LC3 immunoreactivity showed a strong and bright LC3 puncta in the neuronal cell bodies and processes of neurons in the somatosensory/parietal cortex of Tg mice and to a lesser extent, in the wt mice fed with OLE, as compared to age-matched untreated Tg and wt mice (n=5/group). Scale bars=50μm for the low magnification images and 20 μm for the high magnification images of untreated and OLE-fed 6-month-old Tg mice.
*(B)Western blotting analysis of LC3 levels in cortical tissue, exemplified for 3.5 month-old mice, normalized for β-acton (n=6-7/group). LC3 levels are expressed as LC3-II/LC3-I levels. In the cortex of OLE-fed animals LC3 levels were significantly increased respect to age-matched untreated wt and Tg mice (**p<0.01).*

Overall, our data indicate that OLE triggers autophagy not only in Tg but also in wt mice suggesting a possible beneficial effect of this polyphenol also against age-related neurodegeneration such as that occurring in sporadic AD, with an effect similar, at the molecular level, to that elicited by caloric restriction (Pallauf and Rimbach 2012). In general in the hippocampus autophagy was less evident, some Beclin 1 and LC3 puncta were detected in the CA1, CA3 and dentate gyrus of OLE-fed Tg mice only.

To better assess the protective role of the increased autophagy in OLE-fed mice we checked whether it results in autophagosome-lysosome fusion. An intense cathepsin B immunoreactivity was detected as bright puncta in small-sized lysosomes within neurons in the superficial and deep layers of the somatosensory/parietal and

entorhinal/piriform cortices of 3.5 month-old Tg mice fed with OLE (Fig. 33 A). Significant co-localization was found in these animals by double staining with Abs against cathepsin B and p62, a cargo receptor targeting many cellular substrates for autophagic degradation (Salminen et al., 2012) (Fig. 33 A). A similar staining was found in the wt mice treated or untreated with OLE, independently of animal age, as exemplified for 3.5- month-old mice (Fig. 33 A), and the quantitative analysis confirmed that cathepsin B reached similar levels in all these mice (Fig. 33 B).

Altogether, these findings suggest that the autophagosome-lysosome fusion needed for cargo degradation is under way in OLE-fed Tg and wt mice whereas a marked reduction of the cathepsin B expression is present in the same cortical areas of untreated 3.5-month-old Tg mice (Fig. 33 B), with no co-localization between cathepsin B and p62, indicating a transgene-associated dysfunction of the autophagic pathway. In the cortex of untreated 6-month-old Tg mice (Fig. 33 C), bright cathepsin B immunoreactivity occurred mostly in enlarged lysosomal compartments within neuronal soma (arrows) as already reported for cathepsin D staining in the affected brain regions of 6- month-old Tg mice (Yang et al., 2011). As in young Tg mice fed with OLE, also in 6-month-old Tg mice fed with OLE a bright cathepsin B immunoreactivity appeared in small-sized lysosomes, whereas cathepsin B-positive giant lysosomes were almost absent (Fig. 33 C). Moreover, in wt mice, either untreated or treated with OLE (Fig. 33 A), and in OLE-fed Tg mice of both ages, p62 immunoreactivity was markedly greater than in age-matched untreated Tg mice (Fig. 33 A and C), as confirmed by Western blotting of brain extracts, here shown for 3.5-month-old mice (Fig. 33 B). A substantial lack of co-localization between cathepsin B and p62 was found in 6-month-old untreated Tg mice (Fig. 33 C). As shown for younger mice, also in this case OLE feeding was associated with a recover of p62-cathepsin B co-localization (Fig. 33 C, yellow staining), indicating that OLE administration rescues autophagosome-lysosome function also in advanced stages of amyloid deposition.

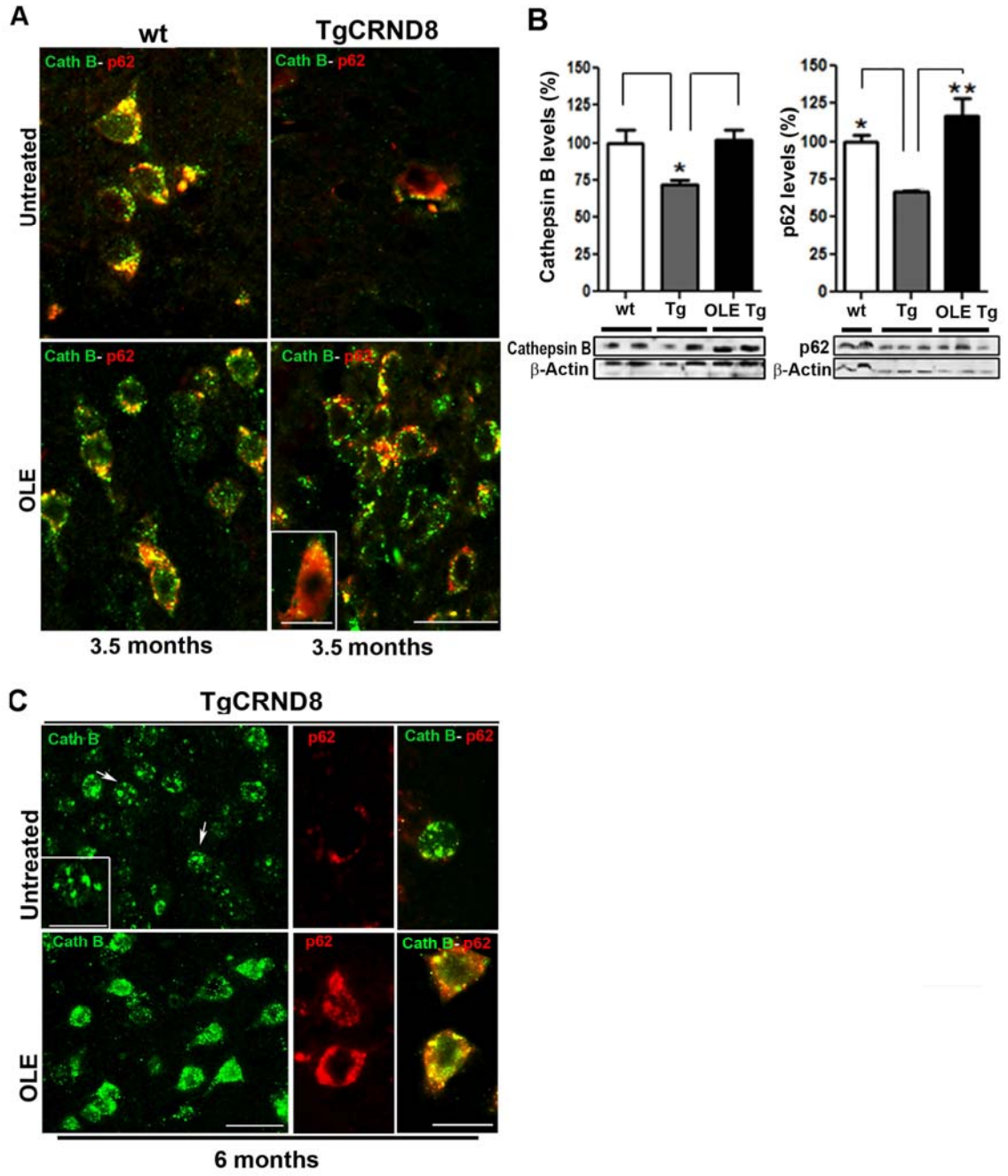


Figure 33. Autophagosome-lysosome fusion in the cortex of untreated and OLE-fed wt and Tg mice.

(A) Merge of cathepsin B (green) and p62 (red) immunoreactivity in 3.5-month-old wt and Tg mice. Co-localization between cathepsin B and p62 staining was detected as bright yellow puncta in small-sized lysosomes in the cortex of untreated wt mice and OLE-fed Tg and wt mice (n = 6/group). Scale bar = 25 μm. Inset: high magnification of a p62 and cathepsin B positive neuron. Scale bar = 14 μm.

*(B) Western blotting analysis of cathepsin B and p62 levels in the cortex of 3.5-month-old wt and Tg mice; wt = pool of untreated and OLE-fed wt mice (n = 10); Tg = untreated, and OLE-fed Tg mice (n = 6/group). Both cathepsin B and p62 levels were significantly increased in OLE-fed Tg mice, as compared to untreated Tg mice. Data are representative of four experiments and are normalized for β-actin and reported as mean values ±S.E.M. *p<0.05, **p<0.01.*

(C) Single and double fluorescent immunohistochemistry with cathepsin B (green) and p62 (red) antibodies in the cortex of untreated and OLE-fed Tg mice. In the untreated Tg mice, bright cathepsin B immunoreactivity occurred in enlarged lysosomal compartments (arrows), p62 immunoreactivity was light and no co-localization between cathepsin B and p62 was found. Inset: high magnification of a cell with cathepsin B-positive giant lysosomes. In the OLE-fed Tg mice, a bright cathepsin B immunoreactivity appeared in small-sized lysosomes, p62 immunoreactivity was greater than in untreated Tg mice and a significant co-localization between cathepsin B and p62 was evident. (n = 6). Scale bars = 25 µm applies for single cathepsin B staining and 10 µm applies to the inset and high magnification images.

1.5. Neuroinflammation

Amyloid deposits in the brain activate an inflammatory response that contributes to cell sufferance and functional decline (Fuhrmann et al., 2010). Accordingly, we investigated whether OLE-treatment reduced the inflammatory response in our Tg mice. We detected hypertrophic astrocytes with long and thick branches in the cortex and in all the subregions of the hippocampus of untreated Tg mice (Fig. 34). By contrast, in the OLE-fed animals the astrocyte reaction was considerably milder (Fig. 34), indicating reduction of inflammation.

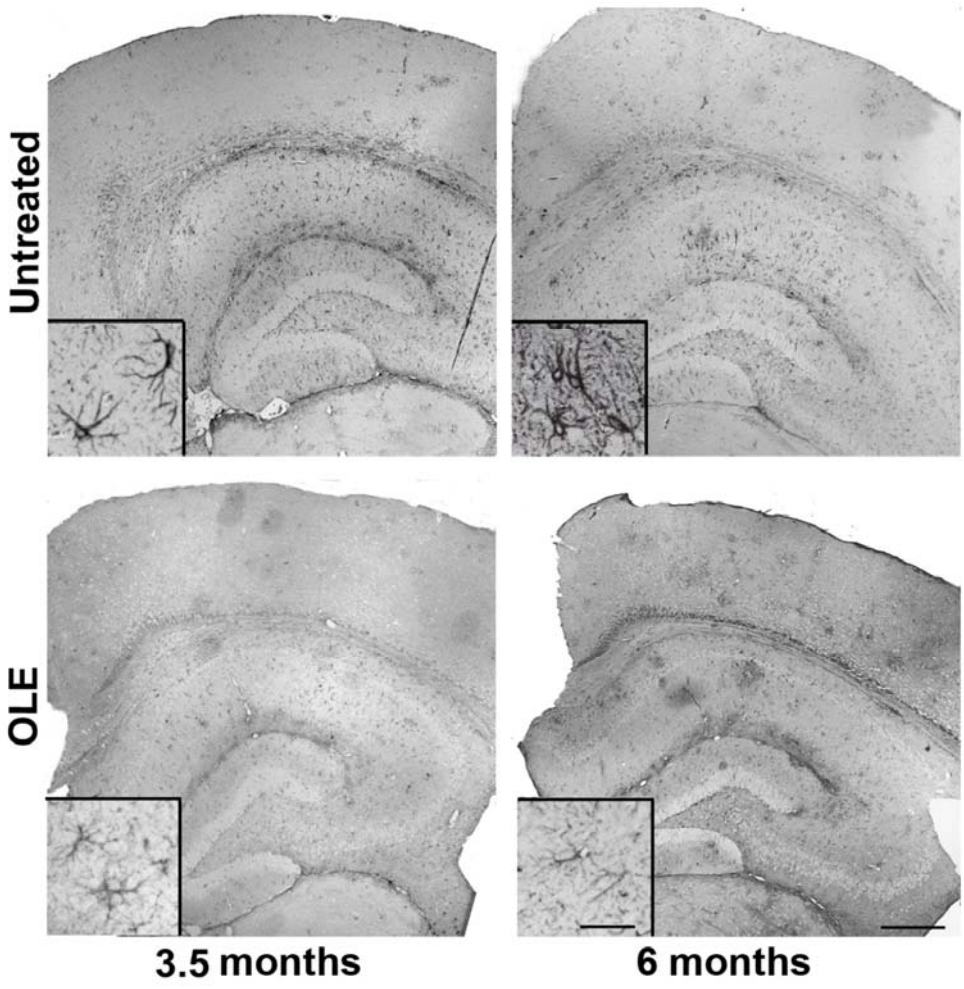


Figure 34. Astrocyte reaction in the brain of untreated and OLE-fed TgCRND8 mice.
Reconstruction of representative photomicrographs of GFAP immunoreactivity in the cortex and hippocampus. Hypertrophic astrocytes with long and thick branches were detected in the brain of untreated Tg mice. In the OLE-fed animals the astrocyte reaction was considerably milder. Insets: high magnification of GFAP-positive astrocytes (n = 4–5/group). Scale bars: 500 μm for all reconstructed images and 25 μm for all insets.

Then we investigated whether the latter was the result of the antioxidant power of OLE; TBARS assay showed that the lipid peroxidation in the cortex of 3.5-month-old Tg mice was not significantly reduced by OLE treatment (Fig. 35).

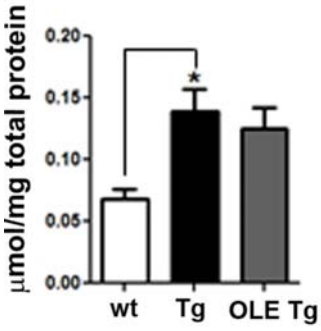


Figure 35. Lipid peroxidation in the cortex of wt, untreated and OLE-fed TgCRND8 mice.
*TBARS assay results in cortical homogenates of 3.5-month-old mice showed that lipid peroxidation in transgenic mice was not significantly reduced by OLE treatment. *P<0.05. Data are reported as mean values ± S.E.M. (n = 3–4 mice/group). Each sample was analyzed in two replicates.*

Next, we investigated the effect of OLE administration on microglia morphology. Surprisingly, activated microglia with enlarged cell bodies, thickened and retracted processes or losses of branches were detected in the hippocampus (not shown) and cortex of OLE-fed 6-month-old Tg mice (Fig. 36). Such a scenario coincided and agreed with the presence of the less dense ‘‘fluffy’’ amyloid plaques described above, suggesting activated microglia involvement in plaque remodelling and phagocytosis. In untreated Tg mice some morphologically activated microglia were detected only in 6-month-old animals while in 3.5-month-old mice resting microglia with thin cell bodies and elongated branches were mostly detected (Fig. 36). These data support protection by OLE against the inflammatory response to amyloid deposits mainly at the astrocyte level, while microglia activation by OLE can mainly contribute to plaque clearance.

