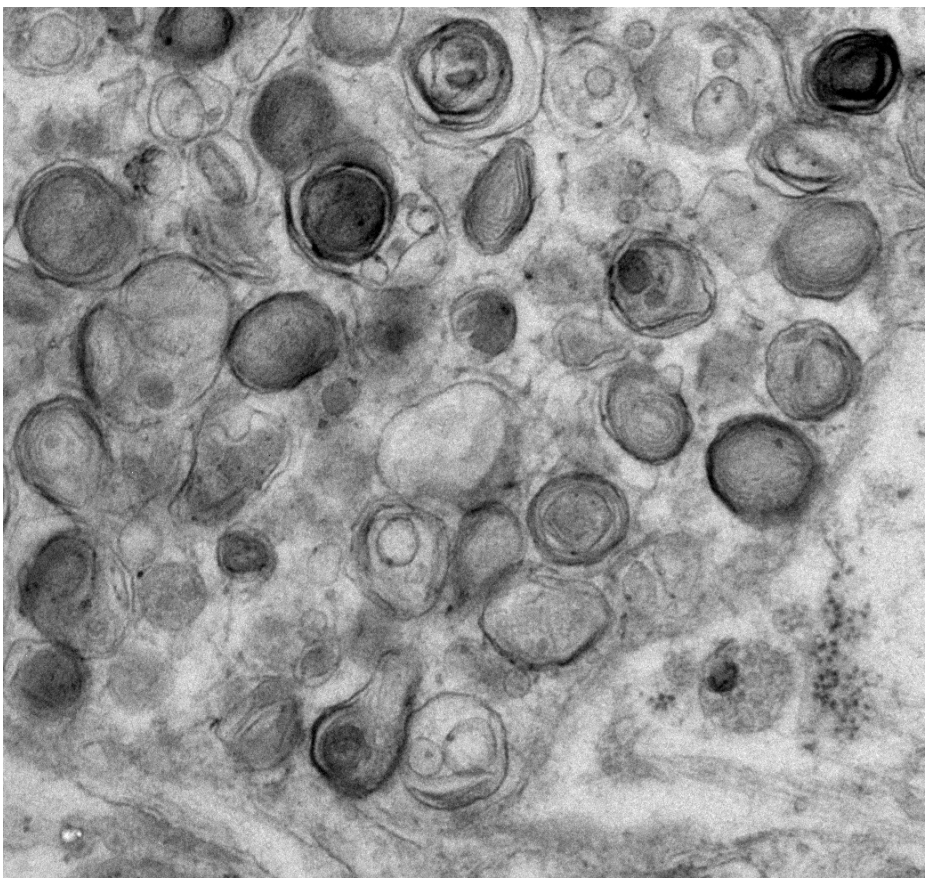
Thesis for doctoral degree (Ph.D.)
2022

Interplay between autophagy and amyloid beta metabolism in Alzheimer's disease



Richeng Jiang,
姜日成,
강일성

Karolinska Institutet

From Department of Neurobiology, Care Sciences and Society
Karolinska Institutet, Stockholm, Sweden

INTERPLAY BETWEEN AUTOPHAGY AND AMYLOID BETA METABOLISM IN ALZHEIMER'S DISEASE

Richeng Jiang

姜日成

강일성

Karolinska Institutet

Stockholm 2022

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022

ISBN 978-91-8016-649-2
Cover illustration: *Can you find me and Per?* by Richeng Jiang

Interplay between autophagy and amyloid beta metabolism in Alzheimer's disease
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Richeng Jiang
姜日成
강일성

The thesis will be defended in public at Atrium, Ground floor in Wargentinhuset, Nobels väg 12A, Solna, Sweden, Monday the 30th of May at 13:00

Principal Supervisor:
Associate Professor Per Nilsson
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society
Division of Neurogeriatrics

Co-supervisor(s):
Dr. Simone Tambaro
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society
Division of Neurogeriatrics

Professor Bengt Winblad
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society
Division of Neurogeriatrics

Opponent:
Associate Professor Joakim Bergström
Uppsala University
Department of Public Health and Caring Sciences

Examination Board:
Professor Per Svenningsson
Karolinska Institutet
Department of Clinical Neuroscience

Associate Professor Henrietta Nielsen
Stockholm University
Department of Biochemistry and Biophysics

Associate Professor Claes Andreasson
Stockholm University
Department of Molecular Biosciences

To my family and all the people that helped me

ABSTRACT

Alzheimer's disease (AD), the most common neurodegenerative disease, is characterized by two pathological hallmarks: extracellular amyloid-beta peptide (Aβ) plaque depositions and neurofibrillary tangles (NFTs) composed of intracellular hyperphosphorylated tau aggregations. A cellular degradation system, autophagy is additionally dysfunctional in AD and plays a key role in Aβ and tau metabolisms.

Paper I and II. To investigate the molecular mechanisms of autophagy alterations induced by Aβ amyloidosis, we characterized the autophagy status in amyloid precursor protein (*App*) knock-in mouse models exhibiting robust Aβ pathology. Interestingly, impaired autophagy was a general phenomenon in the brains which was specifically pronounced in the surrounding regions of Aβ plaques, especially in neurites and pre-synapses in *App* knock-in mice. The region-specific autophagy impairment was substantiated by electron microscopy imaging showing autophagic vacuole accumulation in dystrophic neurites around Aβ plaques. Time course bulk RNA sequencing from hippocampi of *App* knock-in mouse brains further revealed alterations of autophagy-associated gene expression.

Paper III. Cerebrospinal fluid (CSF) is an important body fluid source to study brain-derived biomarkers for AD diagnosis. Comparing the CSF proteomes from *App* knock-in mice and AD human subjects revealed an extracellular matrix protein, decorin, as significantly increased in preclinical AD subjects having abnormal CSF-Aβ42 but normal CSF-total-tau (a+t-) levels and in App^{NL-F} mice exhibiting mild Aβ pathology. In a+t- preclinical AD subjects, CSF-decorin levels positively correlated with CSF-Aβ42 levels and negatively correlated with CSF phosphorylated and total tau levels. Increase of CSF-decorin could predict an AD subtype having innate immune activation and potential choroid plexus dysfunction in the brain with high sensitivity and specificity. Consistently, increased CSF-decorin in App^{NL-F} mice correlated with the decorin levels in choroid plexus and Aβ plaque load.

Paper IV. To directly investigate the role of autophagy in Aβ metabolism, we generated autophagy-deficient AD mouse models by crossing *App* knock-in mice with autophagy-related gene 7 (*Atg7*) conditional knockout mice. Loss of autophagy in excitatory neurons lowered Aβ plaque load but raised intracellular Aβ levels. Severe Aβ pathology together with lack of autophagy led to an autistic-like behavior, decreased anxiety and memory deficits which potentially was related to activated programmed cell death, synaptic impairment, and degraded gamma oscillation power. However, a mild Aβ amyloidosis in autophagy-deficient

App^{NL-F} mice ameliorated the autistic-like behavior driven by loss of autophagy. Notably, proteomic analysis of CA1 pyramidal cell layer in hippocampus unveiled that loss of autophagy upregulated cell transport but downregulated protein translation which can be alleviated by a mild Aβ amyloidosis.

Paper V. Limitations of *App* knock-in mice include a less pronounced tau pathology and lack of neurodegeneration. Generation of an *App* knock-in rat model, *App*$^{NL-G-F}$, circumvented above mentioned shortages by inducing phosphorylated tau aggregations and neuronal loss. *App*$^{NL-G-F}$ rats additionally exhibited enhanced gliosis, impaired spatial learning, and memory deficits including episodic-like memory.

LIST OF SCIENTIFIC PAPERS

I. **Richeng Jiang**, Makoto Shimozawa, Johanna Mayer, Simone Tambaro, Rakesh Kumar, Axel Abelein, Bengt Winblad, Nenad Bogdanovic, Per Nilsson
Autophagy impairment in *App* knock-in Alzheimer model mice
Frontiers in Aging Neuroscience, 2022, Accepted.

II. Luana Naia, Makoto Shimozawa, Erika Bereczki, Xidan Li, Jianping Liu, **Richeng Jiang**, Nuno Santos Leal, Catarina Moreira Pinho, Erik Berger, Viktoria Lim Falk, Giacomo Dentoni, Maria Ankarcrona, Per Nilsson
Early mitochondrial dysfunction proceeds synaptic alteration, neuroinflammation and autophagy inhibition in hippocampus of *App* knock-in Alzheimer mouse models
Manuscript in review.

III. **Richeng Jiang**, Una Smailovic, Hazal Haytural, Betty M. Tijms, Robert Mihai Haret, Ganna Shevchenko, Gefei Chen, Axel Abelein, Johan Gobom, Susanne Frykman, Misaki Sekiguchi, Ryo Fujioka, Naoto Watamura, Hiroki Sasaguri, Sofie Nyström, Per Hammarström, Takaomi C. Saido, Vesna Jelic, Stina Syvänen, Henrik Zetterberg, Bengt Winblad, Jonas Bergquist, Pieter Jelle Visser, Per Nilsson
Increased CSF-decorin predicts brain pathological changes driven by Alzheimer's Aβ amyloidosis
Manuscript in review.

IV. **Richeng Jiang**, Simone Tambaro, Arturo G Isla, Lovisa Johansson, Valerie Sackmann, Delia-Denisa Dunca, Yuxi Guo, Hazal Haytural, Ganna Shevchenko, Makoto Shimozawa, Fengna Chu, Luis E Arroyo-García, Hugo Balleza-Tapia, Bengt Winblad, Jonas Bergquist, André Fisahn, Martin Hallbeck and Per Nilsson
Degree of Aβ amyloidosis has distinct effects on mouse phenotypes induced by loss of autophagy
Manuscript.

V. Keliang Pang, **Richeng Jiang**, Wei Zhang, Zhengyi Yang, Lin-Lin Li, Makoto Shimozawa, Simone Tambaro, Johanna Mayer, Baogui Zhang, Man Li, Jiesi Wang, Hang Liu, Ailing Yang, Xi Chen, Jiazheng Liu, Bengt Winblad, Hua Han, Tianzi Jiang, Weiwen Wang, Per Nilsson, Wei Guo and Bai Lu
An *App* knock-in rat model for Alzheimer's disease exhibiting Aβ and tau pathologies, neuronal death and cognitive impairments
Cell Research, 2021, 32, 157–175.

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AICD	APP intracellular domain
AMPK	Adenosine monophosphate-activated protein kinase
APP	Amyloid precursor protein
ATG	Autophagy-related
AVs	Autophagic vacuoles
Aβ	Amyloid-β peptides
BACE1	β-site APP-cleaving enzyme 1
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
CA	Cornu ammonis
CAA	Cerebral amyloid angiopathy
CaMKII	Calcium/calmodulin-dependent kinase II
cDNA	Complementary deoxyribonucleic acid
ChP	Choroid plexus
CSF	Cerebrospinal fluid
CSF-p-tau	CSF-phosphorylated-tau
CSF-t-tau	CSF-total-tau
CTF	C-terminal fragment
ECM	Extracellular matrix
EEA1	Early endosome antigen 1
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMIF-AD MBD	European Medical Information Framework for Alzheimer's Disease Multimodal Biomarker Discovery
EPM	Elevated plus maze
ER	Endoplasmic reticulum
EVs	Extracellular vesicles
FAD	Familial AD
FIP200	Focal adhesion kinase family interacting protein of 200 kD

LAMP	Lysosomal-associated membrane protein
LC3	Microtubule-associated protein 1 light chain 3
LMD	Laser microdissection
MB	Marble burying
MCI	Mild cognitive impairment
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MVBs	Multivesicular bodies
MWM	Morris water maze
NC	Normal cognition
NFTs	Neurofibrillary tangles
NTA	Nanoparticle tracking analysis
OF	Open field
OI	Object interaction
PBS	Phosphate-buffered saline
PE	Phosphatidylethanolamine
PEA	Proximity extension assay
Peg3	Paternally expressed gene 3
PI3K	Phosphoinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PS	Presenilin
RT-PCR	Real-time polymerase chain reaction
TFEB	Transcription factor EB
Tg	Transgenic
ULK	Unc51-like kinase
VEGFR2	Vascular endothelial growth factor receptor 2
WT	Wildtype

1 INTRODUCTION

In 2021, World Alzheimer Report has reported that over 55 million people are suffering from dementia worldwide and this number is increasing and is estimated to be around 78 million by 2030 (1). One of the major challenges for dementia is diagnosis. Alzheimer's Disease International estimates that up to 75% of people having dementia were not yet diagnosed (1). Alzheimer's disease (AD) is the most common cause of dementia but only one Federal Drug Administration approved disease-modifying treatment, aducanumab, has emerged until now. Prior to aducanumab, only symptomatic treatment was available for mild to moderate AD patients to control the cognitive and behavioral symptoms. Hence, finding new treatment targets and early diagnosis are urgently needed.

Fortunately, a growing number of biomarker studies using different techniques has led to the development of new imaging and fluid biomarkers which increases the possibilities of early diagnosis for AD and further helps the early therapeutic interventions. Nowadays, lumber puncture is adopted as a safe procedure to sample cerebrospinal fluid (CSF) for laboratory tests for AD. Since CSF is directly produced in the brain and carries the proteins that are released from the brain, it is believed to be one of the most reflective resources for brain disease diagnosis. Although three core CSF biomarkers, 42-aminoacid form of amyloid-β peptides (Aβ42), phosphorylated tau and total tau, for AD diagnosis have been established (2), more CSF biomarkers for early diagnosis and for subtyping AD need to be further investigated which could later contribute to an individual therapy.

Autophagy, as a cellular degradation system, plays an important role in Aβ and tau metabolisms in AD progression. However, how autophagy regulates Aβ production, secretion, and extracellular Aβ plaque formation is not fully understood, and it is difficult to elucidate the detailed mechanisms using human postmortem brain tissues or CSF samples. To solve this question, choosing proper animal models is critical. Therefore, in our study, we use amyloid precursor protein (*App*) knock-in AD mouse models (3) that closely mimicking the human Aβ pathology to evaluate the autophagy status in these mouse models. As very few CSF proteomic studies on AD mouse models have been reported (4), our CSF proteomic analysis of *App* knock-in mice is important for further AD biomarker explorations for Aβ amyloidosis-induced changes. Especially, comparing the CSF proteomics from mouse and human may reveal potential CSF biomarkers which we can further study in the AD mouse model brains to unveil their function in healthy and AD brains. To elucidate the effect of autophagy-deficiency on Aβ metabolism, we crossed the autophagy-deficient mouse models

with *App* knock-in mouse models and analyzed the Aβ pathological changes as well as its potential mechanisms in both autophagy-deficient and autophagy-competent *App* knock-in mice. Our work could provide clues for understanding the interplay between autophagy and Aβ metabolism in AD.

2 LITERATURE REVIEW

2.1 ALZHEIMER'S DISEASE

2.1.1 Definition and epidemiology

Over 55 million people in the world are living with dementia (1) and the annual expenditure for dementia in 2019 was approximately 1 trillion US dollars which are about to be doubled by 2030 (5). AD, as the most common cause of dementia, is yet not curable even since the first case was reported by Alois Alzheimer in 1907. The first Federal Drug Administration approved disease-modifying treatment, Aduhelm (aducanumab) emerged in 2021 which is an Aβ-specific antibody aiming at reducing Aβ plaques (6-8). Although the clinical benefits for the future need to be proved, it gives a positive sign for the AD therapeutic development.

AD is a chronic neurodegenerative disease, and the brain changes are thought to start 20 years or more before the onset of symptoms (9). The life expectancy after diagnosis varies widely from as short as three years up to 20 years or more with an average of 10 years. AD is divided into four stages, i) pre-dementia, ii) early, iii) moderate, and iv) advanced according to the progressive cognitive and functional decline. The symptoms mainly start with short-term memory loss in the pre-dementia stage. In early stage of AD, the learning and memory impairment is increased which might be accompanied by language problems. The memory loss deteriorates in moderate stage and the long-term memory becomes impaired. Finally, when the disease reaches advanced stage, the patients not only suffer from poor memory but also have speech loss and they must depend on caregivers.

2.1.2 Neuropathology of AD brain

AD brains have two cardinal pathological hallmarks, amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are composed of extracellular Aβ deposition and NFTs are formed by intercellular aggregation of hyperphosphorylated tau. However, the correlation between Aβ and tau is still unclear. One hypothesis is that tau pathology is driven by Aβ pathology because Aβ levels start to increase before onset of tau pathology, whereas another hypothesis advocates that tau develops independently of Aβ which is supported by the fact that memory impairment correlates better with NFTs than Aβ plaques. Aβ not only aggregates into the plaques, but also deposits in vessel walls causing cerebral amyloid angiopathy (CAA) (10). These Aβ depositions in the vasculature affect the composition of blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) and lead to the impairment of peripheral Aβ clearance which may further worsen the AD progression and

induces a vicious cycle (11). In addition to Aβ and tau, other pathologies are present in parallel like neuroinflammation and synaptic loss (12, 13). Moreover, numerous studies reported that the autophagy-lysosomal system is altered in AD (14, 15). These pathologies separately or together induce the severe neurodegeneration of cortex and hippocampus in AD.

2.1.3 Cerebrospinal fluid and biomarkers

Since AD is a chronic neurodegenerative disease with limited disease-modifying treatments currently available, finding and validating biomarkers is necessary for both early and correct diagnosis and for disease-modifying drug development. Except from brain surgery, it is extremely difficult to get a brain biopsy from living individuals for diagnosis. Instead, other techniques to investigate the brain status have been developed. For example, neuroimaging techniques and body fluid biomarkers were developed to fill the knowledge gap of diagnosis. Emergence of Aβ and tau positron emission tomography imaging as well as the development of pathology-specific radiotracers have an important contribution to the clinical AD diagnosis (16). Recently, Karikari et al. established a promising blood biomarker p-tau181 which could predict the tau and Aβ pathologies and differentiate AD from other neurodegenerative disorders (17).

Currently, two body fluids, plasma and CSF are the main sources of biomarker investigations for AD, though until present more established for CSF (18). CSF is produced from arterial blood by the choroid plexus (ChP) in the ventricles of the brain. It has a close physical interaction with the brain meaning that the proteins present in CSF may directly reflect the brain pathology (18). Plasma in the blood penetrates from fenestrated capillaries into the interstitial space that is filled with interstitial fluid. ChP-derived CSF is produced by the interstitial fluid passing through the epithelial cells in ChP and the tight junctions between the epithelial cells control the substances reaching the CSF. However, this classical hypothesis was challenged by Orešković et al. proposing a new hypothesis that there is no net CSF formation, but CSF is a permanent fluid with substances exchanging between the CSF system and surrounding tissues (19).

