



**HAL**  
open science

# The implication of the $\eta$ CTF fragment in Alzheimer's Disease: Study of its expression, localization, and degradation

Elissa Afram

► **To cite this version:**

Elissa Afram. The implication of the  $\eta$ CTF fragment in Alzheimer's Disease: Study of its expression, localization, and degradation. Molecular biology. Université Côte d'Azur, 2023. English. NNT : 2023COAZ6002 . tel-04099809

**HAL Id: tel-04099809**

**<https://theses.hal.science/tel-04099809>**

Submitted on 17 May 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# THÈSE DE DOCTORAT

L'implication du fragment  $\eta$ CTF dans la  
Maladie d'Alzheimer : étude de son expression,  
sa localisation et sa dégradation

**Elissa AFRAM**

Institut de Pharmacologie Moléculaire et Cellulaire (IPMC)

Présentée en vue de l'obtention du grade de  
**Docteur en Sciences de la Vie et de la Santé**  
d'Université Côte d'Azur

Dirigée par : **Dr. Raphaëlle PIQUARD-  
PARDOSSI** et **Dr. Frédéric CHECLER**

Soutenue le : 30 Janvier 2023

**Devant le jury, composé de :**

Dr. Santiago RIVERA, INP, Marseille  
Dr. Julien CHAPUIS, Institut Pasteur de Lille  
Dr. Marie-Claude POTIER, ICM, Paris  
Dr. Nicolas SERGEANT, LiNCog, Lille  
Dr. Frédéric CHECLER, IPMC, Sophia Antipolis  
Dr. Raphaëlle PARDOSSI-PIQUARD, IPMC,  
Sophia Antipolis

« L'implication du fragment  $\eta$ CTF dans la Maladie d'Alzheimer :  
étude de son expression, sa localisation et sa dégradation »

« The Implication of the  $\eta$ CTF Fragment in Alzheimer's Disease:  
Study of its Expression, Localization, and Degradation »

**Jury:**

Président du jury:

✚ Dr. Santiago RIVERA, Directeur de recherche, INP, Marseille

Rapporteurs:

✚ Dr. Marie-Claude POTIER, Directrice de recherche, ICM, Paris

✚ Dr. Nicolas SERGEANT, Directeur de recherche, LilNCog, Lille

Examineurs:

✚ Dr. Santiago RIVERA, Directeur de recherche, INP, Marseille

✚ Dr. Julien CHAPUIS, Maître de conférences, Université de Lille/Institut Pasteur de  
Lille

Directeurs de thèse:

✚ Dr. Frédéric CHECLER, Directeur de recherche, IPMC, Sophia Antipolis

✚ Dr. Raphaëlle PARDOSSI-PIQUARD, Chargée de recherche, IPMC, Sophia Antipolis

## Résumé

---

Le peptide amyloïde- $\beta$  ( $A\beta$ ) qui est le constituant principal des plaques séniles est formé suite aux clivages canoniques de la protéine amyloïde précurseur amyloïde (APP) par les activités  $\beta$ - et  $\gamma$ -sécrétases. Dans les conditions physiologiques, l'APP est clivée par les activités  $\alpha$ , et  $\gamma$ -sécrétases, limitant ainsi la production d' $A\beta$ . Récemment, la métalloprotéinase matricielle MT5-MMP, appelée  $\eta$ -sécrétase, a été identifiée comme une nouvelle enzyme de clivage de l'APP. L' $\eta$ -sécrétase clive la protéine APP dans sa partie amino-terminale et produit un fragment soluble, le sAPP $\eta$ , et un fragment transmembranaire, l' $\eta$ CTF qui est aussi substrat de l'activité  $\alpha$ - ou  $\beta$ -sécrétase générant respectivement les fragments solubles  $A\eta\alpha$  ou  $A\eta\beta$ . Ces fragments  $A\eta\alpha$  et  $A\eta\beta$  ont récemment été décrits comme pouvant altérer l'activité synaptique mais la fonction physiopathologique du fragment  $\eta$ CTF n'a pas encore été décrite. Mon projet de thèse a consisté à étudier par différentes approches le catabolisme, la biologie et la localisation cellulaire de ce fragment pour évaluer sa contribution dans la pathologie associée à la maladie d'Alzheimer (MA).

En utilisant des approches pharmacologiques, nous avons d'abord montré que l' $\eta$ CTF est dégradé par les voies du protéasome et de l'autophagie. Nous avons aussi établi pour la première fois que l' $\eta$ CTF est un précurseur des APP-CTFs (C99 et C83), et du peptide  $A\beta$ . En effet, sa surexpression dans des fibroblastes dépourvus d'APP induit non seulement comme attendu la sécrétion d' $A\eta\alpha$  mais aussi la sécrétion du peptide  $A\beta_{40}$ , démontrant que l' $\eta$ CTF se comporte comme un nouveau précurseur véritable d' $A\beta$ . De manière importante, nous avons également développé un nouvel anticorps nommé  $\eta$ CTF-Nter qui interagit spécifiquement avec la partie N-terminale de l' $\eta$ -CTF en ciblant l' $\eta$ -CTF, l' $A\eta\beta$ , l' $A\eta\alpha$  mais pas le C99, le C83 ni l' $A\beta$ . Des expériences d'immunomarquage, utilisant ce nouvel anticorps, *in vitro*, ainsi qu'*ex vivo*, ont révélé une localisation majeure de l' $\eta$ CTF dans le Golgi et une proportion plus faible dans les endosomes. De manière très intéressante et en accord avec cette localisation endosomale, nous avons démontré la présence de l' $\eta$ CTF dans les exosomes purifiés à partir du milieu de culture de cellules SH-SY5Y exprimant l'APP sauvage ainsi que dans les exosomes obtenus à partir de souris sauvages exprimant l' $\eta$ CTF suite à l'injection intracérébroventriculaire de virus exprimant l' $\eta$ CTF (AAV-  $\eta$ CTF). De plus, un marquage spécifique de l' $\eta$ CTF a été détecté autour des plaques amyloïdes par immunomarquage réalisé sur des coupes de cerveau de souris 3xTgAD âgées de 20 mois. Enfin, des expériences préliminaires de potentialisation à long terme (LTP) réalisées sur des coupes de cerveau de souris sauvages ont montré une activité synaptique altérée en présence des peptides recombinants  $A\eta\alpha$  et  $A\eta\beta$ .

$A\beta$  n'est certainement pas le seul acteur pathologique de la MA, puisque d'autres catabolites de l'APP peuvent également être impliqués. En effet, indépendamment de l' $A\beta$ , notre laboratoire a établi que le fragment C99 pouvait contribuer aux dysfonctionnements moléculaires et cellulaires observés dans la MA. Dans une revue dont je suis co-auteur, nous avons proposé que la  $\gamma$ -sécrétase soit considérée comme une enzyme bénéfique inactivant le C99 et avons expliqué comment la toxicité du C99 peut être l'une des raisons pour lesquelles l'inhibition de la  $\gamma$ -sécrétase a pu échouer dans les essais cliniques.

**Mots clés:** Maladie d'Alzheimer,  $\eta$ CTF, Production  $A\beta$ , Vesicules Extracellulaires, Endosomes, Autophagie

## Abstract

---

The amyloid- $\beta$  ( $A\beta$ ) peptides, which accumulate in amyloid plaques, are formed by canonical cleavages of the amyloid precursor protein (APP) by  $\beta$ - and  $\gamma$ -secretases. Under physiological conditions, APP is cleaved by  $\alpha$ - and  $\gamma$ -secretases, precluding the production of  $A\beta$ . Recently, the matrix metalloproteinase MT5-MMP, referred to as  $\eta$ -secretase, has been identified as a novel APP cleaving enzyme. The  $\eta$ -secretase targets the APP protein in its amino-terminal part and produces a soluble fragment, the sAPP $\eta$ , and a transmembrane fragment, the  $\eta$ CTF, which can then be a substrate of the  $\alpha$ - or  $\beta$ -secretase activity generating the soluble fragments  $A\eta\alpha$  or  $A\eta\beta$ , respectively. Although  $A\eta\alpha$  and  $A\eta\beta$  fragments have recently been described as being able to affect synaptic activity, the pathophysiological function of the  $\eta$ CTF fragment has not yet been studied. My PhD project has consisted in studying by different approaches the fate, biology and cellular localization of this fragment and in evaluating its contribution in the pathology associated with Alzheimer's disease (AD).

Using pharmacological approaches, we first showed that  $\eta$ CTF undergoes degradation in the autophagic and proteasomal pathways. We established that  $\eta$ CTF is a precursor for APP-CTFs (C99 and C83), and we also revealed for the first time that overexpressing  $\eta$ CTF in fibroblasts lacking APP promotes the secretion of  $A\eta\alpha$  as well as the production of  $A\beta$  peptides, demonstrating that  $\eta$ CTF operates as a novel genuine precursor of  $A\beta$ . Remarkably, we designed a novel immunological probe referred to as  $\eta$ CTF-Nter that specifically interacts with the N-terminal part of  $\eta$ -CTF, thus targeting  $\eta$ -CTF,  $A\eta\alpha$ ,  $A\eta\beta$  but not C99, C83 nor  $A\beta$ . Immunostaining experiments, using this novel antibody, *in vitro*, as well as *ex vivo*, revealed a major Golgi localization of  $\eta$ CTF and a lower proportion in endosomes. Interestingly, and in agreement with this endosomal localization, we demonstrated the presence of  $\eta$ CTF in exosomes purified from culture medium of SH-SY5Y cells expressing wild-type APP as well as in exosomes obtained from wild type mice expressing  $\eta$ CTF after intracerebroventricular injection of AAV-  $\eta$ CTF. Additionally,  $\eta$ CTF fragments were found surrounding amyloid plaques by immunostaining performed on brain slices of 20 months old 3xTgAD mice. Finally, preliminary long-term potentiation (LTP) experiments performed on brain sections from wild-type mice showed impaired synaptic activity in the presence of recombinant  $A\eta\alpha$  and  $A\eta\beta$  peptides.

$A\beta$  is certainly not the only pathological actor in AD, as other APP catabolites may also be involved. Indeed, independently of  $A\beta$ , the C99 fragment has been shown to contribute to the molecular and cellular dysfunctions in AD. In a review in which I contributed, we proposed that  $\gamma$ -secretase should be considered as a beneficial C99-inactivating enzyme and highlighted how the toxicity of C99 may be one of the reasons why inhibiting  $\gamma$ -secretase failed in clinical trials.

Much research is undoubtedly still needed in order to fully understand the implication of  $\eta$ CTF in AD; however, as a precursor of C99 and of  $A\beta$ ,  $\eta$ CTF displays a theoretical toxic potential. The accumulation of  $\eta$ CTF, its detection around amyloid plaques, as well as its presence in exosomes, which may be responsible for the spread of neuropathology, may all support its potential contribution to AD pathology. Conclusively, this demonstrates the complexity of AD, the multifactorial disease with various toxic factors that are still being investigated, which likely explains the challenges in discovering a curative and effective treatment.

**Keywords:** Alzheimer's Disease,  $\eta$ CTF,  $A\beta$ -Production, Extracellular Vesicles, Endosomes, Autophagic Degradation

## Publications et communications

---

### Papers:



“Is  $\gamma$ -secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99?” J Biol Chem 296, 100489, doi:10.1016/j.jbc.2021.100489 (2021).

Frédéric Checler, **Elissa Afram**, Raphaëlle Pardossi-Piquard, Inger Lauritzen  
-Review-



“The  $\eta$ -secretase-derived APP fragment  $\eta$ CTF is localized in Golgi, endosomes and extracellular vesicles and contributes to  $A\beta$  production.”

**Elissa Afram**, Inger Lauritzen, Alexandre Bourgeois, Wejdane El Manaa, Eric Duplan, Mounia Chami, Audrey Valverde, Bauer Charlotte1, Raphaëlle Pardossi-Piquard and Frederic Checler.

- First-author article- Under Review at CMLS

### Communications :



Réunion Francophone sur la Maladie d'Alzheimer et les Syndromes Apparentés « RFMASA » (September 2021) – Oral presentation of my supervisor Dr. Raphaëlle Pardossi-Piquard



International Conference on Alzheimer's and Parkinson's Diseases « AD/PD<sup>TM</sup> » (March 2022) – Poster presentation « New tools to explore the biological function of  $\eta$ -secretase-derived APP-C-Terminal fragment »



Journée de l'ED85 SVS « JEDN » (September 2022) – Oral presentation «  $\eta$ -secretase-derived APP fragments: Production, proteolysis, biology, and development of a novel immunological probe »

## Acknowledgments

---

First of all, I would like to express my sincere gratitude to the members of my jury, **Dr. Marie-Claude POTIER**, **Dr. Nicolas SERGEANT**, **Dr. Santiago RIVERA**, and **Dr. Julien CHAPUIS**, for accepting to evaluate my work.

Secondly, I want to convey my appreciation to **Dr. Frederic Checler** for placing his trust in me with a project that is so important to him. Without you, Dr. Frédéric, I would'nt have had the chance to earn my PhD. I can't thank you enough for this opportunity, and I hope I've reached your expectations.

I would like to deeply thank my thesis director, **Dr Raphaëlle Pardossi-Piquard**. I consider myself quite blessed to have had you as my thesis supervisor, Raphaëlle, as you combine maternal tenderness with research expertise. I genuinely thank your invaluable guidance during my three years of thesis, your constant availability, and your constructive remarks. All of this has helped me mature, and I will always be extremely grateful. Without forgetting to thank you for helping me with my thesis's posters, paper, manuscript, and oral defense. Thank you for everything you did! I'll miss you dearly.

I would like to thank **Dr. Inger Lauritzen** for teaching me how to perform the immunostaining and exosome purification experiments, as well as for always being available to answer to my never-ending questions. Inger, you have always been a bundle of positivity, and I have always enjoyed seeing your contagious and motivating smile in the office.

**Dr. Julie Dunys**, first, thank you for your help with the LTP experiments, and most importantly, thank you for being a delight to work with. **Dr. Cristine Alves Da Costa** and **Dr. Mounia Chami**, the two future team leaders with scientific and leadership abilities; you are excellent role models and inspirations.

**Dr. Eric Duplan**, first, I would like to thank you for all of your assistance with the molecular biology experiments! And, of course, thank you for our occasional scientific and non-scientific talks. I've always looked forward to our small hallway discussions and laughs.

**Dr. Bruno Vincent**, it was a pleasure to meet someone with such a youthful spirit.

Thank you, **Charlotte**, for everything you do! Especially for dealing with orders and maxipreps. All of the projects rely on you, and you certainly handle a lot while working on your PhD! And best of luck with your thesis.

**Audrey**, having you next to me in the office when I first started my thesis was a blessing. You were always there for me, morally and scientifically, to support and help me. I value the knowledge you've given to me, the assistance you've offered me with various techniques, and our chats, which I've always loved.

**Fanny**, thank you for all the pleasant evenings. Thank you, **Aurore**, for always being such a sweetheart. Thank you **Loan** for training me for the lentivirus production, and I will never forget your calm attitude and amazing sense of humor. **Arnaud**, thank you for always being helpful anytime I came into your office to ask a lot of questions, which happened quite frequently! **Julien**, thank you for believing in my babyfoot skills despite the fact that I absolutely suck. Thank you, **Paula and Ligia**, for being the first to welcome me when I arrived, which made it easier for me to adapt to a new environment. **Céline**, thank you for being a delightful and adorable office coworker. **Juliane** and **Gwendoline**, although I have only known you for a few months, our small talks have always been pleasant and cheerful.

Thank you, **Team Checler**, for being my second family, for being such nice, sweet, and loving people. Thank you for providing a fantastic work atmosphere, and thank you for all of the memories that will bring a smile to my face for the rest of my life.

To all my friends in Lebanon, you've always been on my mind. The thought of having such supportive and caring friends back home motivates me to keep going. **Pamela**, you're more than just a best friend; you're a sister. I can't thank you enough for everything you've done for me; for being the best listener and most supportive person on the planet! Thank you for seeing me at my worst and yet being there to support and help me.

To my friends in Antibes: **Joe Kh, Sacha, Elio, Wassim, George** and **Cynthia**, I'll be forever grateful for having you as a second family. Thank you for sticking with me over the past three years.

**Joe**, Jouv Habibi. Even in the most difficult times, talking to you always made everything better. I appreciate how well you always handle my attitude and anxiety. Thank you for believing in me, and never giving up on me. Thank you for being the most considerate and loving partner I could ever ask for. You bring joy to my heart. I love you.

**Mom, Dad, Micha**, and **Paul**, I consider myself extremely lucky to have family members like you. I wish I could tell you how much I love and value you. Mom and Dad, I can't thank you enough for being the most encouraging and loving parents anyone could have wished for.