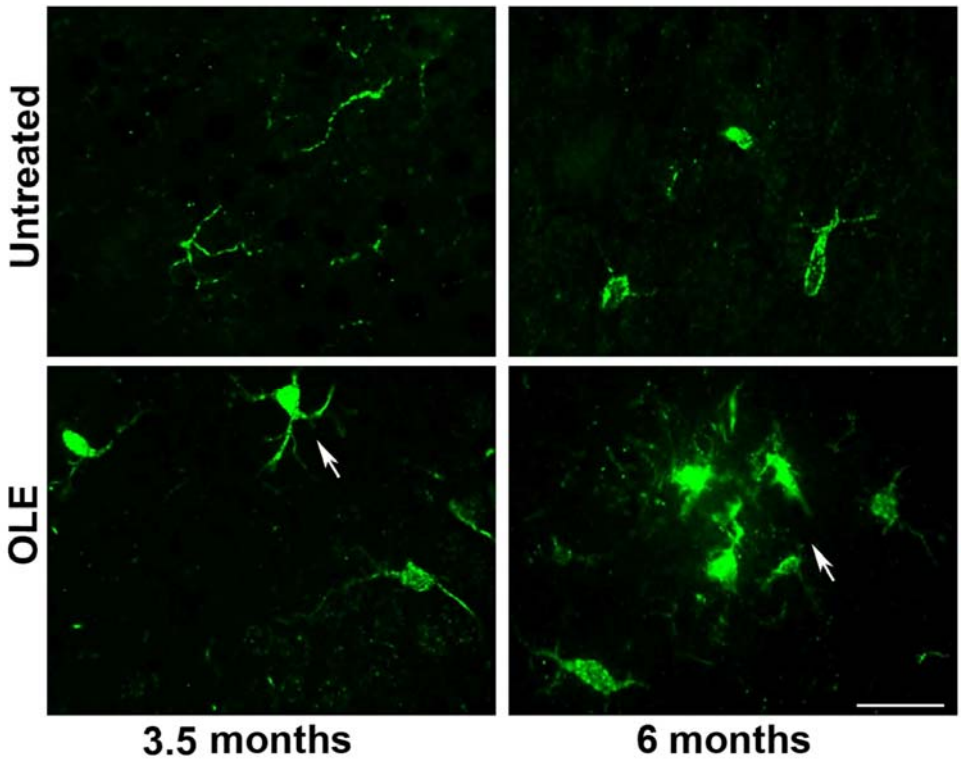


Figure 36. Microglia reaction in the brain of untreated and OLE-fed TgCRND8 mice.
Iba1 immunopositive microglial cells in the cortex. Note the presence of microglia with enlarged cell bodies, thickened and retracted processes (n = 4–5/group) in the cortex of OLE-fed 6-month-old Tg mice. Scale bar: 25 μm applies to all images .

2. Discussion

The present research provides a compelling evidence that, in young/middle-aged TgCRND8 mice, diet supplementation for 8 weeks with OLE remarkably improves animal behavior in two memory tests respect to normally fed littermates, with scores reaching those displayed by age-matched wt mice. Improved behavior is accompanied by a significant reduction in Aβ40 and Aβ42 levels as well as in size and compactness of Aβ plaques and by the presence of fluffy deposits in the older Tg mice. Altogether, our data suggest that OLE treatment reduces *de novo* Aβ deposition and favors preformed plaque disassembly. These results agree with findings on a recombinant *C. elegans* strain expressing human Aβ42 in the cytoplasm of muscle cells, showing that worms grown on an OLE-supplemented medium displayed remarkably improved motility and reduced plaque deposition (Diomede et al., 2013). It has been already reported that the inability to acquire the step down-inhibitory response and to explore a novel object over a familiar one in this mouse model reflects dysfunction of cortical areas (Francis et al., 2012). Functional disruption in the neuronal network has

repeatedly been reported in AD mouse models (Rudinskiy et al., 2012). Aberrant neuronal activity and significant reduction of the number of active neurons is particularly present near amyloid plaques, whose presence causes disturbances resulting in abnormalities of whole neuronal networks both in animal models (Grienberger et al., 2012) and in asymptomatic patients with amyloid deposits (Sperling et al., 2009). Our data demonstrated that OLE treatment of young/middle-aged Tg mice results in a concomitant improvement of non-spatial episodic memory and working memory together with an increased hippocampal neurogenesis, amelioration of cortical neuropathological aspects and a remarkable induction of autophagy underscores a tight link between these effects.

TgCRND8 mice develop a pattern of Aβ deposition recalling several aspects of human AD. Small size Aβ42 immunopositive plaques appear in various brain areas, including the cortex and the hippocampus. As a function of age, they become small-medium to big in size and acquire a compact core, becoming numerous and reaching the maximum roughly by 7-8 months of age (Chishti et al., 2001). OLE administration with diet to pre-plaque TgCRND8 mice for 8 weeks results in a remarkable reduction of the Aβ load, with a significant decrease of levels, plaque number and area, in agreement with the *in vitro* anti-aggregation effect of OLE already reported (Rigacci et al., 2011). In the OLE-treated older Tg mice, the Aβ cortical levels are reduced and the plaques appear less compact displaying ribbon-like and fluffy morphologies in both the cortex and the hippocampus, indicating the occurrence of dual concomitant effects of OLE, prevention of amyloid deposition and disaggregation of preformed plaques. It is increasingly recognized that autophagy protects neurons from Aβ-induced cytotoxicity and that autophagy dysfunction is a molecular link between brain ageing, Aß accumulation in the brain parenchyma and cognitive impairment (Rubinsztein et al., 2011; Yang et al., 2011). Recently, brain inflammation and Aβ deposition following some triggering insult have been proposed to establish a self-reinforcing cycle integrating the amyloid cascade hypothesis presently considered at the basis of cerebral impairment in AD (Herrup 2010). Deletion of Beclin 1 in mice has been reported to increase Aβ deposits, to decrease neuronal autophagy and to promote neuronal degeneration, while gene therapy using lentivirus expressing Beclin 1 reduces amyloid pathology in APP transgenic mice (Pickford et al., 2008). Accordingly, we associate the amelioration of cognitive function of young/middle-aged animals and of

neuropathology with a remarkable induction of the autophagic pathway in the cortex of OLE-treated Tg mice. A strong punctuate immunoreactivity and higher levels of autophagosome-lysosome markers, from Beclin-1 to LC3 and cathepsin B, are detected in the cortex and, to a lesser extent, in hippocampal areas of OLE-fed Tg mice at both stages of amyloid deposition, as compared to untreated age-matched Tg mice. In the Tg mice, OLE administration triggers the autophagic machinery to the level detected in control wt mice and leads to the delivery of autophagosome substrates to lysosomes for degradation. By contrast, in the untreated Tg mice autophagosome-lysosome markers analysis reveals a clear dysfunction of the autophagic pathway. The cortical and hippocampal astrocyte reaction detected in untreated Tg mice is strongly ameliorated by OLE administration. This anti-inflammatory activity of OLE treatment is not associated with the known OLE anti-oxidant activity, which plays a minor role, if any, in all the effects we have found. In fact, the OLE-induced migration of microglia to the amyloid plaques favoring phagocytosis of amyloid deposits could maintain/produce an oxidant environment thus underlying the absence of significant antioxidant effects of OLE administration. In addition, since OLE is highly prone to oxidation, it probably loses its anti-oxidant power before reaching mice brain. These data strongly support the idea that OLE treatment combats Aβ neurotoxicity and Aβ-induced cognitive impairment in our mouse model through reduction of plaque load and consistency resulting from a strong induction of autophagy, concomitantly with a recovery of the lysosomal system (whose dysfunction is one of the earliest disturbances that occur in AD and from microglia activation (Ginsberg et al., 2010). The two responses could co-operate to the final protective effect; in fact, the migration of the activated microglia to the Aβ deposits would result in plaque disassembly and fragmentation; the activated autophagy would complete the protective response explaining reduced plaque burden, dimensions and compactness. The beneficial effects of the MD on attenuating cognitive impairment, AD-like pathology and neurodegenerative diseases has been repeatedly reported (Scarmeas et al., 2006; Féart et al., 2009).

Overall, the behavioral and histological data presented in our study support the notion that the beneficial effects of the MD can, at least in part, be traced back to the intake of extra virgin olive oil and its main polyphenol, OLE. We also provide a molecular explanation of the protective effects elicited by OLE against age-related and AD-type neurodegeneration. Our findings are strengthened by previous data indicating

that in rat and humans, orally administered olive oil phenols, including OLE, its glycoside and/or one of its derivatives arising from tissue metabolism, are intestinally absorbed skipping degradation by microorganisms in the colon, cross the blood-brain barrier and are found inside brain parenchyma (Serra et al., 2012; Vissers et al., 2002). In conclusion, our results support the possibility that dietary supplementation of OLE may prevent or delay the occurrence of AD and may reduce the severity of its symptoms.

ZEBRAFISH EXPERIMENTS

The implication of autophagy-related genes in serious neural diseases have been well documented. Recently has been reported the important role of atg5 gene in zebrafish neurogenesis and organogenesis: knockdown studies demonstrated that zebrafish atg5 is required for normal morphogenesis of brain regionalization and body plan (Hu et al., 2011).

In order to study the role of the autophagy in the embryonic zebrafish development, we aim to generate of Atg5-knockout zebrafish using TALEN technology.

1. Experimental design

First, we designed the TALEN to target the gene to mutate all the known splice variants. We chose a pair of TALENs (*www.talendesign.org/mojohand*) targeting the 3rd exon of *atg5* gene: this exon showed the same sequence in both the splice transcriptional variants, it is upstream to protein family domains (Fig. 37) and the region between the two TALENs designed for this exon could be targeted by the specific restriction enzyme BspHI.

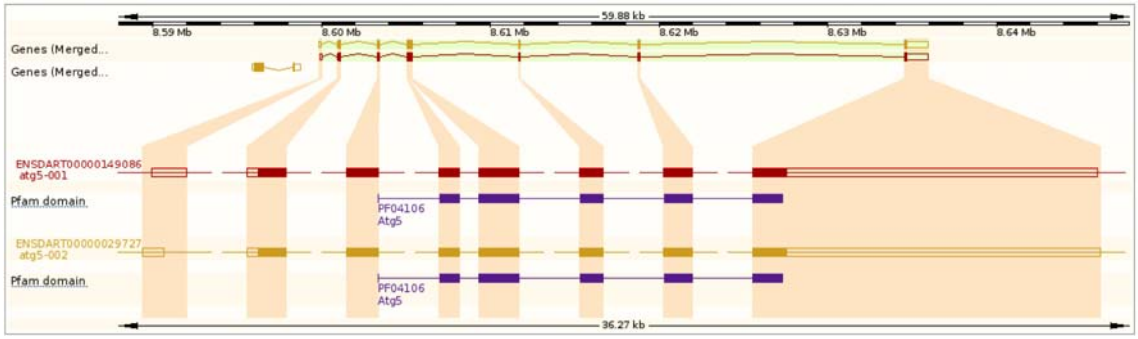


Figure 37. Atg5 splice variants transcripts. *This image show both the spliced transcripts for Atg5 gene,including Expressed Sequence Tag (EST) transcripts and non-coding RNAs (ncRNAs). Transcripts are drawn as boxes (exons) and lines connecting the boxes (introns); filled boxed represent coding sequence and unfilled boxes represent UnTranslated Region (UTR). For coding transcript (gold and red transcripts), protein motif and domains are shown in purple.*

DNA fragments encoding engineered TALENs arrays were cloned into plasmid to create the TALEN expression vectors; TALEN Atg5 encoding RNA was transcribed *in vitro* from TALEN expression vectors using the mMESSAGE mMACHINE® T7 Ultra (*ambion, by life technologies*) and then introduced into early zebrafish embryos by

microinjection. Subsequently, genomic DNA was isolated from a small number of the injected embryos and the somatic mutation efficiencies of the TALEN was assessed by sequencing or through restriction-enzyme digestion analysis: the genomic DNA sequence surrounding the TALEN-cut site was analysed to determine whether an indel mutation had destroyed the recognition site of a restriction enzyme. If such a site was identified, PCR product encompassing the TALEN-target site was digested with the BspHI restriction enzyme and analysed using gel electrophoresis.

If TALEN-induced somatic mutations were detected with high frequency, the remaining injected embryos were raised to adulthood and screened as potential founders. Then we can detect the TALEN-induced indels mutations in the progeny of potential founders through restriction-enzyme digestion analysis to check if the TALEN-induced mutations are in the germ cells, and so the mutation could be hereditary.

The offspring of the positive founders were raised to adulthood and then crossed with wt in order to establish a stable mutated fish line. The injected fish will show a mosaic pattern of mutation, and the following generations will be heterozygous: heterozygous fish can be then incrossed to obtain the homozygous, that will show the full knock out of the *atg5* gene (Fig. 38).

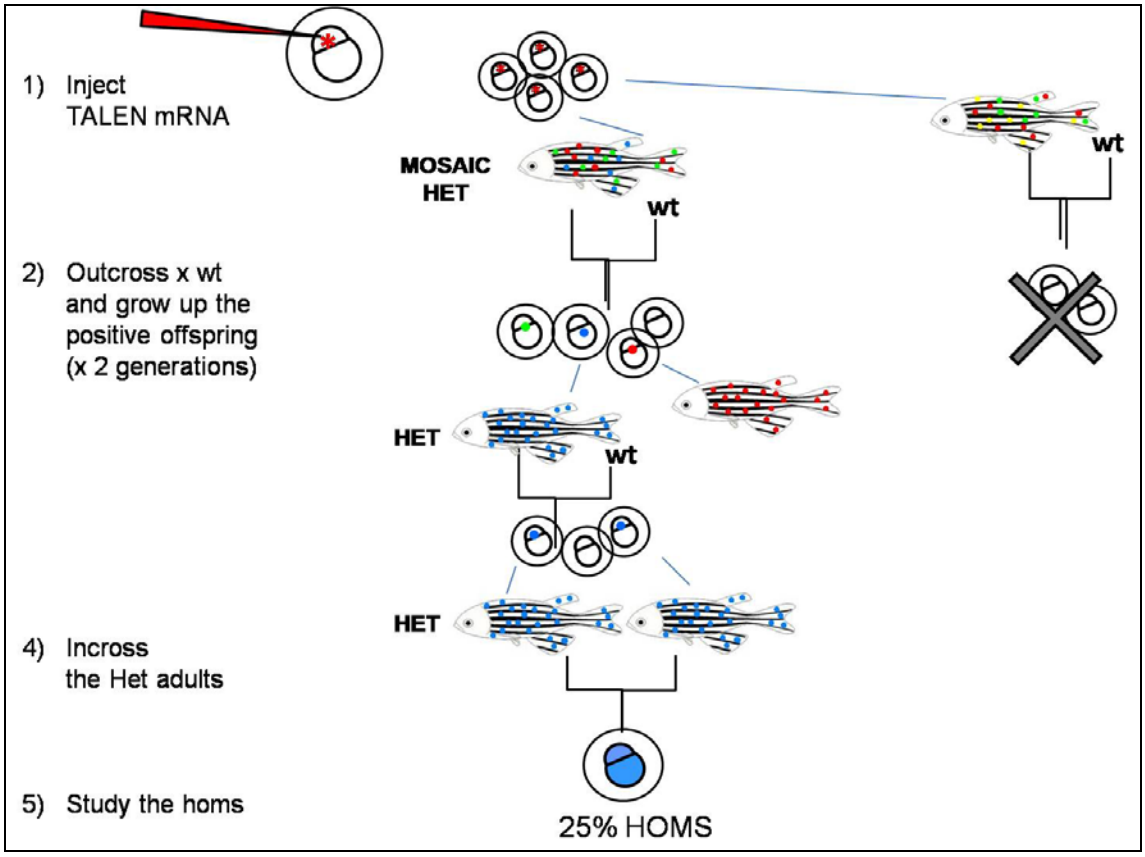


Figure 38. Overview of the project. *First TALEN mRNA was injected in one-cell stage wt embryos that were raised. Each spot of different colour indicates a different mutation in the target gene: since TALEN can just cut, the repair system can introduce different mutations in each cut (a stop codon, a frameshift, etc). So the injected fish showed a mosaic pattern of mutation: several cells had different genome. When the fish are 3-months-old, were crossed with wild type and the offspring was analysed: if it shows no mutation, it means that TALEN hasn't worked or at least it had not mutated cells of the germ line; if the offspring is mutated, the babies were raised, and each embryos was heterozygous and showed just one kind of mutation.*
When the first generation grew up, the fish were crossed with a wt in order to establish a stable expressing fish line. The second generation will be heterozygous, and then they can be incrossed to obtain the homs.

2. Results

Restriction enzyme digestion of the Atg5 PCR products showed a variety of digested product in the different samples, indicating that TALEN could be more or less effective.