Three core CSF biomarkers, total tau, phosphorylated tau and Aβ42, correlate well with AD pathology and can therefore differentiate AD and healthy controls (2, 20). Aβ42 is decreased in the CSF of AD while total tau and phosphorylated tau are increased (2, 20). The combination of these core CSF biomarkers has a high predictive value for AD diagnosis with 86% sensitivity and 97% specificity (21). However, to understand the biological complexity

of AD and its correct disease stratification, further investigations on CSF biomarkers are needed both from clinical samples and AD mouse models (22).

2.1.4 APP processing and Aβ

APP is a type I transmembrane protein expressed in many tissues. APP has several different isoforms produced by alternative splicing of its messenger ribonucleic acid (mRNA) and the 695 amino acid isoform is highly expressed in neurons (23). As a part of APP, Aβ is partially located within the ectodomain and the transmembrane domain. In AD, Aβ plaques are mainly composed of misfolded Aβ40, Aβ42 and Aβ43 (24). There are two main different pathways of APP processing, amyloidogenic and non-amyloidogenic, which control the production of APP fragments and the pathways are determined by the predominant secretase enzyme activity involved in the cleavage. Aβ is produced in the amyloidogenic pathway controlled by the β- and γ-secretases which sequentially cleave at the N- and C-terminus of the Aβ domain. The major β-secretase, β-site APP-cleaving enzyme 1 (BACE1), releases sAPPβ from transmembrane domain C-terminal fragment (CTF)-β (C99) which is further cleaved by γ-secretase to produce Aβ and APP intracellular domain (AICD). Familial AD (FAD)-linked mutations in presenilin (PS)1 and PS2, the catalytic components of γ-secretase, increase the Aβ generation through amyloidogenic processing and leads to autosomal dominant AD (25). On the other hand, in the non-amyloidogenic pathway, APP is cleaved by α- secretase within the Aβ domain at amino acid 17 which precludes the Aβ production by forming other APP fragments, sAPPα and CTF-α (C83) (**Figure 1A**).

Healthy individuals also generate Aβ, the physiological function of which is not well understood. However, Aβ may aggregate and initiate disease progression which is influenced by genetic variants and environmental exposure. Initially, researchers in the field believed that extracellular Aβ deposits are associated with neurotoxic properties which cause the neuronal cell death and reduction of Aβ aggregation is accompanied by the corresponding decrease of toxicity (26). On the other hand, Aβ is generated inside the cells where it may also be toxic. A growing number of studies have shown that intermediate molecular forms of Aβ, e.g., oligomers and protofibrils, are more toxic to the neurons (27) and soluble forms of Aβ better correlate with synaptic dysfunction and severity of neurodegeneration (28, 29).

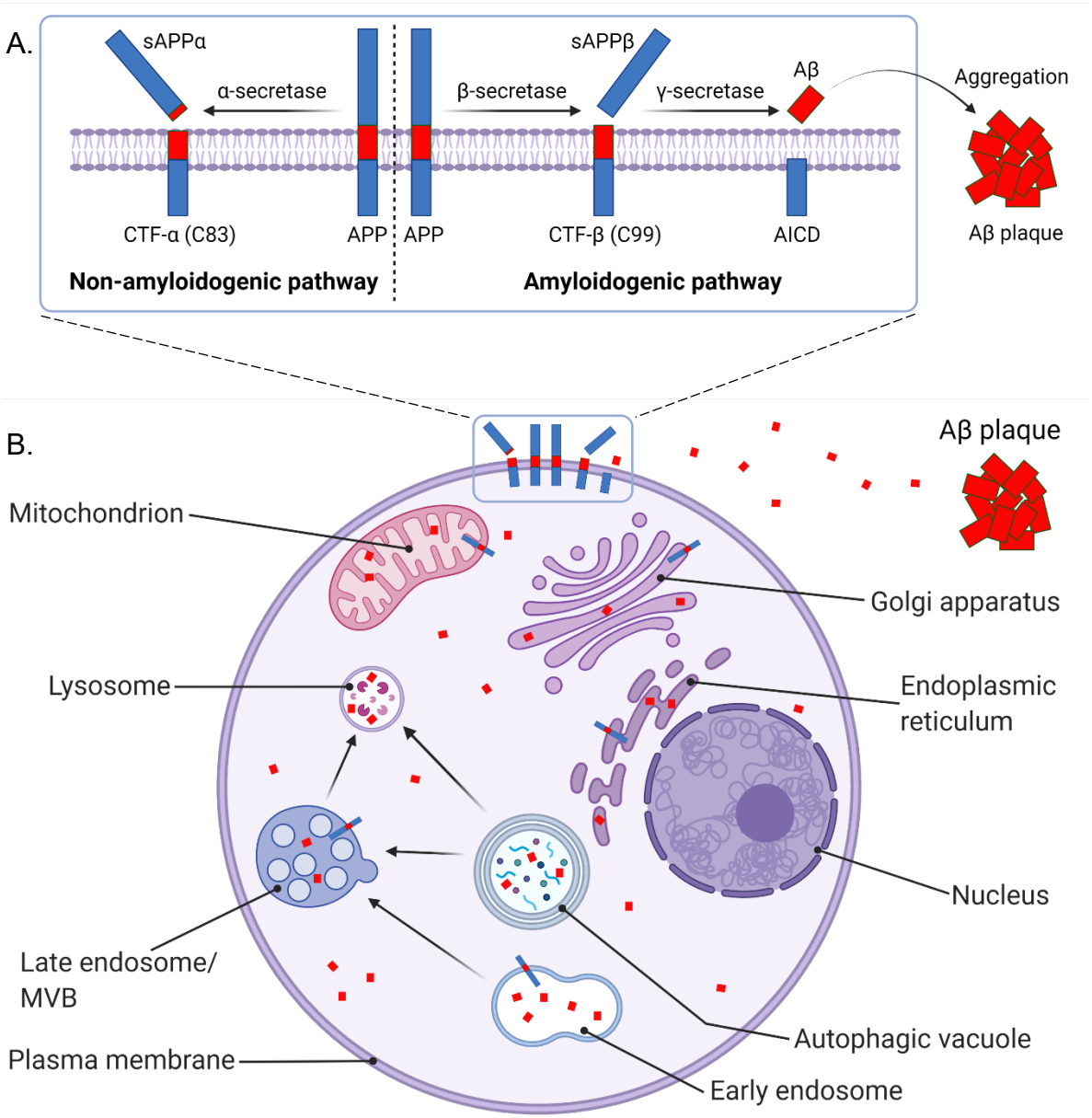


Figure 1. *(A) Amyloidogenic and non-amyloidogenic pathways in APP processing. (B) Sites for APP location and intracellular Aβ generation. (Created with BioRender.com)*

2.1.5 Intracellular Aβ

Intracellular Aβ accumulation is preceding extracellular Aβ aggregation in AD (30). This hypothesis is supported by a large number of transgenic mouse model studies which describe that intracellular Aβ accumulation appears as an early event of Aβ pathogenesis (31-36). Even though there is a debate on the exact mechanism of how intracellular Aβ is released to the extracellular space, it is clear that intracellular Aβ accumulation exists in the human brains (37-42). However, a key question that remains to be addressed is where the intracellular Aβ is generated and how it is metabolized in the cell.

Since 1993, when Wertkin et al. (43) first reported that Aβ is generated intracellularly, researchers started to work on the identification of the site of intracellular Aβ generation. To understand where Aβ is generated, it is important to know where APP, β- and γ- secretases are located and moreover where β- and γ- secretases are active and can cleave APP. APP has been reported to be located not only on the plasma membrane (44), but also in endoplasmic reticulum (ER) (45, 46), Golgi network (47), early/late endosomes (44, 48), mitochondrial membranes (49, 50) and autophagic vacuoles (AVs) (51-53). Therefore, it is likely that Aβ is generated in the above-mentioned cell compartments or in a pathway where these compartments are involved. However, it seems that Aβ is predominantly generated at the plasma membrane or in compartments of the secretory pathway that mediates Aβ release to extracellular interstitial fluid (30) (**Figure 1B**).

2.1.6 Tau and phosphorylated tau

As one of the key hallmarks in AD, NFTs are formed by hyperphosphorylated tau protein aggregation and fibrilization in neurons (54-57). Tau, a microtubule-associated protein, plays a pivotal role in microtubule assembly by interacting with tubulin which is disturbed by the abnormal phosphorylation of tau in AD (58). A postmortem brain tissue study has revealed that the levels of phosphorylated tau in AD brains are 3-4 times higher than in healthy control brains (59). Approximately 45 phosphorylation residues on insoluble tau were identified experimentally in AD brains (60).

Tau is expressed as six different isoforms in the human adult brain due to alternative splicing of exons 2, 3 and 10. Zero, one or two inserts of 29 amino acids at the N-terminus of exons 2 and 3 form 0N, 1N and 2N isoforms of tau while three or four repeats of C-terminal domain in exon 10 constitute 3R or 4R of tau isoforms. Different species express different levels of 3R and 4R tau isoforms and these two types of isoforms are developmentally regulated. For instance, there are six tau isoforms in the brain of adult humans and adult rats although the ratios of 4R/3R tau protein levels are different (nearly equal in human, 9:1 in rats). However, almost exclusively 4R tau isoforms are expressed in adult mouse brains (61-63). Moreover, 3R tau isoforms are predominant in fetal or neonatal brains of those three species and the 4R tau isoforms are dominant in adult brains, especially in rodents (62).

2.2 AUTOPHAGY

2.2.1 Definition and history

The word “autophagy” (auto-self; phagy-eating) was invented by Christian de Duve who discovered the lysosome in 1956. He and his colleagues described autophagy as an intracellular clearing system that digests material of endogenous origin by lysosomes together with related vacuolar structures (64). In the 1990s, several researchers from all over the world separately discovered autophagy-related genes and pathways using gene knockout studies in yeast system (65-69). However, they soon realized that the pathways they described independently are essentially the same resulting in a variety of gene names given by different researchers (68-75). Eventually, a unified nomenclature for autophagy genes and proteins were given as ATG and Atg respectively, as short of “autophagy-related” (76).

Depending on the cargo delivery route, three different general forms of autophagy are described; macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy, commonly referred to as autophagy, is the main subtype of autophagy that degrades damaged cell organelles and proteins including protein aggregates. In this pathway, substrates are enwrapped by isolation membranes (or phagophores) that further form double membraned autophagosomes after elongation and closure (77). Subsequently, autophagosomes fuse either with lysosomes (autolysosomes) or selectively fuse with endosomes (amphisomes) followed by lysosomal fusion to digest the autophagosomal contents by the acidic lysosomal hydrolases. In eukaryotic cells, it is recommended to use the general term “autophagic vacuole” to represent autophagosome, amphisome, and autolysosome since it is difficult to differentiate these three structures in some cases (**Figure 2**). Macroautophagy can be selective and non-selective. Depending on the autophagy target, selective macroautophagy can be divided into mitophagy, aggrephagy, ribophagy, ER-phagy and others (78). For example, mitophagy is the selective autophagic degradation of mitochondria.

Microautophagy is a process in which lysosomes directly engulf the cytoplasmic materials by invaginations. Chaperone-mediated autophagy is a specific autophagy pathway in which substrates are selectively recognized by a chaperone complex which translocates them to lysosomes for degradation (79, 80).

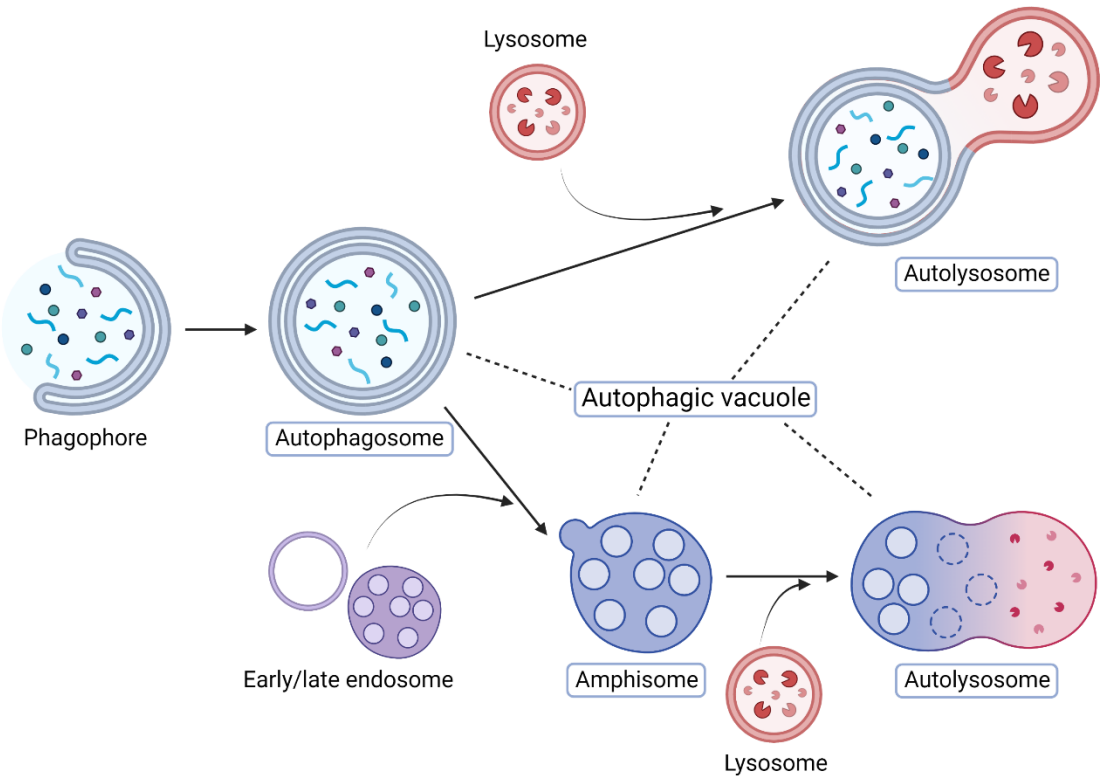


Figure 2. *Macroautophagy process. Substrates are sequestered by phagophores. After phagophore elongation, it forms a closed double membraned structure - autophagosomes. Autophagosomes either fuse with lysosomes to form autolysosomes or selectively fuse with early/late endosomes to form amphisomes followed by lysosomal fusion. The contents of autolysosomes are degraded by the acidic lysosomal hydrolases. Autophagic vacuole represents autophagosomal, autolysosomal and amphisomal stages in macroautophagy. (Created with BioRender.com)*

2.2.2 Molecular pathways and key molecules

Mechanistically, macroautophagy (hereafter referred to as autophagy) can be categorized into four stages including: initiation, phagophore elongation, autophagosome formation, and degradation (81). Autophagy induction can be initiated by the mammalian target of rapamycin (mTOR) inhibition or adenosine monophosphate-activated protein kinase (AMPK) activation which results in different phosphorylation states of Atg1/Unc51-like kinase (ULK) 1 complex including ULK1, Atg13, Atg101, and focal adhesion kinase family interacting protein of 200 kD (FIP200) (82, 83). The coordination of different phosphorylation sites of ULK1 plays an important role in autophagy induction since some of the phosphorylation sites activate while others inhibit ULK1 activity (84, 85). Thereafter,

ULK1 activates class III phosphoinositide 3-kinase (PI3K) complex composed of Beclin-1, Vps34, Vps15, and Atg14, enabling this complex to relocate from the cytoskeleton to the phagophore for the nucleation process. Activated Vps34 stimulates the generation of phosphatidylinositol 3-phosphate (PI3P) which is involved in phagophore elongation. Meanwhile, the only transmembrane autophagy protein Atg9 transports lipid components to the phagophore which is essential for phagophore elongation (86-89). In addition, during phagophore elongation, Atg5 and Atg12 are conjugated catalyzed by Atg7. This Atg5-12 conjugations interact with Atg16L to form Atg5-Atg12-Atg16L complexes that are indispensable in autophagosome formation in the presence of Atg7 and Atg10 (90). As a ubiquitin-like molecule, microtubule-associated protein 1 light chain 3 (LC3)/Atg8 is involved in the closure of the phagophore by interacting with phosphatidylethanolamine (PE) (91). LC3-I, which is generated from precursor LC3 through cleavage by the protease Atg4B, is conjugated to PE to form LC3-II (92, 93). Moreover, this LC3 lipidation process requires Atg7 (94) (**Figure 3**). Importantly, LC3-II is a specific autophagosome-associated marker (95) which enables monitoring of autophagic flux by analyzing LC3-II levels (96). However, only measuring the steady-state levels of LC3-II is not sufficient to monitor autophagic flux, because an increase of endogenous LC3-II levels can be caused by accelerated lipidation of LC3-I to LC3-II, associated with autophagy induction, or lysosomal inhibition leading to decreased degradation of LC3-II (97). Therefore, concomitant analysis of cargo receptor p62 is required. After autophagosomes are formed by the closure of phagophore edges, the completed autophagosomes fuse with lysosomes to form autolysosomes that degrade the autophagic contents.