Thank you for always prioritizing my happiness before yours. Thank you for going above and beyond my needs. I'll be forever grateful, and I pray that God gives you health, happiness, and everything else you desire in this life.

Last but not least, **Jesus**, thank you for holding my hand through every step of the way. You've blessed me more than I deserve!

## Abbreviations

---

**A $\beta$** :  $\beta$ -amyloid peptides

**ACh**: Acetylcholine

**AcD**: Acidic subdomain

**AD**: Alzheimer's Disease

**ADAM**: A Disintegrin And Metalloproteinase

**ADAMTS**: ADAMs with Thrombospondin motifs

**ADSCs**: Adipose Tissue-Derived Mesenchymal Stem Cells

**AEP**: Asparagine Endopeptidase

**AICD**: APP Intracellular Domain

**ALID**: APLP Intracellular Domain

**AMP**: Adenosine Mono-Phosphate

**AMPK**: AMP-Activating Protein Kinase

**aNP**: acidic Nanoparticles

**APA**: Aminopeptidase A

**APH-1**: Anterior pharynx-defective 1

**APLP**: APP-Like Proteins

**APOE**: Apolipoprotein E

**APP**: Amyloid Precursor Protein

**APP-FL**: APP Full Length

**APP<sup>swe</sup>**: APP with the Swedish double mutation

**APP<sup>w</sup>**: Wild-type APP

**ATP**: Adenosine Tri-Phosphate

**BACE:**  $\beta$ -site APP cleaving enzyme

**BafA1:** Bafilomycin A

**BBB:** Blood-Brain Barrier

**BCSFB:** Blood-CSF Barrier

**cAMP:** cyclic-AMP

**CHL1:** Cell adhesion molecule L1

**CMA:** Chaperone-Mediated Autophagy

**CP:** Core Particle

**CRD:** Cysteine-Rich Domain

**CSF:** Cerebrospinal Fluid

**C-terminal:** Carboxy-terminal

**CTF:** C-terminal Fragment

**DMT:** Disease-Modifying Therapy

**DPP4:** Dipeptidyl Peptidase 4

**ECD:** Extracellular Domain

**ECM:** Extracellular Matrix

**ED:** Extension Domain

**EEA1:** Early Endosome Antigen 1

**ELN:** Endo-Lysosomal Network

**EOAD:** Early-Onset AD

**ER:** Endoplasmic Reticulum

**ESCRT:** Endosomal Sorting Complex Required for Transport

**EVs:** Extracellular Vesicles

**FAD:** Familial forms of AD

**FDA:** Food and Drug Administration

**FDG-PET:** Fluorodeoxyglucose-Positron Emission Tomography

**GABABR1a:** Gamma-Aminobutyric Acid type B Receptor subunit 1a

**GFLD:** Growth Factor Like Domain

**GPI:** Glycosyl Phosphatidylinositol

**GSK-3 $\beta$ :** Glycogen Synthase Kinase-3 $\beta$

**HBD:** Heparin Binding Domains

**ICD:** Intracellular Domain

**I-CliPs:** Intramembrane-Cleaving Proteases

**IL-1 $\beta$ :** Interleukine-1 $\beta$

**ILVs:** Intraluminal Vesicles

**iPSCs:** induced Pluripotent Stem Cells

**Jag1:** Jagged 1

**JMR:** Juxtamembrane Region

**KPI:** Kunitz Protease Inhibitor

**LAMP:** Lysosomal-Associated Membrane Proteins

**LBPA:** Lysobisphosphatidic Acid

**LOAD:** Late-Onset AD

**LTP:** Long-Term Potentiation

**MAPT:** Microtubule Associated Protein Tau

**MCI:** Mild Cognitive Impairment

**miR:** microRNA

**MMPs:** Matrix Metalloproteinases

**MT-MMPs:** Membrane-Type MMPs

**MRI:** Magnetic Resonance Imaging

**MVBs:** Multivesicular Bodies

**NFL:** Neurofilament Light

**NFTs:** Neurofibrillary Tangles

**NICD:** Notch IntraCellular Domain

**NMDA:** N-Methyl-D-aspartate

**NRG1:** Neuregulin 1

**NSCs:** Neural Stem Cells

**N-terminal:** Amino-terminal

**PADK:** Phe-Ala-diazomethylketone

**PDEs:** Phosphodiesterases

**PEN2:** Presenilin Enhancer Protein 2

**PET:** Positron Emission Tomography

**PIP3:** Phosphatidylinositol-3-Phosphate

**PKA:** Protein Kinase A

**PLGA:** Poly (Lactic-co-Glycolic Acid)

**PS:** Presenilin

**P-Tau:** Phosphorylated Tau

**Rab:** Ras-associated binding

**RIP:** Regulated Intramembrane Proteolysis

**RP:** Regulatory Particle

**SAD:** Sporadic form of AD

**sAPP:** soluble fragment of APP

**SEZ6:** Seizure protein 6

**TACE:** TNF- $\alpha$  converting enzyme

**TFEB:** Transcription Factor EB

**TGN:** Trans-Golgi Network

**TMs:** Transmembrane

**TNF- $\alpha$ :** Tumour Necrosis Factor- $\alpha$

**TORC1:** Target-Of-Rapamycin Complex 1

**T-tau:** Total Tau

**UPS:** Ubiquitin-Proteasome System

**2xTgAD:** Double Transgenic mouse model of AD (APP<sup>swe</sup>, TauP301L)

**3xTgAD:** Triple Transgenic mouse model of AD (APP<sup>swe</sup>, TauP301L, PS1M146V)

**5xFAD:** Transgenic mouse model of AD with 5 familial mutations on APP and PSEN1 genes

## List of Tables and Figures

---

<b>Table 1: Signs of Alzheimer’s dementia compared with typical age-related changes...</b>	<b>26</b>
<b>Table 2 : Demographic data and neuropathological findings related to human brain samples used in SDS-PAGE analyses, and brain-derived slices used in immunohistochemistry analyses. ....</b>	<b>128</b>
<b>Figure 1 : Projected Number of People Age 65 and Older (Total and by Age) in the U.S. Population with Alzheimer’s Dementia, 2020 to 2060 .....</b>	<b>21</b>
<b>Figure 2: Physiological structure of the brain and neurons in healthy persons (left) and in Alzheimer’s disease (right) patients. ....</b>	<b>22</b>
<b>Figure 3 : An overview of genes which have been implicated in Alzheimer’s disease. ....</b>	<b>31</b>
<b>Figure 4 : Domain architecture, functional segments and conservation of APP-family proteins.....</b>	<b>35</b>
<b>Figure 5 : Non-amyloidogenic and amyloidogenic APP-pathways.....</b>	<b>37</b>
<b>Figure 6 : <math>\beta</math> or <math>\beta'</math>/<math>\gamma</math>-secretase pathway.....</b>	<b>44</b>
<b>Figure 7 : Progressive cleavages of the APP transmembrane domain by the Presenilin/<math>\gamma</math>-secretase complex.....</b>	<b>46</b>
<b>Figure 8 : Subunits of <math>\gamma</math>-secretase and substrate recognition.....</b>	<b>49</b>
<b>Figure 9: Schematic representation of APP trafficking and its convergence with canonical secretases in the endo-lysosomal network.....</b>	<b>51</b>
<b>Figure 10 : The ubiquitin proteasome system.....</b>	<b>55</b>
<b>Figure 11: Turnover of APP and CTFs via Endosomal Exosomal-Lysosomal-Autophagic network.....</b>	<b>58</b>
<b>Figure 12: Scheme of the increased endocytic production of A<math>\beta</math> due to LOAD genetic risk factors.....</b>	<b>60</b>
<b>Figure 13: Non-canonical APP-cleavages. ....</b>	<b>74</b>
<b>Figure 14: The <math>\eta</math>-secretase pathway.....</b>	<b>77</b>
<b>Figure 15: Metzincins superfamily members and classification of MMPs based on their domain arrangement.....</b>	<b>79</b>
<b>Figure 16 Domain organization in MT5-MMP.....</b>	<b>83</b>
<b>Figure 17: <math>\eta</math>CTF-Nter immunostaining in human AD hippocampi. ....</b>	<b>122</b>
<b>Figure 18 : The detection of human APP-FL in different cell and mouse models, using <math>\eta</math>CTF-Nter antibody.....</b>	<b>123</b>
<b>Figure 19 : <math>\eta</math>CTF is not detected in AD brains. ....</b>	<b>124</b>

**Figure 20: A $\eta$  $\alpha$  and A $\eta$  $\beta$  impair hippocampal LTP. ....125**



# TABLE OF CONTENTS

---

I.	Introduction .....	19
1)	Alzheimer's disease .....	20
A)	Definition and Prevalence .....	20
B)	Neuropathological Changes in AD and Biomarkers .....	21
1.	Macroscopic Brain Atrophy.....	22
2.	Microscopic Features .....	22
2.1.	Neurofibrillary Tangles (NFTs) .....	22
2.2.	Amyloid Plaques .....	23
3.	Biomarkers.....	23
C)	AD Progression and Symptoms .....	25
D)	Treatments and Ongoing Clinical Trials .....	26
E)	AD Etiology.....	27
1.	Familial Forms of AD (FAD) .....	28
1.1.	APP Mutations .....	28
1.2.	PSEN1 and PSEN2 Mutations .....	29
2.	Sporadic Forms of AD (SAD) .....	29
2.1.	Genetic Predisposition .....	29
2.1.1.	APOE.....	29
2.1.2.	Other Genetic Risk Factors for AD .....	30
2.2.	Non-Genetic Factors .....	31
2.2.1.	Age .....	31
2.2.2.	Sex.....	31
2.2.3.	Other Environmental Risk Factors for AD.....	32
3.	The Amyloid Cascade Hypothesis.....	32
2)	APP Protein.....	34
A)	APP: Gene, Structure and Function.....	34
1.	Gene.....	34
2.	Structure .....	34
3.	Function .....	35
B)	Canonical APP-Processing Pathway .....	36
1.	The Amyloidogenic Pathway .....	36
1.1.	A $\beta$ peptides .....	37
1.2.	AICD .....	38
1.3.	C99 .....	39
1.4.	sAPP $\beta$ .....	40
2.	The Non-Amyloidogenic Pathway.....	41
2.1.	sAPP $\alpha$ .....	41

2.2.	C83 .....	41
2.3.	P3 .....	42
3.	APP Cleaving Enzymes .....	42
3.1.	$\alpha$ -secretase .....	42
3.2.	$\beta$ -secretase .....	43
3.3.	$\gamma$ -secretase .....	45
4.	APP Localization .....	50
4.1.	APP Trafficking and Co-Residence with Canonical Secretases.....	50
4.2.	Endo-Lysosomal Network (ELN) – Generalities.....	51
C)	APP and APP-Fragments Degradation and Elimination .....	53
1.	Proteasome and its Dysfunction in AD .....	53
1.1.	The Ubiquitin-Proteasome System (UPS) .....	53
1.2.	Dysfunctions of Proteasome in AD .....	54
2.	Lysosomal and Autophagic Pathway and their Dysfunctions in AD .....	56
2.1.	Lysosomal Degradation.....	56
2.2.	Autophagic Pathway.....	57
2.3.	Dysfunctions of ELN and Autophagy in AD .....	59
2.3.1.	PS1 Mutations and AD-Risk Factors Contribute to ELN Dysfunctions .....	59
2.3.2.	C99 Accumulation Contribute to ELN and Autophagic Dysfunctions .....	61
3.	Secretion of Exosomes and their Dysfunction in AD.....	62
3.1.	Secretion of Exosomes .....	62
3.2.	Exosomal Dysfunction in AD .....	63
D)	Therapeutic Approaches and Clinical Trials for AD .....	65
1.	Targeting A $\beta$ .....	65
1.1.	Neutralizing the Formed A $\beta$ .....	65
1.1.1.	Active Immunotherapy.....	66
1.1.2.	Passive Immunotherapy.....	66
1.2.	Preventing A $\beta$ production – Targeting $\beta$ and $\gamma$ -secretases .....	67
1.2.1.	BACE1 Inhibitors.....	67
1.2.2.	$\gamma$ -secretase Inhibitors/Modulators.....	67
2.	Modulation of the ELN.....	68
2.1.	Activating Hydrolases.....	69
2.2.	Normalization and Restoration Of Lysosomal Activity.....	69
2.3.	Autophagy Activation.....	70
3.	Exosomes as Drug Carriers and Biomarkers.....	71
3.1.	Exosomes as Drug Carriers .....	71
3.2.	Exosomes as Biomarkers.....	72
E)	Non-Canonical APP-Processing Pathways .....	73
1.	$\delta$ -secretase .....	73

2.	Meprin- $\beta$ .....	74
3.	Caspases .....	75
4.	Cathepsins .....	76
5.	$\eta$ -secretase .....	76
F)	Biology of Matrix Metalloproteinases .....	78
A)	Matrix Metalloproteinases (MMPs) Overview .....	78
B)	MT-MMP and APP Cleavage .....	80
C)	MT5-MMP .....	81
1.	Overview .....	81
2.	Structure .....	82
3.	Physiological Role .....	83
4.	MT5-MMP in AD.....	84
5.	MT5-MMP as $\eta$ -secretase .....	84
II.	Objectives .....	86
III.	Results .....	90
1)	The study of expression, processing, fate, and subcellular localization of $\eta$ CTF in different in vitro, ex vivo, and in vivo models.....	91
2)	Additional investigations on the detection of $\eta$ CTF in human AD patients and functional implication of A $\eta\alpha$ and A $\eta\beta$ on synaptic activity .....	121
2.1.	Investigations on the detection of $\eta$ CTF in human AD patients.....	121
2.2.	The functional implication of A $\eta\alpha$ and A $\eta\beta$ on synaptic activity .....	124
2.3.	Materials and Methods .....	126
IV.	Discussion .....	130
1)	Can $\eta$ CTF be produced under pathophysiological conditions? .....	131
2)	Can $\eta$ CTF be compared to C99?.....	131
3)	$\eta$ CTF: A new AD player?.....	132
4)	What does the presence of $\eta$ CTF in exosomes lead us to believe?.....	133
V.	Conclusion and Perspectives .....	136
VI.	References .....	139
VII.	Annex .....	162

# I. INTRODUCTION

---

# 1) ALZHEIMER'S DISEASE

---

## A) DEFINITION AND PREVALENCE

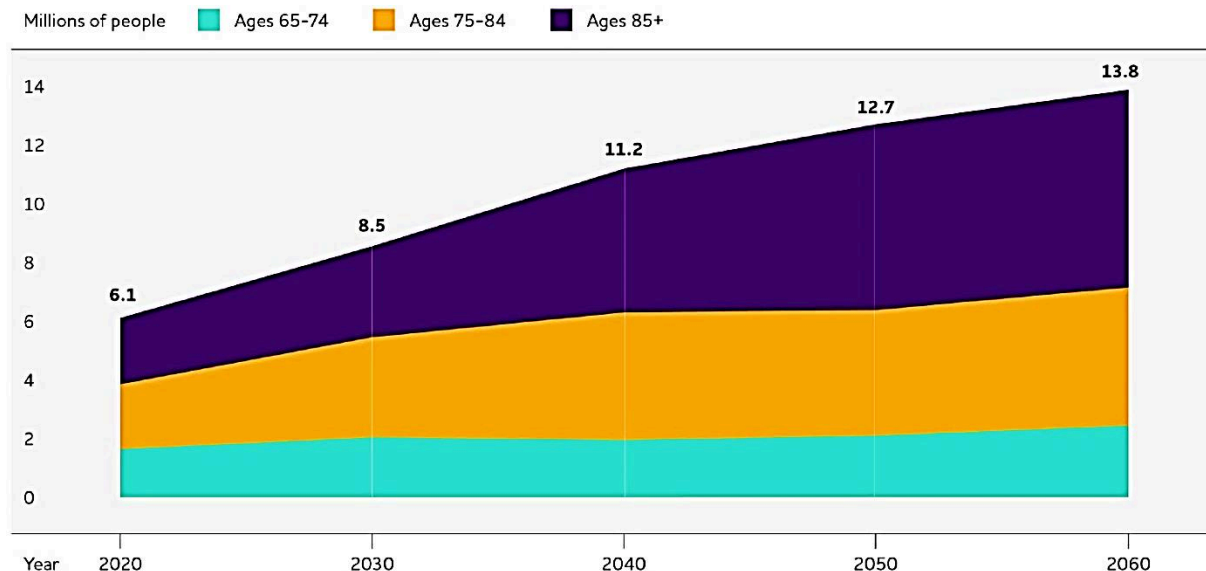
---

Alzheimer's disease (AD) is an neurodegenerative disorder named by Dr. Emil Kraepelin, in reference of the German psychiatrist Alois Alzheimer, who was the first to identify the disease in 1906 (Moller and Graeber, 1998). The disease is induced by neuropathological alterations that majorly affect the cerebral cortex and the hippocampus and but also spread to different areas of the brain, at rates that vary considerably from one patient to another. The term AD is used today by specialists to represent a clinical entity that typically begins with a progressive amnesic disorder and then progresses to various cognitive, behavioral, and neuropsychiatric alterations that affect social function and daily living tasks.