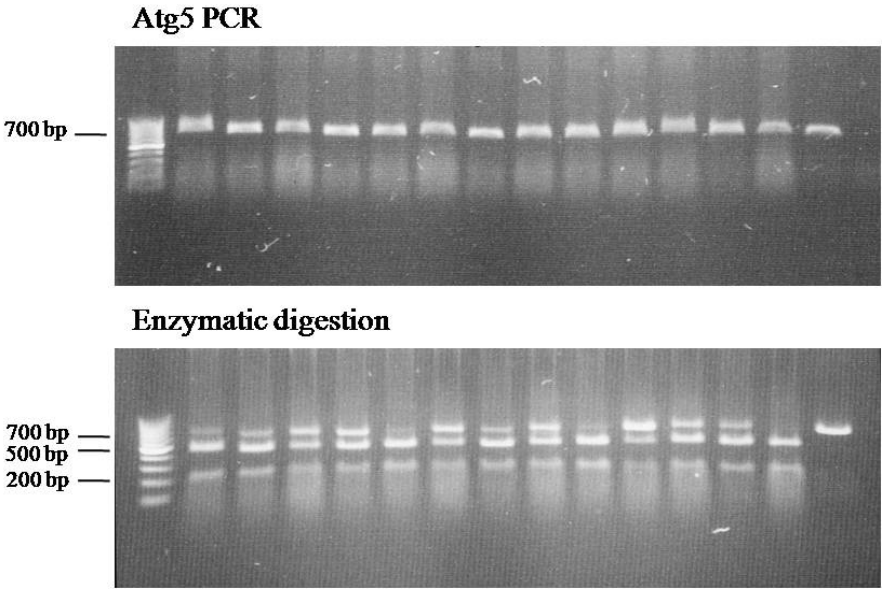


Figure 39. PCR for Atg5 gene and digestion with BspII restriction enzyme. *Once the PCR reaction for Atg5 gene was performed,, the PCR products were digested; in wild type embryos the digestion produces two fragments (500 bp and 200 bp), in mutated embryos an undigested band (700 bp) is shown.*

Comparing the brightness of the undigested and digested products, it is possible to roughly estimate how effective is the TALEN:

- very strong: the product is almost undigested (34% of the samples)
- strong: more than 50% of the product is undigested (48%)
- moderate: about 50% of the product is undigested (32%)
- mild: less than 50% of the product is digested (1%)

These results show that the effect of TALEN Atg5 is quite strong: 82% of the examined samples showed more than the 50% of undigested product, so TALEN had mutated the Atg5 gene in a high percentage of injected embryos.

3. Discussion

These preliminary data show a high percentage of mutation fish in TALEN Atg5 injected fish and provide promising prospects for the production of two more generations of *atg5* $^{+/-}$ fish in order to obtain the homozygous animals by incrossing heterozygoses.

Studying the full knockout of Atg5, one of the most important genes involved in starting autophagic process, will provide to clear the role of autophagy in the development of central nervous system and zebrafish body plan.

Autophagy has been suggested as potential therapeutic target in pathologies characterized by aggregates, and further studies on this process will provide an insight of the role of this process in the brain.

REFERENCES

Abdel-Hafez AA, Meselhy MR, Nakamura N, Hattori M, Watanabe H, Murakami Y, El Gendy MA, Mahfouz NM and Mohamed TA (1998) Effects of paeoniflorin derivatives on scopolamine-induced amnesia using a passive avoidance task in mice; structure-activity relationship. *Biol Pharm Bull* 21(11):1174-1179

Abramov AY, Canevari L and Duchen MR (2004) β-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J Neurosci* 24(2):565-575

Adeyemo A, Gerry N, Chen G, Herbert A, Doumatey A, Huang H, Zhou J, Lashley K, Chen Y, Christman M and Rotimi C (2009) A genome-wide association study of hypertension and blood pressure in African Americans. *PLoS Genet* 5(7):e1000564

Ahlberg J and Glaumann H (1985) Uptake—microautophagy—and degradation of exogenous proteins by isolated rat liver lysosomes: effects of pH, ATP, and inhibitors of proteolysis. *Exp Mol Pathol* 42(1):78-88

Akhtar MW, Kim MS, Adachi M, Morris MJ, Qi X, Richardson JA, Bassel-Duby R, Olson EN, Kavalali ET and Monteggia LM (2012) In vivo analysis of MEF2 transcription factors in synapse regulation and neuronal survival. *PLoS ONE* 7(4):e34863

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, et al. (2000) Inflammation and Alzheimer's disease. Neuroinflammation Working Group. *Neurobiol Aging* 21(3):383-421

Al-Azzawie HF and Alhamdani MS (2006) Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits. *Life Sci* 78(12):1371-1377

Allbaugh LG (1953) Crete: a case study of an underdeveloped area. *Princeton, NJ: Princeton University Press*

Altomare R, Cacciabaudo F, Damiano G, Palumbo VD, Gioviale MC, Bellavia M, Tomasello G and Lo Monte AI (2013) The Mediterranean diet: a history of health. *Iranian J Publ Health* 42(5):449-457

Alzheimer A (1907) Uber eine eigenartige Erkrankung der Hirnrinde. *Allgemeine Zeits Psychiat Y Gerichtlich Med* 64:146-148

Amiot MJ, Fleuriet A and Macheix JJ (1986) Importance and evolution of phenolic compounds in olive during growth and maturation. *J Agric Food Chem* 34:823-826

Amiot MJ, Fleuriet A and Macheix JJ (1989) Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry* 28:67-70

Andersen K, Launer LJ, Ott A, Hoes AW, Breteler MM and Hofman A (1995) Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? *The Rotterdam Study. Neurology* 45(8):1441-1445

Andreadou I, Iliodromitis EK, Mikros E, Constantinou M, Agalias A, Magiatis P, Skaltsounis AL, kamber E, Tsantili-Kakoulidou A and Kremastinos DT (2006) The olive constituent oleuropein exhibits anti-ischemic, antioxidative, and hypolipidemic effects in anesthetized rabbits. *J Nutr* 136(8):2213-2219

Andreadou I, Sigala F, Iliodromitis EK, Papaefthimiou M, Sigalas C, Aligiannis N, Savvari P, Gorgoulis V, Papalabros E and Kremastinos DT (2007) Acute doxorubicin cardiotoxicity is successfully treated with the phytochemical oleuropein through suppression of oxidative and nitrosative stress. *J Mol Cell Cardiol* 42(3):549-58.

Andrikopoulos NK, Kaliora AC, Assimopoulou AN and Papageorgiou VP (2002) Inhibitory activity of minor polyphenolic and nonpolyphenolic constituents of olive oil against *in vitro* low-density lipoprotein oxidation. *J Med Food* 5(1): 1-7

Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP and Blagoskionny MV (2010) Rapamycin extends maximal lifespan in cancer-prone mice. *Am J Pathol* 176(5):2092-2097

Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P and Ogier-Denis E (2001) The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 276(38):35243-35246

Arienti G and Fidanza F (1998) Ruoli e richieste di energia e nutrienti energetici. Alimentazione e nutrizione umana. *Guido Gnocchi Editore*

Armstrong D and Browne R (1994) The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory. *Adv Exp Med Biol* 366:43-58.

Ashford TP and Porter KR (1962) Cytoplasmic components in hepatic cell lysosomes *J Cell Biol* 12:198-202

Asuni AA, Boutajangout A, Scholtzova H, Knudsen E, Li YS, Quartermain D, Frangione B, Wisniewski T and Sigurdsson EM (2006) Vaccination of Alzheimer's model mice with Abeta derivative in alum adjuvant reduces Abeta burden without microhemorrhages. Eur J Neurosci 24(9):2530-2542

Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ and Gibson SB (2008) Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy* 4(2):195-204

Baglioni S, Casamenti F, Bucciantini M, Luheshi LM, Taddei N, Chiti F, Dobson CM and Stefani M (2006) Prefibrillar amyloid aggregates could be generic toxins in higher organisms. *J Neurosci* 26(31):8160-8167

Bales KR, Du Y, Dodel RC, Yan GM, Hamilton-Byrd E and Paul SM (1998) The NF-κB/Rel family of proteins mediates Aβ-induced neurotoxicity and glial activation. *Brain Res Mol Brain Res* 57(1):63-72

Bao XH, Naomoto Y, Hao HF, Watanabe N, Sakurama K, Noma K, Motoki T, Tomono Y, Fukazawa T, Shirakawa Y, Yamatsuji T, Matsuoka J and Takaoka M (2010) Autophagy: can it become a potential therapeutic target? *Int J Mol Med* 25(4):493-503

Bao M, Hanabichi S, Facchinetti V, Du Q, Bover L, Plumas J, Chaperot L, Cao W, Qin J, Sun SC and Liu YJ (2012) CD2AP/SHIP1 complex positively regulates plasmacytoid dendritic cell receptor signaling by inhibiting the E3 ubiquitin ligase Cbl. *J Immunol* 189(2):786-792

Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenki D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D and Yednock T (2000) Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med* 6(8):916-919

Barnett A and Brewer GJ (2011) Autophagy in aging and Alzheimer's disease: pathologic or protective? *J Alzheimers Dis* 25(3):385-394

Bartolini M, Bertucci C, Bolognesi ML, Cavalli, A, Melchiorre C and Andrisano V (2007) Insight into the kinetic of amyloid beta (1-42) peptide self-aggregation: elucidation of inhibitors' mechanism of action. *Chembiochem* 8(17):2152-2161

Bazoti FN, Bergquist J, Markides KE and Tsarbopoulos A (2006) Noncovalent interaction between amyloid-β-peptide (1-40) and oleuropein studied by electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom* 17(4):568-575

Bazoti FN, Bergquist J, Markides K and Tsarbopoulos A (2008) Localization of the noncovalent binding site between amyloid-β-peptide and oleuropein using electrospray ionization FT-ICR mass spectrometry. *J Am Soc Mass Spectrom* 19(8):1078-1085

Becher B, Prat A and Antel JP (2000) Brain-immune connection: immuno-regulatory properties of CNS-resident cells. *Glia* 29(4):293-304

Belanger M and Magistretti PJ (2009) The role of astroglia in neuroprotection. *Dialogues Clin Neurosci* 11:281-295

Bellucci A, Luccarini I, Scali C, Prosperi C, Giovannini MG, Pepeu G and Casamenti F (2006) Cholinergic dysfunction, neuronal damage and axonal loss in TgCRND8 mice. *Neurobiol Dis* 23(2):260-272

Bellucci A, Rosi MC, Grossi C, Fiorentini A, Luccarini I and Casamenti F (2007) Abnormal processing of tau in the brain of aged TgCRND8 mice. Neurobiol Dis 27(3):328-338

Benaki D, Stathopoulou K, Leondiadis L, Ferderigos N, Pelecanou M and Mikros E (2009) Detection of interactions of the beta-amyloid peptide with small molecules employing transferred NOEs. *J Pept Sci* 15(6):435-441

Berg TO, Fengsrud M, Stromhaug PE, Berg T and Seglen PO (1998) Isolation and characterization of rat liver amphisomes. Evidence for fusion of autophagosomes with both early and late endosomes. *J Biol Chem* 273(34):21883-21892

Bernales S, McDonald KL and Walter P (2006) Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* 4(12):e4234

Bessis A, Bechade C, Bernard D and Roumier A (2007) Microglial control of neuronal death and synaptic properties. *Glia* 55(3):233-238

Billings LM, Oddo S, Green KN, McGaugh JL and LaFerla FM (2005) Intraneuronal A-beta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* 45(5):675-688

Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst Rev* (1):CD005593

Bissler JJ, McCormack FX, Young LR, Elwing JM, Chuck G, Leonard JM, Schmithorst VJ, Laor T, Brody AS, Bean J, Salisbury S and Franz DN (2008) Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N Engl J Med* 358(2):140-151

Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Øvervatn A, Stenmark H and Johansen T (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 171(4):603-614

Boccio P, DiDeo A, De Curtis A, Celli N, Iacoviello L and Rotilio D (2003) Liquid chromatography-tandem mass spectrometry analysis of oleuropein and its metabolite hydroxytyrosol in rat plasma and urine after oral administration. *J Chromatogr B* 785:47-56

Bogdan C (2008) Mechanisms and consequences of persistence of intracellular pathogens: Leishmaniasis as an example. *Cell Microbiol* 10(6):1221-1234

Bohrmann B, Baumann K, Benz J, Gerber F, Huber W, Knoflach F, Messer J, Oroszlan K, Rauchenberger R, Richter WF et al. (2012) Gantenerumab: a novel human anti-Aβ antibody demonstrates sustained cerebral amyloid-β binding and elicits cell-mediated removal of human amyloid-β. *J Alzheimers Dis* 28(1):49-69

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

Bolmont T, Haiss F, Eicke D, Radde R, Mathis CA, Klunk WE, Kohsaka S, Jucker M and Calhoun ME (2008) Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. *J Neurosci* 28(16):4283-4292

Bondareff W, Mountjoy CQ, Roth M, Rossor MN, Iversen LL and Reynolds GP (1987) Age and histopathologic heterogeneity in Alzheimer's disease. Evidence for subtypes. *Arch Gen Psychiatry* 44(5):412-417

Braak H, Rub U, Schultz C and Del Tredici K (2006) Vulnerability of cortical neurons to Alzheimer's and Parkinson's diseases. *J Alzheimers Dis* 9(3 Suppl):35-44

Brandenburg LO, Konrad M, Wruck C, Koch T, Pufe T and Lucius R (2008) Involvement of formyl-peptide-receptor-like-1 and phospholipase D in the internalization and signal transduction of amyloid β 1-42 in glial cells. *Neuroscience* 156(2):266-276

Brauer H, Strauss J, Wegner W, Müller-Tidow C, Horstmann M and Jücker M (2012) Leukemia-associated mutations in SHIP1 inhibit its enzymatic activity, interaction with the GM-CSF receptor and Grb2, and its ability to inactivate PI3K/AKT signaling. *Cell Signal* 24(11):2095-2101

Brayne C, Richardson K, Matthews FE, Fleming J, Hunter S, Xuereb JH, Paykel E, Mukaetova-Ladinska EB, Huppert FA, O'Sullivan A, Dening T and Cambridge City Over-75s Cohort Cc75c Study Neuropathology Collaboration (2009) Neuropathological correlates of dementia in over-80-year-old brain donors from the population-based Cambridge city over-75s cohort (CC75C) study. *J Alzheimers Dis* 18(3):645-658

Breitner JC, Gau BA, Welsh KA, Plassman BL, McDonald WM, Helms MJ and Anthony JC(1994) Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study. *Neurology* 44(2):227-232

Brenes M, García A, García P, Rios JJ and Garrido A (1999) Phenolic compounds in Spanish olive oils. *J Agric Food Chem* 47(9):3535-3540

Breunig JJ, Sarkisian MR, Arellano JI, Morozov YM, Ayoub AE, Sojitra S, Wang B, Flavell RA, Rakic P and Town T (2008) Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling. *Proc Natl Acad Sci USA* 105(35):13127-13132

Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW and Kaelin WG Jr (2004) Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* 18(23):2893-2904

Caldero J, Brunet N, Ciutat D, Hereu M and Esquerda JE (2009) Development of microglia in the chick embryo spinal cord: implications in the regulation of motoneuronal survival and death. *J Neurosci Res* 87(11):2447-2466

Cameron B and Landreth GE (2010) Inflammation microglia, and Alzheimer's disease. *Neurobiol Dis* 37(3):503-509

Carlsson CM, Xu G, Wen Z, Barnet JH, Blazel HM, Chappell RJ, Stein JH, Asthana S, Sager MA Alsop DC, Rowley HA, Fain SB and Johnson SC (2012) Effects of atorvastatin on cerebral blood flow in middle-aged adults at risk for Alzheimer's disease: a pilot study. *Curr Alzheimer Res* 9(8):990-997

Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A and De Caterina R (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of mediterranean diet phytochemicals. *Arterioscler Thromb Vasc Biol* 23(4):622-629

Cartier L, Hartley O, Dubois-Dauphin M and Krause KH (2005) Chemokine receptors in the central nervous system: Role in brain inflammation and neurodegenerative diseases. *Brain Res Brain Res Rev* 48(1):16-42

Casadesus G, Milliken EL, Webber KM, Bowen RL, Lei Z, Rao CV, Perry G, Keri RA and Smith MA (2007) Increases in luteinizing hormone are associated with declines in cognitive performance. *Mol Cell Endocrinol* 269(1-2):107-111

Castellani RJ, Lee HG, Zhu X, Perry G and Smith MA (2008) Alzheimer disease pathology as a host response. *J Neuropathol Exp Neurol* 67(6):523-531