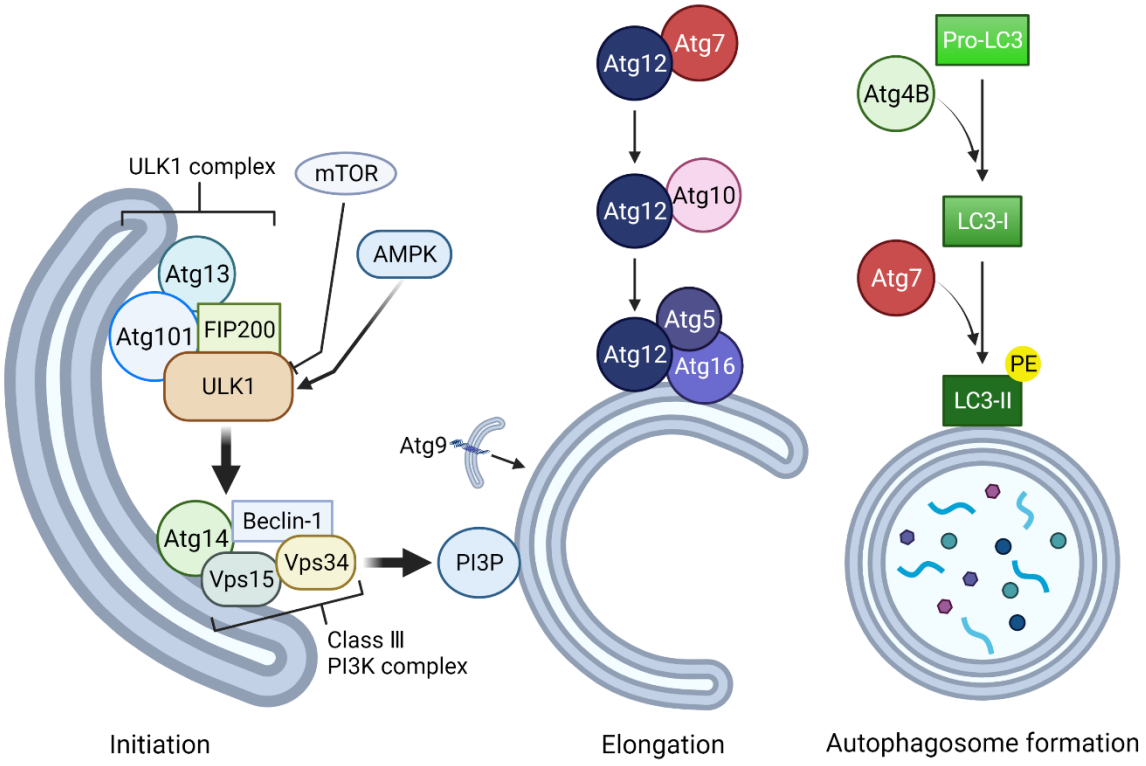


Figure 3. *Molecular pathways and key molecules involved in autophagy initiation, elongation, and autophagosome formation. Autophagy is initiated by the mTOR inhibition or AMPK activation which induces the activity of ULK1 complex consisting of ULK1, Atg13, FIP200 and Atg101. Thereafter, ULK1 activates class III PI3K complex composed of Atg14, Beclin-1, Vps15, and Vps34 and enables this complex to the phagophores for the elongation process. Activated Vps34 stimulates PI3P generation which is involved in phagophore elongation. Atg9, the only transmembrane autophagy protein, transports lipids components to the phagophore. In addition, Atg12 is conjugated to Atg5 by the presence Atg7 and Atg10, and further interacts with Atg16L to form Atg5-Atg12-Atg16L complexes. In autophagosome formation, LC3-I that is produced from precursor LC3 (pro-LC3) by the cleavage of Atg4B conjugates with PE to form LC3-II in the presence of Atg7. (Created with BioRender.com)*

Although autophagy was originally considered as a non-selective process, a large number of studies now support that selective autophagy specifically targets substrates (98, 99). Sequestosome 1/p62, being one of the selective autophagy cargo receptors, tethers ubiquitinated protein aggregates to autophagosomes by interacting with LC3 to facilitate the degradation of the aggregates, along with p62 that is continuously degraded by lysosomes

(100). Therefore, p62 is also used as a marker protein for evaluation of autophagic flux in the selective autophagy pathway.

2.2.3 Autophagy in AD

Although the classical pathological hallmarks of AD are extracellular Aβ plaques and intracellular tau accumulation, the underlying biological mechanisms that cause those proteins to aggregate are still unclear. Nixon et al. provided the first evidence that AVs are accumulated in dystrophic neurites both in AD human brains and APP transgenic (Tg) AD mouse models (shown by electron microscopy (EM)) (14, 53). This together with the finding of endosomal impairment and increased lysosomal proteases shows that the autophagy-lysosomal system is extensively involved in the AD pathological process (101, 102). Therefore, autophagy dysregulation may contribute to Aβ and tau pathologies in AD (103). However, it remains elusive whether autophagy is upregulated or downregulated, and if it differs depending on different stages of AD progression. Even though the findings of autophagic dysfunction are different depending on the disease stages, the accumulation of autophagosomes in dystrophic neurites indicates a lysosomal proteolysis disruption (104, 105) or blockage of autophagosome maturation before fusion to the lysosome (14, 53, 102). However, the increase in autophagosome number could also be due to, at least to some extent, an induction of autophagosome formation which is supported by the increase in gene expression of many autophagy genes in early-stage AD (106-108). Overall, autophagic activity could be upregulated in the early stage of AD, and it seems to collapse at the later stage.

Since increasing evidence have implicated the dysfunction of autophagy-lysosomal system in AD (109), the proteins involved in this pathway could potentially be used as biomarkers for diagnosis. Currently, few studies were published about autophagic body fluid biomarkers in AD. In 2014, one study (110) reported increased levels of six lysosomal network proteins (early endosome antigen 1 (EEA1), lysosomal-associated membrane protein (LAMP)-1, LAMP-2, LC3, Rab3, and Rab7) in CSF of AD indicating the possibility to use them as CSF biomarkers for AD. Most recently, a blood biomarker study (111) reported that plasma ATG5 is increased in AD compared to the control group. Hence, finding biomarkers related to autophagy could help to evaluate autophagy alterations in AD.

Autophagy, as a degradation system of the cell, may be a crucial degradation mechanism to clear misfolded proteins in AD. Since only one disease-modifying treatment, aducanumab, has been approved by Federal Drug Administration after hundreds of times failure of drug

development and growing number of studies have shown that multi-target or treatment combination can be a trend for the future, autophagy is a potential new target for AD therapy. Multiple studies have provided evidence that enhancing the efficacy of autophagy by targeting different autophagic mechanisms could be a promising therapeutic strategy. Inhibition of mTOR by rapamycin can ameliorate Aβ and tau pathologies and cognitive deficits by increasing autophagy in transgenic mouse models of AD (103, 112). Pharmacological agents that induce autophagy in an mTOR independent manner have also been studied by many labs. For example, the mood stabilizer lithium reduced soluble tau levels and NFTs and attenuated phosphorylated-tau-induced motor disturbance in AD mouse models (113). However, given the biological complexity of AD, a combinational treatment instead of a single treatment may be the future of therapeutic strategies.

2.2.4 Aβ metabolism by autophagy

Autophagy plays a key role in Aβ metabolism by degrading Aβ and therefore potentially contributes to AD pathology (103, 114, 115). As previously described, Aβ is present in different cell organelles (ER, Golgi apparatus, endosomes), and some studies provided evidence that intracellular Aβ can also be found and potentially produced in AVs in AD (14, 51-53). In addition to degrading intracellular Aβ, autophagy also mediates Aβ secretion and therefore influences the extracellular Aβ plaque formation (116). When autophagy is deleted in excitatory neurons of transgenic AD mouse models, Aβ plaque load is drastically reduced by decreased Aβ secretion (116). However, the possibility of decreased Aβ production by deleting autophagy cannot be excluded since autophagosomes may generate Aβ.

The possible mechanism for the decreased Aβ secretion could be that autophagy mediates the Aβ trafficking from Golgi to multivesicular bodies (MVBs) since autophagy deficiency induces Aβ accumulation in Golgi whereas it reduces the Aβ levels in MVBs (117). MVBs are specialized endosomal compartments consisting of intraluminal vesicles that are generated by inward budding of the outer endosomal membrane. MVBs, as late endosomes matured from early endosomes, can fuse with lysosomes to degrade the contents of MVBs or fuse with the plasma membrane to release those intraluminal vesicles as exosomes (118, 119).

Extracellular vesicles (EVs) are lipid bilayer delimited vesicles, and they can be broadly divided into three subtypes, exosomes, microvesicles and apoptotic bodies according to their size and biogenesis (120). Exosomes are small EVs with approximately 30 to 150 nm in diameter. These small EVs, derived from MVBs, could carry and transfer proteins, lipids, and

mRNA between cells indicating their important roles in cell-to-cell communications (121, 122). Recently, studies have implied that there is an interplay between exosomes and autophagy by showing the shared molecular machinery and regulatory mechanisms (123-125). In addition, exosomes isolated from AD brains contain increased levels of Aβ oligomers and could transfer Aβ from neuron to neuron in culture (126). Hence, exosomes can be an important vehicle to carry Aβ and autophagy alterations in AD may affect Aβ secretion via exosome release which may further influence the extracellular Aβ depositions and intracellular Aβ cell-to-cell transmissions.

2.3 ANIMAL MODELS

2.3.1 *App* knock-in AD mouse models

Due to the pathophysiological complexity of AD and that extremely limited disease-modifying treatments exist so far, developing and using animal models of AD to deeply understand disease mechanisms, as well as to test newly developed drugs, are needed. Among different kinds of animals, mouse models are one of the most highly developed animal models because of the well-developed genetic manipulation tools for mice, though it is evidently a challenge translating treatments from mouse models to human. To recapitulate the Aβ pathology of AD, APP overexpression paradigms have been previously used. However, the unphysiological levels of APP may cause additional phenotypes unrelated to AD (127). To circumvent this problem a new type of *App* knock-in mice has been generated, which produce pathogenic Aβ without overexpressing APP. Based on mutations of the *APP* gene found in FAD, Saito et al. (3) generated two different *App* knock-in mouse models. One is $App^{NL\text{-}F}$ mice which harbor the Swedish (KM670/671NL) and Beyreuther/Iberian (I716F) mutations, whereas the other, $App^{NL\text{-}G\text{-}F}$ mice, has an additional Arctic (E693G) mutation. In addition, because of Aβ sequence differences between mouse and human, the mouse Aβ sequence was humanized through changing three amino acids (G676R, F681Y, and R684H) (3) (**Figure 4**).

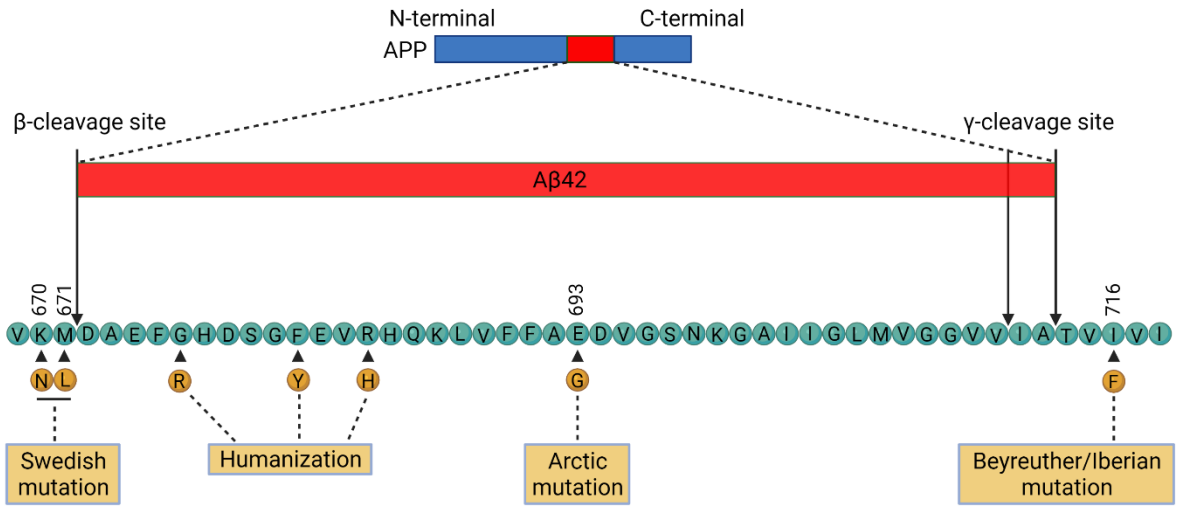


Figure 4. *Gene manipulation design for generating App knock-in mouse and rat models. (Created with BioRender.com)*

The pathophysiological difference between the two models is that the Arctic mutation in $App^{NL\text{-}G\text{-}F}$ mice makes arctic Aβ more oligomerization/fibrillization-prone which induces an earlier and stronger Aβ pathology (128, 129). The Aβ deposition in $App^{NL\text{-}F}$ mice starts around nine months of age and is mainly located in cortex and hippocampus, whereas, in $App^{NL\text{-}G\text{-}F}$ mice, the Aβ deposition starts to form already from two months of age and the plaques are detected not only in cortex and hippocampus but also in subcortical regions (3). This more aggressive Aβ pathology induces greater neuroinflammation, including microgliosis and astrocytosis, and synaptic loss which further leads to memory impairment much faster in $App^{NL\text{-}G\text{-}F}$ mice as compared to $App^{NL\text{-}F}$ mice (3). However, one limitation of *App* knock-in mouse models as well as APP Tg mice is the absence of NFTs and neurodegeneration which prompt further manipulation and is expected to be overcome by new mouse models in the future.

2.3.2 Autophagy-deficient mouse models

In yeast, more than 40 *ATG* genes were identified so far whereof 14 *Atg* genes have been used for knocking out autophagy in mice (130, 131). However, many of the systemic *Atg* knockout mice are lethal in neonatal or embryonic stage indicating the pivotal physiological role of autophagy in the mice (131). Therefore, instead of conventional knockout of *Atg* genes, researchers started to use tissue and/or time specific conditional knockout of these genes which delayed the early death in the mice. In addition, conditional knockout of *Atg* genes could help understand the role of autophagy in specific tissue or cell types as well as in different stages of the life span including embryonic stage.

In the current study, we used two different *Atg7* conditional knockout mouse models, *Atg7*$^{flox/flox}$; Calcium/calmodulin-dependent kinase II (CaMKII)-*Cre* and *Atg7*$^{flox/flox}$; Nestin-*Cre* mice. *Atg7*$^{flox/flox}$; CaMKII-*Cre* mice were generated by crossing *Atg7*$^{flox/flox}$ mice (132) with CaMKII-*Cre* Tg mice (133) to specifically knockout *Atg7* in excitatory forebrain neurons from postnatal stage. *Atg7*$^{flox/flox}$; Nestin-*Cre* mice were generated by crossing *Atg7*$^{flox/flox}$ mice with Nestin-*Cre* Tg mice to exhibit the deficiency of *Atg7* in the central nervous system already from embryonic stage (134). The *Atg7*$^{flox/flox}$; Nestin-*Cre* mice have been well described by Komatsu et al. (134), showing a shortened life span (Max. 28 weeks) and neurodegeneration, which again indicates the essential pathophysiological role of *Atg7 in vivo*.

To study the role of autophagy in AD, especially in Aβ metabolism, we crossed the autophagy-deficient mouse models with *App* knock-in AD mouse models. *Atg7*$^{flox/flox}$; CaMKII-*Cre* x *App* knock-in mice were used for studying the role of autophagy *in vivo*, whereas the *Atg7*$^{flox/flox}$; Nestin-*Cre* x *App* knock-in mice were used for primary neuronal culture obtaining from embryos.

2.3.3 *App* knock-in AD rat models

One of the major goals for generating animal models is recapitulating the pathologies of the human disease in animals and trying to find biomarkers for diagnosis as well as testing new drugs on the animal models before clinical trials. However, the major hurdles for developing animal models for AD is the inability to resemble all the pathologies since AD is a complex disease with many different pathologies appearing together. Having two pathological hallmarks, Aβ and tau, established in AD, animal models were mainly generated by recapitulating either of those two pathologies. Indeed, during past decades, hundreds of experimental AD models have been developed including mice, rats and non-human primates (135, 136). Due to the shortage of genetic manipulation tools in the rats as compared to mice, generation of AD rat models has been lagged (137-139). Recently, however, a growing number of studies show that rat models can be a valuable tool for studying AD (139).

Several transgenic AD rat models have been developed and exhibit some aspects of the AD pathologies including Aβ and tau pathologies as well as neuroinflammation, synaptic loss and memory impairment (140-145). Since transgenic models have an overexpression of certain genes that are not physiologically expressed in the usual circumstance, it is difficult to evaluate the phenotypes shown in these models are related to AD pathologies or other downstream effects. For instance, APP Tg rat models exhibit an overexpression of *App* genes

with certain mutations to increase the Aβ42 levels but, meanwhile, other *App* fragments will be increased which might cause pathological effects on the rats.

Knock-in technology overcomes this shortage of APP Tg models to instead increase Aβ42 levels at physiological level of the *App* gene expression. Recently, although several *App* knock-in rat models were generated, limited AD pathologies were observed (146-148). However, the *App* knock-in rat model, $App^{NL\text{-}G\text{-}F}$, generated in Bai Lu's laboratory exhibits robust AD-like pathologies (149). The gene manipulations in $App^{NL\text{-}G\text{-}F}$ rat model are the same as in the $App^{NL\text{-}G\text{-}F}$ mouse model harboring the Swedish (KM670/671NL), Beyreuther/Iberian (I716F) and Arctic (E693G) mutations with humanized rat Aβ sequence (3, 149). Importantly, this $App^{NL\text{-}G\text{-}F}$ rat model not only exhibits Aβ pathology, but also tau pathology, neuronal loss and cognitive impairment which resembles human AD pathologies from many aspects (149).

2.4 DECORIN

Decorin, a secreted proteoglycan, belongs to the class I family of small leucine-rich proteoglycans. Decorin is composed of a single glycosaminoglycan chain that contains either dermatan or chondroitin sulfate and a 42-kDa conserved protein core containing 12 leucine-rich repeats (150). Many studies have explored the functions of decorin including roles in autophagy (151-155), mitophagy (155-157), angiogenesis (158-160), wound healing (159, 161), carcinogenesis (162), inflammation (163, 164) and myocardial infarct (165).

2.4.1 Decorin in AD

Recently few studies are investigating the role of decorin in neurodegenerative diseases, especially AD. Three decades ago, Snow et al. (166) described that decorin is not only distributed in the periphery of Aβ plaques and within NFTs but also associated with collagen fibrils surrounding blood vessels. One study has reported that decorin is significantly reduced in the skin fibroblasts of FAD patients as compared to control (167).

BBB is formed by tightly conjunct endothelial cells of cerebral microvessels which protect the brain from blood-derived neurotoxic substances. A neuroimaging study has shown a BBB breakdown in the hippocampus of mild cognitive impairment (MCI) which may contribute to early AD (168). Extracellular matrix (ECM) is a three-dimensional network of extracellular macromolecules that contribute to the BBB structure and its dysfunction further affects the progression of neurologic disease (169). As one key component of the ECM, decorin was initially thought to regulate collagen fibrillogenesis and maintain the structural integrity of

ECM (170-173). There are some correlations between decorin upregulation and BBB dysfunction from the analysis of gene expression in the models of seizure, multiple sclerosis, traumatic brain injury and stroke (174). In addition, decorin is highly distributed in a newly identified cell type in the vasculature, the fibroblast-like cells (175). In situ hybridization experiments have shown the expression of decorin mRNA in the rat nervous system (176). However, the exact function of decorin in the central nervous system is still unclear.

2.4.2 Decorin and autophagy

Some studies have described that soluble decorin induces autophagy in endothelial cells (151-153) and mitophagy in tumor cells (155, 156, 177). Moreover, decorin has been shown to inhibit the migration of glioma cells by autophagy activation and TGFβ signaling suppression (178). However, there is a knowledge gap in the relationship between decorin and autophagy function in neurons.