Involved in 60 to 80% of demented cases in people over 65, AD is the most prevalent neurodegenerative dementia from middle age to the elderly. Dementia is a syndrome with multiple causes, characterized by deterioration in cognitive ability, behavior, social abilities and capacity for independent living. While AD is the leading cause of dementia in the elderly, other neurodegenerative disorders such as vascular dementia, Lewy body, and frontotemporal dementia (also termed frontotemporal lobar degeneration) can also be associated to dementia. Non-neurodegenerative diseases such as tumors, vitamin deficiencies or infections for example, may also contribute to patients' dementia syndrome (Gale et al., 2018).

In 2020, 55 million people across the world were diagnosed with dementia. Due to longer life spans, cases will almost double every 20 years. By 2050, the number of people aged over 60 years will have increased by 1.25 billion, and the number of people with dementia is expected to reach 139 million cases. It is worth noting that the global prevalence of AD is between 5 and 6% at age 65 and 30% in individuals over 85 (**Figure 1**).

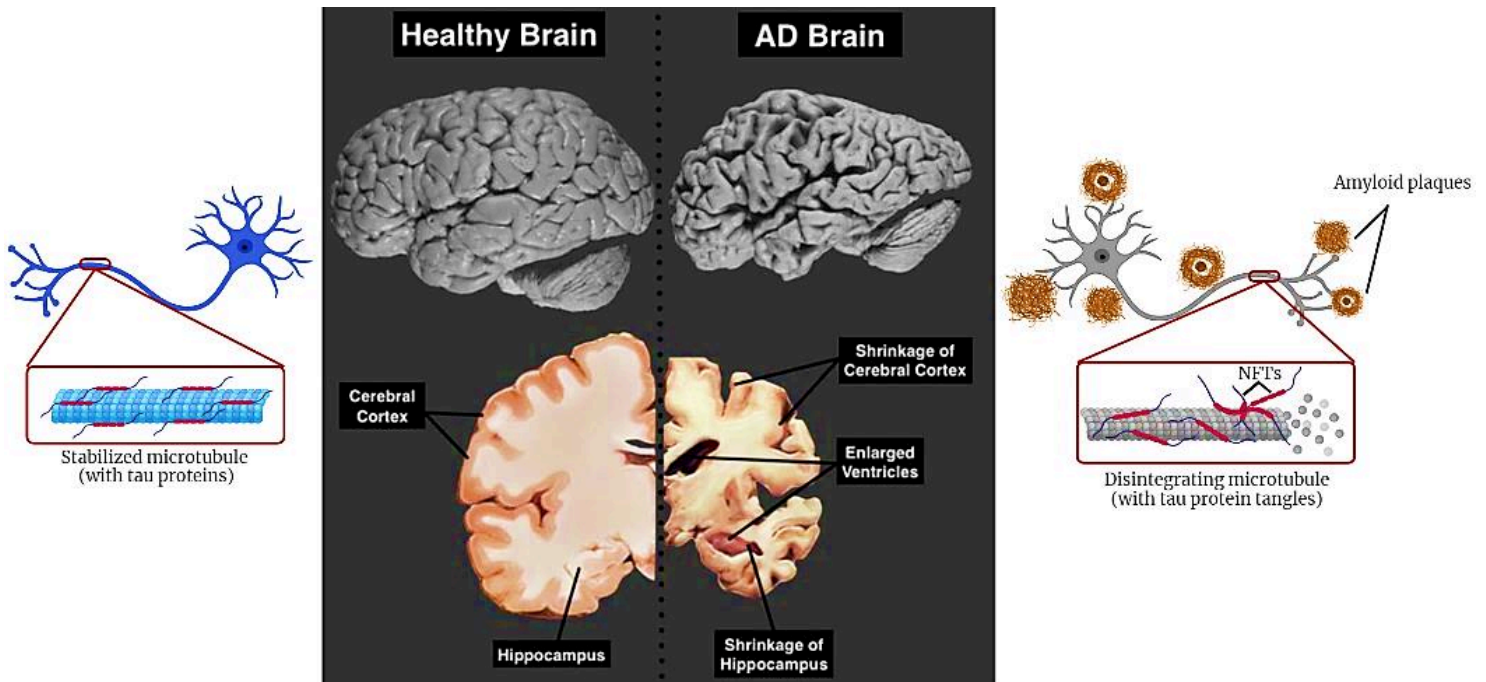
Without effective pharmacologic treatments for Alzheimer disease to date, social and economic burden on families, society and health care systems will continue to grow due to the needs of persistent care and therapy. Total estimated cost of dementia today for health care, is 1.3 trillion dollars and is expected to rise to 2.8 trillion dollars by 2030. (<https://www.alzint.org/about/dementia-facts-figures/dementia-statistics/>)



**Figure 1 :** Projected Number of People Age 65 and Older (Total and by Age) in the U.S. Population with Alzheimer’s Dementia, 2020 to 2060  
<https://www.alz.org/media/Documents/alzheimers-facts-and-figures>

## B) NEUROPATHOLOGICAL CHANGES IN AD AND BIOMARKERS

Prior to breakthroughs in technology and science in the 80s, AD was only diagnosed after the patient died, when a marked reduction in brain volume associated with microscopic histopathological hallmarks such as senile plaques (Glenner and Wong, 1984; Masters et al., 1985) and neurofibrillary tangles (Braak and Braak, 1991; Brion et al., 1985; Grundke-Iqbal et al., 1986) were observed.



**Figure 2: Physiological structure of the brain and neurons in healthy persons (left) and in Alzheimer's disease (right) patients.**

Macroscopic brain atrophy in AD with the two histopathological markers: extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFTs). Created with BioRender.com free software.

## 1. Macroscopic Brain Atrophy

Typical macroscopic alteration of AD is cerebral cortical atrophy, primarily affecting the medial temporal lobes, including the entorhinal cortex, the hippocampus and the amygdala (Dickerson and Sperling, 2008; Jack et al., 2004). This atrophy is accompanied of widened sulcal spaces and gyri shrinking (**Figure 2** middle part).

Both neuronal and synaptic losses contribute to this brain atrophy, and as a result of this cortical thinning, the frontal and temporal horns of the lateral ventricles appear prominently dilated (Apostolova et al., 2012).

## 2. Microscopic Features

### 2.1. Neurofibrillary Tangles (NFTs)

Neurofibrillary tangles (NFT) were one of the key brain abnormalities highlighted by Alois Alzheimer when he originally characterized AD in 1906. However, it took decades for Tau protein to be identified (Brion et al., 1985; Grundke-Iqbal et al., 1986) as the primary component of NFTs (Naseri et al., 2019).

The MAPT (Microtubule Associated Protein Tau) gene, which is found on chromosome 17q21, encodes tau, a microtubule-associated protein that is mostly produced in the axons of neurons. Since microtubules are the primary components of the cytoskeleton, Tau's binding to microtubules helps to assemble and stabilize them, controlling the cytoskeleton's dynamics as well as axonal transport (**Figure 2** left part) (Barbier et al., 2019). Mutations and post-translational modifications, principally phosphorylation, control Tau's physiological function. Tau's conformation is altered by hyperphosphorylation or phosphorylation on proline-rich regions, which facilitates the formation of neurotoxic fibrils and reduces its affinity for microtubules, resulting in the formation and accumulation of NFTs (**Figure 2** right part) (Wegmann et al., 2021).

Tauopathies are neurodegenerative diseases caused by intracellular accumulations of abnormal tau filaments that form NFTs (Takashima et al., 2019).

## 2.2. Amyloid Plaques

---

Unlike NFTs, extracellular amyloid plaques, also known as senile plaques, are a more specific neuropathological diagnostic criterion for AD. Amyloid plaques are composed primarily of  $\beta$ -amyloid peptides ( $A\beta$ ) (Glennner and Wong, 1984; Masters et al., 1985), which are produced by the proteolytic processing of a larger glycoprotein known as amyloid precursor protein (APP). APP metabolism and  $A\beta$  peptides will be discussed in greater detail later.

Amyloid plaques can range from 10 up to 120  $\mu\text{m}$  in cross-sectional dimension, and the density and degree of compaction of the  $A\beta$  fibrils that constitute the extracellular core vary greatly among plaques (**Figure 2** right part). Both inside of this amyloid deposit and immediately around it, dystrophic neurites can be seen. These neurites are frequently tangled and twisted, and are characterized by ultrastructural abnormalities such as enlarged lysosomes, numerous mitochondria, and paired helical filaments (Selkoe, 2001).

## 3. Biomarkers

---

Newer techniques for early AD diagnosis, based on the detection of the abnormal accumulation amyloid  $\beta$  and Tau, are currently in use (Anoop et al., 2010). For instance, methods for measuring the levels of total tau (T-tau) and neurofilament light (NFL) as biomarker of neurodegeneration, phosphorylated tau (P-Tau), and amyloid  $\beta$  ( $A\beta_{42}$  and  $A\beta_{42/40}$  ratio) in cerebrospinal fluid (CSF) have been established. Lumbar puncture is used to collect CSF in the search for the "Alzheimer CSF profile," which is characterized by elevated levels of T-tau and



NFL (reflecting neurodegeneration) and phosphorylated tau (marker for tangles), as well as decreased levels of A $\beta$ 42 and A $\beta$ 42/40 ratio (reflecting brain amyloidosis and plaques).

Multiple studies have found a decrease in CSF A $\beta$ 42 in AD patients (Andreasen et al., 1999; Fagan et al., 2007). Given that A $\beta$ 42 is a significant component of senile plaques, reduced CSF-A $\beta$ 42 levels are believed to be induced by high plaque deposition of A $\beta$ 42 in brains.

Since the 1980s, imaging techniques like magnetic resonance imaging (MRI) and fluorodeoxyglucose-positron emission tomography (FDG-PET) have been used to assist with the diagnosis of AD. Although extremely expensive, amyloid positron emission tomography (PET), which first became available in 2004 (Klunk et al., 2004), and tau PET, which followed a few years later (Chien et al., 2014), are the first true disease-specific imaging biomarkers for AD. They mostly consist of the detection of AD biomarkers, which are used to assess patients' overall health and pathological state (Khan et al., 2020; Lashley et al., 2018).

These biomarkers appear to be valuable elements, allowing early detection of the disease, as well as monitoring the disease's evolution and tracking the efficacy of a treatment.

Dr. Dubois and his colleagues proposed the "A/T/N" system in 2016 (Jack et al., 2016), which divides AD biomarkers into three categories: "A" refers to the value of  $\beta$ -amyloid (amyloid PET or CSF A $\beta$ 42); "T," the value of a Tau (CSF P-Tau, or Tau PET); and "N," neurodegeneration biomarkers (FDG-PET, structural MRI, or CSF T-Tau). This A/T/N approach is not influenced by an individual's cognitive condition and avoids syndromic or clinical labeling, making it an unbiased strategy suited mainly to the diagnostic of AD, but also to cognitive aging research in general. However, biomarker data interpretation without a clinical investigation can be challenging, and positive AD biomarkers can be confused with other age-related diseases. So, while the A/T/N biomarker system allows the detection of the disease in the preclinical phase, it will require clinical information about each individual and must be used in conjunction with a complementary system for a complete diagnosis.

## C) AD PROGRESSION AND SYMPTOMS

---

The findings of PET scanning and CSF analysis showed that the pathological alterations begin two decades before symptoms occur. In fact, AD progresses on a spectrum of three major phases (2022):

1. The preclinical asymptomatic phase in which neuropathological changes have already taken place in the brain, without any clinical symptoms leading to diagnosis. This phase is relatively long, several years or decades before any symptoms start developing (Sperling et al., 2011).
2. The prodromal phase also referred to as mild cognitive impairment (MCI) in some references. Patients can be diagnosed with AD during this mild symptomatic stage based on their clinical presentation and biomarker-confirmed Alzheimer's pathology. Patients start losing the ability to carry out everyday activities, but still manage to be independent. Symptoms may include episodic memory loss, mild decline in semantic memory and working memory, motivation, mood and behavioral disorders (Albert et al., 2011).
3. The final dementia stage - the more severe form of AD - is characterized by severe impairment in talking, walking, sitting and, swallowing, and severe decline in memory and cognitive skills. Patients at this stage lose their motivation, their ability to communicate and their complete autonomy. At this point, 24-hour care should be provided, and hospitalization might also be required since patients become more vulnerable to infections.

Clinical phenotypes are extremely complex and difficult to understand, and it's crucial to remember that each person may experience symptoms differently and go through the stages in a different way (**TABLE 1**).

Signs of Alzheimer's Dementia	Typical Age-Related Changes
<b>Memory loss that disrupts daily life:</b> One of the most common signs of Alzheimer's dementia, especially in the early stage, is forgetting recently learned information. Others include asking the same questions over and over, and increasingly needing to rely on memory aids (for example, reminder notes or electronic devices) or family members for things that used to be handled on one's own.	Sometimes forgetting names or appointments, but remembering them later.
<b>Challenges in planning or solving problems:</b> Some people experience changes in their ability to develop and follow a plan or work with numbers. They may have trouble following a familiar recipe or keeping track of monthly bills. They may have difficulty concentrating and take much longer to do things than they did before.	Making occasional errors when managing finances or household bills
<b>Difficulty completing familiar tasks:</b> People with Alzheimer's often find it hard to complete daily tasks. Sometimes, people have trouble driving to a familiar location, organizing a grocery list or remembering the rules of a favorite game.	Occasionally needing help to use microwave settings or record a television show.
<b>Confusion with time or place:</b> People living with Alzheimer's can lose track of dates, seasons and the passage of time. They may have trouble understanding something if it is not happening immediately. Sometimes they forget where they are or how they got there.	Getting confused about the day of the week but figuring it out later.
<b>Trouble understanding visual images and spatial relationships:</b> For some people, having vision problems is a sign of Alzheimer's. They may also have problems judging distance and determining color and contrast, causing issues with driving.	Vision changes related to cataracts.
<b>New problems with words in speaking or writing:</b> People living with Alzheimer's may have trouble following or joining a conversation. They may stop in the middle of a conversation and have no idea how to continue or they may repeat themselves. They may struggle with vocabulary, have trouble naming a familiar object or use the wrong name (e.g., calling a watch a "hand clock").	Sometimes having trouble finding the right word.
<b>Misplacing things and losing the ability to retrace steps:</b> People living with Alzheimer's may put things in unusual places. They may lose things and be unable to go back over their steps to find them. They may accuse others of stealing, especially as the disease progresses.	Misplacing things from time to time and retracing steps to find them.
<b>Decreased or poor judgment:</b> Individuals may experience changes in judgment or decision-making. For example, they may use poor judgment when dealing with money or pay less attention to grooming or keeping themselves clean.	Making a bad decision or mistake once in a while, such as neglecting to schedule an oil change for a car.
<b>Withdrawal from work or social activities:</b> People living with Alzheimer's disease may experience changes in the ability to hold or follow a conversation. As a result, they may withdraw from hobbies, social activities or other engagements. They may have trouble keeping up with a favorite sports team or activity.	Sometimes feeling uninterested in family and social obligations.
<b>Changes in mood, personality and behavior:</b> <i>The mood and personalities of people living with Alzheimer's can change. They can become confused, suspicious, depressed, fearful or anxious. They may be easily upset at home, at work, with friends or when out of their comfort zones.</i>	Developing very specific ways of doing things and becoming irritable when a routine is disrupted.

**Table 1: Signs of Alzheimer's dementia compared with typical age-related changes.** Adapted from (<https://www.alz.org/media/documents/alzheimers-facts-and-figures>)

## D) TREATMENTS AND ONGOING CLINICAL TRIALS

With AD being now a global health concern, finding a cure is crucial to easing the severe physical, emotional, and financial burdens placed on patients and society.

Only four medications were approved by the Food and Drug Administration (FDA) for the treatment of AD until 2021; all four are only for treating symptoms but not curative drugs.

Donepezil, Rivastigmine, and Galantamine were three of them that were acetylcholinesterase enzyme inhibitors. Acetylcholine (ACh) is crucial for cognitive function, and in AD, cholinergic synaptic loss and cholinergic neuronal degeneration were reported. So these inhibitors prevent acetylcholinesterase from degrading ACh, allowing it to be persistently preserved and activate cholinergic receptors. Memantine, an N-Methyl-D-aspartate (NMDA) receptor antagonist, is the fourth medicine to receive FDA approval. It protects against the overstimulating and toxic stimulation of these receptors that occurs in AD. Unfortunately, these medications neither cure nor prevent the disease; they simply serve to improve the symptoms (Breijyeh and Karaman, 2020).