Castellani RJ, Rolston RK and Smith MA (2010) Alzheimer disease. 56(9):484-546

Cataldo AM, Peterhoff CM, Schmidt SD, Terio NB, Duff K, Beard M, Mathews PM and Nixon RA (2004) Presenilin mutations in familial Alzheimer disease and transgenic mouse models accelerate neuronal lisosomal pathology. *J Neuropathol Exp Neurol* 63(8):821-830

Cecconi F and Levine B (2008) The role of autophagy in mammalian development: cell makeover rather than cell death. *Dev Cell* 15(3):344-357

Chakrabarty P, Jansen-West K, Beccard A, Ceballos-Diaz C, Levites Y, Verbeeck C, Zubair AC, Dickson D, Golde TE and Das P (2010) Massive gliosis induced by interleukin-6 suppresses Aβ deposition *in vivo*: evidence against inflammation as a driving force for amyloid deposition. *FASEB J* 24(2):548-559

Chan WY, Kohsaka S and Rezaie P (2007) The origin and cell lineage of microglia: new concepts. *Brain Res Rev* 53(2):344-354

Chan EY, Longatti A, McKnight NC and Tooze SA (2009) Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Mol Cell Biol* 29(1):157-171

Chang YY and Neufeld TP (2009) An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol Biol Cell* 20(7):2004-2014

Chang WP, Downs D, Huang XP, Da H, Fung KM and Tang J (2007) Amyloid-beta reduction by memapsin 2 (beta-secretase) immunization. *FASEB J* 21(12):3184-3196

Chen JL, Lin HH, Kim KJ, Lin A, Forman HJ and Ann DK (2008) Novel roles for protein kinase Cδ-dependent signaling pathways in acute hypoxic stress-induced autophagy. *J Biol Chem* 283(49):34432-34444

Chen Y, McMillan-Ward E, Kong J, Israels SJ and Gibson SB (2007) Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *J Cell Sci* 120(Pt 23):4155-4166

Chen Y, McMillan-Ward E, Kong J, Israels SJ and Gibson SB (2008) Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ* 15(1):171-182

Chishti MA, Yang DS, Janus C, Phinney AL, Horne P, Pearson J, Strome R, Zuker N, Loukides J, French J et al. (2001) Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. *J Biol Chem* 276(24):21562-21570

Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z and Lipton SA (2009) S-Nitrosylation of Drp1 mediates beta amyloid-related mitochondrial fission and neuronal injury. *Science* 324(5923):102-105

Chorover SL and Schiller PH (1965) Short-term retrograde amnesia in rats. *J Comp Physiol Psychol* 59:73-78

Chow SK, Yu D, Macdonald CL, Buibas M and Silva GA (2010) Amyloid β-peptide directly induces spontaneous calcium transients, delayed intercellular calcium waves and gliosis in rat cortical astrocytes. *ASN Neuro* 2(1):e00026

Christensen DD (2007) Changing the course of Alzheimer's disease: anti-amyloid disease modifying treatments on the horizon. *Prim Care Companion J Clin Psychiatry* 9(1):32-41

Cicerale S, Conlan XA, Sinclair AJ and Keast RSJ (2009) Chemistry and health of olive oil phenolics. *Crit Rev Food Sci Nutr* 49(3):218-236

Clark SL Jr (1957) Cellular differentiation in the kidneys of new born mice studied with the electron microscope. *J Biophys Biochem Cytol* 3(3):349-362

Codogno P and Meijer AJ (2005) Autophagy and signaling: Their role in cell survival and cell death. *Cell Death Differ* 12 (Suppl. 2):1509-1518

Colacicco AM, Solfrizzi V, D'Introno A, Capurso C, Kehoe PG, Seripa D, Pilotto A, Santamato A, Capurso A and Panza F (2009) Alpha-2-macroglobulin gene, oxidized low-density lipoprotein receptor-1 locus, and sporadic Alzheimer's disease. *Neurobiol Aging* 30(9):1518-1520

Colton CA (2009) Heterogeneity of microglial activation in the innate immune response in the brain. *J Neuroimmune Pharmacol* 4(4):399-418

Copp AJ, Greene ND and Murdoch JN (2003) The genetic basis of mammalian neurulation. *Nat Rev Genet* 4(10):784-793

Coraci IS, Husemann J, Berman JW, Hulette C, Dufour JH, Campanella GK, Luster AD, Silverstein SC and El-Khoury JB (2002) CD36, a class B scavenger receptor, is expressed on microglia in Alzheimer's disease brains and can mediate production of reactive oxygen species in response to β-amyloid fibrils. *Am J Pathol* 160(1):101-112

Covas MI (2007) Olive oil and the cardiovascular system. *Pharmacol Res* 55(3):175-186

Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T and Ryan KM (2006) DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* 126(1):121-134

Criollo A, Maiuri MC, Tasdemir E, Vitale I, Fiebig AA, Andrews D, Molgo J, Diaz J, Lavandero S, Harper F, Pierron G, di Stefano D, Rizzuto R, Szabadkai G and Kroemer G (2007) Regulation of autophagy by the inositol trisphosphate receptor. *Cell Death Differ* 14(5):1029-1039

Cuervo AM (2004) Autophagy: in sickness and in health. *Trends Cell Biol* 14(2):70-77

D'Ambrosio AL, Pinsky DJ and Connolly ES (2001) The role of the complement cascade in ischemia/reperfusion injury: Implications for neuroprotection. *Mol Med* 7(6):367-382

Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y and Kondo S (2004) Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res* 64(12):4286-4293

Damtoft S, Franzyk H and Jensen SR (1992) Excelsioside, a secoiridoid glucoside from *Fraxinus excelsior*. *Phytochemistry* 31:4197-4201

Damtoft S, Franzyk H and Jensen SR (1993) Biosynthesis of secoiridoid glucosides in *Oleaceae*. *Phytochemistry* 34:1291-1299

Damtoft S, Franzyk H and Jensen SR (1995) Biosynthesis of iridoids in Syringa and Fraxinus: carbocyclic iridoid precursors. *Phytochemistry* 40:785-792

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML and Gan WB (2005) ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat Neurosci* 8(6):752-758

De Duve C and Wattiaux R (1966) Functions of lysosomes. *Annu Rev Physiol* 28:435-492

De la Puerta R, Guttierrez VR and Hoult JRS (1999) Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem Pharmacol* 57(4):445-449

Deane R, Du Yan S, Submamaryan RK, LaRue B, Jovanovic S, Hogg E, Welch D, Manness L, Lin C, Yu J et al. (2003) RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nat Med* 9(7):907-913

Del Villar K and Miller CA (2004) Down-regulation of DENN/MADD, a TNF receptor binding protein, correlates with neuronal cell death in Alzheimer's disease brain and hippocampal neurons. *Proc Natl Acad Sci USA* 101(12):4210-4215

Delgado MA, Elmaoued RA, Davis AS, Kyei G and Deretic V (2008) Toll-like receptors control autophagy. *EMBO J* 27(7):1110-1121

Demarchi F, Bertoli C, Copetti T, Tanida I, Brancolini C, Eskelinen E-L and Schneider C (2006) Calpain is required for macroautophagy in mammalian cells. *J Cell Biol* 175(4):595-605

Dewil M, Van Den Bosch L and Robberecht W (2007) Microglia in amyotrophic lateral sclerosis. *Acta Neurol Belg* 107(3):63-70

Di Bartolomeo S, Nazio F and Cecconi F (2010) The role of autophagy during development in higher eukaryotes. *Traffic* 11(10):1280-1289

Dice FJ (2007) Chaperone-mediated autophagy. *Autophagy* 3(4):295-299

Dickson DW, Lee SC, Mattiace LA, Yen SH and Brosnan C (1993) Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia* 7(1):75-83

Ding WX, Ni HM, Gao W, Hou YF, Melan MA, Chen X, Stolz DB, Shao ZM and Yin XM (2007) Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. *J Biol Chem* 282(7):4702-4210

Diomede L, Rigacci S, Romeo M, Stefani M and Salmona M (2013) Oleuropein Aglycone protects transgenic *C. elegans* strains expressing Aβ42 by reducing plaque load and motor deficit . *PLoS ONE* 8(3): e58893

Donahue JE, Flaherty SL, Johanson CE, Duncan JA 3rd, Silverberg GD, Miller MC, Tavares R, Yang W, Wu Q, Sabo E et al. (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. *Acta Neuropathol* 112(4):405-415

Doody RS, Aisen PS and Iwatsubo T (2013) Semagacestat for treatment of Alzheimer's disease. *N Engl J Med* 369(17):1661

El Khoury J and Luster AD (2008) Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. *Trends Pharmacol Sci* 29(12):626-632

Ennaceur A and Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioural data. *Behav Brain Res* 31(1):47-59

Ennaceur A, Michalikova S, Bradford A and Ahmed S (2005) Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. *Behav Brain Res* 159(2):247-266

Epple UD, Suriapranata I, Eskelinen E-L and Thumm M (2001) Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *J Bacteriol* 183(20):5942-5955

Eriksdotter, Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M et al. (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dement Geriatr Cogn Disord* 9(5):246-257

Errafiy R, Aguado C, Ghislat G, Esteve JM, Gil A, Loutfi M and Knecht E (2013) PTEN increases autophagy and inhibits the ubiquitin-proteasome pathway in glioma cells independently of its lipid phosphatase activity. *PLoS One* 8(12):e83318

Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V, Covas MI, Fiol M, Gómez-Gracia E, López-Sabater MC, Vinyoles E et al. (2006) Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med* 145(1):1-11

Farlow MR and Evans RM (1998) Pharmacologic treatment of cognition in Alzheimer's dementia. *Neurology* 51(1 Suppl 1):S36-S44; discussion S65–S67

Farr SA, Price TO, Dominguez LJ, Motisi A, Saiano F, Niehoff ML, Banks WA, Ercal N and Barbagallo M (2012) Extra virgin olive oil improbe learning and memory in SAMP8 mice J Alzheimers Dis 28(1):81-92

Farré JC and Subramani S (2004) Peroxisome turnover by micropexophagy: an autophagy-related process. *Trends Cell Biol* 14(9):515-523

Farzan M, Schnitzler CE, Vasilieva N, Leung D and Choe H (2000) BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. *Proc Natl Acad Sci USA* 97(17): 9712-9717

Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K et al. (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta-amyloid peptides Abeta 42 and Abeta 40 *in vitro* and *in vivo*. *Proc Natl Acad Sci* USA 98(10):5856-5861

Féart C, Sanieri C, Rondeau V, Amieva H, Portet F, Dartigues JF, Scarmeas N and Barberger-Gateau P (2009) Adherence to a Mediterranean diet, cognitive decline and risk of dementia. *JAMA* 302(6):638-648

Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S and Gage FH (1987) Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 329(6134):65-68

Fitó M, Guxens M, Corella D, Sáez G, Estruch R, de la Torre R, Francés F, Cabezas C, López-Sabater Mdel C et al. (2007). Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. *Arch Intern Med* 167(11):1195-1203

Fonseca MI, Kawas CH, Troncoso JC and Tenner AJ (2004) Neuronal localization of C1q in preclinical Alzheimer's disease. *Neurobiol Dis* 15(1):40-46

Fraser DA, Pisalyaput K and Tenner AJ (2010) C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. *J Neurochem* 112(3):733-743

Fratiglioni L, De Ronchi D and Agüero-Torres H (1999) Worldwide prevalence and incidence of dementia. *Drugs Aging* 15(5):365-375

Fujita N, Itoh T, Omori H, Fukuda M, Noda T and Yoshimori T (2008) The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell* 19(5):2092-2100

Furuta S, Hidaka E, Ogata A, Yokota S and Kamata T (2004) Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene* 23(22):3898-3904

Gallo JM and Spickett C (2010) The role of CELF proteins in neurological disorders. *RNA Biol* **7**(4):474-479

Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, Fu W, Zhang J, Wu W, Zhang X and Chen YG (2010) Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. *Nat Cell Biol* 12(8):781-790

Garden GA (2002) Microglia in human immunodeficiency virus-associated neurodegeneration. *Glia* 40(2):240-251

Gaspar RC, Villarreal SA, Bowles N, Hepler RW, Joyce JG and Shughrue PJ (2010) Oligomers of beta-amyloid are sequestered into and seed new plaques in the brains of an AD mouse model. *Exp Neurol* 223(2):394-400

Gasque P (2004) Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 41(11):1089-1098

Gasque P, Dean YD, McGreal EP, Beek JV and Morgan BP (2000) Complement components of the innate immune system in health and disease in the CNS. *Immunopharmacology* 49(1-2):171-186

Geng J and Klionsky DJ (2008) The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. "Protein modifications: beyond the usual suspects" review series. *EMBO Rep* 9(9):859-864

Giacobini E (2000) Cholinesterase inhibitors stabilize Alzheimer's disease. *Ann N Y Acad Sci* 920:321-327

Giacobini E (2001) Do cholinesterase inhibitors have disease-modifying effects in Alzheimer's disease? *CNS Drugs* 15(2):85-91

Giacobini E, Mori F and Lai CC (1996) The effect of cholinesterase inhibitors on the secretion of APPS from rat brain cortex. *Ann N Y Acad Sci* 777:393-398

Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette, Orgogozo JM; AN1792(QS-21)-201 Study Team (2005) Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. *Neurology* 64(9):1553-1562

Glabe CG (2006) Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. *Neurobiol Aging* 27(4):570-575

Glabe CG (2008) Structural classification of toxic amyloid oligomers. *J Biol Chem* 283(44):29639-29643

Golde TE, Estus S, Younkin LH, Selkoe DJ and Younkin SG (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* 255(5045):728-730

Gonzalez M, Zarzuelo A, Gamez MJ, Utrilla MP, Jimenez J and Osuna I (1992) Hypoglycemic activity of olive leaf. *Planta Med* 58(6):513-515

Gordon PB and Seglen PO (1988) Prelysosomal convergence of autophagic and endocytic pathways. *Biochem Biophys Res Commun* 151(1):40-47

Gordon S, Taylor PR (2005) Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5(12): 953-964

Goutte C, Tsunozaki M, Hale VA and Priess JR (2002) APH-1 is a multipass membrane protein essential for the Notch signaling pathway in *Caenorhabditis elegans* embryos. *Proc Natl Acad Sci USA* 99(2):775-779

Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, Sabanay H, Mizushima N, Yoshimori T and Kimchi A (2008) DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. *Cell Death Differ* 15(12):1875-1886

Green RC, Schneider LS, Amato DA, Beelen AP, Wilcock G, Swabb EA, Zavitz KH and Tarenflurbil Phase 3 Study Group (2009) Effect of tarenflurbil on cognitive decline and activities of daily

living in patients with mild Alzheimer disease: a randomized controlled trial. *JAMA* 302(23):2557-2564.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS and Wisniewski HM (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem* 261(13): 6084-6089

Gupta S, Rifici V, Crowley S, Brownlee M, Shan Z and Schlondorff D (1992) Interactions of LDL and modified LDL with mesangial cells and matrix. *Kidney Int* 41(5):1161-1169

Haass C, Hung AY, Schlossmacher MG, Oltersdorf T, Teplow DB and Selkoe DJ (1993) Normal cellular processing of the beta-amyloid precursor protein results in the secretion of the amyloid beta peptide and related molecules. Ann NY Acad Sci 695: 109-116.

Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK and Lippincott-Schwartz J (2010) Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* 141(4):656-667

Hajós M, Morozova E, Siok C, Atchison K, Nolan CE, Riddell D, Kiss T and Hajós-Korcsok E (2013) Effects of the γ-secretase inhibitor semagacestat on hippocampal neuronal network oscillation. *Front Pharmacol* 4:72

Halassa MM, Fellin T, Takano H, Dong JH and Haydon PG (2007) Synaptic islands defined by the territory of a single astrocyte. *J Neurosci* 27:6473-6477

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S and Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. *Nat Neurosci* 11(3):277-284

Hanger DP, Brion JP, Gallo JM, Cairns NJ, Luthert PJ and Anderton BH (1991) Tau in Alzheimer's disease and Down's sindrome is insoluble and abnormaly phosphorylated. *Biochem Journ* 275:99-104

Hara T, Takamura A, Kishi C, Iemura S, Natsume T, Guan JL and Mizushima N (2008) FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol* 181(3):497-510

Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC et al (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet* 41(10):1088-1093

Hardy J (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J Neurochem* 110(4):1129-1134

Hardy J and Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297(5580):353-356

Harvey RP (2002) Patterning the vertebrate heart. *Nat Rev Genet* 3(7):544-556

Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T and Yamamoto A (2009) A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nat Cell Biol* 11(12):1433-1437

He C and Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 43:67-93

He P, Zhong Z, Lindholm K, Berning L, Lee W, Lemere C, Staufenbiel M, Li R and Shen Y (2007) Deletion of tumor necrosis factor death receptor inhibits amyloid β generation and prevents learning and memory deficits in Alzheimer's mice. *J Cell Biol* 178(5):829-841

He C, Baba M, Cao Y and Klionsky DJ (2008) Self-interaction is critical for Atg9 transport and function at the phagophore assembly site during autophagy. *Mol Biol Cell* 19(12):5506-5516

He F, Umehara T, Saito K, harada T, watanabe S, Yabuki T, Kigawa T, Takahashi M, Kuwasako K, Tsuda K, matsuda T, Aoki M, Seki E, Kobayashi N, Güntert P, Yokoyama S and Muto Y (2010) Structural insight into the zinc finger CW domain as a histone modification reader. *Structure* 18(9):1127-1139

Hebert LE, Scherr PA, Beckett LA, Albert MS, Pilgrim DM, Chown MJ, Funkenstein HH and Evans DA (1995) Age-specific incidence of Alzheimer's disease in a community population. *JAMA* 273(17):1354-1359

Hebert LE, Scherr PA, Bienias JL, Bennett DA and Evans DA (2003) Alzheimer disease in the US population: prevalence estimates using the 2000 census. *Arch Neurol* 60(8):1119-1122

Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, Johnson AB, Kress Y, Vinters HV, tabaton M, Shimohama S, cash AD, Siedlak SL, harris PL, Jones PK, Petersen RB, Perry G and Smith MA (2001) Mitochondrial abnormalities in Alzheimer's disease. *J Neurosci* 21(9): 3017-3023

Hiramatsu M, Sasaki M and Kameyama T (1995) Effects of dynorphin A-(1-13) on carbon monoxide-induced delayed amnesia in mice studied in a step-down type passive avoidance task. *Eur J Pharmacol* 282(1-3):185-191

Holers VM (1996) Complement. *In Clinical immunology principles and practice (ed. Rich RR)*, pp.363-391 *Mosby-Year Book*, St. Louis, MO.

Holtzman DM (2004) In vivo effects of ApoE and clusterin on amyloid-beta metabolism and neuropathology. *J Mol Neurosci* 23(3):247-254

Honson NS, Johnson RL, Huang W, Inglese J, Austin CP and Kuret J (2007) Differentiating Alzheimer disease-associated aggregates with small molecules. *Neurobiol Dis* 28(3):251-260

Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, Bianchi K, Fehrenbacher N, Elling F, Rizzuto R et al. (2007) Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-β, and Bcl-2. *Mol Cell* 25(2):193-205

Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N et al. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex requie for autophagy. *Mol Biol Cell* 20(7):1981-1991

Hu WT, Chen-Plotkin A, Arnold SE, Grossman M, Clark CM, Shaw LM, Pickering E, Kuhn M, Chen Y, McCluskey L et al. (2010) Novel CSF biomarkers for Alzheimer's disease and mild cognitive impairment. *Acta Neuropathol* 119(6):669-678

Hu Z, Zhang J and Zhang Q (2011) Expression pattern and functions of autophagy-related gene atg5 in zebrafish organogenesis. *Autophagy* 7(12):1514-27

Huang Y, Lu W, Ali DW, Pelkey KA, Pitcher GM, Lu YM, Aoto H, Roder JC, sasaki T, salter MW and MacDonald JF (2001) CAKβ/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* 29(2):485-496

Huang Q, Wu YT, Tan HL, Ong CN and Shen HM (2009) A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. *Cell Death Differ* 16(2):264-277

Inoki K, Zhu T and Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115(5):577-590

Inoki K, Corradetti MN and Guan KL (2005) Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 37(1):19-24

INRAN - Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (2003) Linee guida per una Sana Alimentazione Italiana

Ishihara N, Hamasaki M, Yokota S, Suzuki K, Kamada Y, Kihara A, Yoshimori T, Noda T and Ohsumi Y (2001) Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. *Mol Biol Cell* 12(11):3690-3702

Itakura E, Kishi C, Inoue K and Mizushima N (2008) Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell* 19(12):5360-5372

Iwata A, Riley BE, Johnston JA and Kopito RR (2005) HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem* 280(48):40282-40292

Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E and Wyss-Coray T (2010) Regulation of amyloid precursor protein processing by the beclin 1 complex. *PLoS One* 5(6):e11102

Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P and Eskelinen EL (2004) Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* 117(Pt 20):4837-4848

Jahreiss L, Menzies FM and Rubinsztein DC (2008) The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. *Traffic* 9(4):574-587

Jantaratnotai N, Ryu JK, Kim SU and McLarnon JG (2003) Amyloid β peptide-induced corpus callosum damage and glial activation *in vivo*. *Neuroreport* 14(11):1429-1433

Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J et al. (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* 408(6815):979-982

Jiang H, Burdick D, Glabe CG, Cotman CW and Tenner AJ (1994) β-amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J Immunol* 152(10):5050-5059

Jick H, Zornberg GL, Jick SS, Seshadri S and Drachman DA (2000) Statins and the risk of dementia. *Lancet* 356(9242):1627-1631

Jimenez-Sanchez M, Menzies FM, Chang YY, Simecek N, Neufeld TP and Rubinsztein DC (2012) The Hedgehog signalling pathway regulates autophagy. *Nat Commun* 3:1200

John GR, Lee SC and Brosnan CF (2003) Cytokines: Powerful regulators of glial cell activation. *Neuroscientist* 9(1):10-22

Joseph JA, Shukitt-Hale B and Casadesus G (2005) Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Am J Clin Nutr* 81(1 Suppl):313S-316S

Joseph JA, Cole G, Head E and Ingram D (2009) Nutrition, brain aging, and neurodegeneration. *J Neurosci* 29(41):12795-12801

Juhasz G, Hill JH, Yan Y, Sass M, Baehrecke EH, Backer Jm and Neufeld TP (2008) The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. *J Cell Biol* 181(4):655-666

Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M and Kim DH (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* 20(7):1992-2003

Jung CH, Ro SH, Cao J, Otto NM and Kim DH (2010) mTOR regulation of autophagy. *FEBS Lett* 584(7):1287-1295

Juurlink BH (1997) Response of glial cells to ischemia: roles of reactive oxygen species and glutathione. *Neurosci Biobehav Rev* 21(2):151-166

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. *EMBO J* 19(21):5720-5728

Kabeya Y, Kawamata T, Suzuki K and Ohsumi Y (2007) Cis1/Atg31 is required for autophagosome formation in *Saccharomyce cerevisiae*. *Biochem Biophys Res Commun* 356(2):405-410

Kallberg Y, Gustafsson M, Persson B, Thyberg J and Johansson JJ (2001) Prediction of amyloid fibril-forming proteins. *J Biol Chem* 276(16):12945-12950

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* 150(6):1507-1513

Kanazawa T, Taneike I, Akaishi R, Yoshizawa F, Furuya N, Fujimura S and Kadowaki M (2004) Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes. *J Biol Chem* 279(9):8452-8459

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K and Müller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell surface receptor. *Nature* 325(6106):733-736

Katsel P, Tan W and Haroutunian V (2009) Gain in brain immunity in the oldest-old differentiates cognitively normal from demented individuals. *PLoS One* 4(10):e7642

Kayed R and Lasagna-Reeves CA (2013) Molecular mechanisms of amyloid oligomers toxicity. *J Alzheimers Dis* 33(Suppl 1):S67-78

Kawamata T, Kamada Y, Suzuki K, Kuboshima N, Akimatsu H, Ota S, Ohsumi M and Ohsumi, Y (2005) Characterization of a novel autophagy-specific gene, ATG29. *Biochem Biophys Res Commun* 338(4):1884-1889

Keys AB (1980) Seven countries: a multivariate analysis of death and coronary heart disease. *Cambridge, MA: Harvard University Press*

Keys AB and Keys M (1975) How to eat well and stay well, the Mediterranean way. *New York*

Keys A, Menotti A, Karvonen MJ, Aravanis C, Blackburn H, Buzina R, Djordjevic BS, Dontas AS, Fidanza F, Keys MH et al. (1986) The diet and 15-year death rate in the seven countries study. *Am J Epidemiol* 124(6):903-915

Kiffin R, Christian C, Knecht E and Cuervo AM (2004) Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell* 15(11):4829-4840

Kim E, Goraksha-Hicks P, Li L, Neufeld TP and Guan KL (2008) Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol* 10(8):935-945

Kim J, Huang W-P and Klionsky DJ (2001) Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and autophagy conjugation complex. *J Cell Biol* 152(1):51-64

Kim J, Huang WP, Stromhaug PE and Klionsky DJ (2002) Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to *de novo* vesicle formation. *J Biol Chem* 277(1):763-773

Kim HD, Tahara K, Maxwell JA, Lalonde R, Fukuiwa T, Fujihashi K, Van Kampen KR, Kong FK, Tang DC and Fukuchi K (2007) Nasal inoculation of an adenovirus vector encoding 11 tandem repeats

of Abeta1-6 upregulates IL-10 expression and reduces amyloid load in a Mo/Hu APPswe PS1dE9 mouse model of Alzheimer's disease. *J Gene Med* 9(2):88-98

Kinoshita A, Shah T, Tangredi MM, Strickland DK and Hyman BT (2003) The intracellular domain of the low density lipoprotein receptor-related protein modulates transactivation mediated by amyloid precursor protein and Fe65. J Biol Chem 278(42):41182-41188

Kirsch KH, Georgescu MM, Ishimaru S and Hanafusa H (1999) CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc Natl Acad Sci USA* 96(11):6211-6216

Klionsky DJ (2005) The molecular machinery of autophagy: Unanswered questions. *J Cell Sci* 118(Pt 1):7-18

Klionsky DJ (2007) Autophagy: From phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol* 8(11):931-937

Klionsky DJ and Ohsumi Y (1999) Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol* 15:1-32

Klionsky DJ, Cregg JM, Dunn Jr WA, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M and Ohsumi Y (2003) A unified nomenclature for yeast autophagy-related genes. *Dev Cell* 5(4):539-545

Klionsky DJ, Cuervo AM and Seglen PO (2007) Methods for monitoring autophagy from yeast to human. *Autophagy* 3(3):181-206

Knapp MJ, Knopman DS, Solomon PR, Pendlebury WW, Davis CS, Gracon SI (1994) A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. The Tacrine Study Group. *JAMA* 271(13):985-991

Köchl R, Hu XW, Chan EYW and Tooze SA (2006) Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. *Traffic* 7(2):129-145

Koistinaho M, Lin S, Wu X, Esterman M, Koger D, Hanson J, Higgs R, Liu F, Malkani S, Bales KR and Paul SM (2004) Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-β peptides. *Nat Med* 10(7):719-726

Koliatsos VE, Clatterbuck RE, Nauta HJ, Knusel B, Burton LE, Hefti FF, Mobley WC and Price DL (1991) Human nerve growth factor prevents degeneration of basal forebrain cholinergic neurons in primates. *Ann Neurol* 30(6):831-840

Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K and Chiba T (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 169(3):425-434

Kon M and Cuervo AM (2010) Chaperone-mediated autophagy in health and disease. *FEBS Lett.* 584(7):1399-1404

Konno K, Hirayama C, Yasui H and NakamuraM (1999) Enzymatic activation of oleuropein: a protein crosslinker used as chemical defence by privet tree. *Proc Natl Acad Sci USA* 96(16):9159-9162

Kopitz J, Kisen GO, Gordon PB, Bohley P and Seglen PO (1990) Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. *J Cell Biol* 111(3):941-953

Kordower JH, Winn SR, Liu YT, Mufson EJ, Sladek JR Jr, Hammang JP, Baetge EE and Emerich DF (1994) The aged monkey basal forebrain: rescue and sprouting of axotomized basal forebrain neurons after grafts of encapsulated cells secreting human nerve growth factor. *Proc Natl Acad Sci USA* 91(23):10898-10902

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Mufson EJ, Penn R, Goetz CG and Comella CD (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Ann Neurol* 46(3):419-424

Kornhuber J, Bormann J, Retz W, Hübers M and Riederer P (1989) Memantine displaces [3H]MK-801 at therapeutic concentrations in postmortem human frontal cortex. *Eur J Pharmacol* 166(3):589-590

Kostomoiri M, Fragkouli A, Sagnou M, Skaltsounis LA, Pelecanou M, Tsilibary EC and Tzinia AK (2013) Oleuropein, an anti-oxidant polyphenol constituent of olive promotes α-secretase cleavage of the amyloid precursor protein (AβPP). *Cell Mol Neurobiol* 33(1):147-154

Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E and Momoi T (2007) ER stress (PERK/eIF2α phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ* 14(2):230-239

Kromhout D, Keys A, Aravanis C, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M et al (1989) Food consumption patterns in the 1960s in seven countries. *Am J Clin Nutr* 49(5):889-894

Kubanis P and Zornetzer SF (1981) Age-related behavioral and neurobiological changes: a review with an emphasis on memory. *Behav Neural Biol* 31(2):115-172

Kushi LH, Lenart EB and Willett WC (1995) Health implications of Mediterranean diets in light of contemporary knowledge. Plant foods and dairy products; Meat, wine, fats, and oils. *Am J Clin Nutr* 61(6 Suppl):1407S-1427S

La Motta C, Sartini S, Mugnaini L, Simorini F, Taliani S, Salerno S, Marini AM, Da Settimo F, Lavecchia A, Novellino E, Cantore M, Failli P and Ciuffi M (2007) Pyrido[1,2-a]pyrimidin-4-one derivatives as a novel class of selective aldose reductase inhibitors exhibiting antioxidant activity. *J Med Chem* 50(20):4917-4927

LaFerla FM, Green KN and Oddo S (2007) Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci* 8(7):499-509

Lai K, Kaspar BK, Gage FH and Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation *in vitro* and *in vivo*. *Nat Neurosci* 6(1):21-27

Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C and Fahrenholz F (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci USA* 96(7):3922-3927

Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, Jun G, Destefano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thornton-Wells TA, Jones N, Smith AV, Chouraki V, Thomas C, Ikram MA, Zelenika D, Vardarajan BN, Kamatani Y, Lin CF, Gerrish A, Schmidt H, Kunkle B, Dunstan ML, Ruiz A, Bihoreau MT, Choi SH, Reitz C, Pasquier F, Hollingworth P, Ramirez A, Hanon O, Fitzpatrick AL, Buxbaum JD, Campion D, Crane PK, Baldwin C, Becker T, Gudnason V, Cruchaga C, Craig D, Amin N, Berr C, Lopez OL, De Jager PL, Deramecourt V, Johnston JA, Evans D, Lovestone S, Letenneur L, Morón FJ, Rubinsztein DC et al (2013) Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat Genet* 45(12):1452-1458

Landreth GE and Reed-Geaghan EG (2009) Toll-like receptors in Alzheimer's disease. *Curr Top Microbiol Immunol* 336: 137-153

Larsson M, Duffy DL, Zhu G, Liu JZ, Macgregor S, McRae AF, Wright MJ, Sturm RA, Mackey DA, Montgomery GW, Martin NG and Medland SE (2011) GWAS findings for human iris patterns: associations with variants in genes that influence normal neuronal pattern development. *Am J Hum Genet* 89(2):334-343

Làvadas E and Berti A (2002) Neuropsicologia. *Il Mulino, Bologna*

Le Meur N, Holder-Espinasse M, Jaillard S, Goldenberg A, Joriot S, Amati-Bonneau P, Guichet A, Barth M, Charollais A, Journel H et al. (2010) *MEF2C* haploinsufficiency caused by either

microdeletion of the 5q14.3 region or mutation is responsible for severe mental retardation with stereotypic movements, epilepsy and/or cerebral malformations. *J Med Genet* 47(1):22-29