In endothelial cells, the autophagy process is affected by the interaction of decorin with vascular endothelial growth factor receptor 2 (VEGFR2) (151). Subsequently, decorin/VEGFR2 triggers paternally expressed gene 3 (Peg3)-dependent pathway which induces transcription of Beclin 1 and LC3 (151). During decorin/VEGFR2/Peg3 signaling, Vps34 is an upstream kinase required for Peg3 induction (153). In addition, decorin/VEGFR2 inhibits the mTOR signaling similar to rapamycin with concurrent AMPK activation which further induces autophagy (153). Transcription factor EB (TFEB), which is a master transcription factor of lysosomal biogenesis, is involved in decorin-evoked Peg3-dependent autophagy. One study showed that Peg3 is an upstream regulator of TFEB and necessary for decorin-induced TFEB expression (179).

It has been hence established that decorin induces autophagy in certain cell types. Then the subsequent question is whether decorin itself is regulated by autophagy. Gubbiotti et al. reported that decorin expression can be upregulated at the mRNA and protein level by autophagic stimuli, like nutrient deprivation, *in vivo* and *in vitro* (154). However, the mechanism behind is still unknown.

3 RESEARCH AIMS

A key pathological hallmark of AD is Aβ deposition which is one of the earliest pathological changes in AD brains. Autophagy, a lysosome-dependent cellular degradation system, removes dysfunctional cell components including organelles and proteins and produces energy in normal condition. However, autophagy goes awry in AD. Studies have shown that AVs, which are key components of the autophagic system, are mediating degradation and serve as one of the sources for Aβ generation. This indicates that there is a close link between autophagy and Aβ production. Nevertheless, if and how autophagy is further involved in Aβ transport and release is still unclear. To understand the molecular links between autophagy and Aβ transport and secretion, and also the effect of Aβ amyloidosis on the autophagic system, animal models are a powerful tool. Therefore, our study aims to characterize the autophagy status in novel mouse models of Aβ amyloidosis (*App* knock-in) and to find potential CSF biomarkers that reflect brain pathological changes including autophagy alterations in these mice and to compare the results with human CSF proteomic data. Furthermore, we crossed autophagy-deficient mice with Aβ mouse models to unambiguously determine the role of autophagy in Aβ metabolism by analyzing the effect of autophagy absence on Aβ amyloidosis.

The specific aims for each paper are listed below.

Paper I: To characterize the autophagy status in *App* knock-in mice including App^{NL-F} and App^{NL-G-F} mice and compare it with autophagy alterations in human AD brains.

Paper II: To analyze the timewise Aβ-induced pathological changes including mitochondrial alterations, autophagy dysfunction and neuroinflammation in *App* knock-in mice including App^{NL-F} and App^{NL-G-F} mice.

Paper III: To explore potential AD CSF biomarkers induced by Aβ amyloidosis by comparing mouse and human CSF proteomics data.

Paper IV: To investigate the role of autophagy in Aβ metabolism using autophagy-deficient *App* knock-in mice and characterize the behavioral, memory and pathological effect by loss of autophagy and Aβ amyloidosis.

Paper V: To compare the pathological differences induced by Aβ amyloidosis in two rodents, mice and rats, induced by the same *App* knock-in strategy of FAD-linked mutations.

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL MODELS

4.1.1 Human subjects

Human brain slices used for p62 immunostaining in **Paper I** were provided by the brain bank of Karolinska Institutet. Human brain homogenates for p62 immunoblotting in **Paper I** were provided by the Netherland brain bank. The Braak stage for control subjects is 0-I and for AD subjects is V-VI.

The CSF proteomic results from human subjects in **Paper III** have been reported in a previous study (180, 181). Briefly, the human subjects are from a European Medical Information Framework for Alzheimer's Disease Multimodal Biomarker Discovery (EMIF-AD MBD) cohort (n = 310). Individuals having abnormal CSF-Aβ42 (a+) levels were defined as AD pathological group (n = 228) while those having normal CSF-Aβ42 (a-) levels with normal cognition were defined as healthy control group (n = 82). The AD pathological group was subdivided into two groups based on CSF-total-tau (CSF-t-tau) levels, abnormal tau (t+) (n = 151) and normal tau (t-) (n = 77). These two AD pathological groups (a+t+ and a+t-) were further classified in three clinical stages based on cognitive performance, normal cognition (NC), MCI and mild to moderate AD-type dementia (**Table 1**). The NC group is also defined as preclinical AD while the MCI group is defined as prodromal AD. All participants granted their consent before inclusion in the study.

Table 1. ***The classification of EMIF-AD MBD cohort.***

CSF-Aβ42	Healthy (a-) (n = 82)	AD pathological group (a+) (n = 228)					
CSF-t-tau	t-	a+t- (n = 77)			a+t+ (n = 151)		
Cognitive performance	NC	NC (n = 36)	MCI (n = 24)	AD (n = 17)	NC (n = 21)	MCI (n = 68)	AD (n = 62)

EMIF-AD MBD: European Medical Information Framework for Alzheimer's Disease Multimodal Biomarker Discovery; NC: normal cognition, MCI: mild cognitive impairment, a+: abnormal CSF-Aβ42, a-: normal CSF-Aβ42, t+: abnormal CSF-t-tau, t-: normal CSF-t-tau.

4.1.2 Animal models

All animals were maintained in 12:12 light-dark cycle and with *ad libitum* access to food and water.

4.1.2.1 App knock-in animal models

In this thesis, I have used two *App* knock-in mouse models, App^{NL-F} and App^{NL-G-F}, which have been generated and described in Saido's laboratory in RIKEN, Japan (3). In these mice, the mouse Aβ sequence was humanized by replacing the three amino acids that differ between mouse and human Aβ sequence (G676R, F681Y, and R684H). To induce the generation of high levels of Aβ42, App^{NL-F} mice harbor two FAD mutations, the Swedish (KM670/671NL) mutation in exon 16 and the Beyreuther/Iberian (I716F) mutation in exon 17 of the *App* gene, while the App^{NL-G-F} mice contain one additional Arctic (E693G) mutation in exon 17 (3).

The *App* knock-in rat model, App^{NL-G-F}, in **Paper V** has the same knock-in design as the App^{NL-G-F} mouse model. The App^{NL-G-F} rat model was generated in Bai Lu's laboratory at Tsinghua University, China.

4.1.2.2 Autophagy-deficient AD mouse models

The autophagy-deficient mouse models were produced by conditional knockout of an essential autophagy gene *Atg7* that plays a key role in autophagosome formation. Two different *Atg7* conditional knockout mice were generated using Cre-*loxP* technology under the control of two different promoters, either CaMKII or Nestin. $Atg7^{flox/flox}$; CaMKII-*Cre* mice were generated by crossing $Atg7^{flox/flox}$ mice containing lox sequence surrounding exon 14 within the *Atg7* gene (provided by Dr. Komatsu) (132) with CaMKII-*Cre* Tg mice (Dr. Shigeyoshi Itohara in RIKEN Brain Science Institute) (133) to specifically knockout *Atg7* in excitatory neurons in the forebrain. $Atg7^{flox/flox}$; Nestin-*Cre* mice were generated by crossing $Atg7^{flox/flox}$ mice with Nestin-*Cre* Tg mice (purchased from Jackson Laboratory) (182) to exhibit the *Atg7* knockout in the central nervous system bypassing the embryonic lethality (134).

In our laboratory, we generated new autophagy-deficient AD mouse models by crossing the $Atg7^{flox/flox}$; CaMKII-*Cre* or $Atg7^{flox/flox}$; Nestin-*Cre* mice with *App* knock-in mice. $Atg7^{flox/flox}$; CaMKII-*Cre* x App^{NL-F} (*Atg7* cKO x App^{NL-F}) and $Atg7^{flox/flox}$; CaMKII-*Cre* x App^{NL-G-F} (*Atg7* cKO x App^{NL-G-F}) mouse models were used for *in vivo* study to analyze the role of autophagy in Aβ metabolism in the mouse brains. $Atg7^{flox/flox}$; Nestin-*Cre* mice were used for *in vitro*

studies to analyze the role of autophagy in Aβ secretion in mouse primary neurons (**Figure 5**).

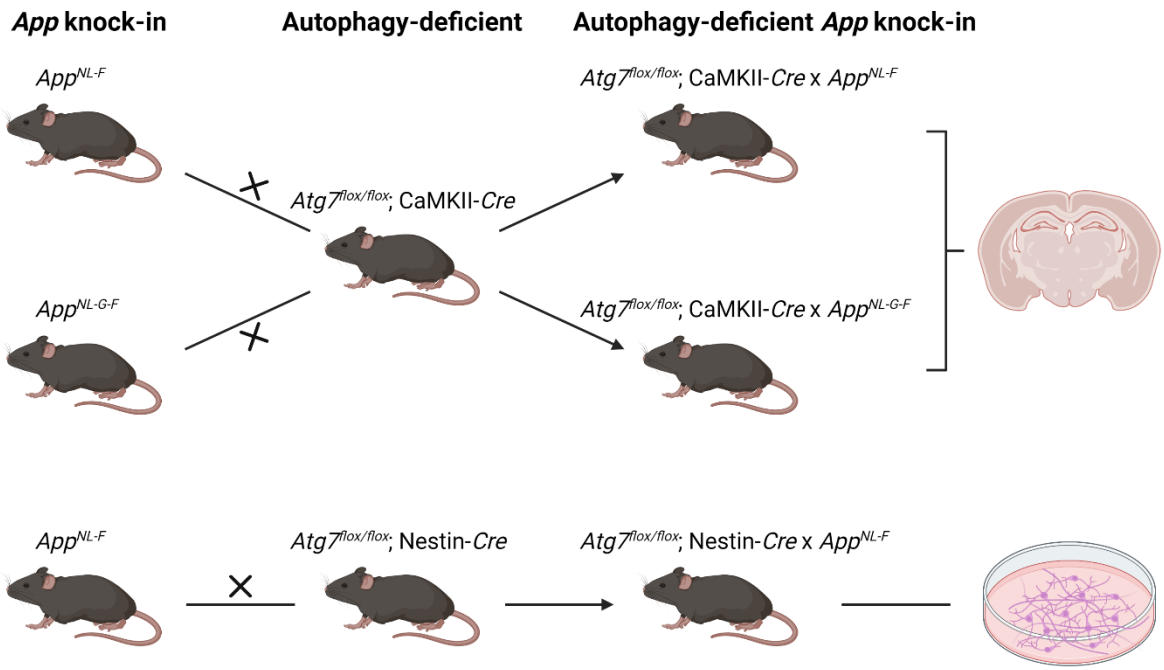


Figure 5. *Crossing scheme to generate autophagy-deficient App knock-in mouse models. CaMKII-Cre mediated deletion leads to the deletion of autophagy in excitatory neurons in postnatal stage whereas Nestin-Cre leads to the deletion of autophagy in central nervous system from prenatal stage. (Created with BioRender.com)*

4.1.2.3 APP Tg mouse models

Two APP Tg mice were used in **Paper III**. APP23 mice containing only the Swedish (KM670/671NL) mutation (183) were kindly provided by Per Hammarström's laboratory at Linköping university. Tg-ArcSwe mice harboring both Swedish (KM670/671NL) and Arctic (E693G) mutations (33) were kindly provided by Stina Syvänen's laboratory at Uppsala University.

4.2 EXPERIMENTAL TECHNIQUES

4.2.1 Mouse brain collection and dissection

Mice were anesthetized by isoflurane and perfused with phosphate-buffered saline (PBS) through cardiac perfusion. Mouse brains were collected after removal of skull bone and cut into two hemispheres. Right hemispheres were fixed in the 10% formalin solution for immunohistology. Left hemispheres were dissected into cortex and hippocampus and kept at

-80°C for immunoblotting or enzyme-linked immunosorbent assay (ELISA). For laser microdissection (LMD), the brains were fixed in OCT compound on dry ice and kept at -80°C.

4.2.2 Mouse CSF collection

Mice were deeply anesthetized by isoflurane and the heads were fixed on the mouse stereotaxis apparatus with an angle of the neck around 135° from the body. After removal of the fur, sagittal incision was performed on the skin of the head. Subcutaneous tissues, fat and muscles were carefully separated under the dissection microscope to expose the dura mater on the cisterna magna and the blood on dura mater was cleaned by PBS-soaked cotton swabs. An area without blood vessels was punctured with a 27-gauge needle carefully to avoid any bleeding. A glass capillary was placed on the punctured region to collect the CSF and transferred to a low affinity Eppendorf tube. After several rounds of collection, around 10-15 µL of CSF was sampled and stored at -80°C (**Figure 6**).

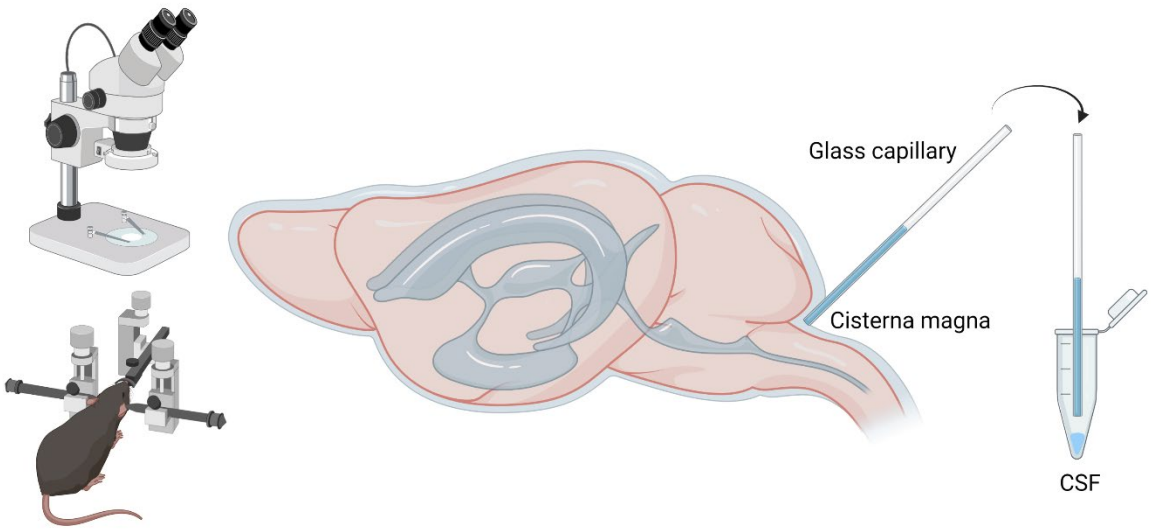


Figure 6. *Mouse CSF collection procedure. CSF is collected through the dura mater from cisterna magna. (Created with BioRender.com)*

4.2.3 Primary neuron culture from mice

Heads from E16-18 embryos were dissected under the dissection microscope followed by the dissection of the brain. Cell suspensions of cortex/hippocampus tissue were prepared by pipetting the tissue up and down in media containing 97% Neurobasal medium + 2% B-27 + 1% Glutamax. Cells from cortex/hippocampus cell suspension were counted by a hematocytometer and co-cultured in Poly-D-lysine coated plates. Cells were collected in

radioimmunoprecipitation assay lysis buffer, sonicated, and centrifuged at 4°C, and kept at -80°C.

4.2.4 Western blot

Cell lysates or tissue homogenates from fresh frozen mouse brains were mixed with β-Mercaptoethanol and 4x Laemmli sample buffer and boiled at 95°C for 5 min. Equal amount of proteins were loaded in 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis for separation and transferred to polyvinylidene difluoride or nitrocellulose membranes. Membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. Next day, membranes were incubated with secondary antibodies for 1 hour at room temperature and images were acquired by a fluorescence imaging system (Odyssey CLx). Images were analyzed using Image Studio Lite software.

4.2.5 Immunohistochemistry

For Immunostaining of human brain tissues, DAB staining was performed with Dako EnVision Systems. Tissue sections from paraffin-embedded brains were deparaffinized and blocked by peroxidase for 5 min. Slides were further blocked with 10% normal goat serum at room temperature for 20 min and incubated with primary antibodies overnight at 4°C. On the following day, slides were incubated with secondary antibodies at room temperature for 30 min and subsequently incubated with chromogen solution at room temperature for 5 min. Thereafter, slides were counterstained with Mayer's Hematoxylin solution for 30 sec and followed by dehydration. Images were acquired by Nikon light microscope.

Paraffin-embedded mouse brain tissue sections were deparaffinized and antigen retrieval was performed if needed. Sections were blocked by blocking buffer and incubated with primary antibodies overnight at 4°C. Next day, slides were incubated with secondary antibodies for 2 hours at room temperature and nuclei were counterstained with Hoechst solution. TSA Fluorescein System was used for amplifying the signals if the amount of antigen was low in the brains. Images were acquired by Nikon fluorescence microscope.

All immunostaining images were analyzed and quantified by ImageJ software.

4.2.6 ELISA

Mouse decorin ELISA was performed following the manufacturer's instructions (Abcam) to measure decorin levels in mouse CSF in **Paper III**. Aβ42 ELISA was performed following

the manufacturer's protocol (Wako) to measure Aβ42 levels in EVs samples from mouse primary neurons in **Paper IV**. The absorbance was read at 450 nm using a microplate reader.

4.2.7 Mouse behavior tests

Batteries of mouse behavioral tests were conducted using 16-18 months old mice and the sequence of behavioral tests follows the least to the most stressful to the mice; Open field (OF), Object interaction (OI), Elevated plus maze (EPM), Modified Y-maze, Marble burying (MB), Morris water maze (MWM). Experimental procedures were performed under white light from 10:00 to 17:00. Mice were transferred to the experimental room 1 hour before starting behavior tests for acclimatization. Apparatuses were cleaned with 70% ethanol between each mouse except for MWM. Video tracking system EthoVision XT 15 (Noldus Information Technology) was used for monitoring, recording, and analyzing the mouse behavioral tests.

4.2.7.1 Open field (OF)

OF consisted of an open square arena with 45 × 45 cm^2 size and mice were allowed to explore the area for 5 minutes after being placed it in the center. Several parameters were obtained from EthoVision XT 15 software including frequency to cross to the center, time spent at the border, time spent in the center, total travel distance and velocity.

4.2.7.2 Object interaction (OI)

OI tests were performed in the same open square arena as used in OF test. Mice were allowed to explore the arena for 5 min in the first day for habituation. Next day, two identical objects were placed in the middle of the arena keeping equal distances to the walls and to each other. Mice were allowed to explore these two objects for 10 min and the total interaction time was obtained and analyzed.

4.2.7.3 Elevated plus maze (EPM)

EPM consisted of two closed arms and two open arms in "+" shape located at 40 cm height. Mice were placed in the center of the plus maze and allowed to move for 5 min. Several parameters were acquired including the number of entries to closed or open arms, time spent in closed or open arms, and frequency of head dips.