In 2021, Aducanumab was the first disease-modifying therapy (DMT) to be approved for AD. Aducanumab is a monoclonal anti-amyloid antibody that targets plaques and oligomers. However, the medicine was approved only in United States by FDA with significant debate, and phase III clinical trials will proceed until 2023 (Walsh et al., 2021).

There are currently 143 other molecules in 172 ongoing trials of treatments for AD. The most common agents being studied are DMTs, and 21 of these agents have reached phase III studies. These DMTs have a wide range of targets, including tau, synaptic plasticity, oxidative stress, metabolism, and amyloid (A $\beta$  monomers, oligomers, protofibrils, and amyloid plaques), etc... The majority of these DMT target amyloid, including Donanemab and Lecanemab, two monoclonal plaque-lowering antibodies that are now being evaluated by the FDA (Cummings et al., 2022). Eisai and Biogen made an exclusive announcement on 27<sup>th</sup> of September 2022, on the positive results of the Phase 3 Clarity trial of Lecanemab. They claimed in this exciting statement that Lecanemab significantly slowed the progression of cognitive decline by 27% over 18 months in patients with very mild to mild AD, and that both companies intend to apply for regulatory approval in the United States, Europe, and Japan. The findings of this trial has been presented at the Clinical Trials on Alzheimer's Congress, which has been held in San Francisco in November, and will be published in a peer-reviewed journal soon (<https://www.alzforum.org/news/research-news/finally-big-win-all-outcomes-lecanemab-phase-3-topline-results>).

## E) AD ETIOLOGY

---

AD exists in two forms: hereditary/familial and sporadic. Only 1 to 2% of patients have the hereditary form, which is caused by one of three monogenic autosomal dominant mutations in one of three genes: APP (amyloid precursor protein), PS1 (presenilin 1), or PS2 (presenilin 2).

The remaining 98–99% of cases, however, are sporadic, in which the disease is not caused by a dominant mutation but rather by a combination of factors including age, genetic predispositions, and environmental factors.

According to the age at which symptoms first appeared, AD patients can also be divided into two groups: those with early-onset AD (EOAD), which develop symptoms before the age of 65, and those with late-onset AD (LOAD) where symptoms are detected after the age of 65.

Dementia and the two histological markers (amyloid plaques and NFTs) are common hallmarks of EOAD and LOAD, indicating that distinguishing the two AD age groups at the end-stage of disease can be challenging.

## 1. Familial Forms of AD (FAD)

---

Patients suffering from familial forms of AD (FAD) start developing symptoms between their 30s and 50s, consequently, FAD is characterized mostly by EOAD. Autosomal dominant mutations in APP, PSEN1 or PSEN2 genes have been generally described to induce an abnormal increase in the production of total A $\beta$  or an aggregated form of A $\beta$ . Interestingly, only 10% of EOAD are explained by a mutation of these genes, suggesting that the remaining 90% of the disease's early manifestations, whose origins are still undetermined, are more likely to have autosomal recessive causes (Wingo, 2012).

### 1.1. APP Mutations

---

Since the aggregation of the A $\beta$  peptide into senile plaques is one of the two histopathological hallmarks of AD, mutations in the APP gene, the precursor of A $\beta$ , are consequently directly linked to the disease.

Currently, 73 APP gene mutations are documented on the Alzforum website, with over 53 of them linked to FAD (<https://www.alzforum.org/mutations/app>). These mutations alter the cleavage of APP by certain secretases (see chapter [APP Cleaving Enzymes](#)), resulting in AD via various mechanisms. For instance, the first APP mutation discovered, known as the 'London' mutation (V717I), causes an increase in A $\beta$ <sub>42</sub> (Goate et al., 1991). However, the arctic mutation (E693G), enhances A $\beta$  aggregation (Kamino et al., 1992; Nilsberth et al., 2001).

In my thesis project, I used cellular and mouse models including the triple transgenic mouse model of AD (3xTgAD) mice that overexpress the human APP with the Swedish double mutation (APP<sup>swe</sup>) (KM670/671NL). This mutation promotes APP cleavage at the  $\beta$  site (see

chapter  $\beta$ -secretase), increasing total A $\beta$  levels (Johnston et al., 1994; Mullan, 1992) , and replicating amyloid pathology with senile plaque deposits in mice brains.

Not all APP mutations are pathogenic; for example, the Icelandic mutation (A673T) has been identified as protective, reducing A $\beta_{40}$  and A $\beta_{42}$  by 40% in HEK293 cells (Jonsson et al., 2012). Furthermore, since the APP gene is located on chromosome 21 (21q21.3), a duplication of this gene results in autosomal dominant EOAD (Rovelet-Lecrux et al., 2006). Thus, the presence of an extra copy of APP in Down Syndrome patients (due to a trisomy on chromosome 21) leads to development of typical AD phenotypes by the age of 40 (Lott and Head, 2019).

## 1.2. PSEN1 and PSEN2 Mutations

---

PSEN1 and PSEN2 encode PS1 and PS2 respectively, which are subunits of  $\gamma$ -secretase, a key enzyme in APP processing and A $\beta$  production (see chapter  $\gamma$ -secretase). To date, 87 mutations in PSEN2, located on chromosome 1 (1q42.13), have been discovered and described as an uncommon cause of EOAD, whereas more than 300 mutations in PSEN1, located on chromosome 14 (14q24.2), have been reported and represent the most common cause of EOAD (<https://www.alzforum.org/mutations>).

PSEN1 mutations mostly increase the A $\beta_{42/40}$  ratio, either by decreasing A $\beta_{40}$  levels or raising A $\beta_{42}$  levels. For instance, the first reported PSEN1 mutation (M146V), identified in 1995 (Alzheimer's Disease Collaborative, 1995), results in increased levels of secreted A $\beta_{42}$  and an elevated A $\beta_{42/40}$  ratio. This is one of the most common mutations reported in FAD patients, and it is also present in the 3xTgAD mouse model.

## 2. Sporadic Forms of AD (SAD)

---

The sporadic form of AD (SAD), unlike FAD, is not caused by an autosomal dominant mutation, but it can be genetically impacted. Mainly referred to as LOAD because symptoms occur after the age of 65, it is a multifactorial disorder influenced mostly by environmental factors, genetic predispositions, patient's sex, and age, with age being the primary risk factor (Riedel et al., 2016).

### 2.1. Genetic Predisposition

---

#### 2.1.1. APOE

The Apolipoprotein E (APOE) is glycoprotein, important for transporting lipids and for the metabolism of cholesterol. The central nervous system (CNS) contains this glycoprotein, which

is widely expressed in astrocytes, and to a lesser level in stressed neurons. By binding to cell-surface APOE receptors, this glycoprotein redistributes cholesterol and other lipids to neurons, critical for the maintenance of myelin and neuronal membranes (Riedel et al., 2016; Yamazaki et al., 2019).

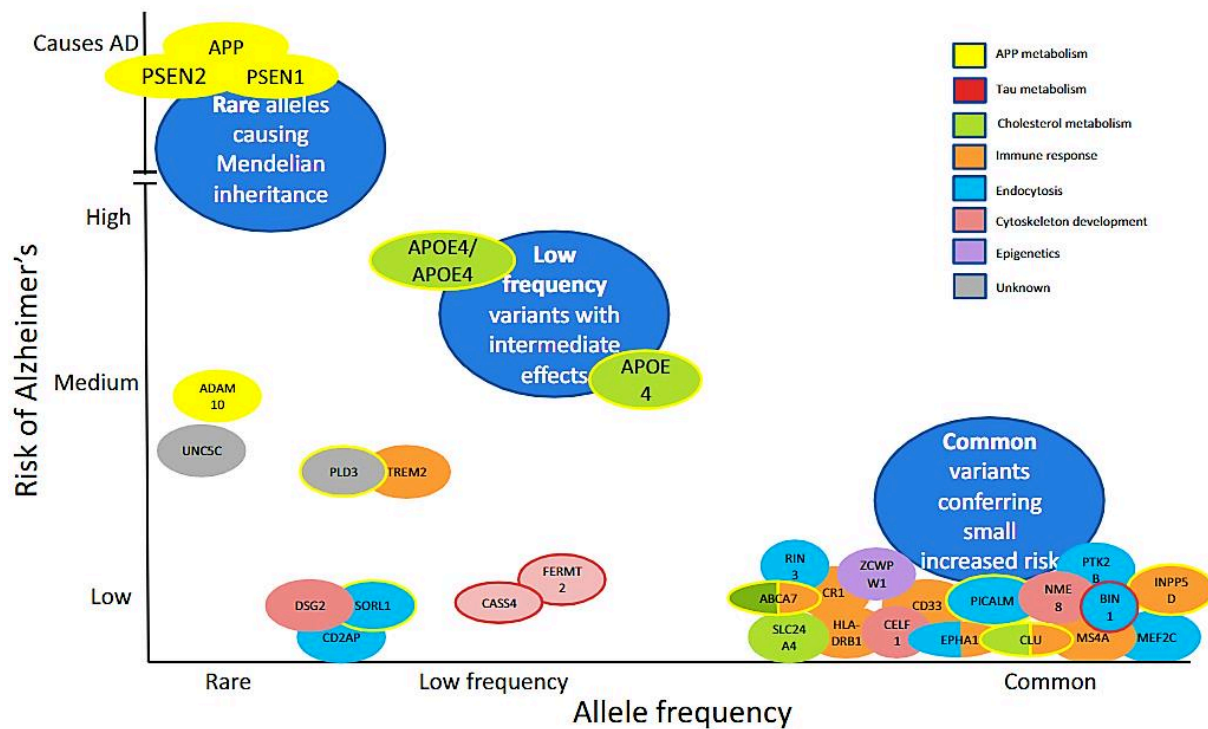
APOE is found on chromosome 19 and consists of three allelic variants: APOE- $\epsilon$ 2 (Cys-112, Cys-158), APOE- $\epsilon$ 3 (Cys-112, Arg-158), and APOE- $\epsilon$ 4 (Arg-112, Arg-158). Each APOE isoform's functionality is impacted by the structural changes brought by the various amino acids at these sites (Verghese et al., 2011). This polymorphism has been identified as the most important genetic risk factor for LOAD. The replacement of cysteine for arginine at position 112 and 158 in APOE4, raises plasma cholesterol and LDL levels and puts the person greater risk for cardiovascular and neurodegenerative diseases like AD (Riedel et al., 2016).

One copy of the  $\epsilon$ 4 allele increases risk by fourfold, while two copies increase risk by fourteen (Corder et al., 1993), with each copy decreasing onset by eight years (Verghese et al., 2011).

The processes through which APOE4 is related to AD are reviewed in Yamazaki's article (Yamazaki et al., 2019). In summary, it appears to interfere with inflammation and synaptic plasticity, as well as the phosphorylation of the Tau protein, and the clearance and aggregation of the A $\beta$  peptide in senile plaques. In fact, APOE4 interacts with A $\beta$  and decreases its perivascular drainage, its phagocytosis and its clearance by enzymatic degradation or through the blood-brain barrier (BBB).

### 2.1.2. Other genetic risk factors for AD

APOE was assumed to be the only genetic predisposition factor associated to AD until 2009, but today, 75 independent loci for AD and related dementias have been reported (Bellenguez et al., 2022). These genetic risk factors are mainly involved in cholesterol metabolism, endosome/lysosome trafficking/function, or innate immunity and microglial activation (**Figure 3**).



**Figure 3 : An overview of genes which have been implicated in Alzheimer's disease.** The internal color corresponds to their understood function; where there are two internal colors, the gene has been implicated in more than one pathway. Genes circled in yellow are also thought to influence amyloid precursor protein metabolism; genes circled in red are thought to influence tau metabolism (Lane, Hardy and Schott, 2018).

## 2.2. Non-Genetic Factors

### 2.2.1. Age

Age continues to be the most important risk factor for AD and is a key element in the progression of the illness. However, phenotypes comparable to those reported in AD brains, such as reduced brain volume, enlarged ventricles, synapses loss, and even amyloid plaques, have been observed in normal aging and cognitively normal brains. This means that AD is not the consequence of normal aging alone, and that age without other factors, is insufficient to develop the disease (A. Armstrong, 2019).

### 2.2.2. Sex

When it comes to gender as a risk factor of AD, females are more affected than men after the age of 80. This was explained by the fact that women have a longer life expectancy than men, and it was believed that when men's lives are estimated to be as long as women's in a few years, this AD risk factor would be spread equally between the two sexes (Riedel et al., 2016).



However, hormones and post-menopausal hormonal changes have been proven to have an impact on increasing AD risk in women (Mosconi et al., 2017; Scheyer et al., 2018). Similar sex difference is also observed in AD mouse models such as 3xTgAD brain.

### 2.2.3. Other environmental risk factors for AD

LOAD can also be influenced by *environmental* and *lifestyle* factors. These include mainly: diet and malnutrition, obesity, education, alcohol consumption, education, and physical exercises. The risk of AD can also be increased by other events including traumatic brain injury, and some diseases such as diabetes and vascular diseases can also increase risk for AD. All these factors are reviewed in (A. Armstrong, 2019).

## 3. The Amyloid Cascade Hypothesis

---

The amyloid cascade hypothesis, introduced by John Hardy and Gerald Higgins in 1992, places A $\beta$  peptide at the root of the disease. According to this theory, A $\beta$  accumulation is the initial cause of AD, and that the formation of neurofibrillary tangles is triggered by the aggregation of A $\beta$ , and dementia arise later as a direct result of these deposits (Hardy and Higgins, 1992; Selkoe and Hardy, 2016).

The amyloid hypothesis dominates the AD study field since it is supported by a significant number of evidence such as genetics and histopathology (Pimplikar et al., 2010). Indeed, A $\beta$  plaques first appear in the cortical regions and then spread to the remainder of the brain as the disease progresses, and histopathologically, they are regarded as one of the diagnostic hallmarks of AD and a key element of AD pathogenesis. Moreover, genetic research served as a foundation for the amyloid theory. Strong evidence in favor of the amyloidogenic hypothesis, for example, comes from mutations in FAD that increase the production of the A $\beta$  peptide or modify the A $\beta$ 42/A $\beta$ 40 ratio, as well as from the fact that individuals with Down's syndrome develop typical Alzheimer neuropathology due to an increased APP copy number. Besides the APP mutations inducing the pathological phenotypes of AD, there is also the Icelandic mutation on APP, identified as protective, since it reduces  $\beta$ -secretase cleavage and A $\beta$  production. Along with the autosomal dominant mutations, genome-wide association studies (GWAS) studies have identified risk factors for SAD that are related directly or indirectly to APP metabolism and A $\beta$  clearance.

In spite of being the most widely accepted hypothesis, it is heavily debated, and even rejected by some scientists due to some of its weaknesses. However, this hypothesis has evolved as studies and research on the disease have progressed (Selkoe and Hardy, 2016).

For instance, it has been reported that many healthy individuals have amyloid deposits, whereas many AD patients present very few amyloid deposits (Edison et al., 2007), hence implying that the accumulation  $A\beta$  is related to aging rather than the development of AD. Moreover, it was claimed that NFTs correlate better than amyloid plaques with the progression of cognitive defects in AD (Braak and Braak, 1991). But then as counterarguments, Selkoe and Hardy explained that  $A\beta_{42}$  oligomers start to accumulate very early in the brain, inhibiting long-term potentiation (LTP), impairing memory and causing neuronal dysfunction even before the formation of plaques. Thus, intracellular  $A\beta$  oligomers instead of  $A\beta$  deposits are now considered as the initial trigger responsible for AD. Furthermore, the deposits detected in healthy individuals are frequently diffuse plaques with little  $A\beta$  oligomers and have a lower neurotoxic state.

In addition to all this hypothesis' flaws, the failure of drugs targeting amyloid plaques or  $\beta$  and  $\gamma$ -secretases in clinical trials raised doubts concerning the amyloidogenic hypothesis. Despite optimistic outcomes in mouse models and the recent but contested Aducanumab FDA approval, the large majority of trials with anti-amyloid drugs conducted in humans with AD have failed to improve cognitive impairment in spite of their abilities to remove completely the brain from  $A\beta$  deposits. Until the plaque-lowering antibody Lecanemab Clarity trial revealed encouraging results in favor of the amyloidogenic hypothesis in September 2022.

Despite  $A\beta$ 's neurotoxic properties, the peptide could not be the only factor triggering AD. Without a doubt, AD is a complex pathology, and  $A\beta$ , Tau, and even multiple APP C-terminal fragments participate in the neuropathological cascade of AD; hence, a multitargeting treatment for AD is required.