Le Tutour B and Guedon D (1992) Antioxidant activities of *Olea europaea* leaves and related phenolic compounds. *Phytochemistry* 31:1173-1178

Leal MC, Dorfman VB, Gamba AF, Frangione B, Wisniewski T, Castano EM, Sigurdsson EM and Morelli L (2006) Plaque-associated overexpression of insulin-degrading enzyme in the cerebral cortex of aged transgenic tg2576 mice with Alzheimer pathology. *J Neuropathol* Exp Neurol 65(10):976-987

Lee JT, Xu J, Lee JM, Ku G, Han X, Yang DI, Chen S and Hsu CY (2004) Amyloid-β peptide induces oligodendrocyte death by activating the neutral sphingomyelinase-ceramide pathway. *J Cell Biol* 164(1):123-131

Lee SB, Kim S, Lee J, Park J, Lee G, Kim Y, Kim JM and Chung J (2007) ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. *EMBO Rep* 8(4):360-365

Lee JA and Gao FB (2009) Inhibition of autophagy induction delays neuronal cell loss caused by dysfunctional ESCRT-III in frontotemporal dementia. *J Neurosci* 29(26):8506-8511

Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, Wolfe DM, Martinez-Vicente M, Massey AC, Sovak G et al. (2010) Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell* 141(7):1146-1158

Lee JY, Koga H, Kawaguchi Y, Tang W, Wong E, Gao YS, Pandey UB, Kaushik S, Tresse E, Lu J et al. (2010) HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. *EMBO J* 29(5):969-980

Legakis JE, Yen WL and Klionsky DJ (2007) A cycling protein complex required for selective autophagy. *Autophagy* 3(5):422-432

Levine B and Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 6(4):463-477

Levine B and Kroemer G (2008) Autophagy in the pathogenesis of disease. *Cell* 132(1):27-42

Li R, Strohmeyer R, Liang Z, Lue LF and Rogers J (2004) CCAAT/enhancer binding protein delta (C/EBPdelta) expression and elevation in Alzheimer s disease. *Neurobiol Aging* 25(8):991-999

Li G, Larson EB, Sonnen JA, Shofer JB, Petrie EC, Schantz A, Peskind ER, Raskind MA, Breitner JC and Montine TJ (2007) Statin therapy is associated with reduced neuropathologic changes of Alzheimer disease. *Neurology* 69(9):878-885

Li J, Ni M, Lee B, Barron E, Hinton DR and Lee AS (2008) The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ* 15(9):1460-1471

Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH and Jung JU (2006) Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* 8(7):688-699

Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL et al. (2007) The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 9(2):218-224

Ling Y, Morgan K and Kalsheker N (2003) Amyloid precursor protein (APP) and the biology of proteolytic processing: relevance to Alzheimer's disease. *Int J Biochem Cell Biol* 35(11):1505-1535

Long X, Ortiz-Vega S, Lin Y and Avruch J (2005) Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J Biol Chem* 280(25):23433-23436

Lue LF, Rydel R, Brigham EF, Yang LB, Hampel H, Murphy GM, Brachova L, Yan SD, Walker DG, Shen Y et al. (2001a) Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia *in vitro*. *Glia* 35(1):72-79

Lue LF, Walker DG and Rogers J (2001b) Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures. *Neurobiol Aging* 22(6):945-956

Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T and Thompson CB (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 120(2):237-248

Luttazzi C, Denes G and Pizzamiglio L (1996) I disturbi del linguaggio nella demenza. Manuale di neuropsicologia. *Zanichelli, Bologna*

Ma Y and Hendershot LM (2001) The unfolding tale of the unfolded protein response. *Cell* 107(7):827-830

MacDonald BT, Tamai K and He X (2009) Wnt/β-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17(1):9-26

Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N et al. (2007) Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J* 26(10):2527-2539

Maiuri MC, Malik SA, Morselli E, Kepp O, Criollo A, Mouchel PL, Carnuccio R and Kroemer G (2009) Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle* 8(10):1571-1576

Majumdar A, Chung H, Dolios G, Wang R, Asamoah N, Lobel P and Maxfield FR (2008) Degradation of fibrillar forms of Alzheimer's amyloid β-peptide by macrophages. *Neurobiol Aging* 29(5):707-715

Mandel RJ (2010) CERE-110, an adeno-associated virus-based gene delivery vector expressing human nerve growth factor for the treatment of Alzheimer's disease. *Curr Opin Mol Ther* 12(2):240-247

Mandrekar-Colucci S, Landreth GE (2010) Microglia and inflammation in Alzheimer's disease. *CNS Neurol Disord Drug Targets* 9(2):156-167

Manning BD, Tee AR, Logsdon MN, Blenis J and Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/Akt pathway. *Mol Cell* 10(1):151-162

Maria Fimia G, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P et al. (2007) Ambra1 regulates autophagy and development of the nervous system. *Nature* 447(7148):1121-1125

Mariani Costantini A, Cannella C and Tommassi G (2006). Alimenti e nutrizione umana. Il Pensiero Scientifico Edizione, Roma.

Mattson FH and Grundy SM (1985) Comparison of effects of dietary saturated, monounsaturated and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J Lipid Res* 26(2):194-202

Markowska AL, Koliatsos VE, Breckler SJ, Price DL and Olton DS (1994) Human nerve growth factor improves spatial memory in aged but not in young rats. *J Neurosci* 14(8):4815-4824

Martinez-Vicente M and Cuervo AM (2007) Autophagy and neurodegeneration:when the cleaning crew goes on strike. *Lancet Neurol* 6(4):352-361

Massague J (2012) TGFβ signalling in context. *Nat Rev Mol Cell Biol* 13(10):616-630

Massey AC, Zhang C and Cuervo A (2006) Chaperone-mediated autophagy in aging and disease. *Curr Top Dev Biol* 73:205-235

Matsuura A, Tsukada M, Wada Y and Ohsumi Y (1997) Apg1p, a novel protein kinase requie for the autophagic process in *Saccharomyces cerevisiae*. *Gene* 192(2):245-250

McCoy MK, Martinez TN, Ruhn KA, Szymkowski DE, Smith CG, Botterman BR, Tansey KE and Tansey MG (2006) Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. *J Neurosci* 26(37):9365-9375

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI and Masters CL (1999) Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* 46(6):860-866

Menotti A, Keys A, Blackburn H, Kromhout D, Karvonen M, Nissinen A, Pekkanen J, Punsar S, Fidanza F, Giampaoli S et al. (1996) Comparison multivariate predictive power of major risk factors for coronary heart diseases in different countries: results from eight nations of the seven countries study, 25-year follow-up. *J Cardiov Risk* 3(1):69-75

Mensink RP and Katan MB (1987) Effect of monounsaturated fatty acids versus complex carbohydrates on high-density lipoproteins in healthy men and women. *Lancet* 1(8525):122-125

Mercer CA, Kaliappan A and Dennis PB (2009) A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy* 5(5):649-662

Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, de Calignon A, Rozkalne A, Koenigsknecht-Talboo J, Holtzman DM, Bacskai BJ and Hyman BT (2008) Rapid appearance and local toxicity of amyloid-β plaques in a mouse model of Alzheimer's disease. *Nature* 451(7179):720-724

Mills KR, Reginato M, Debnath J, Queenan B and Brugge JS (2004) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation *in vitro*. *Proc Natl Acad Sci* 101(10):3438-3443

Mishra R, Sellin D, Radovan D, Gohlke A and Winter R (2009) Inhibiting islet amyloid polypeptide fibril formation by the red wine compound resveratrol. *Chembiochem* 10(3):445-449

Mitew S, Kirkcaldie MT, Halliday GM, Shepherd CE, Vickers JC and Dickson TC (2010) Focal demyelination in Alzheimer's disease and transgenic mouse models. *Acta Neuropathol* 119(5):567-577

Mizushima N (2004) Methods for monitoring autophagy. *Int J Biochem Cell Biol* 36(12):2491-2502

Mizushima N (2005) The pleiotropic role of autophagy: from protein metabolism to bactericide. *Cell Death Differ* 12(Suppl. 2):1535-1541

Mizushima N (2007) Autophagy: process and function. *Gene Dev* 21(22):2861-2873

Mizushima N, Sugita H, Yoshimori T and Ohsumi Y (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J Biol Chem* 273(51):33889-33892

Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y and Yoshimori T (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 152(4):657-668

Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, Ohsumi Y and Yoshimori T (2003) Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci* 116(Pt 9):1679-1688

Mizushima N and Klionsky DJ (2007) Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr* 27:19-40

Mizushima N, Levine B, Cuervo AM and Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. *Nature* 451(7182):1069-1075

Mizushima N and Levine B (2010) Autophagy in mammalian development and differentiation. *Nat Cell Biol* 12(9):823-830

Moore FE, Reyon D, Sander JD, Martinez SA, Blackburn JS, Khayter C, Ramirez CL, Joung JK and Langenau DM (2012) Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). *PLoS One* 7(5):e37877

Mora A, Sabio G, González-Polo RA, Cuenda A, Alessi DR, Alonso JC, Fuentes JM, Soler G and Centeno F (2001) Lithium inhibits caspase 3 activation and dephosphorylation of PKB and GSK3 induced by K^+ deprivation in cerebellar granule cells. *J Neurochem* 78(1):199-206

Mordier S, Deval C, Bechet D, Tassa A and Ferrara M (2000) Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. *J Biol Chem* 275(38):29900-29906

Moreau K, Ravikumar B, Renna M, Puri C and Rubinsztein DC (2011) Autophagosome precursor maturation requires homotypic fusion. *Cell* 146(2):303-317

Moreau K, Renna M and Rubinsztein DC (2013) Connections between SNAREs and autophagy. *Trends Biochem Sci* 38(2):57-63

Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D et al. (2000) A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 408(6815):982-985

Moriyasu Y and Ohsumi Y (1996) Autophagy in tobacco suspension-cultured cells in response to sucrose starvation. *Plant Physiol* 111(4):1233-1241

Mortimore GE and Pösö AR (1987) Intracellular protein catabolism and its control during nutrient deprivation and supply. *Annu Rev Nutr* 7:539-564

Moscat J, Diaz-Meco MT and Wooten MW (2007) Signal integration and diversification through the p62 scaffold protein. *Trends Biochem Sci* 32(2):95-100

Moynagh PN (2005) The interleukin-1 signalling pathway in astrocytes: A key contributor to inflammation in the brain. *J Anat* 207(3):265-269

Mrak RE, Sheng JG and Griffin WS (1996) Correlation of astrocytic S100 β expression with dystrophic neurites in amyloid plaques of Alzheimer's disease. *J Neuropathol Exp Neurol* 55(3):273-279

Mulugeta E, Karlsson E, Islam A, Kalaria R, Mangat H, Winblad B and Adem A (2003) Loss of muscarinic M4 receptors in hippocampus of Alzheimer patients. *Brain Res* 960(1-2):259-262

Muñoz-Gamez JA, Rodríguez-Vargas JM, Quiles-Pérez R, Aguilar-Quesada R, Martin-Oliva D, de Murcia G, Menissier de Murcia J, Almendros A, Ruiz de Almodóvar M and Oliver FJ (2009) PARP-1 is involved in autophagy induced by DNA damage. *Autophagy* 5(1):61-74

Muzio L, Martino G and Furlan R (2007) Multifaceted aspects of inflammation in multiple sclerosis: the role of microglia. *J Neuroimmunol* 191(1-2):39-44

Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M et al. (2007) The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat Med* 13(5):619-624

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):165-178

Nair U, Jotwani A, Geng J, Gammoh N, Richerson D, Yen WL, Griffith J, Nag S, Wang K, Moss T, Baba M, McNew JA, Jiang X, Reggiori F, Melia TJ and Klionsky DJ (2011) SNARE proteins are required for macroautophagy. *Cell* 146(2):290-302

Nalls MA, Plagnol V, Hernandez DG, Sharma M, Sheerin UM, Saad M, Simón-Sánchez J, Schulte C, Lesage S, Sveinbjörnsdóttir S et al. (2011) Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. *Lancet* 377(9766):641-649

Namba Y, Tomonaga M, Kawasaki H, Otomo E and Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541(1):163-166

NDHSRG – National Diet Heart Study Research Group. National Diet Heart Study final report. Circulation 1968; 37(S):1-428

Netto CA and Izquierdo I (1985) On how passive is inhibitory avoidance. *Behav Neural Biol* 43(3):327-330

Nikolic WV, Bai Y, Obregon D, Hou H, Mori T, Zeng J, Ehrhart J, Shytle RD, Giunta B, Morgan D et al. (2007) Transcutaneous beta-amyloid immunization reduces cerebral beta-amyloid deposits without T cell infiltration and microhemorrhage. *Proc Natl Acad Sci USA* 104(7):2507-2512

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science* 308:1314-1318

Nixon RA (2007) Autophagy, amyloidogenesis and Alzheimer disease. *J Cell Sci* 120(Pt 23):4081-4091

Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A and Cuervo AM (2005) Extensive involvement of autophagy in Alzheimer's disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* 64(2):113-122

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL et al. (2005) Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci* 102(40):14238-14243

Noda T and Ohsumi Y (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 273(7):3963-3966

Noda NN, Kumeta H, Nakatogawa H, Satoo K, Adachi W, Ishii J, Fujioka Y, Ohsumi Y and Inagaki F (2008) Structural basis of target recognition by Atg8/LC3 during selective autophagy. *Genes Cells* 13(12):1211-1218

Novakovic D, Feligioni M, Scaccianoce S, Caruso A, Piccinin S, Schepisi C, Errico F, Mercuri NB, Nicoletti F, Nisticò R (2013) Profile of gantenerumab and its potential in the treatment of Alzheimer's disease. *Drug Des Devel Ther* 7:1359-64

Novikoff AB (1959) The proximal tubule cell in experimental hydronephrosis. *J Biophys Biochem Cytol* 6(1):136-138

Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S and Smith MA (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci* 19(6):1959-1964

Obara K, Sekito T, Niimi K and Ohsumi Y (2008) The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. *J Biol Chem* 283(35):23972-23980

Oberstein A, Jeffrey PD and Shi Y (2007) Crystal structure of the Bcl-XL–Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J Biol Chem* 282(17):13123-13132

Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K et al. (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 26(24):9220-9231

Okun E, Mattson MP, Arumugam TV (2010) Involvement of Fc receptors in disorders of the central nervous system. *Neuromolecular Med* 12(2):164-178

Ono K, Condron MM, Ho L, Wang J, Zhao W, Pasinetti GM and Teplow DB (2008) Effects of grape seed-derived polyphenols on amyloid beta-protein self-assembly and cytotoxicity. *J Biol Chem* 283(47):32176-32187

Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S et al. (2003) Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. *Neurology* 61(1):46-54

Orvedahl A, Alexander D, Tallóczy Z, Sun Q, Wei Y, Zhang W, Burns D, Leib DA and Levine B (2007) HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 1(1):23-35

Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B and Bartsch H (2000) Olive oil consumption and health: the possible role of antioxidants. *Lancet Oncol* 1:107-112

Palou A, Remesar X, Arola L, Herrera E and Alemany M (1981) Metabolic effects of short term food deprivation in the rat. *Horm Metab Res* 13(6):326-330

Pangalos MN, Jacobsen SJ and Reinhart PH (2005) Disease modifying strategies for the treatment of Alzheimer's disease targeted at modulating levels of the beta-amyloid peptide. *Biochem Soc Trans* 33: 553-558.