4.2.7.4 Modified Y-maze

Modified Y-maze consisted of three identical arms in a “Y” shape. In the training session, one arm was closed by a door and mice were allowed to explore the other two arms for 10 min. After one hour rest, the closed arm was opened which is a “novel unexplored arm” for the mice. Mice were placed at the same position as the training session and the entering chance to the new arm in the first turn was recorded.

4.2.7.5 Marble burying (MB)

MB tests were performed in a rat cage with dimensions of 395 x 346 x 213 mm and filled with 5 cm deep fresh wood chips for bedding. 20 glass marbles (5 × 4) were placed evenly, and mice were allowed to explore the marbles for 30 minutes. The number of buried marbles was recorded.

4.2.7.6 Morris water maze (MWM)

MWM test follows the protocol published by Vorhees et al (184). Briefly, mice were placed in a circular water pool colored with white dye and trained to find a hidden platform according to the direction of the four different cues. The training session consists of five consecutive days, four trials per day, and 60 sec for each trial. In the sixth day (probe test), the platform was removed, and the mice were allowed to swim for 1 min for spatial memory recall. The latency to escape in each training day and time spent in target quadrant in probe test were obtained and analyzed.

4.2.8 Laser microdissection (LMD)

Frozen mouse hippocampal tissues were cryo-sectioned into 20-30 µm thick slices using a Leica3050CM cryostat at -21°C. Sections were placed on polyethylene naphthalate membrane slides and stained with toluidine blue for 30 sec. Tissues from Cornu Ammonis (CA)1 pyramidal cell layers were micro-dissected by an UV laser and collected automatically into the tube caps containing Milli-Q water. The size of the cut area was recorded. Samples were dried using Speedvac and thereafter, used for label-free mass spectrometry (MS) (**Figure 7**).

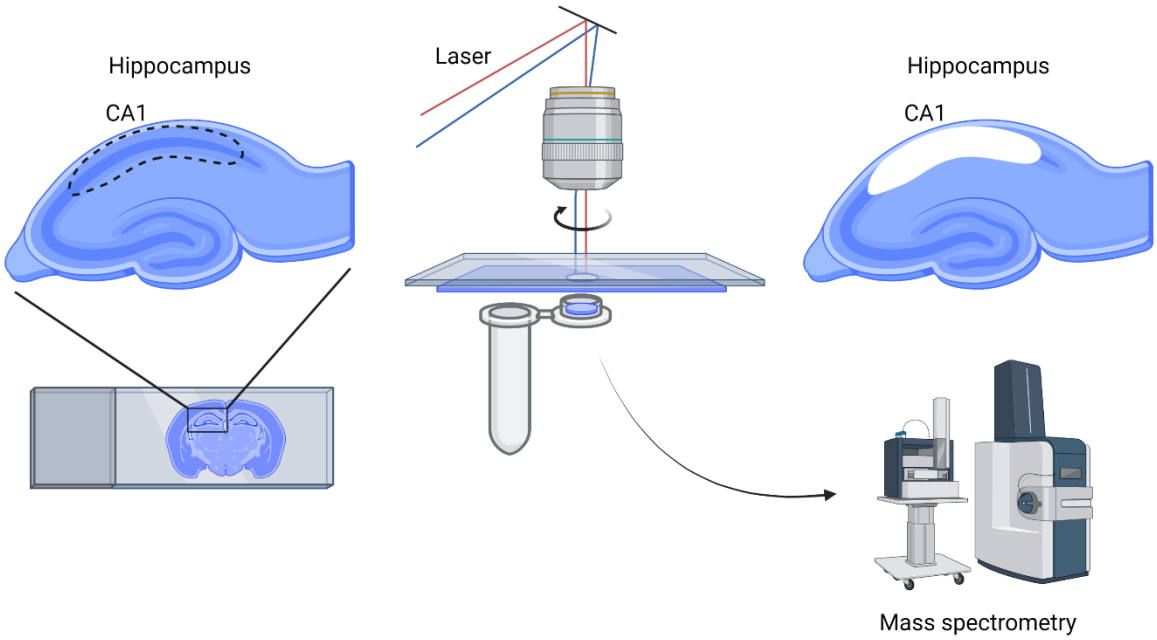


Figure 7. *LMD of mouse hippocampal CA1 regions for MS analysis. (Created with BioRender.com)*

4.2.9 Mass spectrometry

Mouse samples were lysed in lysis buffer with urea followed by sonication and mixing with dithiothreitol. Tryptic digestion of the samples by trypsin was further performed and the peptides were separated by a nano-liquid chromatography-MS/MS which is a Q Exactive Orbitrap mass spectrometer equipped with a nano electrospray ion source. Peptides were eluted with a 150 min linear gradient and the 10 most intense ions were selected for higher-energy collisional dissociation fragmentation. The mass spectrometer was operated in data-dependent mode. Acquired data were processed by MaxQuant and the database searches were performed by Andromeda against the UniProt Mus musculus database. At least two matching peptides were used for protein identification and the protein intensity values were used for label-free quantification.

4.2.10 Transmission electron microscopy

Mice were deeply anaesthetized by isoflurane and cardiac perfusion was performed with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde solution which was further used on tissue fixation. Coronal brain tissue sections were of 1 mm thickness and the hippocampi were dissected under a dissection microscope. Tissues were dehydrated and embedded in LX-112. Prior to performing ultrathin sections, tissue slices were stained with toluidine blue

solution and the region of interest in hippocampus was further selected. Images were acquired by Hitachi HT7700 transmission electron microscope operated at 80 kV equipped with a 2kx2k Veleta CCD camera.

4.2.11 Quantitative real-time polymerase chain reaction (RT-PCR)

RNA was extracted from fresh mouse brain tissues kept in RNA protect tissue reagent. After RNA concentration measurement, 200 ng of RNA was used for synthesizing complementary deoxyribonucleic acid (cDNA). Thereafter, targeted DNA was amplified by RT-PCR and the acquired data were quantified.

4.2.12 Proximity extension assay

Due to the limitation of sample amount, e.g., mouse CSF samples, highly sensitive techniques are required for protein biomarker discovery. Proximity extension assay (PEA) invented by Olink is a powerful technology to discover and quantify proteins with high sensitivity and specificity. Although only one mouse exploratory panel has been established so far, this mouse panel could simultaneously analyze 92 proteins from only 1 μL of biological samples. When a matched antibody-pair containing unique DNA sequences binds to the targeted protein, the unique DNA sequences were allowed to hybridize to each other and further amplified by RT-PCR to increase the sensitivity and specificity.

4.2.13 EV isolation and characterization

EVs were isolated from fresh frozen mouse brain tissue or conditioned media of mouse primary neuronal cultures by several steps of differential ultracentrifugation as described previously (185). Thereafter, EVs were characterized by two methods, immunoblotting and nanoparticle tracking analysis (NTA). For immunoblotting characterization, two EV marker proteins e.g., flotillin-1 and CD63 were used for EV identification and two intracellular proteins were used for evaluating the contaminations. For nanoparticle tracking analysis, a nanoparticle tracking analyzer Nanosight NS300 was used for measuring the particle size and the abundance of those particles.

4.2.14 Electrophysiology

To obtain hippocampal slices, mouse brains were horizontally sectioned in ice-cold artificial cerebrospinal fluid. Local field potential recordings were performed in CA3 stratum pyramidale. By applying kainic acid, gamma oscillations were elicited and recorded. The

power of gamma oscillation was calculated as the integrated power spectrum between 20 and 80 Hz.

4.3 ETHICAL CONSIDERATIONS

All the experiments conducted on human and rodent materials strictly followed the ethical permits that were approved by the respective regional ethical committees.

The ethical boards and ethical permit numbers are listed below.

Human brain slides for immunohistochemistry in **Paper I**: Brain bank of Karolinska Institutet (Dnr. 2013/1301-31/2).

Human brain homogenates for p62 immunoblotting in **Paper I**: Netherlands Brain Bank (Dnr EPN 2011/962-31/1 and 2018/1993-32 to Karolinska Brain Bank).

Mouse CSF collection from APP23 mice in **Paper III**: Linköping animal ethical board (Dnr. 10925-2020).

Mouse CSF collection from tg-ArcSwe mice in **Paper III**: Uppsala County animal ethics committee, the Swedish Board of Agriculture (Dnr. 5.8.18-20401/2020).

App knock-in rat experiments in **Paper V**: Institutional Animal Care and Use Committee (IACUC) of Tsinghua University (15-LB5).

App knock-in mouse experiments in **Paper V**: Stockholm's animal research ethical board (Dnr 15758-2019).

All the mouse experiments (except for APP23 and tg-ArcSwe mice) in **Paper I-IV**: Linköping animal ethical board (ID 407) and Stockholm animal ethical board (Dnr. 12570-2021).

5 RESULTS AND DISCUSSION

5.1 PAPER I AND II: AUTOPHAGY IS IMPAIRED IN *APP* KNOCK-IN AD MOUSE MODELS

As mentioned previously, compelling data from previous studies have shown a dysfunctional autophagy-lysosomal system in AD (14, 105). Autophagy dysfunction has also been reported in PS1/APP AD mice, an APP Tg mouse model, by describing autophagy induction in early stage and subsequent impairment in late stage (53). However, APP Tg mice have some limitations including overproduced non-Aβ APP fragments which may cause non-AD related phenotypes and mutations in PS1 may further contribute to unrelated phenotypes since PS1 has over 100 substrates (127, 186). Therefore, we first characterized the autophagy status in *App* knock-in mice, a model that overcomes above limitation in APP Tg mice.

In **Paper I**, we first investigated an autophagy marker, p62 in AD postmortem brains by immunostaining and immunoblotting and the data clearly showed an increase of p62 levels in AD as compared to healthy control. Interestingly, p62 was not only increased in the neurons in cortex and hippocampus, including in axonal beadings, but also in the vasculature and corpora amylacea. These p62 accumulations further support previous data on an impairment of the autophagy-lysosomal pathway in neurons and opens up a potential autophagy impairment also in the vessels in AD.

Next, we analyzed the autophagy status in *App* knock-in mice to evaluate whether autophagy dysfunction is also present in these Aβ mouse models. We first investigated p62 levels in 12-month-old App^{NL-F} and App^{NL-G-F} mice which exhibit drastic differences in Aβ plaque depositions. Interestingly, p62 was significantly increased in App^{NL-G-F} mice whereas no changes were observed in App^{NL-F} mice as compared to wildtype (WT). In addition, LC3-II, a specific autophagosomal marker, was significantly increased in App^{NL-G-F} mice whereas the levels were not changed in cortex and even slight decreased in hippocampus of App^{NL-F} mice. Taken these data together indicate a potential inhibition of autophagy-lysosomal pathway at 12-month-old App^{NL-G-F} mice.

To better characterize the autophagy alterations taking into account the whole autophagy process, we investigated the molecules that are critical for autophagy initiation and autophagosome formation. Notably, p-Ulk1 S555 (phosphorylation site for Ulk1 activation) was increased in the cortex of 12-month-old App^{NL-G-F} mice whereas p-Ulk1 S757 (phosphorylation site for Ulk1 inhibition) was not altered. In addition, both Atg7 and Atg9A

were significantly increased in *App*$^{NL-G-F}$ mice while no changes of Atg5-Atg12 conjugation and Atg16L were observed. All these above autophagy markers, however, were not changed in 12-month-old *App*$^{NL-F}$ mice. Taken together, an increase of autophagy initiation may occur in *App*$^{NL-G-F}$ mice to compensate the inhibition of the end stage autophagosomal-lysosomal degradation.

Considering the autophagy impairment in *App*$^{NL-G-F}$ mice being driven by severe Aβ amyloidosis, we further investigated old *App*$^{NL-F}$ mice that exhibit more pronounced Aβ plaque depositions as compared to younger mice. Although p62 and LC3-II levels were not changed in brain homogenates of 18- and 24-month-old *App*$^{NL-F}$ mice as compared to WT, we did observe LC3-positive puncta around Aβ plaques by double immunostaining. Indeed, those LC3-positive puncta most likely represent accumulated AVs in dystrophic neurites which was indeed also revealed by EM imaging similar to what is also seen in AD brains (14). Hence, the dysfunction of autophagy-lysosomal pathway in *App*$^{NL-F}$ mice is more specifically related to the regions, around Aβ plaques, and this effect may be masked in the total brain homogenate.

In **Paper II**, the autophagy dysfunction in *App* knock-in mice was further investigated and substantiated at gene expression levels by time course transcriptome analysis of hippocampus. The pathway analysis of transcriptomic data showed a slight increase in activity of the autophagic pathway at two-month-old *App*$^{NL-G-F}$ mice which was followed by a decrease from six months of age. The transcriptional upregulation of autophagy in AD brains has been reported in AD brains by Lipinski et al. and they claimed that there might be a compensatory autophagic regulation to remove those harmful effects of Aβ exposure (106).

Overall, the autophagy related genes were mainly altered in *App*$^{NL-G-F}$ mice from six months of age. The mTOR signaling pathway, a negative regulator of autophagy, was activated in 12-month-old *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mice whereas the expression of *Trim30a* (a substrate for ubiquitin-autophagy pathway), *Rubcnl* (a positive autophagic regulator for autophagosome formation), *Vamp8* (a lysosomal associated gene), *Lamp2* (a lysosomal associated gene) and *Rab7b* (a negative autophagic regulator) were significantly increased in six-month-old *App*$^{NL-G-F}$ mice which was confirmed by quantitative RT-PCR. Moreover, EM imaging analysis revealed that an accumulation of AVs in dystrophic neurites and pre-synaptic regions was associated with an increased number of synaptic vesicles and decreased post synaptic densities around Aβ plaques in 12-month-old *App*$^{NL-G-F}$ mice. Taking all these data together indicate that autophagy is mainly altered in *App*$^{NL-G-F}$ mice exhibiting the most severe pathology and those autophagy alterations may be closely related to synaptic dysfunction.

Therefore, we further focused on the autophagy status in synapses in 12-month-old *App*$^{NL-G-F}$ mice. A triple immunostaining showed that surrounding Aβ plaques, synaptophysin, a pre-synaptic marker, was accumulated and partially colocalized with LC3-positive puncta which support the EM data. This data indicates that the autophagy impairment in pre-synapses may contribute to the accumulation of synaptic vesicles leading to synaptic dysfunction in AD brains.

In agreement with the data in **Paper I**, p62 and LC3-II were significantly increased in hippocampal synaptosomal fraction of 12-month-old *App*$^{NL-G-F}$ mice. However, these changes were not observed in two-month-old *App*$^{NL-G-F}$ mice as well as in primary neurons from *App*$^{NL-G-F}$ mice indicating that autophagy alteration may occur after two months of age. Furthermore, in **Paper II**, a link between altered energy metabolism evaluated by mitochondrial functional experiments and autophagy alterations in *App*$^{NL-G-F}$ mice was suggested and it is tempting to speculate that this involves mitophagy. One more interesting finding of this paper is that the genes related to neuroinflammation were significantly altered mainly in *App*$^{NL-G-F}$ mice including an increase of *Ccl3*. Interestingly, the Ccl3 protein levels were found to be increased in the CSF of *App*$^{NL-G-F}$ mice measured by PEA. One study has reported that the Ccl3 levels are increased in AD brains (187). All these together open the possibilities that inflammatory proteins can be utilized as potential CSF biomarkers in AD.

5.2 PAPER III: INCREASED CSF-DECORIN PREDICTS BRAIN PATHOLOGICAL CHANGES DRIVEN BY ALZHEIMER'S Aβ AMYLOIDOSIS

App knock-in mouse models exhibiting robust Aβ pathologies, resemble to some extent the autophagy alterations in AD brains (**Paper I**) and, furthermore, there are potential opportunities that mouse brain pathological changes could be reflected in CSF (**Paper II**). This together prompted us to investigate the CSF of *App* knock-in mice in more detail and to find possible CSF AD biomarkers including autophagy-associated biomarkers.

In **Paper III**, we performed label-free MS to analyze CSF from 12-month-old *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mice which exhibit a different degree of Aβ amyloidosis and autophagy alterations. Notably, up to 703 proteins were identified from the limited volume of CSF samples and 246 proteins that were identified and quantified in all the individuals (n = 4/group, WT, *App*$^{NL-F}$ and *App*$^{NL-G-F}$) were included in the downstream analysis. Principal component analysis of these 246 proteins showed a separation of protein clusters between the different models confirming the differences of *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mouse models. Analyzing the

significantly changed proteins, several ECM proteins were differently altered in *App* knock-in mice as compared to WT mice. In App^{NL-F} mice, decorin and lumican, which are also autophagy-associated proteins, were upregulated while SPARC-like protein 1 and fibronectin were downregulated as compared to WT. In App^{NL-G-F} mice, however, most ECM proteins were downregulated including SPARC-like protein 1, fibronectin, basement membrane-specific heparan sulfate proteoglycan core protein, collagen alpha-1(I) chain, vitronectin, fibulin-1 and ecm1 protein. In addition, PEA analysis additionally revealed two ECM proteins, CCN family member 4 and matrilin-2 as significantly ($p < 0.05$) altered in *App* knock-in mice. Taken together, ECM proteins are significantly and differently altered in the CSF of *App* knock-in mice which may be caused by the severity of Aβ pathologies in the brains.

We next compared our mouse CSF proteomic data with human CSF proteomic data from EMIF-AD MBD cohort published previously (180) (detail information for this cohort described in the section MATERIALS AND METHODS). Interestingly, protein alterations in the CSF of *App* knock-in mice matched to a higher degree the alterations in the CSF of a+t- human subjects than to that of a+t+ human subjects. This can be explained by *App* knock-in mouse models being Aβ models with less pronounced tau pathology. Therefore, we mainly focused on comparing the data from mouse with a+t- human subjects.

Notably, only one protein, decorin, was significantly increased in both 12-month-old App^{NL-F} mice and a+t- NC subjects. A time course measurement clearly showed that CSF-decorin level was already increased in three-month-old App^{NL-F} mice and decreasing upon aging. In contrast to the MS data, the CSF-decorin levels were also increased in 13-month-old App^{NL-G-F} mice as compared to WT measured by ELISA. The reason could be due to the detection of different proteolytic fragments of decorin by the two methods. In addition, several different mRNA transcripts of decorin exists and the protein products of these may be differently identified by the two methods. Most importantly, CSF-decorin level was significantly increased in a+t- NC subjects as compared to healthy control and the level was decreased in a+t- MCI and AD subjects which showed a similar pattern as App^{NL-F} mice. Interestingly, in a+t- NC subjects, CSF-decorin levels positively correlated with CSF-Aβ42 levels whereas a negative correlation was observed between CSF-decorin and CSF-t-tau and CSF-phosphorylated-tau (CSF-p-tau). To evaluate the potential for CSF-decorin as biomarker for early AD diagnosis, we performed receiver operating characteristic analysis in a+t- preclinical AD subjects vs healthy controls. The results indicated not high enough specificity and sensitivity. However, CSF-decorin indeed classified an AD subtype (180) characterized

by innate immune activation and a potential ChP dysfunction with high sensitivity and specificity (area under the curve = 0.70, $p = 1.73\times10^{-6}$). Although the correlation of CSF-decorin and ChP pathological changes in this subtype need to be warranted and confirmed in a second cohort, the increase of CSF-decorin levels, to some extent, could predict the innate immune activation and ChP dysfunction in the brain.