## 2) APP PROTEIN

---

### A) APP: GENE, STRUCTURE AND FUNCTION

---

#### 1. Gene

---

APP is a type I integral membrane protein that belongs to a family that includes APP and two mammalian homologs known as APP-like proteins, APLP-1 and APLP-2. The three have similar domain structures, with APP and APLP-2 being evolutionarily more closely related, and APLP-1 representing the APP gene family members' ancestral form (Coulson et al., 2000). The different secretases process all three proteins in a similar manner, but the A $\beta$  sequence is unique to APP and FAD mutations were only found on APP genes but not on those of its homologs.

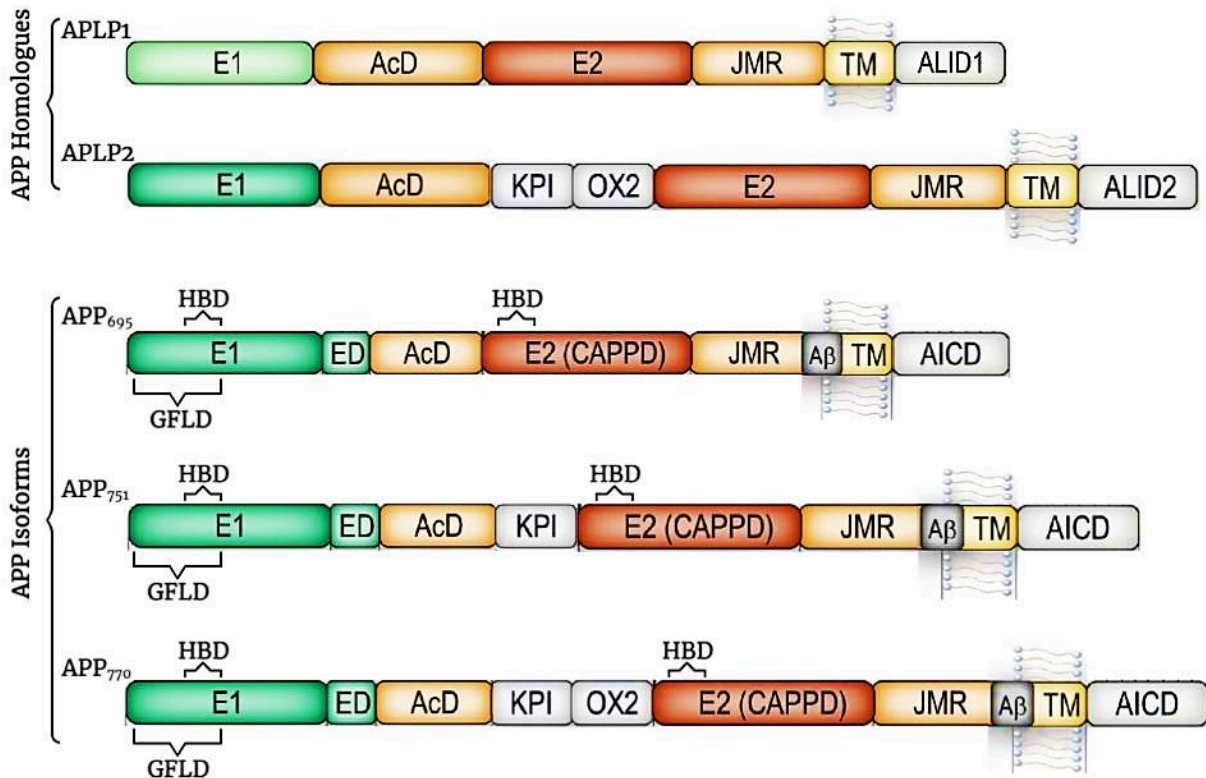
The APP gene, which is found on chromosome 21, has 18 exons. Multiple isoforms of APP are produced as a result of various alternative splicings of this gene, each with a different size and expression levels depending on the organ. APP<sub>770</sub>, APP<sub>751</sub>, and APP<sub>695</sub> are those expressed in the CNS, with APP<sub>695</sub> being the predominant neuronal form from which A $\beta$  is preferentially produced (Nalivaeva and Turner, 2013).

#### 2. Structure

---

Structurally, as presented in (**Figure 4**) these proteins share a long N-terminal extracellular domain whose length varies according to the isoforms, a hydrophobic transmembrane domain, containing the A $\beta$  sequence, and a short domain C-terminal intracellular tail. The extracellular N-terminal part contains a cysteine-rich globular E1 domain, an extension domain (ED), an acidic subdomain (AcD), a helix-rich E2 domain, and a juxtamembrane region (JMR). The E1 and E2 domains of APP contain heparin binding domains (HBD), which are extracellular matrix (ECM) components. The first HBD on E1, known as the GFLD (Growth Factor Like Domain) can form three disulfide bridges to stabilize APP on the cell surface.

APP contains the A $\beta$  sequence extending into the transmembrane domain. APP<sub>770</sub> and APP<sub>751</sub> contain a Kunitz protease inhibitor (KPI) subdomain following the AcD. In addition, APP<sub>770</sub> contain an OX2 domain following the KPI domain. At the C-terminus part, all APP isoforms and homologs possess the intracellular domain, the APP intracellular domain (AICD) or the APLP1/APLP2 intracellular domain (ALID1/ALID2) and contain a YENPTY motif controlling their transport and metabolism. These different domains are reviewed in (Coburger et al., 2014).



**Figure 4 : Domain architecture, functional segments and conservation of APP-family proteins.** APP and its mammalian homologues APLP1 and APLP2 share on overall a similar domain architecture. The A $\beta$ -peptide and the extension domain (ED) are only present in all isoforms of APP, the KPI-insertion only in APP<sub>751</sub>, the KPI/OX2-insertion only in APP<sub>770</sub> and APLP2 and the E1-domain is less conserved in APLP1 as indicated by its light green color. Additional functional units in APP isoforms are indicated by brackets [heparin binding domain (HBD); growth factor like domain (GFLD)]. Adapted from (Coburger et al., 2014).

### 3. Function

APP can form homodimers or heterodimers with its homologues APLP1 and APLP2. These dimers promote cell adhesion, synaptogenesis, and synapse stabilization and can act as cell surface receptors for adaptors and signaling proteins (Baumkötter et al., 2014; Deyts et al., 2016; Soba et al., 2005). Due to its cysteine-rich domain (CRD) within the E1, APP was recently recognized as a Wnt receptor, and this function actually regulates the levels and trafficking of APP protein. When APP binds to Wnt3a, it promotes the recycling of APP full length (APP-FL), whereas binding to Wnt5a enhances the targeting of APP to lysosomal compartments (Liu et al., 2021).

APP dimerizes primarily in the ectodomain via the E1 domain and disulfide bridges that stabilize the dimerization; the E2 domain can also dimerize but with lower affinity. The

transmembrane domain, on the other hand, contains three consecutive GXXXG motifs that would promote dimerization via helix-helix interaction.

The study of mice invalidated for the APP gene yielded important insights into the *in vivo* functions of APP. Single-knockout (KO) APP mice (APP<sup>-/-</sup>) exhibited subtle phenotypes such as reduced brain, body weight, and age-dependent impairments in neuronal morphology, synaptic plasticity, and behavior while double (APP<sup>-/-</sup> and APLP2<sup>-/-</sup>) or triple- (APP<sup>-/-</sup>, APLP1<sup>-/-</sup> and APLP2<sup>-/-</sup>) KO are lethal after birth. Intriguingly, the APP<sup>-/-</sup> mice remained viable, most likely due to APP/APLP redundancy. However, despite the fact that its homologs can compensate for some of its function, APP clearly appears to have a physiological function of its own. Aged APP KO mice showed synaptic defects, LTP and memory impairments, suggesting the involvement of APP in the synaptic plasticity and memory. Moreover, it participates in the development of the nervous system at the embryonic stage, particularly at the level of neuronal migration, neurogenesis and axonal guidance (Müller et al., 2017; Muller and Zheng, 2012). Lastly, it has been recently highlighted that Wnt pathway plays an important role in regulating neurite outgrowth through CRD of the APP (Liu et al, 2022).

Given that each proteolytic fragment of APP has one or more distinct roles of its own (see chapters [A \$\beta\$  peptides](#), [AICD](#), [C99](#), [sAPP \$\beta\$](#) , [sAPP \$\alpha\$](#) , [C83](#), [P3](#)), all the precise potential functions of APP are challenging to determine and require further investigation.

## B) CANONICAL APP-PROCESSING PATHWAY

---

For many years, cleavages of APP have been investigated to better understand A $\beta$  generation. These cleavages can be classified into two main canonical proteolytic pathways: the amyloidogenic pathway, which produces A $\beta$  following  $\beta$ -secretase and  $\gamma$ -secretase and the non-amyloidogenic pathway, in which APP is first cleaved by  $\alpha$ -secretase then by  $\gamma$ -secretase, thus precluding A $\beta$  production (**Figure 5**).

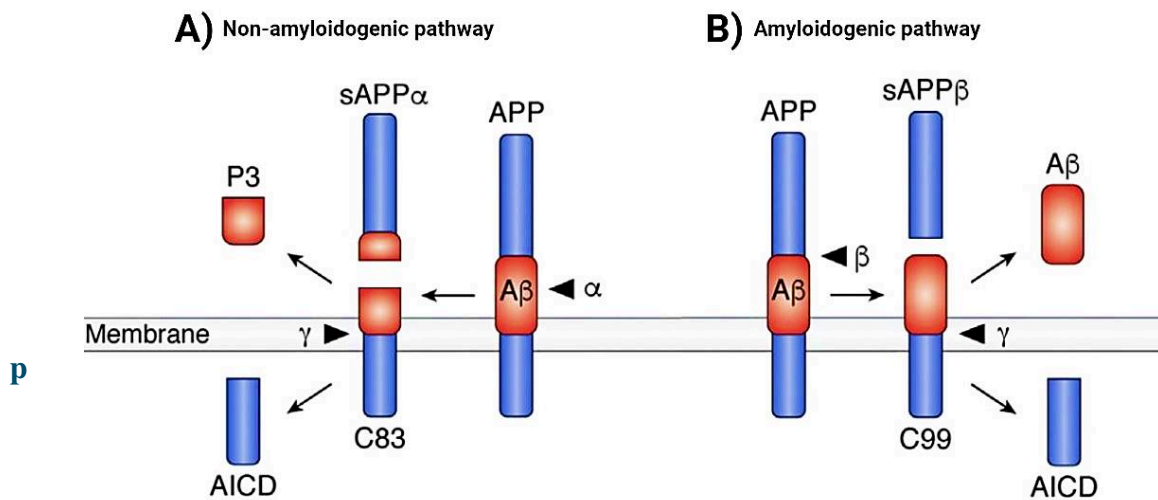
Numerous non-canonical APP cleavage pathways have been identified and were found to generate novel fragments; some of these are emerging as new players in the pathophysiology of AD.

### 1. The Amyloidogenic Pathway

---

In the amyloidogenic pathway where A $\beta$  is formed,  $\beta$ -secretase cleaves APP at the N-terminus of A $\beta$  sequence, releasing sAPP $\beta$ , a 100 kDa soluble amino-terminal (N-terminal) fragment,

and C99, a 12 kDa carboxy-terminal (C-terminal) fragment that remains membrane bound. C99 can be further cleaved by  $\gamma$ -secretase, resulting in the production and secretion of  $A\beta$ , and a cytosolic Cter fragment AICD (**Figure 5-B**).



**Figure 5 : Non-amyloidogenic and amyloidogenic APP-pathways.**

A) represents the non-amyloidogenic pathway in which APP is first cleaved by the  $\alpha$ -secretase producing the soluble fragment sAPP $\alpha$  and the membrane-embedded C-terminal fragment, C83 that is then cleaved by  $\gamma$ -secretase to release the cytosolic AICD fragment and p3 peptide. B) represents the amyloidogenic pathway in which APP is first cleaved by the  $\beta$ -secretase to liberate the soluble fragment sAPP $\beta$  and the membrane-embedded C99 fragment, respectively. Then  $\gamma$ -secretase cleavage releases the cytosolic AICD fragment and  $A\beta$  peptides (Checler et al., 2021).

### 1.1. $A\beta$ peptides

The cleavage of  $\gamma$ -secretase results in the generation of several  $A\beta$  species ranging from 37 to 42 amino acids. The majority of the  $A\beta$  generated under physiological conditions is  $A\beta_{40}$ , with only a small percentage attributed to  $A\beta_{42}$ . Although  $A\beta_{40}$  is the most prominent form,  $A\beta_{42}$  is thought to be the most pathogenic due to its high tendency for aggregation.

Until 1992, it was assumed that  $A\beta$  generation was exclusively pathological. Nonetheless, despite the fact that amyloid plaques are neuropathological characteristic of AD,  $A\beta$  is produced throughout life in physiological levels under entirely normal cellular conditions. In fact, picomolar doses of  $A\beta$ , appear to physiologically promote LTP, and improve synaptic plasticity, learning, and memory (Morley et al., 2010; Puzzo et al., 2011; Puzzo et al., 2008). Other physiological effects of  $A\beta$  include lowering neuronal apoptosis and oxidative damage as well as enhancing blood-brain barrier function (Morley et al., 2019).

However, in pathological conditions, A $\beta$  peptides accumulate abnormally in AD brains due to either an increase in production or a problem with their clearance. A $\beta$  monomers can aggregate into soluble oligomers, protofibrils, and eventually insoluble amyloid fibrils that assemble into amyloid plaques (Serpell, 2000). A $\beta$  accumulation and aggregation, through an amyloid cascade, induce synaptic alterations, microglial and astrocytic activation, progressive neuronal death, and cognitive impairment (Haass and Selkoe, 2007).

Although A $\beta$ 40 and A $\beta$ 42 have gained the most attention, N- and C-terminally shortened A $\beta$  peptides have received far less attention. These shortened A $\beta$  molecules are produced by either direct enzymatic cleavage of APP or subsequent processing of A $\beta$  by exopeptidases, and many of these peptides have been shown to be involved in the pathology of AD (Dunys et al., 2018). For instance, human CSF fluids and blood plasma samples from AD patients have been found to contain the C-terminally truncated A $\beta$ 37, A $\beta$ 38, and A $\beta$ 39.

In addition to full-length A $\beta$ 1-42, research has shown the presence of N-truncated A $\beta$  variants in amyloid plaques (Bayer, 2021); the predominant variants were A $\beta$ 4-42 and A $\beta$ pE3-42, a pyroglutamate-modified A $\beta$  where the first two residues are removed and the glutamate in position three is converted to pyroglutamate. Both A $\beta$ 1-42 and A $\beta$ pE3-42 have been found to cause neurodegeneration and memory impairments. Given the toxicity of these N-truncated A $\beta$  variants, their capacity to aggregate, and their role in AD pathogenesis, therapies targeting these variants are being studied. For example, targeting aminopeptidase A (APA) or dipeptidyl peptidase 4 (DPP4), both enzyme contributing to the synthesis of A $\beta$ pE3-42, leads to reduced levels of plaque-associated A $\beta$ pE3-X peptides, as well as improved memory deficits in 3xTg-AD mice (Valverde et al., 2021a; Valverde et al., 2021b).

## 1.2. AICD

---

AICD is the cytosolic counterpart of A $\beta$  produced in the amyloidogenic pathway but also theoretically generated in the non-amyloidogenic pathways when  $\gamma$ -secretase cleaves C99 or C83 respectively. However, research has shown that the transcriptionally active form of AICD is mostly generated via the amyloidogenic pathway, implying its possible contribution to AD pathology (Flammang et al., 2012; Goodger et al., 2009). After being highly debated for several years, AICD's ability to act as a transcription factor has now been established (Pardossi-Piquard and Checler, 2012). In fact, (von Rotz et al., 2004) showed that AICD may interact with the APP-adaptor protein Fe65, and the AICD-Fe65 complex can translocate into the nucleus, where it assembles into the AFT trimeric complex with histone acetyl-transferase Tip60 (AICD-Fe65-Tip60). In this study, they discovered an AICD-mediated increase in APP and  $\beta$ -site APP

cleaving enzyme (BACE) transcripts, showing that AICD might indirectly enhance C99 and A $\beta$  production. However, another study from my laboratory found that AICD could help eliminate A $\beta$  by promoting the transcription of the A $\beta$ -degrading enzyme neprilysin (Pardossi-Piquard et al., 2005).

On the other hand, AICD has been found to produce pathological features of AD in an A $\beta$ -independent manner, where AICD up-regulates the glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), the latter being involved in neuronal apoptosis and responsible for hyperphosphorylation and age-dependent aggregation of tau and neurodegeneration (Ghosal et al., 2009; Kim et al., 2003; Ryan and Pimplikar, 2005).

Additionally, it has been demonstrated that AICD physically interacts with the p53 promoter, leading to p53-dependent cell death (Alves da Costa et al., 2006). The AICD-mediated cell death has been proved to be neuron-specific (Ohkawara et al., 2011), or both, neuronal and non-neuronal specific when promoting FoxO-induced transcription of the pro-apoptotic gene Bim (Wang et al., 2014).