Pandey P, Avraham S, Kumar S, Nakazawa A, Place A, Ghanem L, Rana A, Kumar V, Majumder PK, Avraham H et al. (1999) Activation of p38 mitogen-activated protein kinase by PYK2/related adhesion focal tyrosine kinase–dependent mechanism. *J Biol Chem* 274(15):10140-10144

Pankiv S, Hoyvarde Clausen T, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn A, Bjørkøy G and Johansen T (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282(33):24131-24145

Papandreou I, Lim AL, Laderoute K and Denko NC (2008) Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. *Cell Death Differ* 15(10):1572-1581

Pardossi-Piquard R, Dunys J, Yu G, St George-Hyslop P, Alves da Costa C and Checler F (2006) Neprilysin activity and expression are controlled by nicastrin. *J Neurochem* 97(4):1052-1056

Passafiume D, Di Giacomo D and De Federicis LS (2002) Emotion processing nella demenza di Alzheimer. *Nuova rivista di neurologia* 12

Passafiume D and Di Giacomo D (2006) La demenza di Alzheimer. Guida all'intervento di stimolazione cognitiva e comportamentale. *Franco Angeli, Milano*

Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD and Levine B (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122(6):927-939

Paxinos G and Watson C (1997) The Rat Brain. Third Edition

Perri E, Raffaelli A and Sindona G (1999) Quantitation of oleuropein in virgin olive oil by ionspray mass spectrometry-selected reaction monitoring. *J Agric Food Chem* 47(10):4156-4160

Perrig WJ, Perrig P and Stähelin HB (1997) The relation between antioxidants and memory performance in the old and very old. *J Am Geriatr Soc* 45(6):718-724

Perry RH, Perry EK, Smith CJ, Xuereb JH, Irving D, Whitford CA, Candy JM and Cross AJ (1987) Cortical neuropathological and neurochemical substrates of Alzheimer's and Parkinson's diseases. *J Neural Transm Suppl* 24:131-136

Petersen RC (2004) Mild cognitive impairment as a diagnostic entity. *J Intern Med* 256(3):183-194

Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* 275(2):992-998

Petkov V and Manolov P (1978) Pharmacological studies on substances of plant origin with coronary dilatating and antiarrhythmatic action. *Comp Med East West* 6(2):123-130

Petralia RS, Schwartz CM, Wang YX, Kawamoto EM, Mattson MP and Yao PJ (2013) Sonic hedgehog promotes autophagy in hippocampal neurons. *Biol Open* 2(5):499-504

Petroni A, Blasevich M, Salami M, Papini N, Montedoro GF and Galli C (1995) Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb Res* 78(2):151-160

Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B and Wyss-Coray T (2008) The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J Clin Invest* 118(6):2190-2199

Pimplikar SW (2009) Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int J Biochem Cell Biol* 41(6):1261-1268

Pluskota E, Dowling JJ, Gordon N, Golden JA, Szpak D, West XZ, Nestor C, Ma YQ, Bialkowska K, Byzova T and Plow EF (2011) The integrin coactivator kindlin-2 plays a critical role in angiogenesis in mice and zebrafish. *Blood* 117(18):4978-4987

Porat Y, Abramowitz A and Gazit E (2006) Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. *Chem Biol Drug Des* 67(1):27-37

Postina R (2008) A closer look at alpha-secretase. *Curr Alzheimer Res* 5(2):179-186

Pottier C, Hannequin D, Coutant S, Rovelet-Lecrux A, Wallon D, Rousseau S, Legallic S, Paquet C, Bombois S et al. (2012) High frequency of potentially pathogenic *SORL1* mutations in autosomal dominant early-onset Alzheimer disease. *Mol Psychiatry* 17(9):875-879

Priault M, Salin B, Schaeffer J, Vallette FM, di Rago JP and Martinou JC (2005) Impairing the bioenergetic status and the biogenesis of mitochondria triggers mitophagy in yeast. *Cell Death Differ* 12(12):1613-1621

Prickaerts J, van Goethem NP, Chesworth R, Shapiro G, Boess FG, Methfessel C, Reneerkens OA, Flood DG, Hilt D, Gawryl M et al. (2012) EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacology* 62(2):1099-10110

Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, Cho DH, Choi B, Lee H, Kim JH et al. (2005) Essential Roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem* 280(21):20722-20729

Rasool CG, Svendsen CN and Selkoe DJ (1986) Neurofibrillary degeneration of cholinergic and noncholinergic neurons of the basal forebrain inAlzheimer's disease. *Ann Neurol* 20(4):482-488

Rasool S, Albay R 3rd, Martinez-Coria H, Breydo L, Wu J, Milton S, Misra S, Tran A, Pensalfini A, Laferla F et al. (2012) Vaccination with a non-human random sequence amyloid oligomer mimic

results in improved cognitive function and reduced plaque deposition and micro hemorrhage in Tg2576 mice. *Mol Neurodegener* 7:37

Raught B, Gingras AC and Sonenberg N (2001) The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci USA* 98(13):7037-7044

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ and Rubinsztein DC (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nature Genet* 36(6):585-595

Ravikumar B, Moreau K, Jahreiss L, Puri C and Rubinsztein DC (2010) Plasma membrane contributes to the formation of pre-autophagosomal structure. Nat Cell Biol 12(8):747-757

Raya A and Izpisua Belmonte JC (2006) Left–right asymmetry in the vertebrate embryo: from early information to higher-level integration. *Nat Rev Genet* 7(4):283-293

Reaven P, Parthasarathy S, Grasse BJ, Miller E, Almazan F, Mattson FH, Khoo JC, Steinberg D and Witztum JL (1991) Feasibility of using oleate-rich diet to reduce the susceptibility of LDL to oxidative modification in humans. *Am J Clin Nutr* 54(4):701-706

Reef S, Zalckvar E, Shifman O, Bialik S, Sabanay H, Oren M and Kimchi A (2006) A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. *Mol Cell* 22(4):463-475

Reggiori F, Monastyrska I, Shintani T and Klionsky DJ (2005) The actin cytoskeleton is required for selective types of autophagy, but not nonspecific autophagy, in the yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* 16(12):5843-5856

Reggiori F, Tucker KA, Stromhaug PE and Klionsky DJ (2004) The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev Cell* 6(1):79-90

Reggiori F, Wang C-W, Nair U, Shintani T, Abeliovich H and Klionsky DJ (2004) Early stages of the secretory pathway, but not endosomes, are required for Cvt vesicle and autophagosome assembly in *Saccharomyces cerevisiae*. *Mol Biol Cell* 15(5):2189-2204

Rezaie P and Male D (2002) Mesoglia & microglia–a historical review of the concept of mononuclear phagocytes within the central nervous system. *J Hist Neurosci* 11(4):325-374

Ribeiro Rde A, Fiuza de Melo MM, De Barros F, Gomes C and Trolin G (1986) Acute antihypertensive effect in conscious rats produced by some medicinal plants used in the state of Sao Paulo. *J Ethnopharmacol* 15(3):261-269

Rigacci S, Guidotti V, Bucciantini M, Parri M, Nediani C, Cerbai E, Stefani M and Berti A (2010) Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin. *J Nutr Biochem* 21(8):726-735

Rigacci S, Guidotti V, Bucciantini M, Nichino D, Relini A, Berti A and Stefani M (2011) Aβ(1-42) aggregates into non-toxic amyloid assemblies in the presence of the natural polyphenol oleuropein aglycon. *Curr Alzheimer Res* 8(8):841-852

Rivière C, Richard T, Quentin L, Krisa S, Mérillon JM and Monti JP (2007) Inhibitory activity of stilbenes on Alzheimer's beta-amyloid fibrils *in vitro*. *Bioorg Med Chem* 15(2):1160-1167

Rodriguez JJ, Olabarria M, Chvatal A and Verkhratsky A (2009) Astroglia in dementia and Alzheimer's disease. *Cell Death Differ* 16(3):378-385

Rogaeva E, Meng Y, Lee JH, Gu Y, Kawarai T, Zou F, Katayama T, Baldwin CT, Cheng R, Hasegawa H et al. (2007) The neuronal sortilin-related receptor *SORL1* is genetically associated with Alzheimer disease. *Nat Genet* 39(2):168-177

Rogers J, Luber-Narod J, Styren SD and Civin WH (1988) Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol Aging* 9(4):339-349

Rogers J, Mastroeni D, Leonard B, Joyce J and Grover A (2007) Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? *Int Rev Neurobiol* 82:235-246

Roth M, Tomlinson BE and Blessed G (1966) Correlation between scores for dementia and counts of 'senile plaques' in cerebral grey matter of elderly subjects. *Nature* 209(5018):109-110

Roth AD, Ramirez G, Alarcon R and Von Bernhardi R (2005) Oligodendrocytes damage in Alzheimer's disease: β amyloid toxicity and inflammation. *Biol Res* 38(4):381-387

Rubinsztein DC, Codogno P and Levine B (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discovery* 11:709-730

Rusten TE, Vaccari T, Lindmo K, Rodahl LM, Nezis IP, Sem-Jacobsen C, Wendler F, Vincent JP, Brech A, Bilder D and Stenmark H (2007) ESCRTs and Fab1 regulate distinct steps of autophagy. *Curr Biol* 17(20):1817-1825

Sagné C, Agulhon C, Ravassard P, Darmon M, Hamon M, El Mestikawy S, Gasnier B and Giros B (2001) Identification and characterization of a lysosomal transporter for small neutral amino acids. *Proc Natl Acad Sci* 98(13):7206-7211

Sahu A and Lambris J (2001) Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev* 180:35-48

Sakaki K,Wu J and Kaufman RJ (2008) Protein kinase Cθ is required for autophagy in response to stress in the endoplasmic reticulum. *J Biol Chem* 283(22):15370-15380

Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L and Sabatini DM (2008) The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320(5882):1496-1501

Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK and Yeh JR (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol* 29(8):697-698

Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M and Wang J (2008) Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 454(7201):232-235

Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S and Green DR (2007) Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 450(7173):1253-1257

Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E et al. (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* 336(17):1216-1222

Sano M, Bell KL, Galasko D, Galvin JE, Thomas RG, van Dyck CH and Aisen PS (2011) A randomized, double-blind, placebo-controlled trial of simvastatin to treat Alzheimer disease. *Neurology* 77(6):556-563

Santiago-Mora R, Casado-Díaz A, De Castro MD and Quesada-Gómez JM (2011) Oleuropein enhances osteoblastogenesis and inhibits adipogenesis: the effect on differentiation in stem cells derived from bone marrow. *Osteoporos Int* 22(2):675-684

Santos RX, Correia SC, Wang X, Perry G, Smith MA, Moreira PI and Zhu X (2010) A synergistic dysfunction of mitochondrial fission/fusion dynamics and mitophagy in Alzheimer's disease. *J Alzheimers Dis* 20(Suppl 2):S401-S412

Sarbassov DD, Ali SM and Sabatini DM (2005) Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 17(6):596-603

Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, Cook LJ and Rubinsztein DC (2005) Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol* 170(7):1101-1111

Sarkar S, Perlstein EO, Imarisio S, Pineau S, Cordenier A, Maglathlin RL, Webster JA, Lewis TA, O'Kane CJ, Schreiber SL and Rubinsztein DC (2007) Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. *Nat Chem Biol* 3(6):331-338

Sarkar S and Rubinsztein DC (2008) Small molecule enhancers of autophagy for neurodegenerative diseases. *Mol Biosyst* 4(9):895-901

Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA et al. (2011) Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 476(7359):214-219

Scarmeas N, Stern Y, Tang M, Mayeux R and Luchsinger JA (2006) Mediterranean diet and risk for Alzheimer's disease. *Ann Neurol* 59(6):912-921

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K et al. (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400(6740):173-177

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 J* 26(7):1749-1760

Schmelzle T, Beck T, Martin DE and Hall MN (2004) Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol Cell Biol* 24(1):338-351

Schworer CM, Shiffer KA and Mortimore GE (1981) Quantitative relationship between autophagy and proteolysis during graded amino acid deprivation in perfused rat liver. *J Biol Chem* 256(14):7652-7658

Scislowski V, Bauchart D, Gruffat D, Laplaud PM and Durand D (2005) Effect of dietary n-6 and n-3 polyunsaturated fatty acids on peroxidizability of lipoproteins in steers. *Lipids* 40(12):1245-1256

Sedgwick JD, Schwender S, Imrich H, Dorries R, Butcher GW and ter Meulen V (1991) Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci* 88(16):7438-7442

Seglen PO (1987) Lysosomes: their role in protein breakdown . *eds Glaumann H and Ballard FJ* 371-414 *Academic Press*, Florida

Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* 6(4):487-98

Selkoe DJ (1994) Normal and abnormal biology of the b-amyloidprecursor protein. *Annual Review Neuroscience* 17:489-517

Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399(6738 Suppl):A23-A31

Selkoe DJ (2001) Clearing the brain's amyloid cobwebs. *Neuron* 32(2):177-180

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. *Science* 298(5594):789-791

Serra-Majem L, Roman B and Estruch R (2006) Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev* 64(2 Pt 2):S27-S47

Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro G and Morozzi G (2004) Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J Chromatogr A* 1054(1-2):113-27

Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE and O'Banion MK (2007) Sustained hippocampal IL-1 β overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. *J Clin Invest* 117(6):1595-1604

Shen Y, Lue L-F, Yang L-B, Roher A, Kuo Y-M, Strohhmeyer R, Goux WJ, Lee V, Johnson GVW, Webster SD et al. (2001) Complement activation by neurofibrillary tangles in Alzheimer's disease. *Neurosci Lett* 305(3):165-168

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375(6534):754-760

Shintani T, Huang WP, Stromhaug PE and Klionsky DJ (2002) Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. *Dev Cell* 3(6):825-837

Shintani T and Klionsky DJ (2004) Autophagy in health and disease: a double-edged sword. *Science* 306(5698):990-995

Singh SB, Davis AS, Taylor GA and Deretic V (2006) Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 313(5792):1438-1441

Singh B, Parsaik AK, Mielke MM, Erwin PJ, Knopman DS, Petersen RC and Roberts RO (2014) Association of Mediterranean diet with mild cognitive impairment and Alzheimer's disease: a systematic review and meta-analysis. *J Alzheimers Dis* 39:271-282

Sir D, Chen WL, Choi J,Wakita T, Yen TS and Ou JH (2008) Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. *Hepatology* 48(4):1054-1061

Small GW, Rabins PV, Barry PP, Buckholtz NS, DeKosky ST, Ferris SH, Finkel SI, Gwyther LP, Khachaturian ZS, Lebowitz BD et al. (1997) Diagnosis and treatment of Alzheimer disease and related disorders. Consensus statement of the American Association for Geriatric Psychiatry, the Alzheimer's Association, and the American Geriatrics Society. *JAMA* 278(16):1363-1371

Smith DE, Roberts J, Gage FH and Tuszynski MH (1999) Age-associated neuronal atrophy occurs in the primate brain and is reversible by growth factor gene therapy. *Proc Natl Acad Sci USA* 96(19):10893-10898

Sofi F, Abbate R, Gensini GF and Casini A (2010) Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. *Am J Clin Nutr* 92(5):1189-1196

Sofroniew MV and Vinters HV (2010) Astrocytes: Biology and pathology. *Acta Neuropathol* 119:7-35

Soler-Rivas C, Espin JC and Wichers HJ (2000) Oleuropein and related compounds. *J Sci Food Agric* 80(7):1013-1023

Solfrizzi V, Panza F, Torres F, Mastroianni F, Del Parigi A, Venezia A and Capurso A (1999). High monounsaturated fatty acids intake protects against age-related cognitive decline. *Neurology* 52(8):1563-1569

Solfrizzi V, Panza F, Frisardi V, Seripa D, Logroscino G, Imbimbo BP and Pilotto A (2011) Diet and Alzheimer's disease risk factors or prevention: the current evidence. *Expert Rev Neurother* 11(5):677-708

Solomon B (2007) Intravenous immunoglobulin and Alzheimer's disease immunotherapy. *Curr Opin Mol Ther* 9(1):79-85

Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R and Galvan V (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One* 5(4):e9979

Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI and O'Callaghan J (2002) Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *FASEB J* 16(11):1474-1476

Stahl SM (2000) The new cholinesterase inhibitors for Alzheimer's disease, part 2: illustrating their mechanisms of action. *J Clin Psychiatry* 61(11):813-814

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F and Hawkins PT (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 277(5325):567-570

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS and Roses AD (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 90(50):1977-1981

Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Magnusson KP, Manolescu A, Karason A, Palsson A, Thorleifsson G et al. (2007) Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet* 39(12):1443-1452

Suriapranata I, Epple UD, Bernreuther D, Bredschneider M, Sovarasteanu K and Thumm M (2000) The breakdown of autophagic vesicles inside the vacuole depends on Aut4p. *J Cell Sci* 113(Pt 22):4025-4033

Suzuki K and Ohsumi Y (2007) Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae. FEBS Lett* 581(11):2156-2161

Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T and Ohsumi Y (2001) The pre-autophagosomal structure organized by concerted functions of *APG* genes is essential for autophagosome formation. *EMBO J* 20(21):5971-5981