To understand mechanistically the correlation of CSF-decorin and ChP dysfunction, we analyzed decorin expression in mouse brains. Interestingly, decorin expression in ChP in *App*$^{NL-F}$ mice was significantly higher than in WT. Moreover, decorin was expressed in parvalbumin-positive interneurons and the expression levels were decreased in *App* knock-in mice especially in *App*$^{NL-G-F}$ mice. Human Aβ42 treatment *in vitro* significantly increased the decorin secretion from mouse primary neurons indicating a direct link between decorin alterations and Aβ amyloidosis. Taken together, early Aβ amyloidosis may increase the decorin expression in ChP as well as increase the secretion of neuron-derived decorin to interstitial fluid which can be a potential cause for an increase of CSF-decorin levels.

As several studies reported decorin could activate autophagy in endothelial cells, we were interested in the relationship between decorin and autophagy in a neuronal setting. By analyzing autophagy markers and phospho-explorer antibody array in decorin treated mouse primary neurons, decorin may stimulate the autophagy-lysosomal pathway via activating lysosomal degradation rather than increasing autophagy initiation.

5.3 PAPER IV: DEGREE OF Aβ AMYLOIDOSIS HAS DISTINCT EFFECTS ON MOUSE PHENOTYPES INDUCED BY LOSS OF AUTOPHAGY

Loss of autophagy in excitatory neurons reduces extracellular Aβ plaques and induces intracellular Aβ accumulation in APP Tg mice (116). We were thus interested if these Aβ pathological changes are still valid in other AD mouse models free of APP overexpression. Therefore, we generated autophagy-deficient AD mouse models by crossing *Atg7* cKO (*Atg7*$^{flox/flox}$; CaMKII-*Cre*) mice with *App* knock-in mice to achieve a conditional knockout of *Atg7* in excitatory neurons in the forebrain. Indeed, significantly less Aβ plaque depositions were observed in *Atg7* cKO x *App*$^{NL-F}$ and *Atg7* cKO x *App*$^{NL-G-F}$ as compared to *App*$^{NL-F}$ and *App*$^{NL-G-F}$ respectively whereas more intracellular Aβ were detected in CA1 pyramidal cell layer of *Atg7* cKO x *App*$^{NL-F}$ mouse hippocampus as compared to *App*$^{NL-F}$ mice. Studies have shown that intracellular Aβ is toxic to the neurons which may affect the cellular physiological function and contribute to the neuronal loss (188, 189). On the other hand, the clinical

benefits from a reduction of Aβ plaques by monoclonal antibody aducanumab treatment indicate that extracellular Aβ depositions harm the brain (8).

Considering EVs may play a role in Aβ secretion via autophagy, we purified small EVs (mainly exosomes) from cortex and hippocampus of the same age of mice that we found having a reduction of Aβ plaques. The EV isolation samples were characterized by presence of two typical EV markers, transmembrane protein CD63 and the cytosolic protein flotillin-1 by immunoblotting. Interestingly, both p62 and LC3-II were also detected in EV samples indicating that those isolated EVs were associated with the autophagic pathway in the cells. NTA of EVs clearly showed that the vesicle size corresponding to the highest concentration was less than 150 nm which is a typical for small EV size. Unexpectantly, small EV abundances in the brains of autophagy-deficient mice and autophagy-competent mice were similar. One potential reason is that the small EVs isolated from brain tissues can be derived from many different cell types including neurons, microglia, and astrocytes and the autophagy-deficiency is specific to excitatory neurons. Therefore, we next studied the small EVs derived from neurons *in vitro*. We generated a new mouse model $Atg7^{flox/flox}$; Nestin-*Cre* x App^{NL-F} which exhibits autophagy-deficiency and Aβ pathologies from prenatal stage allowing us to study the role of autophagy in mouse primary neurons. After purification of small EVs from conditioned media of $Atg7^{flox/flox}$; Nestin-*Cre* x App^{NL-F} mouse primary neurons, EV samples were characterized by immunoblotting and NTA which confirmed the successful purification. Intriguingly, the concentration of small EVs from $Atg7^{flox/flox}$; Nestin-*Cre* or $Atg7^{flox/flox}$; Nestin-*Cre* x App^{NL-F} neurons were higher than those from WT and App^{NL-F} neurons whereas the Aβ42 levels per million EVs were similar between $Atg7^{flox/flox}$; Nestin-*Cre* x App^{NL-F} and App^{NL-F} neurons. This reveals that loss of autophagy in neurons may increase the release of small EVs in an autophagy-independent manner to compensate for the inhibited autophagy degradation to reduce the waste material such as Aβ inside the neurons. However, *in vivo*, those secreted Aβ in small EVs could be easily phagocytosed by other cell types including, neurons and glial cells, rather than deposits in the extracellular space.

The alterations of intracellular and extracellular Aβ depositions induced by loss of autophagy prompted us to investigate mouse behavioral and memory phenotypes as well as other pathological changes which may be caused by this changed pattern of the Aβ pool. Firstly, we observed a shorter life span of autophagy-deficient mice as compared to autophagy-competent mice and especially the autophagy-deficient App^{NL-G-F} mice have the shortest life span; only approximately 25% of mice are alive at 20 months of age. In addition, the body weight of autophagy-deficient App^{NL-G-F} mice was lower whereas autophagy-deficient App^{NL-}

^{F} mice were almost the same weight as WT mice. OF and EPM tests clearly showed that *Atg7* cKO mice exhibited thigmotaxis and less anxiety behavior and these behavior phenotypes were worsened in *Atg7* cKO x App^{NL-G-F} mice whereas interestingly an improvement was noted in *Atg7* cKO x App^{NL-F} mice. This thigmotaxis behavior was further confirmed by OI and MB tests. These data together indicate that loss of autophagy in combination with the severe Aβ pathology in App^{NL-G-F} mice induces most striking behaviors including less curiosity, decreased anxiety, and autistic-like behavior which tends to be improved in autophagy-deficient App^{NL-F} mice having less pronounced Aβ pathology. Furthermore, the memory was most severely impaired in *Atg7* cKO x App^{NL-G-F} mice measured by modified Y-maze and MWM. All these behavior impairments in *Atg7* cKO x App^{NL-G-F} mice can be partially explained by decreased gamma oscillatory network activity and synaptic density in hippocampal CA3 as well as increased onset of apoptosis and necroptosis in CA1 pyramidal cell layer evaluated by cleaved caspase-3, RIPK1 and RIPK3.

Since we found intracellular Aβ accumulation and increased programmed cell death in CA1 pyramidal cell layer in *Atg7* cKO x App^{NL-F} mice, we were interested in this specific region. Therefore, we isolated CA1 pyramidal cell layer by LMD from WT, App^{NL-F}, *Atg7* cKO and *Atg7* cKO x App^{NL-F} mice and performed label-free MS which identified up to 2753 proteins in total. Among those, 742 proteins were identified in all the individuals and 106 proteins were significantly altered (false discovery rate < 0.05) in autophagy-deficient mice as compared to autophagy-competent mice. Gene ontology biological pathway analysis of these significantly altered proteins revealed that the biological process “transport” was upregulated whereas the “translation” was downregulated in *Atg7* cKO and *Atg7* cKO x App^{NL-F} mice. To investigate the protein alterations induced by one single pathological factor, Aβ amyloidosis or autophagy-deficiency, or a combination of these two factors, we compared every two groups to each other. Interestingly, while autophagy-deficiency induced a downregulation of ribosomal proteins in *Atg7* cKO mice, Aβ amyloidosis increased the levels of ribosomal proteins in App^{NL-F} mice. This led to only one significantly downregulated ribosomal protein in *Atg7* cKO x App^{NL-F} mice indicating that the combination of autophagy-deficiency and Aβ amyloidosis even out the ribosomal protein alterations. Taking these data together, lack of autophagy increases cellular transport and decreases translation which is ameliorated by a milder Aβ amyloidosis. It is tempting to speculate that an early Aβ amyloidosis induces compensatory cell mechanisms counterbalancing the negative effects driven by lack of autophagy.

5.4 PAPER V: AN *APP* KNOCK-IN RAT MODEL FOR ALZHEIMER'S DISEASE EXHIBITING Aβ AND TAU PATHOLOGIES, NEURONAL DEATH AND COGNITIVE IMPAIRMENTS

A limited number of AD rat models, mainly Tg models have been generated because of a shortage of gene manipulation tools. In this paper, Bai Lu's laboratory at Tsinghua University generated a novel AD rat model, *App* knock-in, that has the same genetic mutations as the $App^{NL\text{-}G\text{-}F}$ mouse model. We next compared the pathological differences between rat and mouse $App^{NL\text{-}G\text{-}F}$ models. The $App^{NL\text{-}G\text{-}F}$ rat model exhibited a robust Aβ pathological development during aging, similar to the $App^{NL\text{-}G\text{-}F}$ mouse model. In contrast to the mouse model, $App^{NL\text{-}G\text{-}F}$ rats expressed a more pronounced tauopathy. In $App^{NL\text{-}G\text{-}F}$ rats, both AT8 (S202/T205) and AT180 (T231) antibodies recognizing different phosphorylation sites in tau proteins detected that these sites were significantly increased whereas, in $App^{NL\text{-}G\text{-}F}$ mice, AT180 was unaltered as compared to WT mice. In agreement with a previous study (190), the AT8 levels were increased in $App^{NL\text{-}G\text{-}F}$ mice as compared to WT mice showing a slight tau pathology also in the $App^{NL\text{-}G\text{-}F}$ mice. Notably, different intermediate forms of tau aggregates detected by antibodies, APN-mab005, MC1, APN-1607, were significantly increased in $App^{NL\text{-}G\text{-}F}$ rat brains as compared to WT rats. However, no typical structure of NFTs were detected in 22-month-old $App^{NL\text{-}G\text{-}F}$ rat brains. These results demonstrated that $App^{NL\text{-}G\text{-}F}$ knock-in mutations could lead to tau hyperphosphorylation and aggregation in rats but not in mice. This may be caused by lacking 3R tau isoforms in adult mice whereas, in adult rats, there are 3R tau isoforms exist with a lower 3R/4R ratio (1:9) than human (1:1).

Like the $App^{NL\text{-}G\text{-}F}$ mice, enhanced gliosis and synaptic degeneration were observed in $App^{NL\text{-}G\text{-}F}$ rat brains. Importantly, the levels of apoptosis and necroptosis markers were significantly increased in $App^{NL\text{-}G\text{-}F}$ rats but not in $App^{NL\text{-}G\text{-}F}$ mice. It was also noticed that neuronal death and brain atrophy occurred in $App^{NL\text{-}G\text{-}F}$ rats measured by neuronal counting and magnetic resonance imaging. In addition, $App^{NL\text{-}G\text{-}F}$ rats not only exhibited spatial learning and memory deficits from six-month-old similar to $App^{NL\text{-}G\text{-}F}$ mice (191), but also had episodic-like memory impairment that is a typical AD symptoms (192). Overall, compared to the $App^{NL\text{-}G\text{-}F}$ mouse model, the $App^{NL\text{-}G\text{-}F}$ rat model has advantages of resembling AD pathologies, thus making it an ideal rat model for future AD research.

6 CONCLUSIONS

All in all, this thesis contributes to the autophagy and AD fields through studying both how autophagy is regulated by Aβ amyloidosis and how autophagy controls Aβ metabolism. The CSF proteomic data from *App* knock-in mice provide an important database for exploring potential CSF biomarkers for AD. The new *App* knock-in rat model can be a useful tool for understanding the correlation between Aβ and tau pathologies and their interplay with autophagy.

The major findings of this thesis are:

i) Autophagy dysfunction in *App* knock-in mice revealed by the alterations of autophagy-related protein and gene expression levels indicates these mouse models can be an important tool for understanding the correlations between autophagy and Aβ amyloidosis.

ii) Increased CSF-decorin levels at an early stage of AD may mirror the ChP pathological changes in the brain induced by Aβ amyloidosis and also has implications for autophagy regulation.

iii) Loss of autophagy in excitatory neurons in *App* knock-in mice reduces extracellular Aβ plaque depositions, increases intracellular Aβ levels and leads to synaptic degeneration, memory loss and autistic-like behavior. In addition, autophagy-deficiency increases cell transport and decreases protein translation which can be ameliorated by a mild Aβ amyloidosis.

iv) The $App^{NL\text{-}G\text{-}F}$ rat model has some advantages such as hyperphosphorylated and aggregated tau, increased cell death, neuronal loss and episodic-like memory impairment as compared to the $App^{NL\text{-}G\text{-}F}$ mouse model. The limitation of this rat model is an absence of NFTs which is a key hallmark for AD.

7 POINTS OF PERSPECTIVE

The next step is to analyze the autophagy markers that were used in *App* knock-in mice in **Paper I** in AD postmortem brain samples to evaluate how many degrees of autophagy alterations are similar in the Aβ mouse models. It will be interesting to additionally investigate the autophagy status in tau mouse models and *App* knock-in rat model that exhibit pronounced tau pathologies induced by Aβ. This will give a more thorough perspective of autophagy evaluation in AD related to both Aβ or tau or a combination of Aβ and tau pathologies. Taking the data from **Paper I** and **Paper II** into account, $App^{NL\text{-}G\text{-}F}$ mice seem to have more alterations similar to the autophagy dysfunction in AD brains. $App^{NL\text{-}G\text{-}F}$ mice can be potentially used as a model for drug tests targeting Aβ amyloidosis to validate the drug effectiveness from an autophagy perspective. Moreover, drugs aiming at activating or restoring autophagy can be tested.

The CSF proteomic data from *App* knock-in mice provide an important database to the biomarker field. By comparing the CSF proteomics of AD mouse models and AD human, it will give more specific and robust protein targets that are changed similarly in the mice and human and reflect a certain aspect of the disease e.g., amyloidosis. Moreover, an extremely important advantage is that those proteins can be studied on the same mice and analyze those protein expression in the brains which is impossible to be performed in the human brains. This will help us to understand the potential cause of those protein alterations in the CSF. On the other hand, the protein targets, like an ECM protein decorin in **Paper III** can be further studied on postmortem brain tissues with different Braak stages to assess and speculate decorin alterations in the brains in AD spectrum. Decorin is a key component of the ECM that maintains the structure and function of the BBB and BCSFB. The changes of decorin levels may indicate an alteration of BBB and BCSFB functions which is dysfunctional in AD (168, 193). Therefore, before being established as biomarkers in clinics, it is prudent to confirm the validity of those protein targets on other human cohorts as well as the correlation between those proteins and AD core biomarkers.

Having found a clear reduction of extracellular Aβ depositions in autophagy-deficient AD mouse models (both APP Tg and *App* knock-in) reveals autophagy indeed has a crucial role in the Aβ metabolism including degradation and secretion. Surprisingly, in **Paper IV**, a relatively mild Aβ amyloidosis in $App^{NL\text{-}F}$ mice seems have a beneficial effect on autophagy-deficiency induced phenotypes whereas the severe Aβ pathology in $App^{NL\text{-}G\text{-}F}$ mice exhibits a worse effect. Especially the MS results from CA1 pyramidal cells indicate that a mild Aβ

amyloidosis has an opposite effect on protein translation to that of autophagy-deficiency. This attempt us to rethink about the effect of Aβ amyloidosis at an early stage of AD, i.e., preclinical AD or even before, which may be not as negative as we believed. Moreover, it is still not clear what the physiological role of Aβ is, which is constantly generated in the cells. However, severe Aβ amyloidosis at the late stage of AD harms the brain.

From species' evolutionary point of view, rats are closer to humans than mice. Moreover, along with the development of gene manipulation tools in rats, rat models start to draw attention in the AD field. The appearance of an *App* knock-in rat model in **Paper V** exhibiting robust Aβ and tau pathologies as well as neuronal loss and cognitive impairments will provide a beneficial platform to carry out biomarker studies and pharmaceutical trials. Elucidation of autophagy alterations in this model will also be of great importance. The advantages of using rat models instead of mice enable us to obtain more material especially CSF samples which are limited from the mice. Furthermore, rats behave better than mice in memory tests, which is important for the validation of therapeutic effectiveness and has a more elaborated cognitive performance.

8 ACKNOWLEDGEMENTS

At this stage, I would like to say “Thank you!” to all the people that helped and supported me during my PhD journey. Without any of you, this thesis wouldn’t be that wonderful.

No doubt, first of all, I owe a debt of gratitude to all my family. **Meiying**, thank you for being my wife and coming with me here in Sweden. I can’t imagine how my PhD life would be without you and the yummy food that you made. **Junhao**, thank you for being my son and growing up happily and healthily during these 5 years. You are the person who brings a lot of happy viruses to our family. Both of you are my energy sources that drive me to go forward no matter the difficulties I have encountered. Although I have unlimited words to say, this thesis cannot afford it. Therefore, I want to put all my deepest gratitude in three words “I Love You!”.

To my main supervisor **Per**. Tack så mycket! Thank you for accepting me as your PhD student and it’s my great honor also being your first PhD student. I am very proud of it. I still remember how we met in the first time in Changchun, and how we went to the Changbai mountain. Prior to being my supervisor, thank you for being an English teacher. Because of your good teaching with a lot of patience, I can eventually pass the English test and open my PhD journey. As you know, I start my PhD study with almost zero research basis, so thank you for always keeping patient to teach me everything from doing experiments to writing manuscripts and so on. All these efforts you put on me will positively influence my whole research life I believe.

To my co-supervisor **Simone**. It was a lot of fun doing mouse behavior tests with you in the summer 2019. Thanks for teaching me all those technologies and the valuable knowledge. It’s my pleasure to have you as my co-supervisor.

To my co-supervisor **Bengt**. Thank you for inviting me to your summer house every year and enjoying sauna and Surströmming!!! That’s how and where I start to love Surströmming. It was an unforgettable experience. Of course, your expert knowledge and feedbacks are always very helpful.