Many more target genes of AICD, that are involved in DNA repair, cell cycle re-entry, cytoskeletal dynamics, and synaptic activity are reviewed and discussed in some articles (Bukhari et al., 2017; Müller et al., 2008).

### 1.3. C99

---

The C99 transmembrane fragment includes both the A $\beta$  and AICD sequences. However, C99 is not only the precursor of two fragments known to be involved in AD, but it may also have its own pathogenicity, especially since the fragment's aggregation can be found in individuals with the disease. In fact, a recent study found that the accumulation of C99, rather than A $\beta$ , correlated with neuronal death in AD patients (Pulina et al., 2020).

Several research conducted by my team highlighted the role of C99 in the etiology of AD. First, C99 was found to accumulate in the hippocampus of 3xTgAD mice, in the early stages, even before A $\beta$ , raising the possibility that C99 could be an early contributor to the disease (Lauritzen et al., 2012). C99 were detected in the endosomal-lysosomal network, and by pharmacologically inhibition of  $\gamma$ -secretase, in absence of A $\beta$  formation, the accumulation of C99 was linked to lysosomal dysfunction (Lauritzen et al., 2019; Lauritzen et al., 2016). Since C99 can be degraded by the lysoso-autophagic system, its accumulation is both, the cause and the consequence of impaired lysosomal-autophagic function (Lauritzen et al., 2016). Furthermore, C99 was discovered to promote endocytic pathway impairment in DS and AD patients (Jiang et al., 2010).



Importantly, C99 accumulation has also been associated to endolysosomal dysfunction in more physiological cellular models devoid of any overexpression such as induced pluripotent stem cells (iPSCs) harboring AD-related APP or PS1 mutations (Kwart et al., 2019) and iPSCs derived neurons obtained from AD patients harboring APP or PS1 mutations (Hung and Livesey, 2018).

In addition to endosomal and lysosomal alterations, high levels of C99 expression were related with inflammatory responses and synaptic dysfunctions in C99 expressing mice when A $\beta$  formation was also prevented (Lauritzen et al., 2016). In addition to synaptic dysfunction, the intraneuronal accumulation of C99 was also found to contribute to spatial learning deficits and apathy-like behavior, in 3xTgAD mice, and more importantly in double transgenic AD mouse model (2xTgAD)(APP<sup>swe</sup>, TauP301L) that show the same accumulation of C99 as 3xTgAD mice without any A $\beta$  accumulation (Bourgeois et al., 2018). Oligomers of C99 were not only detected in the endo-lysosomal network (ELN), but in exosomes as well, which indeed originate from endosomes (Lauritzen et al., 2019). Exosomes are known to transmit neurotoxic molecules from cell to cell in the brain, and since microglia were shown to take neuron-derived extracellular vesicles (EVs) (Lauritzen et al., 2019), C99 might be undergoing neuron-neuron propagation causing LTP impairment and behavioral defects, or neuron-glia cell propagation implying the neuroinflammation caused by accumulation of C99.

In addition to these findings, and with mitochondrial dysfunction being one of the key characteristics of AD, a recent study from our team envisioned that C99 accumulation causes mitophagy disruption by interfering with multiple steps in the mitophagic process (Vaillant-Beuchot et al., 2021).

#### 1.4. sAPP $\beta$

---

Only a few studies were interested in this soluble fragment of APP generated by the  $\beta$ -secretase cleavage but it has been demonstrated that sAPP $\beta$  stimulates the differentiation of human embryonic stem cells toward a neuronal fate (Freude et al., 2011). Additionally, it has been shown that sAPP $\beta$  binds to the gamma-aminobutyric acid type B receptor subunit 1a (GABABR1a), leading to a suppressed synaptic transmission and an enhanced short-term facilitation in hippocampal synapses (Rice et al., 2019).

As reviewed in the article from Chasseigneaux and colleagues (Chasseigneaux and Allinquant, 2011), sAPP $\beta$  can perform similar roles to sAPP $\alpha$ , such as: promoting axon outgrowth both during development and in response to brain injury (Chasseigneaux and Allinquant, 2011), and triggering microglial activation (Barger and Harmon, 1997). However, since sAPP $\beta$  lacks the

last 16 residues of sAPP $\alpha$ , its neuroprotective properties are significantly reduced compared with sAPP $\alpha$  (Copanaki et al., 2010; Furukawa et al., 1996; Taylor et al., 2008).

## 2. The Non-Amyloidogenic Pathway

---

The predominant and physiological cleavage mechanism of APP is the non-amyloidogenic pathway.  $\alpha$ -secretase first cleaves APP, resulting in the soluble N-terminal fragment sAPP $\alpha$  and the 83 amino acid C-terminal transmembrane protein C83. The latter can be then cleaved by  $\gamma$ -secretase, generating a 3kDa Nter peptide, p3, and AICD (**Figure 5-A**).

### 2.1. sAPP $\alpha$

---

The sAPP $\alpha$  fragment released after the  $\alpha$ -secretase cleavage of APP has been found to exhibit neurotrophic and neuroprotective characteristics, contributing to neuron physiology (Müller et al., 2017). In fact, neurons produce sAPP $\alpha$  as a survival and defense strategy in response to stress caused by elevated A $\beta$  levels (Rose et al., 2018). Interestingly, sAPP $\alpha$  inhibits BACE1 by direct interaction, shifting and modulating APP processing, and thus decreasing A $\beta$  production. Indeed, the overexpression of sAPP $\alpha$  in an AD mouse model reduced amyloid plaques, as well as A $\beta$  oligomeric forms (Obregon et al., 2012). Another study that demonstrates how sAPP $\alpha$  prevents the loss of dendritic spines and protects neurons from A $\beta$  and phospho-tau-dependent toxicity in AD provides more evidence for the neuroprotective properties of sAPP $\alpha$  (Tackenberg and Nitsch, 2019). Furthermore, sAPP $\alpha$  reduces phospho-tau-dependent toxicity by decreasing GSK3 $\beta$  activity, an enzyme that is responsible for Tau phosphorylation (Deng et al., 2015). sAPP $\alpha$  appears also to be capable of binding to APP, interrupting its dimerization (Gralle et al., 2009), and activating the PI3K/Akt/mTor pathway via coupling to a G-protein, a pathway mostly known for its function in cell survival by blocking apoptosis (Milosch et al., 2014). Along with its neuroprotective qualities, sAPP $\alpha$  promotes axonal outgrowth via a separate mechanism that involves the recruitment of  $\alpha$ 3-NKA to the neuronal surface (Dorard et al., 2018).

### 2.2. C83

---

C83's functions have not been fully investigated. What is well known is that C83 can be produced directly from the  $\alpha$ -secretase cleavage of APP full length but also by cleaving C99 with  $\alpha$ -secretase, lowering C99 levels and limiting A $\beta$  production (Jager et al., 2009). C83 can be then cleaved by  $\gamma$ -secretase, resulting in p3 and AICD. However, it has been demonstrated

that the latter is primarily derived from the amyloidogenic pathway (Flammang et al., 2012; Goodger et al., 2009), and that C83's interaction with  $\gamma$ -secretase limits the latter's catalytic ability via feedback inhibition, thereby down-regulating A $\beta$  production (Tian et al., 2010).

### 2.3. P3

---

More research is needed to better understand the P3 peptide's characteristics and effects, as studies on this peptide are often inconsistent. P3 lacks A $\beta$ 's first 16 residues but includes the 17-21 residues essential for fibril assembly and neurotoxicity (Higgins et al., 1996). Despite the fact that it contains residues involved in the formation of  $\beta$  sheets, a previous study indicated that p3 is unlikely to form stable soluble oligomers (Dulin et al., 2008). However, a more recent work published in 2020 found that p3 aggregated to form fibrils and oligomers with a comparable size and distribution as A $\beta$  (Kuhn et al., 2020).

Furthermore, p3 has been detected in some dystrophic neurites, implying that this peptide may play a pathogenic or neurotoxic role (Higgins et al., 1996). Nonetheless, a further investigation found that only A $\beta$  oligomers, and not p3, are synaptotoxic in AD (Walsh et al., 2002).

All of these contradicting findings lead to reassess the p3 and  $\alpha$ -secretase pathways' categorization as "non-amyloidogenic" (Kuhn and Raskatov, 2020).

## 3. APP Cleaving Enzymes

---

### 3.1. $\alpha$ -secretase

---

The  $\alpha$ -secretase cleavage is carried out by three members of the ADAM (a disintegrin and metalloproteinase) family: ADAM9, ADAM10, and ADAM17, also known as TNF- $\alpha$  converting enzyme (TACE) (Allinson et al., 2003). It has been determined that ADAM10 is the essential enzyme responsible for cleaving APP at the  $\alpha$ -secretase site both physiologically and functionally in neurons (Kuhn et al., 2010). Therefore, ADAM10 functions as the constitutive neural  $\alpha$ -secretase under non-stimulated conditions, whereas ADAM17 is known to be the "regulated  $\alpha$ -secretase". This signifies that stimuli such as protein kinase C (PKC) activation or M1 muscarinic receptor induction is required for ADAM17 to cleave APP (Buxbaum et al., 1998; Caccamo et al., 2006). Considering that  $\alpha$ -secretase is involved in the non-amyloidogenic pathway, ADAM17 may, in theory, have a preventative effect on the development of AD. However, due to microglial activation and TNF $\alpha$  induced neuroinflammation, ADAM17 may have the opposite consequences, as reviewed in (Hsia et al., 2019). ADAM9 is also involved in

the  $\alpha$ -secretase cleavage but indirectly by activating ADAM10 (Cissé et al., 2005; Moss et al., 2011).

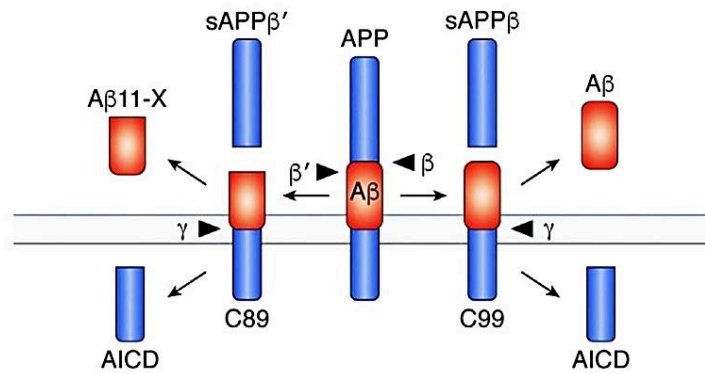
ADAMs are type I integral membrane proteins that belong to the zinc metalloproteinases. In a nutshell, they are synthesized as inactive precursors where the prodomain acts as an inbuilt inhibitor, and the enzyme becomes active only when the inhibitory prodomain is processed by a proprotein convertase in the Trans-Golgi network (TGN) (Allinson et al., 2003; Endres et al., 2003). After being activated, ADAM10 seems to cleave APP within the TGN compartment or predominantly on the plasma membrane (Sisodia, 1992; Tan and Gleeson, 2019).

These ADAMs are not only linked to Alzheimer's disease; for example, ADAM10 and ADAM17 are known to cleave not only APP but over 40 distinct type I and II integral membrane proteins, contributing to neurological diseases, cancers, prion diseases, and inflammatory diseases (Vincent and Checler, 2012).

### 3.2. $\beta$ -secretase

---

The type 1 transmembrane aspartic protease BACE1 ( $\beta$ -site APP cleaving enzyme 1), also known as aspartyl protease 2 and membrane-associated aspartic protease 2, is considered as the main enzyme in charge of the  $\beta$ -secretase cleavage of APP. It cleaves APP695 at the bond between Met596 and the first A $\beta$  residue, Asp597 ( $\beta$ -site) to generate C99, also known as  $\beta$ -CTF (C-terminal fragment). BACE1 also cleaves APP at another bond between Tyr606 and the 11th residue of A $\beta$ , Glu607 ( $\beta'$ -site), generating the non-amyloidogenic C89 fragment, favored in physiological conditions, and in individuals with the protective Icelandic APP mutation (Kimura et al., 2016) (**Figure 6**). Additionally, it has been shown that BACE1 can cleave A $\beta_{40}$  and A $\beta_{42}$  to generate a C-terminally truncated A $\beta_{34}$  form. A $\beta_{34}$  levels are significantly increased in people with MCI and correlate with the overall A $\beta$  clearance rates in amyloid positive individuals. In the early stages of the disease, it is believed that the increase in A $\beta_{34}$  help to protect against A $\beta$ -mediated toxicity; however, it may not be sufficient to counteract the toxicity brought on by increased levels of A $\beta$  in the advanced stages of the pathology (Caillava et al., 2014; Liebsch et al., 2019).



**Figure 6 :  $\beta$  or  $\beta'$ / $\gamma$ -secretase pathway.**

APP is first cleaved by the  $\beta$ -secretase at  $\beta$  or  $\beta'$  site to liberate the soluble fragments sAPP $\beta$  or sAPP $\beta'$  and the membrane-embedded C99 or C89 fragments, respectively. Then  $\gamma$ -secretase cleavage releases the cytosolic AICD fragment and A $\beta$  peptides (A $\beta$ 1-40/42) or A $\beta$ 11-40/42, respectively. (Checler et al., 2021).

BACE1 is produced as a pro-enzyme and is present in the ER in its immature state, already N-glycosylated. It is then delivered to the TGN to be cleaved by furins for maturation (Capell et al., 2000). Nevertheless, research has revealed that even pro-BACE1 can cleave APP and act as a  $\beta$ -secretase in the ER and early Golgi (Creemers et al., 2001). BACE1 can then target the plasma membrane and be internalized to the endosomal system, or it can directly target endocytic compartments from the TGN, completely bypassing the plasma membrane. The C-terminal dileucine motif of BACE is essential for its trafficking to endosomes, where its activity is highest at an acidic pH (4.5). The deletion of this motif results in a twofold increase in recycling to the cell surface and accumulation on the plasma membrane (Huse et al., 2000).

While much focus has been given to its role in the amyloidogenic process, APP is not the only substrate of BACE1; in fact, about 70 BACE1 substrates have been identified to date. Many of the BACE1 substrates are associated with neurological function. The most extensively researched substrates, for example, are Neuregulin 1 (NRG1), cell adhesion molecule L1-like protein (CHL1), Jagged 1 (Jag1), and Seizure protein 6 (SEZ6) which are required for myelination and remyelination, axonal growth and migration, astrogenesis and neurogenesis, and synaptic plasticity, respectively (Yan, 2017).

Through a variety of its non-neuronal substrates, BACE1 has also been linked to inflammation, cardiovascular health, glucose homeostasis, and insulin signaling (Taylor et al., 2022). According to a 2018 study, BACE1 regulates the amount of active insulin receptors in the liver by cleaving their ectodomain in a glucose concentration-dependent manner; and that in diabetes

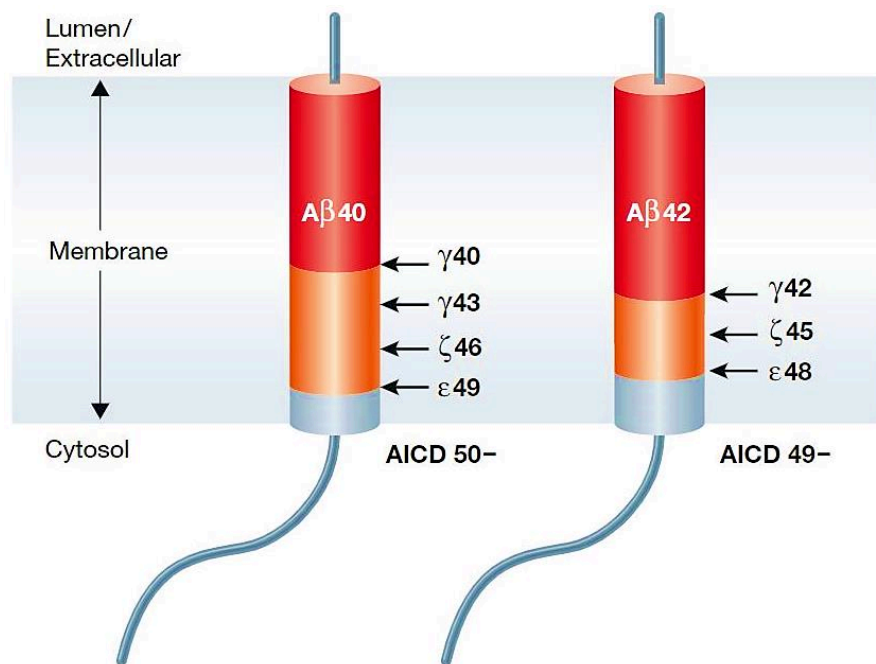
patients, high glucose levels contribute to lower active insulin receptor levels and impair insulin signaling (Meakin et al., 2018).