Suzuki K, Kubota Y, Sekito T and Ohsumi Y (2007) Hierarchy of Atg proteins in pre-autophagosomal structure organization. *Genes Cells* 12(2):209-218

Suzuki HI, Kiyono K and Miyazono K (2010) Regulation of autophagy by transforming growth factor-β (TGF-β) signaling. *Autophagy* 6(5):645-647

Tabata T (2001) Genetics of morphogen gradients. *Nat Rev Genet* 2(8):620-630

Tagliavini F, Giaccone G, Frangione B and Bugiani O (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. *Neurosci Lett* 93(2-3):191-196

Takeshige K, Baba M, Tsuboi S, Noda T and Ohsumi Y (1992) Autophagy in yeast demonstrated with proteinasi-deficient mutants and conditions for its induction. *J Cell Biol* 119(2):301-311

Tallóczy Z, Jiang W, Virgin HWT, Leib DA, Scheuner D, Kaufman RJ, Eskelinen EL and Levine B (2002) Regulation of starvation- and virus-induced autophagy by the eIF2 kinase signaling pathway. *Proc Natl Acad Sci* 99(1):190-195

Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, Mattson MP, Flavell RA and Mullan M (1999) Microglial activation resulting from CD40-CD40L interaction after β-amyloid stimulation. *Science* 286:2352-2355

Tan HW, Tuck KL, Stupans I and Hayball PJ (2003) Simultaneous determination of oleuropein and hydroxytyrosol in rat plasma using liquid chromatography with fluorescence detection. *J Chromatogr B* 785:187-191

Tanida I, Minematsu-Ikeguchi N, Ueno T and Kominami E (2005) Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy* 1(2):84-91

Tanzi RE, Kovacs DM, Kim TW, Moir RD, Guenette SY and Wasco W (1996) The gene defects responsible for familial Alzheimer's disease. *Neurobiol Dis* 3(3):159-168

Tanzi RE, Moir RD and Wagner SL (2004) Clearance of Alzheimer's Abeta peptide: the many roads to perdition. Neuron 43(5):605-608

Tariot PN, Farlow MR, Grossberg GT, Graham SM, McDonald S and Gergel I (2004) Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. *JAMA* 291(3):317-324

Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, Criollo A, Morselli E, Zhu C, Harper F et al. (2008) Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol* 10(6):676-687

Tassa A, Roux MP, Attaix D and Bechet DM (2003) Class III phosphoinositide 3-kinase–Beclin1 complex mediates the amino acid-dependent regulation of autophagy in C2C12 myotubes. *Biochem J* 376(Pt 3):577-586

Taylor PR, Carugati A, Fadok VA, Cook HT, Andrews M, Carroll MC, Savill JS, Henson PM, Botto M and Walport MJ (2000) A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells*in vivo*. *J Exp Med* 192(3):359-366

Tenner AJ (1999) Membrane receptors for soluble defense collagens. *Curr Opin Immunol* 11(1):34-41

Tesseur I, Zou K, Esposito L, Bard F, Berber E, Can JV, Lin AH, Crews L, Tremblay P, Mathews P, et al. (2006) Deficiency in neuronal TGF-β signaling promotes neurodegeneration and Alzheimer's pathology. *J Clin Invest* 116(11):3060-3069

Teter SA, Eggerton KP, Scott SV, Kim J, Fischer AM and Klionsky DJ (2001) Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J Biol Chem* 276(3):2083-2087

TJDA - The Japan Dietetic Association (1992) Nutrition and dietetics in Japan. 6th ed. Tokyo: The Japan Dietetic Association

Tracy K and Baehrecke EH (2013) The role of autophagy in Drosophila metamorphosis. *Curr Top Dev Biol* 103:101-25

Tracy K, Dibling BC, Spike BT, Knabb JR, Schumacker P and Macleod KF (2007) BNIP3 is an RB/E2F target gene required for hypoxia-induced autophagy. *Mol Cell Biol* 27(17):6229-6242

Trichopoulou A, Costacou T, Bamia C and Trichopoulos D (2003) Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med* 348(26):2599-2608

Tsukada M and Ohsumi Y (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae. FEBS Lett* 333(1-2):169-174

Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A and Mizushima N (2008a) Autophagy is essential for preimplantation development of mouse embryos. *Science* 321(5885):117-120

Tsukamoto S, Kuma A and Mizushima N (2008b) The role of autophagy during the oocyte-to-embryo transition. *Autophagy* 4(8):1076-1078

Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G et al. (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med* 11(5):551-555

Ulbricht TL and Southgate DA (1991) Coronary heart disease: seven dietary factors. *Lancet* 338(8773):985-92

USDA - US Department of Health and Human Services, US Department of Agriculture (2005) Dietary guidelines for Americans 2005. 6th ed. Washington, DC: US Government Printing Office (available at *www.healthierus.gov/dietaryguidelines*)

van der Vaart A, Griffith J and Reggiori F (2010) Exit from the golgi is required for the expansion of the autophagosomal phagophore in yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* 21(13):2270-2284

Visioli F, Bellomo G, Montedoro GF and Galli C (1995) Low-density lipoprotein oxidation is inhibited *in vitro* by olive oil constituents. *Atherosclerosis* 117(1):25-32

Visioli F, Bellosta S and Galli C (1998) Oleuropein, the bitter principles of olives, enhances nitric oxide production by mouse macrophages. *Life Sci* 62(6):541-546

Visioli F, Poli A and Galli C (2002) Antioxidant and other biological activities of phenols from olives and olive oil. *Med Res Rev* 22(1):65-75

Vissers MN, Zock PL, Roodenburg AJC, Leenen R and Katan MB (2002) Olive oil phenols are absorbed in humans. *J Nutr* 132(3):409-417

Vissers MN, Zock PL and Katan MB (2004) Bioavailability and antioxidant effects of olive oil phenols in humans: a review. *Eur J Clin Nutr* 58(6):955-965

Wake H, Moorhouse AJ, Jinno S, Kohsaka S and Nabekura J (2009) Resting microglia directly monitor the functional state of synapses *in vivo* and determine the fate of ischemic terminals. *J Neurosci* 29(13):3974-3980

Walker DG and Lue LF (2005) Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases. *J Neurosci Res* 81(3):412-425

Walker DG, Lue LF and Beach TG (2001) Gene expression profiling of amyloid β peptide stimulated human post-mortem brain microglia. *Neurobiol Aging* 22(6):957-966

Wang Z, Wilson WA, Fujino MA and Roach PJ (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol Cell Biol* 21(17):5742-5752

Wang X, Su B, Fujioka H and Zhu X (2008) Dynamin-like protein 1 reduction underlies mitochondrial morphology and distribution abnormalities in fibroblasts from sporadic Alzheimer's disease patients. *Am J Pathol* 173(2):470-482

White JA, Manelli AM, Holmberg KH, Van Eldik LJ and Ladu MJ (2005) Differential effects of oligomeric and fibrillar amyloid-β 1-42 on astrocyte-mediated inflammation. *Neurobiol Dis* 18(3):459-465

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT and Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* 215(4537):1237-1239

Whitehouse PJ (2006) Quality of life: the bridge from the cholinergic basal forebrain to cognitive science and bioethics. *J Alzheimers Dis* 9(3 Suppl):447-453

Willet WC (1994) Diet and health. What should we eat? *Science* 264(5158):532-537

Willett WC (2006) The Mediterranean diet: science and practice. *Public Health Nutr* 9(1A):105-110

Willet WC, Sacks F, Trichopoulou A, Drescher G, Ferro.Luzzi A, Helsing E and Trichopoulos D (1995) Mediterranean diet pyramid: a cultural model for healthy eating. *Am J Clin Nutr* 61(6 Suppl):1402S-1406S

Williams A, Jahreiss L, Sarkar S, Saiki S, Menzies FM, Ravikumar B and Rubinsztein DC (2006) Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. *Curr Top Dev Biol* 76:89-101

Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, Jahreiss L, Fleming A, Pask D, Goldsmith P, O'Kane CJ, Floto RA and Rubinsztein DC (2008) Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nat Chem Biol* 4(5):295-305

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K and Bayer TA (2001) Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. *Neurosci Lett* 306(1-2):116-120

World Cancer Research Fund (1997). Food, nutrition and the prevention of cancer: a global perspective. *American Institute for Cancer Prevention* Washington DC

Wu X, Won H and Rubinsztein DC (2013) Autophagy and mammalian development. Biochem Soc Trans 41(6):1489-94

Wyss-Coray T (2006) Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med* 12(9):1005-1015

Wyss-Coray T and Rogers J (2012) Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature. *Cold Spring Harb Perspect Med* 2(1):a006346

Wyss-Coray T, Lin C, Yan F, Yu G, Rohde M, McConlogue L, Masliah E, Mucke L (2001) TGF-β1 promotes microglial amyloid-β clearance and reduces plaque burden in transgenic mice. *Nat Med* 7(5):612-618

Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC and Husemann J (2003) Adult mouse astrocytes degrade amyloid-β *in vitro* and *in situ*. *Nat Med* 9(4):453-457

Xie Z, Nair U and Klionsky DJ (2008) Atg8 controls phagophore expansion during autophagosome formation. *Mol Biol Cell* 19(8):3290-3298

Wu X, Won H and Rubinsztein DC (2013) Autophagy and mammalian development. *Biochem Soc Trans* 41(6):1489-1494

Xu J, Chen S, Ahmed SH, Chen H, Ku G, Goldberg MP and Hsu CY (2001) Amyloid-β peptides are cytotoxic to oligodendrocytes. *J Neurosci* 21(1):RC118

Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE and Eissa NT (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* 27(1):135-144

Xu G, Fitzgerald ME, Wen Z, Fain SB, Alsop DC, Carroll T, Ries ML, Rowley HA, Sager MA, Asthana S, Johnson SC and Carlsson CM (2008) Atorvastatin therapy is associated with greater and faster cerebral hemodynamic response. *Brain Imaging Behav* 2(2):94

Yamaguchi H, Hirai S, Morimatsu M, Shoji M and Harigaya Y (1988) Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathol* 77(2):113-119

Yan P, Bero AW, Cirrito JR, Xiao Q, Hu X, Wang Y, Gonzales E, Holtzman DM and Lee JM. (2009) Characterizing the appearance and growth of amyloid plaques in APP/PS1 mice. *J Neurosci* 29(34):10706-10714

Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kayed R, Glabe CG, Frautschy SA and Cole GM (2005) Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*. *J Biol Chem* 280(7):5892-5901

Yang Z, Huang J, Geng J, Nair U and Klionsky DJ (2006) Atg22 recycles amino acids to link the degradative and re cycling functions of autophagy. *Mol Biol Cell* 17(12):5094-5104

Yano T, Mita S, Ohmori H, Oshima Y, Fujimoto Y, Ueda R, Takada H, Goldman WE, Fukase K, Silverman N, Yoshimori T and Kurata S (2008) Autophagic control of Listeria through intracellular innate immune recognition in *Drosophila*. *Nat Immunol* 9(8):908-916

Yen WL, Shintani T, Nair U, Cao Y, Richardson BC, Li Z, Hughson FM, Baba M, Klionsky DJ (2010) The conserved oligomeric Golgi complex is involved in double-membrane vesicle formation during autophagy. *J Cell Biol* 188(1):101-114

Yin KJ, Cirrito JR, Yan P, Hu X, Xiao Q, Pan X, Bateman R, Song H, Hsu FF, Turk J et al. (2006) Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism. *J Neurosci* 26(43):10939-10948

Ylä-Anttila P, Vihinen H, Jokitalo E and Eskelinen EL (2009) 3D tomography reveals connections between the phagophore and endoplasmic reticulum. *Autophagy* 5(8):1180-1185

Yorimitsu T, He C, Wang K and Klionsky DJ (2009) Tap42-associated protein phosphatase type 2A negatively regulates induction of autophagy. *Autophagy* 5(5):616-624

Yorimitsu T, Nair U, Yang Z and Klionsky DJ (2006) Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 281(40):30299-30304

Yorimitsu T, Zaman S, Broach JR and Klionsky DJ (2007) Protein kinase A and Sch9 cooperatively regulate induction of autophagy in *Saccharomyces cerevisiae*. *Mol Biol Cell* 18(10):4180-4189

Yoshimoto K, Hanaoka H, Sato S, Kato T, Tabata S, Noda T and Ohsumi Y (2004) Processing of ATG8s, ubiquitinlike proteins, and their deconjugation by ATG4s are essential for plant autophagy. *Plant Cell* 16(11):2967-2983

Yorimitsu T and Klionsky DJ (2005) Autophagy: molecular machinery for self-eating. *Cell Death Differ* 12 Suppl 2:1542-1552

Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, Mohan PS, Mercken M, Farmery MR, Tjernberg LO et al. (2005) Macroautophagy - a novel β-amyloid peptide-generating pathway activated in Alzheimer's disease. *J Cell Biol* 171(1):87-98

Yue Z, Jin S, Yang C, Levine AJ and Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci* 100(25):15077-15082

Zaman S, Lippman SI, Zhao X and Broach JR (2008) How *Saccharomyces* responds to nutrients. *Annu Rev Genet* 42:27-81

Zandi PP, Anthony JC, Khachaturian AS, Stone SV, Gustafson D, Tschanz JT, Norton MC, Welsh-Bohmer KA, Breitner JC and Cache County Study Group (2004) Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. *Arch Neurol* 61(1):82-88

Zhai P and Sadoshima J (2012) Glycogen synthase kinase-3β controls autophagy during myocardial ischemia and reperfusion. *Autophagy* 8(1):138-139

Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ and Semenza GL (2008) Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 283(16):10892-10903

Zhu CW and Sano M (2006) Economic considerations in the management of Alzheimer's disease. *Clin Interv Aging* 1(2):143-154

Zornetzer SF, Thompson R and Rogers J (1982) Rapid forgetting in aged rats. *Behav Neural Biol* 36(1):49-60

ACKNOWLEDGEMENT

Ringraziamenti – Acknowledgement

D*esidero ringraziare le persone che hanno contribuito al raggiungimento di questo importante traguardo e che mi hanno sostenuto, motivato e incoraggiato.*

Ringrazio la mia famiglia: mio padre per la fiducia incondizionata e mia mamma per avermi lasciata andare lontano, prima a Firenze e poi a Cambridge. Ringrazio Stefania, per avermi sostenuta, spronata e anche coccolata;grazie tante a Simone per l'estrema disponibilità e i sempre lucidi consigli; a Davide per l'affetto profondo, a Sara, a Carlo e ai piccoli Fabio e Lisa, nonché la piccolissima Sofia.

Un sentito ringraziamento a chi ha reso possibile questo lavoro: la Prof.ssa Casamenti per avermi accolto nel suo laboratorio, per i suoi insegnamenti e la sua disponibilità; inoltre ringrazio per l'incoraggiamento ad intraprendere l'avventura inglese i professori Casamenti, Pedata e Moroni.

La Prof.ssa Failli per i consigli e gli utili suggerimenti.

Ringrazio le persone che mi hanno affiancato nel lavoro sperimentale di questa tesi e da cui ho imparato molto: Chiara, Cristina, Ilaria e Stefano; i tesisti la cui curiosità e voglia di imparare sono stati molto stimolanti: Heather, Barom, Annamaria, Francesca, Daniela, Maria e le indimenticabili "Brindisi e Maddaluno". Ho potuto lavorare in una atmosfera piacevole grazie a Lucrezia, Alessia, Daniele, Giovanna, Francesca, Elisabetta e le Professoresse Pugliese, Giovannini e Matucci.

I want to thank Professor Rubinsztein for giving me the opportunity to work for 7 months in his group, in the zebrafish unit; thanks to Dr. Fleming for being a wonderful supervisor; thanks to Ana, Marcia, Sarah, Ben, Catherine, Els, Bartek, Steffi and Tomasz for sharing with me nice moments in the lab and during the several "tea-time".

A special thanks to Penny, wonderful landlady and lovely housemate, for being by my side especially during my first and difficult weeks in Cambridge.

In queste poche righe non riesco a citare tutte le persone che hanno contribuito alla mia formazione, ma non le dimentico e a tutti loro va un grazie sincero.