To our autophagy guys. It was a lot of fun in autophagy meeting in Tromsø with a small piece of real northern light. **Makoto**-san, どうもありがとうございます, 私の友人! You really impressed me with your perfect Japanese WB skills and rigorous research thought. **Johanna**, thank you for helping me with primary neurons - separately culturing 16 embryos in one day. You are the only person understanding me why we are crazy about chocolate!

To Nilsson group members. Thank you all for your valuable feedbacks in the group meetings. **Erika**, thanks for organizing the PhD seminars. **Monika**, thanks for sharing the knowledge about zebrafish. **Hao**, good luck with your PhD study. **Robert, Delia, Yuxi**, thank you for being my students and produced a lot of fantastic data. **Gustaf**, thanks for the flowers in my half time. **Shaffi**, thanks for your company in genotyping. **Ljerka**, it is difficult to forget how you squeeze all the food in one lunch box without cover and carry them to our floor 10 with a tray. **Dina, Federico, Caterina G**, **Viktoria, Erik, Riccardo, Luca, Hongjing, Dominik, Caterina T,** thanks for your help in all sorts of thing.

Zhu Jie, thank you so much for all your help and input, bringing me to KI, and your encouraging words that keep me working hard. **Zhulin, Bo**, I am appreciated your kind help in the beginning of my PhD study.

To our Mexican friends. All of you impressed to me with your passion and enthusiasm for doing anything including dancing. **Luis**, thank you for being a tough and professional gym coach. Also, thanks for sharing the pH meter. **Arturo**, thanks for the collaboration and sharing your expert electrophysiology knowledge with me. **Hugo**, thanks for making amazing paper flowers and bringing life to the kitchen. **Erika**, thanks for being very responsible in the PhD course. **Raul**, thank you for sharing your smart ideas.

Maria A, thank you for being a neighbor from Novum to BC. Most impressively, you always keep your consistent personalities whatever positions you have and say hi to me whenever we met.

Helena, thank you for trusting me to take out the stitches for you. You are my first and the only patient in Sweden.

Una, thanks for helping me with the mouse CSF analysis and statistics. It was very interesting to discuss with you about the clinics and research in general.

Ipsit, you are always very cool, calm and friendly. It was my good luck to have you as my neighbor both in the lab and office.

Hazal, I really appreciate your bioinformatic knowledge and laser microdissection technique that you taught me. Also, thanks to your great contributions in our collaboration.

Shaobo, thanks for sharing your great knowledge about vasculature and let me try those nice vessel markers.

Luana, thanks for teaching me the mouse embryo brain dissection.

Tingting, thanks for your company in the gym.

Xiaofei, thanks for sharing the knowledge about scRNA-seq and good luck with your papers.

Johanna W, thanks for organizing all those one hundred mice for my paper revision. It was super helpful.

Lars, Sophia, Yang Y, Gao, thank you for sharing the cool super resolution technology.

Michael, you are a crazy and ambitious clinician whom I can talk to with clinical mind.

Thanks to all the PIs in Neurogeriatrics, **Marianne, Silvia, Erik S, Maria L**, and all the colleagues, **Ying, Ceren, Erik H, Makiko, Julen, Francesca, Maria LL, Vilma, Anna, Pavel, Lisha, Daniel, Wenchao, Katrine, Giacomo, Nuno**, **Lorena, Yang L, Chenhong, Fengna, Sumonto, Gunilla**, for the excellent scientific discussions as well as the good times we have.

Eva and Elin, thanks to you with all the helps in administrations.

Moreover, I would like to say thank you to all our collaborators. **Martin, Lovisa, Valerie**, thanks for your helps in exosome study and sharing your expert knowledges. **Gefei**, thanks for your home made Aβ proteins which work perfectly. Last but not the least, thanks to other collaborators from all over the world, Uppsala University- **Stina, Dag, Ganna, Jonas,** Linköping University- **Sofie, Per H,** Amsterdam UMC- **Betty, Pieter**, RIKEN- **Naoto**, Tsinghua University-**Keliang, Bai**.

献给家人--**爸爸，妈妈，岳父，岳母**，非常感谢你们一直以来对我的信任、关心和支持。我爱你们，希望你们都平安喜乐！等日成回家好好孝顺你们！

献给吉林大学第一医院耳鼻咽喉头颈外科—**祝威、汪欣**主任，谢谢你们对小姜的信任，使得我有这个宝贵的机会在国外闯荡和学习，我会带着满满的硕果回科室报答你们！**尹万忠、王宇声、杜波**副主任，也非常感谢你们对我的默默支持！还有，所有咱们**科室医护人员**，你们抗疫辛苦了！

I would like to thank the **China Scholarship Council** (No. 201700260295), **Hållstens forskningsstiftelse**, and **Gun och Bertil Stohnes Stiftelse** for fundings the projects enclosed in this thesis.

Paper V is reproduced with permission from Springer Nature.

9 REFERENCES

1. Gauthier S, Rosa-Neto P, Morais J, Webster CJAsDI. World Alzheimer Report 2021: Journey through the diagnosis of dementia. 2021.

2. Olsson B, Lautner R, Andreasson U, Öhrfelt A, Portelius E, Bjerke M, et al. CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. Lancet Neurol. 2016;15(7):673-84.

3. Saito T, Matsuba Y, Mihira N, Takano J, Nilsson P, Itohara S, et al. Single App knock-in mouse models of Alzheimer's disease. Nat Neurosci. 2014;17(5):661-3.

4. Wang H, Dey KK, Chen PC, Li Y, Niu M, Cho JH, et al. Integrated analysis of ultra-deep proteomes in cortex, cerebrospinal fluid and serum reveals a mitochondrial signature in Alzheimer's disease. Mol Neurodegener. 2020;15(1):43.

5. International AsD. World Alzheimer Report 2019: Attitudes to dementia. Alzheimer’s Disease Internationals London; 2019.

6. Padda IS, Parmar M. Aducanumab. StatPearls [Internet]: StatPearls Publishing; 2021.

7. Selkoe DJ. Alzheimer disease and aducanumab: adjusting our approach. Nat Rev Neurol. 2019;15(7):365-6.

8. Sevigny J, Chiao P, Bussière T, Weinreb PH, Williams L, Maier M, et al. The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. Nature. 2016;537(7618):50-6.

9. 2022 Alzheimer's disease facts and figures. Alzheimers Dement. 2022;18(4):700-89.

10. Vinters HV. Cerebral amyloid angiopathy. A critical review. Stroke. 1987;18(2):311-24.

11. Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, Fieremans E, et al. Clearance systems in the brain-implications for Alzheimer disease. Nat Rev Neurol. 2015;11(8):457-70.

12. Leng F, Edison P. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? Nat Rev Neurol. 2021;17(3):157-72.

13. Hamos JE, DeGennaro LJ, Drachman DA. Synaptic loss in Alzheimer's disease and other dementias. Neurology. 1989;39(3):355-61.

14. Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, et al. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. J Neuropathol Exp Neurol. 2005;64(2):113-22.

15. Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci. 2007;120(Pt 23):4081-91.

16. Rowley PA, Samsonov AA, Betthauser TJ, Pirasteh A, Johnson SC, Eisenmenger LB. Amyloid and Tau PET Imaging of Alzheimer Disease and Other Neurodegenerative Conditions. Semin Ultrasound CT MR. 2020;41(6):572-83.

17. Karikari TK, Pascoal TA, Ashton NJ, Janelidze S, Benedet AL, Rodriguez JL, et al. Blood phosphorylated tau 181 as a biomarker for Alzheimer's disease: a diagnostic performance and prediction modelling study using data from four prospective cohorts. Lancet Neurol. 2020;19(5):422-33.

18. Aluise CD, Sowell RA, Butterfield DA. Peptides and proteins in plasma and cerebrospinal fluid as biomarkers for the prediction, diagnosis, and monitoring of therapeutic efficacy of Alzheimer's disease. Biochim Biophys Acta. 2008;1782(10):549-58.

19. Oresković D, Klarica M. The formation of cerebrospinal fluid: nearly a hundred years of interpretations and misinterpretations. Brain Res Rev. 2010;64(2):241-62.

20. Blennow K, Zetterberg H. Cerebrospinal fluid biomarkers for Alzheimer's disease. J Alzheimers Dis. 2009;18(2):413-7.

21. Maddalena A, Papassotiropoulos A, Müller-Tillmanns B, Jung HH, Hegi T, Nitsch RM, et al. Biochemical diagnosis of Alzheimer disease by measuring the cerebrospinal fluid ratio of phosphorylated tau protein to beta-amyloid peptide42. Arch Neurol. 2003;60(9):1202-6.

22. Wesenhagen KEJ, Teunissen CE, Visser PJ, Tijms BM. Cerebrospinal fluid proteomics and biological heterogeneity in Alzheimer's disease: A literature review. Crit Rev Clin Lab Sci. 2019:1-13.

23. Nalivaeva NN, Turner AJ. The amyloid precursor protein: A biochemical enigma in brain development, function and disease. FEBS Lett. 2013;587(13):2046-54.

24. Welander H, Frånberg J, Graff C, Sundström E, Winblad B, Tjernberg LO. Abeta43 is more frequent than Abeta40 in amyloid plaque cores from Alzheimer disease brains. J Neurochem. 2009;110(2):697-706.

25. St George-Hyslop PH, Petit A. Molecular biology and genetics of Alzheimer's disease. C R Biol. 2005;328(2):119-30.

26. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J Neurosci. 1993;13(4):1676-87.

27. Walsh DM, Selkoe DJ. Deciphering the molecular basis of memory failure in Alzheimer's disease. Neuron. 2004;44(1):181-93.

28. McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, et al. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol. 1999;46(6):860-6.

29. Sakono M, Zako T. Amyloid oligomers: formation and toxicity of Abeta oligomers. FEBS J. 2010;277(6):1348-58.

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

31. Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, et al. Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. Neurosci Lett. 2001;306(1-2):116-20.

32. Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, et al. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron. 2003;39(3):409-21.

33. Lord A, Kalimo H, Eckman C, Zhang XQ, Lannfelt L, Nilsson LN. The Arctic Alzheimer mutation facilitates early intraneuronal Abeta aggregation and senile plaque formation in transgenic mice. Neurobiol Aging. 2006;27(1):67-77.

34. Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci. 2006;26(40):10129-40.

35. Knobloch M, Konietzko U, Krebs DC, Nitsch RM. Intracellular Abeta and cognitive deficits precede beta-amyloid deposition in transgenic arcAbeta mice. Neurobiol Aging. 2007;28(9):1297-306.

36. Chui DH, Tanahashi H, Ozawa K, Ikeda S, Checler F, Ueda O, et al. Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat Med. 1999;5(5):560-4.

37. LaFerla FM, Troncoso JC, Strickland DK, Kawas CH, Jay G. Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular Abeta stabilization. J Clin Invest. 1997;100(2):310-20.

38. Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, et al. Intraneuronal Abeta42 accumulation in human brain. Am J Pathol. 2000;156(1):15-20.

39. Mochizuki A, Tamaoka A, Shimohata A, Komatsuzaki Y, Shoji S. Abeta42-positive non-pyramidal neurons around amyloid plaques in Alzheimer's disease. Lancet. 2000;355(9197):42-3.

40. D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH. Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology. 2001;38(2):120-34.

41. D'Andrea MR, Nagele RG, Wang HY, Lee DH. Consistent immunohistochemical detection of intracellular beta-amyloid42 in pyramidal neurons of Alzheimer's disease entorhinal cortex. Neurosci Lett. 2002;333(3):163-6.

42. Nagele RG, D'Andrea MR, Anderson WJ, Wang HY. Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience. 2002;110(2):199-211.

43. Wertkin AM, Turner RS, Pleasure SJ, Golde TE, Younkin SG, Trojanowski JQ, et al. Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. Proc Natl Acad Sci U S A. 1993;90(20):9513-7.

44. Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT. Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. J Cell Sci. 2003;116(Pt 16):3339-46.

45. Plácido AI, Pereira CM, Duarte AI, Candeias E, Correia SC, Santos RX, et al. The role of endoplasmic reticulum in amyloid precursor protein processing and trafficking: implications for Alzheimer's disease. Biochim Biophys Acta. 2014;1842(9):1444-53.

46. Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, et al. Alzheimer's Aβ(1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. Nat Med. 1997;3(9):1021-3.

47. Xu H, Greengard P, Gandy S. Regulated formation of Golgi secretory vesicles containing Alzheimer beta-amyloid precursor protein. J Biol Chem. 1995;270(40):23243-5.

48. Das U, Wang L, Ganguly A, Saikia JM, Wagner SL, Koo EH, et al. Visualizing APP and BACE-1 approximation in neurons yields insight into the amyloidogenic pathway. Nat Neurosci. 2016;19(1):55-64.

49. Anandatheerthavarada HK, Biswas G, Robin MA, Avadhani NG. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. J Cell Biol. 2003;161(1):41-54.

50. Lin MT, Beal MF. Alzheimer's APP mangles mitochondria. Nat Med. 2006;12(11):1241-3.

51. Yu WH, Kumar A, Peterhoff C, Shapiro Kulnane L, Uchiyama Y, Lamb BT, et al. Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for beta-amyloid peptide over-production and localization in Alzheimer's disease. Int J Biochem Cell Biol. 2004;36(12):2531-40.

52. Mizushima N. A(beta) generation in autophagic vacuoles. J Cell Biol. 2005;171(1):15-7.

53. Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, et al. Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol. 2005;171(1):87-98.

54. Alonso A, Zaidi T, Novak M, Grundke-Iqbal I, Iqbal K. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. Proc Natl Acad Sci U S A. 2001;98(12):6923-8.

55. Alonso AD, GrundkeIqbal I, Iqbal K. Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. Nat Med. 1996;2(7):783-7.

56. Alonso AD, Mederlyova A, Novak M, Grundke-Iqbal I, Iqbal K. Promotion of hyperphosphorylation by frontotemporal dementia tau mutations. J Biol Chem. 2004;279(33):34873-81.

57. Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem. 1986;261(13):6084-9.

58. Iqbal K, Grundke-Iqbal I, Zaidi T, Merz PA, Wen GY, Shaikh SS, et al. Defective brain microtubule assembly in Alzheimer's disease. Lancet. 1986;2(8504):421-6.

59. Kopke E, Tung YC, Shaikh S, Alonso AC, Iqbal K, Grundke-Iqbal I. Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. J Biol Chem. 1993;268(32):24374-84.

60. Hanger DP, Anderton BH, Noble W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med. 2009;15(3):112-9.

61. Kosik KS, Orecchio LD, Bakalis S, Neve RL. Developmentally regulated expression of specific tau sequences. Neuron. 1989;2(4):1389-97.

62. Takuma H, Arawaka S, Mori H. Isoforms changes of tau protein during development in various species. Dev Brain Res. 2003;142(2):121-7.

63. Hanes J, Zilka N, Bartkova M, Caletkova M, Dobrota D, Novak M. Rat tau proteome consists of six tau isoforms: implication for animal models of human tauopathies. J Neurochem. 2009;108(5):1167-76.

64. De Duve C, Wattiaux R. Functions of lysosomes. Annu Rev Physiol. 1966;28:435-92.

65. Klionsky DJ, Cueva R, Yaver DS. Aminopeptidase I of Saccharomyces cerevisiae is localized to the vacuole independent of the secretory pathway. J Cell Biol. 1992;119(2):287-99.

66. Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol. 1992;119(2):301-11.

67. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Lett. 1993;333(1-2):169-74.

68. Thumm M, Egner R, Koch B, Schlumpberger M, Straub M, Veenhuis M, et al. Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. FEBS Lett. 1994;349(2):275-80.

69. Harding TM, Morano KA, Scott SV, Klionsky DJ. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. J Cell Biol. 1995;131(3):591-602.

70. Titorenko VI, Keizer I, Harder W, Veenhuis M. Isolation and characterization of mutants impaired in the selective degradation of peroxisomes in the yeast Hansenula polymorpha. J Bacteriol. 1995;177(2):357-63.

71. Harding TM, Hefner-Gravink A, Thumm M, Klionsky DJ. Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. J Biol Chem. 1996;271(30):17621-4.

72. Yuan W, Tuttle DL, Shi YJ, Ralph GS, Dunn WA, Jr. Glucose-induced microautophagy in Pichia pastoris requires the alpha-subunit of phosphofructokinase. J Cell Sci. 1997;110 (Pt 16):1935-45.

73. Sakai Y, Koller A, Rangell LK, Keller GA, Subramani S. Peroxisome degradation by microautophagy in Pichia pastoris: identification of specific steps and morphological intermediates. J Cell Biol. 1998;141(3):625-36.

74. Mukaiyama H, Oku M, Baba M, Samizo T, Hammond AT, Glick BS, et al. Paz2 and 13 other PAZ gene products regulate vacuolar engulfment of peroxisomes during micropexophagy. Genes Cells. 2002;7(1):75-90.

75. Scott SV, Hefner-Gravink A, Morano KA, Noda T, Ohsumi Y, Klionsky DJ. Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. Proc Natl Acad Sci U S A. 1996;93(22):12304-8.

76. Klionsky DJ, Cregg JM, Dunn WA, Jr., Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. Dev Cell. 2003;5(4):539-45.

77. Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. Cell Struct Funct. 2002;27(6):421-9.

78. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, Abdellatif M, Abdoli A, Abel S, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)(1). Autophagy. 2021;17(1):1-382.

79. Cuervo AM. Autophagy: many paths to the same end. Mol Cell Biochem. 2004;263(1-2):55-72.

80. Massey AC, Zhang C, Cuervo AM. Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol. 2006;73:205-35.

81. Ntsapi C, Lumkwana D, Swart C, du Toit A, Loos B. New insights into autophagy dysfunction related to amyloid beta toxicity and neuropathology in Alzheimer's disease. Int Rev Cell Mol Biol. 336: Elsevier; 2018. p. 321-61.

82. Chan EY, Longatti A, McKnight NC, Tooze SA. Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol Cell Biol. 2009;29(1):157-71.

83. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol. 2010;22(2):132-9.

84. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol. 2011;13(2):132-41.

85. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science. 2011;331(6016):456-61.

86. Yamamoto H, Kakuta S, Watanabe TM, Kitamura A, Sekito T, Kondo-Kakuta C, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. J Cell Biol. 2012;198(2):219-33.

87. Lang T, Reiche S, Straub M, Bredschneider M, Thumm M. Autophagy and the cvt pathway both depend on AUT9. J Bacteriol. 2000;182(8):2125-33.

88. Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, et al. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. J Cell Biol. 2000;148(3):465-80.

89. Young AR, Chan EY, Hu XW, Köchl R, Crawshaw SG, High S, et al. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci. 2006;119(Pt 18):3888-900.

90. Mizushima N, Yoshimori T, Ohsumi Y. Role of the Apg12 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2003;35(5):553-61.