BACE2 was discovered to be a close homologue of BACE1, exhibiting 64% amino acid sequence identity. In contrast to BACE1, BACE2 is highly expressed in the heart, kidney, and placenta, but modestly in the brain. Furthermore, BACE 2 does not contribute as much as BACE1 to the formation of A $\beta$  because it cuts APP essentially in the A $\beta$  sequence, resulting in the C80 fragment. However more recently BACE2 has been described as a conditional  $\beta$ -secretase cleaving APP at the  $\beta$ -site when its juxtamembrane helix domain is mutated as it is the case for the Flemish and Arctic mutations (Wang et al., 2019).

### 3.3. $\gamma$ -secretase

$\gamma$ -Secretase belongs to a new class of proteases, intramembrane-cleaving proteases (I-CliPs) characterized by an unusual cleavage process occurring in the lipid bilayer of membranes. Like  $\beta$ -secretase,  $\gamma$ -Secretase is an aspartyl protease highly active at acidic pH but with a particularity to require the formation of a high molecular weight proteolytic complex.

After being cleaved by  $\beta$ -secretase, the membrane-bound C99 is cleaved by  $\gamma$ -secretase in a sequential manner in order to generate A $\beta$ . Cleavage at  $\epsilon$ -site is the first, releasing AICD49 or AICD50 and A $\beta$ 48 or A $\beta$ 49 respectively, followed by cleavage at  $\zeta$ -site giving rise to A $\beta$ 45 or A $\beta$ 46, and A $\beta$ 43, then A $\beta$ 40 or A $\beta$ 42 generated at  $\gamma$ -site. A final cleavage can occur, producing even shorter species: A $\beta$ 37 and A $\beta$ 38, the latter being with A $\beta$ 42, the predominant forms after A $\beta$ 40 (Zhang et al., 2014a). Thus, in a more straightforward manner, C99 is cleaved at the  $\epsilon$ -site to produce AICD along with A $\beta$ 48 and A $\beta$ 49, which are then, trimmed every three amino acids along two pathways: A $\beta$ 49→A $\beta$ 46→A $\beta$ 43→A $\beta$ 40 and A $\beta$ 48→A $\beta$ 45→A $\beta$ 42→A $\beta$ 38 (**Figure 7**).



**Figure 7 : Progressive cleavages of the APP transmembrane domain by the Presenilin/ $\gamma$ -secretase complex.**

Following  $\beta$ -secretase cleavage, the resultant C99 goes through a sequence of consecutive cleavages catalyzed by  $\gamma$ -secretase to generate A $\beta$ . C99 first undergoes  $\epsilon$ -cleavage, releasing A $\beta$ 48 or A $\beta$ 49, and a further  $\zeta$ -cleavage, producing A $\beta$ 45 or A $\beta$ 46. Subsequently, at  $\gamma$ -site, A $\beta$ 45 and A $\beta$ 46 are processed into A $\beta$ 42 and A $\beta$ 43, respectively. The resulting A $\beta$ 43 then faces additional processing at  $\gamma$ -site to produce A $\beta$ 40. (Selkoe and Hardy, 2016).

The  $\gamma$ -secretase complex consists of four essential subunits: PS1 or PS2 constitute the catalytic core of  $\gamma$ -secretase, and three interacting proteins partner, anterior pharynx-defective 1 (APH-1), Nicastrin, and Presenilin Enhancer Protein 2 (PEN2), crucial for complex maturation and stabilization (Lu et al., 2014; Wolfe, 2020) (**Figure 8-A**). PS1 and PS2 are two homologues of intramembrane cleaving proteases that are produced as precursor proteins and mature through autocatalysis. In addition to their endoproteolysis, their proper assembly into the multiprotein complex is required for  $\gamma$ -secretase activity to occur (De Strooper et al., 2012).

Composition of  $\gamma$ -secretase complex is of high importance for its activity and can differ depending on its cellular localization. In fact, in addition to the two possible PS, there are two homologs of Aph-1 (Aph-1a and Aph-1b) identified in humans, as well as splice variants for Aph-1a (Aph-1aL and Aph-1aS). Only one of the two PS and one of the three Aph1 isoforms can coexist in a complex, resulting in at least six distinct  $\gamma$ -secretase complexes in humans. A recent study found that APH-1b-containing  $\gamma$ -secretase complexes produce longer A $\beta$  peptides than APH-1a-containing complexes (Zhang et al., 2014a).

$\gamma$ -secretase is located in multiple subcellular compartments, including the plasma membrane, ER, TGN, endosomes, and lysosomes. Interestingly, it has been shown that complexes containing PS2 have a distinct distribution than those containing PS1. PS1/ $\gamma$ -secretase, for example, is localized to the plasma membrane and is more widely distributed in the cell, releasing extracellular A $\beta$ , whereas PS2/ $\gamma$ -secretase is primarily restricted to the TGN, as well as late endosomes and lysosomes, thus creating A $\beta$  in the endosomal pathway (Meckler and Checler, 2016; Sannerud et al., 2016).

Cryo-EM single-particle analysis revealed the three-dimensional structure of the human  $\gamma$ -secretase complex, which includes a horseshoe-shaped transmembrane domain, which contains 20 transmembrane segments (TMs), and a large extracellular domain (ECD) from Nicastrin (Bai et al., 2015; Guner and Lichtenthaler, 2020; Lu et al., 2014) (**Figure 8-B**). This structure is in accordance with Nicastrin's alleged substrate recruitment function, which depends on Glu333 and Tyr337 that are hidden in a hydrophilic pocket. In the absence of a substrate, this pocket is covered by a "Lid" that is positioned directly above the concave side horseshoe, while the cleaving site is found on the convex side. The "Lid" opens upon substrate binding, reorienting the substrate for cleavage, possibly via lining up the hydrophilic pocket and the active site of PS1. The subunits are mostly buried within the membrane. An exception is Nicastrin. It has a large extracellular domain that acts as a gatekeeper which only allows proteins with short ectodomains to be transferred to the active site and provides a mechanism to anchor the substrates in the active site. While the majority of substrates undergo ectodomain shedding to fit under the Nicastrin lid-like structure, a substrate with a naturally short ectodomain may be directly cleaved by  $\gamma$ -secretase without prior shedding (**Figure 8-B**).

Indeed,  $\gamma$ -secretase cleavage is reliant on shedding of the substrate's ectodomain; the enzyme appears to cleave substrates within the transmembrane domain near the cytoplasmic membrane border, releasing a cytosolic fragment, the intracellular domain (ICD), capable of entering the nucleus and controlling the transcription of different genes; a process generally referred to as regulated intramembrane proteolysis (RIP).

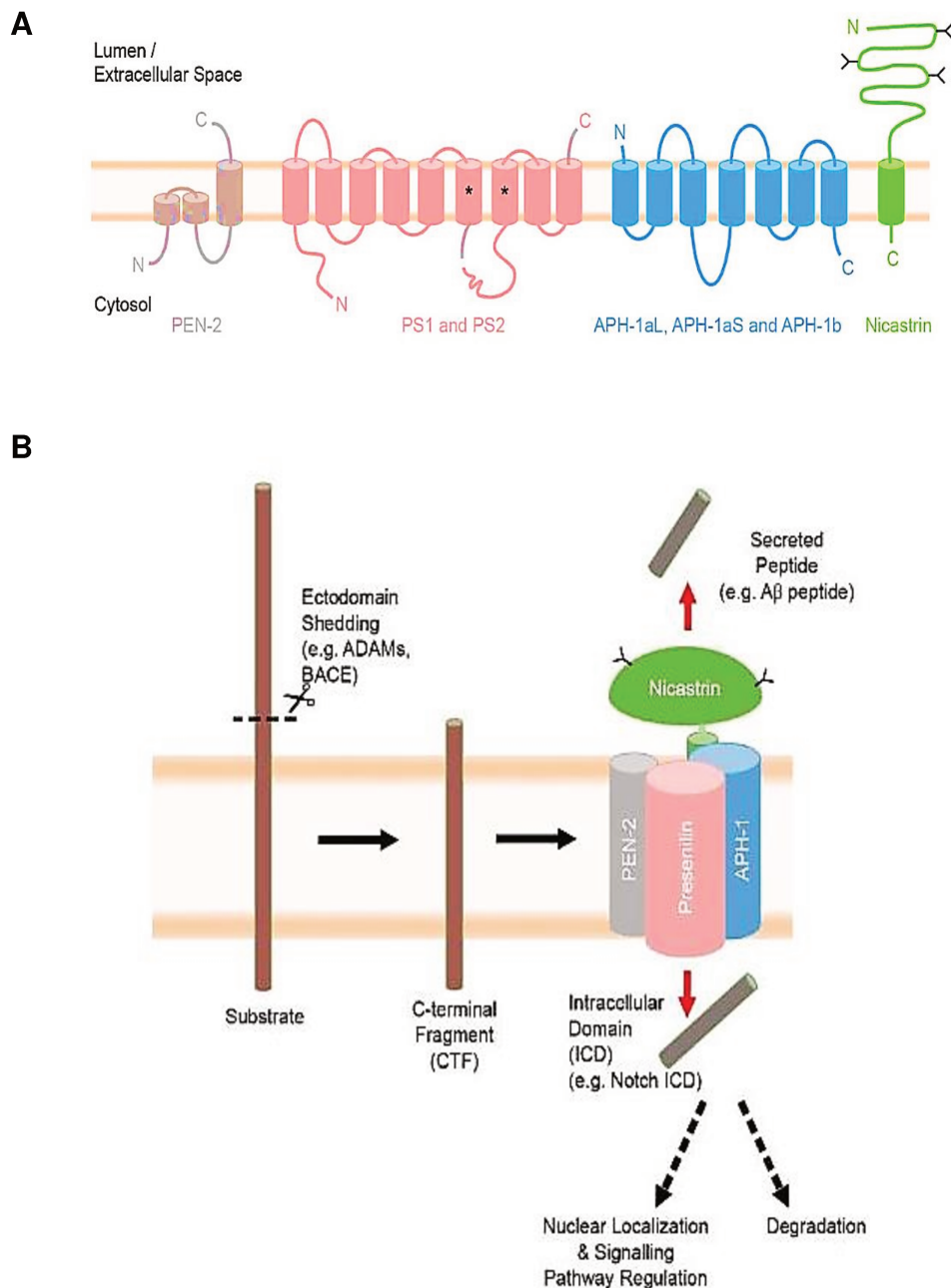
APP is just one of more than 140 type 1 transmembrane proteins that are substrates candidates for  $\gamma$ -secretase (Guner and Lichtenthaler, 2020). Notch was the second, widely known substrate of  $\gamma$ -secretase (Ray et al., 1999). Notch is cleaved by  $\gamma$ -secretase, releasing the Notch IntraCellular Domain (NICD), which can bind to transcription factors and regulate genes implicated in neural differentiation, which is especially crucial during embryonic development.



Moreover, Notch is critical cell differentiation and tissue homeostasis in healthy adults and plays an important role in the regulation of inflammatory responses in many diseases.

Aside from APP and Notch, several of the  $\gamma$ -secretase substrates appear to produce an ICD that regulates cell fate determination, adhesion, migration, neurite outgrowth, axon guidance, or formation and maintenance of synapses, angiogenesis and tumorigenesis, immune responses, etc... (Guner and Lichtenthaler, 2020; Jurisch-Yaksi et al., 2013).

Given the diverse variety of  $\gamma$ -secretase substrates, some may serve as signaling molecules, while others may only be intermediates heading for degradation. It has been hypothesized that  $\gamma$ -secretase cleavage could possibly serve as the membrane's "proteasome", maintaining the membrane homeostasis (Kopan and Ilagan, 2004). According to this theory, ectodomain shedding prepares the  $\gamma$ -secretase to cleave within the membrane-tethered CTF, either producing a biologically active protein fragment or releasing peptides for further degradation by soluble proteases. This is analogous to how ubiquitin conjugation prepares target proteins for proteasomal degradation.



**Figure 8 : Subunits of  $\gamma$ -secretase and substrate recognition.**

A) Presenilins (PS) consist of 9 transmembrane domains, Aph-1 of 7 transmembrane domains, PEN-2 of 3 transmembrane domains, and Nicastrin of a single transmembrane domain and a large, glycosylated extracellular domain. Asterisks indicate the catalytic aspartic acid residues. B)  $\gamma$ -Secretase forms a hetero-tetrameric, horseshoe-shaped complex with one copy of each subunit. The  $\gamma$ -secretase cleavage releases the intracellular domain (ICD) to the cytosol and a short secreted peptide to the extracellular space. (Guner and Lichtenthaler, 2020).

## 4. APP Localization

---

### 4.1. APP Trafficking and Co-Residence with Canonical Secretases

---

At cellular level, APP is located in the endoplasmic reticulum (ER), the TGN, the plasma membrane, endosomes and lysosomes (Thinakaran and Koo, 2008). APP has also been detected in mitochondria, cytosol or nucleus. At the neuronal level, it is found in the soma-dendritic compartment but also at the level of the axon.

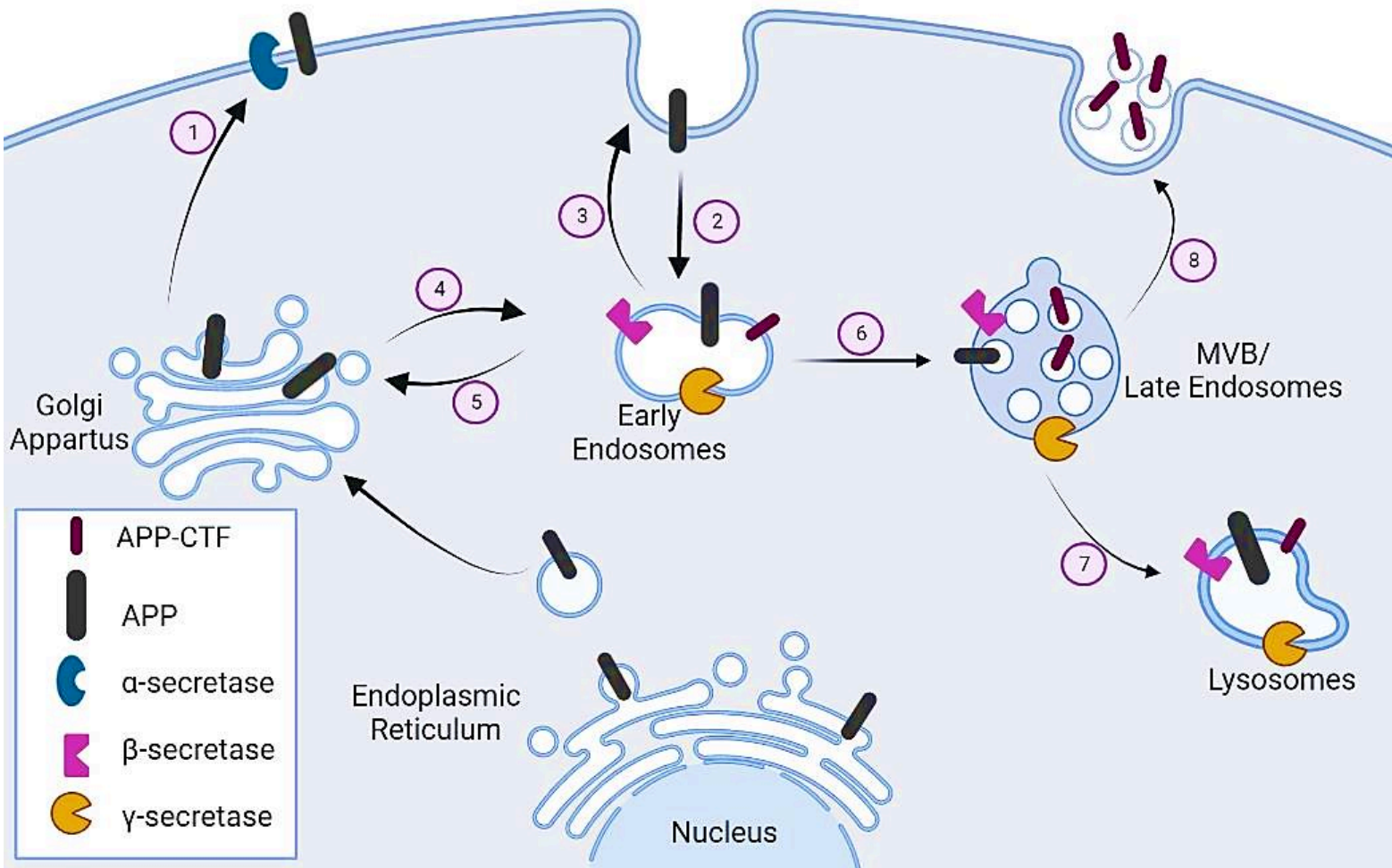
After being synthesized in the membrane-bound polysomes, the signal peptide of APP gets cleaved during its translocation in the ER. APP then transports to the Golgi apparatus, the trans-Golgi network where it undergoes a large number of post-translational modifications (N and O-glycosylations, phosphorylations and sulphations), necessary to obtain its mature form and its three-dimensional structure. Only 10% of APP approximately reaches the plasma membrane, whereas the majority is predominantly localized at steady state in the Golgi apparatus and TGN (**Figure 9**).