91. Fujita N, Hayashi-Nishino M, Fukumoto H, Omori H, Yamamoto A, Noda T, et al. An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. Mol Biol Cell. 2008;19(11):4651-9.

92. Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. J Biol Chem. 2003;278(51):51841-50.

93. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2004;36(12):2503-18.

94. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, et al. A ubiquitin-like system mediates protein lipidation. Nature. 2000;408(6811):488-92.

95. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19(21):5720-8.

96. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol. 2008;445:77-88.

97. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy. 2005;1(2):84-91.

98. Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. Mol Cell. 2009;34(3):259-69.

99. Kraft C, Reggiori F, Peter M. Selective types of autophagy in yeast. Biochim Biophys Acta. 2009;1793(9):1404-12.

100. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem. 2007;282(33):24131-45.

101. Colacurcio DJ, Pensalfini A, Jiang Y, Nixon RA. Dysfunction of autophagy and endosomal-lysosomal pathways: Roles in pathogenesis of Down syndrome and Alzheimer's Disease. Free Radic Biol Med. 2018;114:40-51.

102. Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci. 2008;28(27):6926-37.

103. Caccamo A, Majumder S, Richardson A, Strong R, Oddo S. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. J Biol Chem. 2010;285(17):13107-20.

104. Lee S, Sato Y, Nixon RA. Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's-like axonal dystrophy. J Neurosci. 2011;31(21):7817-30.

105. Wolfe DM, Lee JH, Kumar A, Lee S, Orenstein SJ, Nixon RA. Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification. Eur J Neurosci. 2013;37(12):1949-61.

106. Lipinski MM, Zheng B, Lu T, Yan Z, Py BF, Ng A, et al. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc Natl Acad Sci U S A. 2010;107(32):14164-9.

107. Lipinski MM. Towards the global understanding of the autophagy regulatory network. Autophagy. 2010;6(8):1218-20.

108. Cataldo AM, Barnett JL, Berman SA, Li J, Quarless S, Bursztajn S, et al. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: Evidence for early up-regulation of the endosomal-lysosomal system. Neuron. 1995;14(3):671-80.

109. Orr ME, Oddo S. Autophagic/lysosomal dysfunction in Alzheimer’s disease. Alzheimers Res Ther. 2013;5(5):53.

110. Armstrong A, Mattsson N, Appelqvist H, Janefjord C, Sandin L, Agholme L, et al. Lysosomal network proteins as potential novel CSF biomarkers for Alzheimer's disease. Neuromolecular Med. 2014;16(1):150-60.

111. Cho S-J, Lim HJ, Jo C, Park MH, Han C, Koh YH. Plasma ATG5 is increased in Alzheimer's disease. Sci Rep. 2019;9(1):4741-.

112. Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. PLoS One. 2010;5(4):e9979-e.

113. Shimada K, Motoi Y, Ishiguro K, Kambe T, Matsumoto SE, Itaya M, et al. Long-term oral lithium treatment attenuates motor disturbance in tauopathy model mice: implications of autophagy promotion. Neurobiol Dis. 2012;46(1):101-8.

114. Tian Y, Bustos V, Flajolet M, Greengard P. A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway. FASEB J. 2011;25(6):1934-42.

115. Vingtdeux V, Chandakkar P, Zhao H, d'Abramo C, Davies P, Marambaud P. Novel synthetic small-molecule activators of AMPK as enhancers of autophagy and amyloid-β peptide degradation. FASEB J. 2011;25(1):219-31.

116. Nilsson P, Loganathan K, Sekiguchi M, Matsuba Y, Hui K, Tsubuki S, et al. Aβ secretion and plaque formation depend on autophagy. Cell Rep. 2013;5(1):61-9.

117. Nilsson P, Sekiguchi M, Akagi T, Izumi S, Komori T, Hui K, et al. Autophagy-related protein 7 deficiency in amyloid β (Aβ) precursor protein transgenic mice decreases Aβ in the multivesicular bodies and induces Aβ accumulation in the Golgi. Am J Pathol. 2015;185(2):305-13.

118. Pan BT, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol. 1985;101(3):942-8.

119. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem. 1987;262(19):9412-20.

120. Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. Journal of extracellular vesicles. 2015;4:27066.

121. Record M, Carayon K, Poirot M, Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochim Biophys Acta. 2014;1841(1):108-20.

122. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010;73(10):1907-20.

123. Murrow L, Malhotra R, Debnath J. ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. Nat Cell Biol. 2015;17(3):300-10.

124. Liu J, Zhang Y, Liu A, Wang J, Li L, Chen X, et al. Distinct Dasatinib-Induced Mechanisms of Apoptotic Response and Exosome Release in Imatinib-Resistant Human Chronic Myeloid Leukemia Cells. Int J Mol Sci. 2016;17(4):531.

125. Xu J, Camfield R, Gorski SM. The interplay between exosomes and autophagy - partners in crime. J Cell Sci. 2018;131(15).

126. Sardar Sinha M, Ansell-Schultz A, Civitelli L, Hildesjö C, Larsson M, Lannfelt L, et al. Alzheimer's disease pathology propagation by exosomes containing toxic amyloid-beta oligomers. Acta Neuropathol. 2018;136(1):41-56.

127. Sasaguri H, Nilsson P, Hashimoto S, Nagata K, Saito T, De Strooper B, et al. APP mouse models for Alzheimer's disease preclinical studies. EMBO J. 2017;36(17):2473-87.

128. Cheng IH, Scearce-Levie K, Legleiter J, Palop JJ, Gerstein H, Bien-Ly N, et al. Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. J Biol Chem. 2007;282(33):23818-28.

129. Gessel MM, Bernstein S, Kemper M, Teplow DB, Bowers MT. Familial Alzheimer's disease mutations differentially alter amyloid β-protein oligomerization. ACS Chem Neurosci. 2012;3(11):909-18.

130. Ohsumi Y. Historical landmarks of autophagy research. Cell Res. 2014;24(1):9-23.

131. Kuma A, Komatsu M, Mizushima N. Autophagy-monitoring and autophagy-deficient mice. Autophagy. 2017;13(10):1619-28.

132. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol. 2005;169(3):425-34.

133. Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, et al. Subregion- and cell type-restricted gene knockout in mouse brain. Cell. 1996;87(7):1317-26.

134. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J-i, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880-4.

135. Drummond E, Wisniewski T. Alzheimer's disease: experimental models and reality. Acta Neuropathol. 2017;133(2):155-75.

136. Götz J, Bodea LG, Goedert M. Rodent models for Alzheimer disease. Nat Rev Neurosci. 2018;19(10):583-98.

137. Charreau B, Tesson L, Soulillou JP, Pourcel C, Anegon I. Transgenesis in rats: technical aspects and models. Transgenic Res. 1996;5(4):223-34.

138. Tesson L, Cozzi J, Ménoret S, Rémy S, Usal C, Fraichard A, et al. Transgenic modifications of the rat genome. Transgenic Res. 2005;14(5):531-46.

139. Benedikz E, Kloskowska E, Winblad B. The rat as an animal model of Alzheimer's disease. J Cell Mol Med. 2009;13(6):1034-42.

140. Leon WC, Canneva F, Partridge V, Allard S, Ferretti MT, DeWilde A, et al. A novel transgenic rat model with a full Alzheimer's-like amyloid pathology displays pre-plaque intracellular amyloid-beta-associated cognitive impairment. J Alzheimers Dis. 2010;20(1):113-26.

141. Cohen RM, Rezai-Zadeh K, Weitz TM, Rentsendorj A, Gate D, Spivak I, et al. A transgenic Alzheimer rat with plaques, tau pathology, behavioral impairment, oligomeric aβ, and frank neuronal loss. J Neurosci. 2013;33(15):6245-56.

142. Agca C, Klakotskaia D, Schachtman TR, Chan AW, Lah JJ, Agca Y. Presenilin 1 transgene addition to amyloid precursor protein overexpressing transgenic rats increases amyloid beta 42 levels and results in loss of memory retention. BMC Neurosci. 2016;17(1):46.

143. Hanzel CE, Pichet-Binette A, Pimentel LS, Iulita MF, Allard S, Ducatenzeiler A, et al. Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. Neurobiol Aging. 2014;35(10):2249-62.

144. Qi Y, Klyubin I, Harney SC, Hu N, Cullen WK, Grant MK, et al. Longitudinal testing of hippocampal plasticity reveals the onset and maintenance of endogenous human Ass-induced synaptic dysfunction in individual freely behaving pre-plaque transgenic rats: rapid reversal by anti-Ass agents. Acta neuropathologica communications. 2014;2:175.

145. Petrasek T, Vojtechova I, Lobellova V, Popelikova A, Janikova M, Brozka H, et al. The McGill Transgenic Rat Model of Alzheimer's Disease Displays Cognitive and Motor Impairments, Changes in Anxiety and Social Behavior, and Altered Circadian Activity. Front Aging Neurosci. 2018;10:250.

146. Serneels L, T'Syen D, Perez-Benito L, Theys T, Holt MG, De Strooper B. Modeling the β-secretase cleavage site and humanizing amyloid-beta precursor protein in rat and mouse to study Alzheimer's disease. Mol Neurodegener. 2020;15(1):60.

147. Tambini MD, Yao W, D'Adamio L. Facilitation of glutamate, but not GABA, release in Familial Alzheimer's APP mutant Knock-in rats with increased β-cleavage of APP. Aging Cell. 2019;18(6):e13033.

148. Tambini MD, D'Adamio L. Knock-in rats with homozygous PSEN1(L435F) Alzheimer mutation are viable and show selective γ-secretase activity loss causing low Aβ40/42 and high Aβ43. J Biol Chem. 2020;295(21):7442-51.

149. Pang K, Jiang R, Zhang W, Yang Z, Li LL, Shimozawa M, et al. An App knock-in rat model for Alzheimer's disease exhibiting Aβ and tau pathologies, neuronal death and cognitive impairments. Cell Res. 2022;32(2):157-75.

150. Zhang W, Ge Y, Cheng Q, Zhang Q, Fang L, Zheng J. Decorin is a pivotal effector in the extracellular matrix and tumour microenvironment. Oncotarget. 2018;9(4):5480-91.

151. Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, et al. Decorin causes autophagy in endothelial cells via Peg3. Proc Natl Acad Sci U S A. 2013;110(28):E2582-E91.

152. Neill T, Torres A, Buraschi S, Iozzo RV. Decorin has an appetite for endothelial cell autophagy. Autophagy. 2013;9(10):1626-8.

153. Goyal A, Neill T, Owens RT, Schaefer L, Iozzo RV. Decorin activates AMPK, an energy sensor kinase, to induce autophagy in endothelial cells. Matrix Biol. 2014;34:46-54.

154. Gubbiotti MA, Neill T, Frey H, Schaefer L, Iozzo RV. Decorin is an autophagy-inducible proteoglycan and is required for proper in vivo autophagy. Matrix Biol. 2015;48:14-25.

155. Buraschi S, Neill T, Iozzo RV. Decorin is a devouring proteoglycan: Remodeling of intracellular catabolism via autophagy and mitophagy. Matrix Biol. 2019;75-76:260-70.

156. Neill T, Torres A, Buraschi S, Owens RT, Hoek JB, Baffa R, et al. Decorin induces mitophagy in breast carcinoma cells via peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and mitostatin. J Biol Chem. 2014;289(8):4952-68.

157. Schaefer L, Tredup C, Gubbiotti MA, Iozzo RV. Proteoglycan neofunctions: regulation of inflammation and autophagy in cancer biology. FEBS J. 2017;284(1):10-26.

158. Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. Oncogene. 2002;21(31):4765-77.

159. Järveläinen H, Puolakkainen P, Pakkanen S, Brown EL, Höök M, Iozzo RV, et al. A role for decorin in cutaneous wound healing and angiogenesis. Wound Repair Regen. 2006;14(4):443-52.

160. Järveläinen H, Sainio A, Wight TN. Pivotal role for decorin in angiogenesis. Matrix Biol. 2015;43:15-26.

161. Järvinen TA, Ruoslahti E. Target-seeking antifibrotic compound enhances wound healing and suppresses scar formation in mice. Proc Natl Acad Sci U S A. 2010;107(50):21671-6.

162. Horváth Z, Kovalszky I, Fullár A, Kiss K, Schaff Z, Iozzo RV, et al. Decorin deficiency promotes hepatic carcinogenesis. Matrix Biol. 2014;35:194-205.

163. Merline R, Moreth K, Beckmann J, Nastase MV, Zeng-Brouwers J, Tralhão JG, et al. Signaling by the matrix proteoglycan decorin controls inflammation and cancer through PDCD4 and MicroRNA-21. Science signaling. 2011;4(199):ra75.

164. Bocian C, Urbanowitz AK, Owens RT, Iozzo RV, Götte M, Seidler DG. Decorin potentiates interferon-γ activity in a model of allergic inflammation. J Biol Chem. 2013;288(18):12699-711.

165. Weis SM, Zimmerman SD, Shah M, Covell JW, Omens JH, Ross J, Jr., et al. A role for decorin in the remodeling of myocardial infarction. Matrix Biol. 2005;24(4):313-24.

166. Snow AD, Mar H, Nochlin D, Kresse H, Wight TN. Peripheral distribution of dermatan sulfate proteoglycans (decorin) in amyloid-containing plaques and their presence in neurofibrillary tangles of Alzheimer's disease. J Histochem Cytochem. 1992;40(1):105-13.

167. Brandan E, Melo F, García M, Contreras M. Significantly reduced expression of the proteoglycan decorin in Alzheimer's disease fibroblasts. Clin Mol Pathol. 1996;49(6):M351-6.

168. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, et al. Blood-brain barrier breakdown in the aging human hippocampus. Neuron. 2015;85(2):296-302.

169. Baeten KM, Akassoglou K. Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. Dev Neurobiol. 2011;71(11):1018-39.

170. Weber IT, Harrison RW, Iozzo RV. Model structure of decorin and implications for collagen fibrillogenesis. J Biol Chem. 1996;271(50):31767-70.

171. Keene DR, San Antonio JD, Mayne R, McQuillan DJ, Sarris G, Santoro SA, et al. Decorin binds near the C terminus of type I collagen. J Biol Chem. 2000;275(29):21801-4.

172. Reed CC, Iozzo RV. The role of decorin in collagen fibrillogenesis and skin homeostasis. Glycoconj J. 2002;19(4-5):249-55.

173. Rühland C, Schönherr E, Robenek H, Hansen U, Iozzo RV, Bruckner P, et al. The glycosaminoglycan chain of decorin plays an important role in collagen fibril formation at the early stages of fibrillogenesis. FEBS J. 2007;274(16):4246-55.

174. Munji RN, Soung AL, Weiner GA, Sohet F, Semple BD, Trivedi A, et al. Profiling the mouse brain endothelial transcriptome in health and disease models reveals a core blood-brain barrier dysfunction module. Nat Neurosci. 2019;22(11):1892-902.

175. Vanlandewijck M, He L, Mäe MA, Andrae J, Ando K, Del Gaudio F, et al. A molecular atlas of cell types and zonation in the brain vasculature. Nature. 2018;554(7693):475-80.

176. Hanemann CO, Kuhn G, Lie A, Gillen C, Bosse F, Spreyer P, et al. Expression of decorin mRNA in the nervous system of rat. J Histochem Cytochem. 1993;41(9):1383-91.

177. Neill T, Iozzo RV. The Role of Decorin Proteoglycan in Mitophagy. Cancers (Basel). 2022;14(3).

178. Yao T, Zhang CG, Gong MT, Zhang M, Wang L, Ding W. Decorin-mediated inhibition of the migration of U87MG glioma cells involves activation of autophagy and suppression of TGF-β signaling. FEBS open bio. 2016;6(7):707-19.

179. Neill T, Sharpe C, Owens RT, Iozzo RV. Decorin-evoked paternally expressed gene 3 (PEG3) is an upstream regulator of the transcription factor EB (TFEB) in endothelial cell autophagy. J Biol Chem. 2017;292(39):16211-20.

180. Tijms BM, Gobom J, Reus L, Jansen I, Hong S, Dobricic V, et al. Pathophysiological subtypes of Alzheimer's disease based on cerebrospinal fluid proteomics. Brain. 2020;143(12):3776-92.

181. Bos I, Vos S, Vandenberghe R, Scheltens P, Engelborghs S, Frisoni G, et al. The EMIF-AD Multimodal Biomarker Discovery study: design, methods and cohort characteristics. Alzheimers Res Ther. 2018;10(1):64.

182. Betz UA, Vosshenrich CA, Rajewsky K, Müller W. Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. Curr Biol. 1996;6(10):1307-16.

183. Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, et al. Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A. 1997;94(24):13287-92.

184. Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc. 2006;1(2):848-58.

185. Perez-Gonzalez R, Gauthier SA, Kumar A, Levy E. The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space. J Biol Chem. 2012;287(51):43108-15.

186. Nhan HS, Chiang K, Koo EH. The multifaceted nature of amyloid precursor protein and its proteolytic fragments: friends and foes. Acta Neuropathol. 2015;129(1):1-19.

187. Liao Y, Guan ZZ, Ravid R. [Changes of nuclear factor and inflammatory chemotactic factors in brain of patients with Alzheimer's disease]. Zhonghua Bing Li Xue Za Zhi. 2011;40(9):585-9.

188. Wirths O, Bayer TA. Intraneuronal Aβ accumulation and neurodegeneration: lessons from transgenic models. Life Sci. 2012;91(23-24):1148-52.

189. Li M, Chen L, Lee DH, Yu LC, Zhang Y. The role of intracellular amyloid beta in Alzheimer's disease. Prog Neurobiol. 2007;83(3):131-9.

190. Saito T, Mihira N, Matsuba Y, Sasaguri H, Hashimoto S, Narasimhan S, et al. Humanization of the entire murine Mapt gene provides a murine model of pathological human tau propagation. J Biol Chem. 2019;294(34):12754-65.

191. Mehla J, Lacoursiere SG, Lapointe V, McNaughton BL, Sutherland RJ, McDonald RJ, et al. Age-dependent behavioral and biochemical characterization of single APP knock-in mouse (APP(NL-G-F/NL-G-F)) model of Alzheimer's disease. Neurobiol Aging. 2019;75:25-37.

192. Gallagher M, Koh MT. Episodic memory on the path to Alzheimer's disease. Curr Opin Neurobiol. 2011;21(6):929-34.

193. Ott BR, Jones RN, Daiello LA, de la Monte SM, Stopa EG, Johanson CE, et al. Blood-Cerebrospinal Fluid Barrier Gradients in Mild Cognitive Impairment and Alzheimer's Disease: Relationship to Inflammatory Cytokines and Chemokines. Front Aging Neurosci. 2018;10:245.