APP at the plasma membrane is mainly cleaved by  $\alpha$ -secretase (**Figure 9-1**). Uncleaved APP on the cell surface is internalized into the early endosomes thanks to its YENPTY motif (Haass et al., 2012; Lai et al., 1995) (**Figure 9-2**).

From endosomes, APP can be sorted into different pathways through the ELN: the majority is directed to the endosome-lysosomal pathway for degradation in lysosomes (**Figure 9-7**) or incorporation into exosomes (**Figure 9-8**), the remaining part is recycled to the membrane (**Figure 9-3**) or directed towards the Golgi apparatus via a so-called retrograde transport (**Figure 9-5**). Finally, the APP present in the TGN can also reach the endosomes directly, without passing through the plasma membrane, via so-called anterograde transport (**Figure 9-4**).

While  $\alpha$ -secretase cleavage takes place at the plasma membrane,  $\beta$ -cleavage mainly occurs in the endosomes and lysosomes where the acidic pH is optimal for the activity of  $\beta$ -secretase. And since this acidic pH also suits  $\gamma$ -secretase complex, amyloidogenic cleavage of APP mainly occurs in the endosomal compartments. However, in addition to early endosomes, late endosomes, lysosomes and autophagic vesicles, the  $\gamma$ -secretase complex have been found also in the plasma membrane.

In order to properly understand APP trafficking and metabolism, it is crucial to understand the complex biology of the ELN.



**Figure 9: Schematic representation of APP trafficking and its convergence with canonical secretases in the endo-lysosomal network.**

After synthesis, APP transits along the secretory pathway through the ER to the TGN, then from the TGN to the plasma membrane where it is further metabolized by  $\alpha$ -secretase (1). APP can be also endocytosed into early endosomes vesicles (2) or directly transported from the TGN to early endosomes (4). Early endosomes can be recycled back to the plasma membrane (3) or to the Golgi compartment (5). The maturation of early endosomes generates multivesicular bodies (MVB)/late endosomes (6) containing multiple vesicles. Once in the MVB/endo-somes, APP can be degraded in lysosomes (7) or released through exosomes (8). Non-amyloidogenic processing mainly occurs at the cell surface where  $\alpha$ -secretases are present (1). Amyloidogenic processing involves transit through the endocytic organelles where APP encounters  $\beta$ - and  $\gamma$ -secretases (6 to 8). Figure created with BioRender.com free software.

#### 4.2. Endo-Lysosomal Network (ELN) – Generalities

The ELN consists of complex and highly dynamic vesicles distinguished by their morphology, their pH and by the expression of specific markers, in particular the small GTPases, Rabs — for "Ras-associated binding".

Cargos are internalized and then subsequently sorted by sorting complexes to either reach the cell surface, to be degraded by lysosomes, or to be transported to the trans-Golgi for further

sorting. Furthermore, the ELN engages in exocytosis, which allows cellular contents to be released into the extracellular environment. Thus, this system enables the recycling or destruction of dysfunctional, potentially harmful internal materials. This system also regulates the amount and topography of surface receptors, including those that mediate neurotransmission and synaptic plasticity, through internalization and recycling back to the cell surface. ELN plays an equally significant role in cell signaling by transmitting vast information from the surface and external environment to the nucleus.

Extracellular and plasma membrane proteins are internalized through clathrin-mediated or clathrin-independent endocytosis (**Figure 9-2**). The internalization, or the endocytosis starts with the collecting of the membrane-bound cargos, the formation of endocytic vesicles from the plasma membrane, and the fission of these vesicles, which merge with early endosomes.

Early endosomes have a slightly acidic pH values of  $\approx 6.2$ , and they are mainly marked by Rab5 and by its effector protein early endosome antigen 1 (EEA1) which is a tethering protein required for fusion of endocytic vesicles with early endosomes.

Once cargos reach early endosomes, they can be recycled back to the plasma membrane (**Figure 9-3**), or routed by retrograde transport to the TGN (**Figure 9-5**) (Langemeyer et al., 2018; Scott et al., 2014).

Endosome maturation is the transformation of early endosomes into late endosomes (**Figure-6**) by a sequence of biochemical and morphological changes particularly Rab7 takes the place of Rab5 in late endosomes. In addition, luminal invaginations of the early endosomes membrane can be formed and then detach to form cargo-containing intraluminal vesicles (ILVs). This ILV formation that starts in early endosomes is mainly dependent of the endosomal sorting complex required for transport (ESCRT) machinery. During this ILV formation, some proteins are incorporated into the invaginating membrane, while the cytosolic components are engulfed and enclosed within the ILVs. Vacuolar areas harboring these ILVs also detach and mature from early endosomes, resulting in the formation of free MVBs (multivesicular bodies) (**Figure 9-6**). Late endosomes/MVBs can either fuse with lysosomes for protein degradation (**Figure 9-7**), or with the plasma membrane, releasing ILVs into the extracellular space and resulting in the secretion of exosomes (**Figure 9-8**) (Elkin et al., 2016; Langemeyer et al., 2018; Scott et al., 2014).

## C) APP AND APP-FRAGMENTS DEGRADATION AND ELIMINATION

---

The APP protein and its resulting fragments have been described to be degraded by both the proteasome and the lysosomal and autophagic pathways, and many studies have also revealed the elimination of these fragments via exosomal secretion.

### 1. Proteasome and its dysfunction in AD

---

#### 1.1. The ubiquitin-proteasome system (UPS)

---

In eukaryotic cells, the ubiquitin-proteasome system (UPS) is the principal system for the degradation of short-lived proteins as well as the elimination of damaged soluble proteins. Mostly, intracellular soluble proteins are degraded by the UPS, but transmembrane proteins can occasionally be degraded by this system if released from the membrane into the cytosol (Dederer and Lemberg, 2021; Nakatsukasa et al., 2008).

This process starts with the adenosine tri-phosphate (ATP)-dependent insertion of ubiquitin chains covalently linked to lysine residues in substrate proteins. This step serves as a recognition signal and is then followed by proteolytic degradation of ubiquitinated proteins by the proteasome. The ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) are three types of enzymes that contribute to the multistep process of ubiquitination. First, E1 forms a thiol-ester bond with the carboxyl-terminal glycine of ubiquitin. E1 then passes the ubiquitin to E2 that also binds to the carboxyl terminus of the ubiquitin by a transthioylation reaction. Finally, E3 catalyzes the transfer of ubiquitin from the E2 enzyme to the substrate's lysine residue. The same process attaches additional ubiquitin molecules to the initial one via internal ubiquitin lysines, resulting in the formation of a ubiquitin chain (**Figure 10-A**).

The proteasome, also known as the 26S proteasome, is a hollow barrel-shaped multiprotein structure consisting of two major assemblies: the core particle (CP), or 20S proteasome, which is a central cylindrical structure with peptide hydrolysis activity, and the regulatory particle (RP), also referred to as 19S proteasome, which borders the CP from one or both sides (**Figure 10-B**). The cylindrical structure of the CP is composed by two types of subunits,  $\alpha$  and  $\beta$ , which are organized in four stacked heptameric rings around a central cavity; seven  $\alpha$  subunits constitute the cylinder's two outer rings, while seven distinct  $\beta$  subunits form the two inner rings. Three of the inner ring's  $\beta$ -type subunits have proteolytic active sites facing the inside of the cylinder:  $\beta 1$  has caspase-like specificity, cleaving after acidic residues;  $\beta 2$  is trypsin-like,

cleaving after basic residues; and  $\beta 5$  is chymotrypsin-like, cleaving after hydrophobic residues (**Figure 10-B**).

Once poly-ubiquitinated with at least four ubiquitins, the protein is detected, de-ubiquitinated, and then unfolded in an ATP-dependent process by the RP, allowing ubiquitin molecules to be recycled and the target protein to be delivered to the CP for degradation. The protein gets then processively degraded by the catalytic core to produce heterogeneous mixture of peptides. The proteasome does, however, occasionally subject regulatory proteins to partial proteolysis, activating them by removing their inhibitory domains (Finley, 2009).

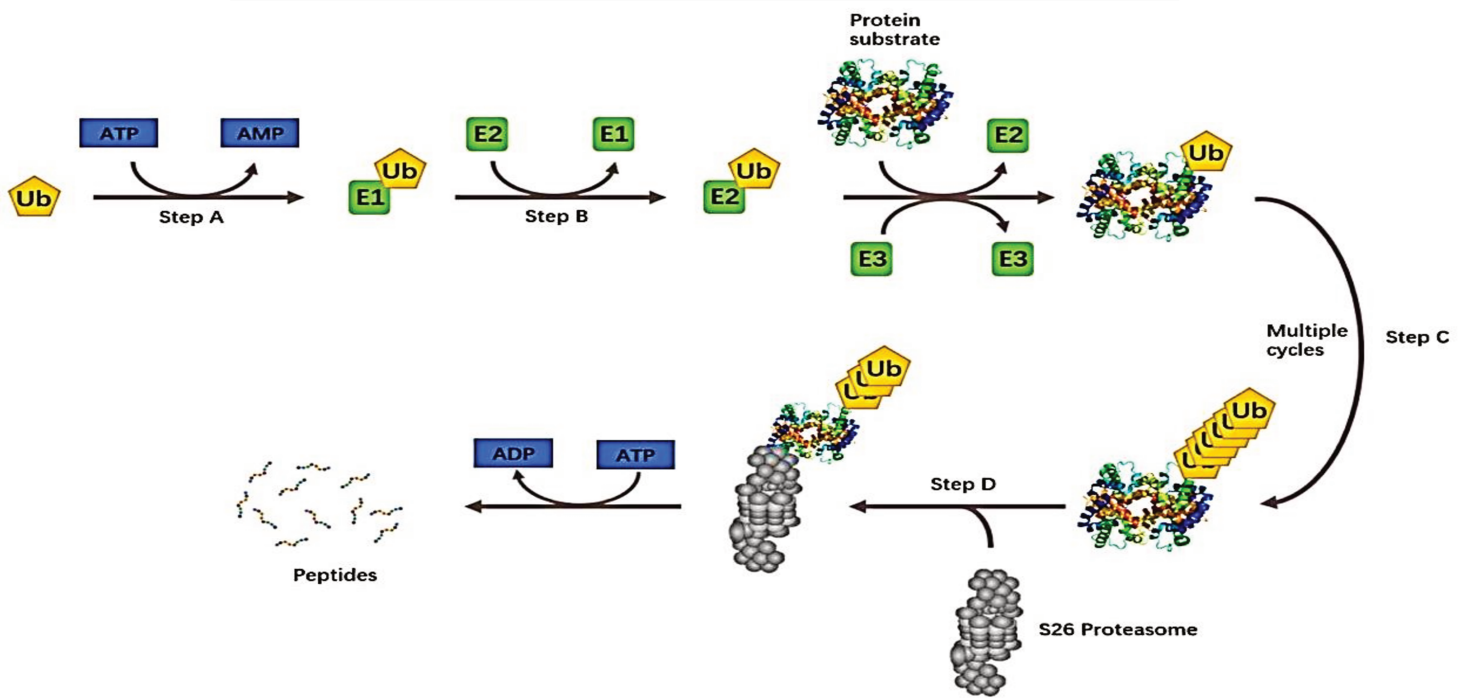
Not only APP is degraded by the proteasome (Hiltunen et al., 2006; Jung et al., 2015; Kaneko et al., 2010) but also the secretases related to the amyloidogenic pathway,  $\beta$ -, and  $\gamma$ -secretases, are potentially degraded by the UPS (Flood et al., 2005; Haapasalo et al., 2010; Qing et al., 2004). As a result, the UPS's activity contributes to the clearance of A $\beta$  peptide. Additionally, some studies suggest that the proteasome may degrade the C99 as well (Bustamante et al., 2013; Evrard et al., 2018; Nunan et al., 2001; Nunan et al., 2003).

## 1.2. Dysfunctions of Proteasome in AD

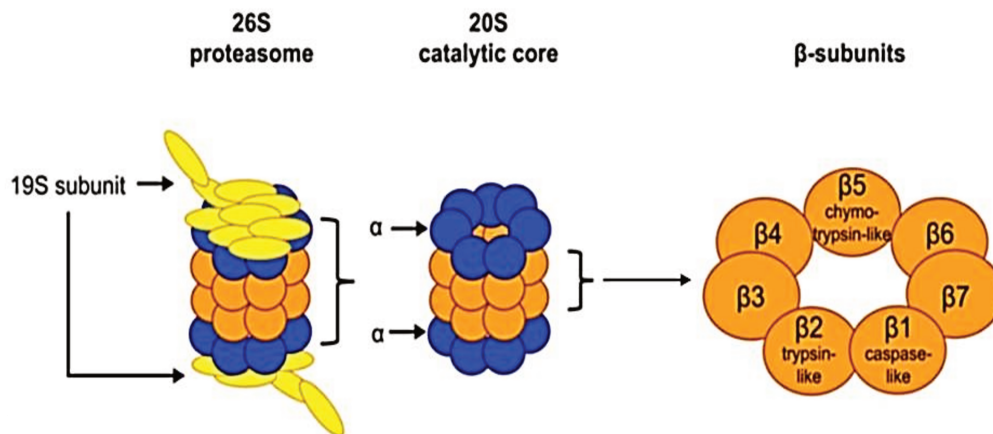
Since aggregated A $\beta$  and tau proteins accumulate, AD is also considered as a proteinopathy and it was speculated that impairments in the UPS play a significant role in the disease's pathogenesis through various mechanisms (Cao et al., 2019; Gadhav et al., 2016). Indeed, as a consequence of processing and degrading critical proteins involved in the pathogenesis of AD, including APP, BACE1, PS1, PS2, and C99, UPS indirectly reduces the produced levels of A $\beta$ . Moreover, A $\beta$  has also been discovered to be a substrate of this enzymatic complex, which enables UPS to directly degrade and eliminate A $\beta$  peptides (Lopez Salon et al., 2003; Zhao and Yang, 2010). However, as it turned out, there is a two-way link between A $\beta$  and UPS, as A $\beta$  is degraded by UPS while also having an impact on this system (Hong et al., 2014). In fact, A $\beta$  may operate as a UPS inhibitor or induce a UPS impairment in AD due to competition of natural proteasomal substrates with rising amounts of toxic oligomeric A $\beta$  peptides. As a consequence of alterations in the proteasome in AD, abnormal accumulations of A $\beta$ 42 and tau occur, both of which are degraded by the proteasome. Proteasome dysfunction, for example, has been found to take place early in the development of pathological events in 3xTg-AD mice, right when A $\beta$  accumulates, A $\beta$  plaques first appear, and tau pathological alterations begin (Tseng et al., 2008). Along with Tau's accumulation, hyperphosphorylated tau oligomers were found accumulated in AD synapses due to the proteasome's impairment, suggesting that these

oligomers may be a key mediator of the proteotoxicity that damages synapses in AD (Tai et al., 2012).

A



B



**Figure 10 : The ubiquitin proteasome system.**

A) Step A, ubiquitin is activated by a ubiquitin-activating enzyme, E1. Step B, activated ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2. In step C, ubiquitin is subsequently conjugated to target proteins in a process mediated by an E3 ubiquitin ligase. Step D, the polyubiquitylated substrate protein is degraded by the 26S proteasome. B) The 20S core is composed of 2 inner  $\beta$ -rings and 2 outer  $\alpha$ -rings. In the constitutive proteasome, the  $\beta$ -rings each contain 3 proteolytic sites named according to their chymotrypsin-like ( $\beta 5$ ), trypsin-like ( $\beta 2$ ), or caspase-like ( $\beta 1$ ) activity. Figure adapted from (Zhao et al, 2017, Yong et al, 2018).



## 2. Lysosomal and autophagic pathway and their dysfunctions in AD

---

### 2.1. Lysosomal Degradation

---

As already mentioned (see chapter [Endo-Lysosomal Network \(ELN\) – Generalities](#)), in the ELN, cargos that are destined for degradation are delivered by late endosomes to lysosomes. Late endosomes merge with lysosomes, exchanging membrane components and solutes to generate a transitory hybrid endo-lysosome, which is later re-converted into secondary lysosomes, where hydrolases are retained. This fusion and exchange often make it difficult to distinguish between late endosomes and lysosomes at the molecular level. The main components of their membranes are lysosomal-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) which create a glycocalyx-like layer that shields the rest of the cell from the aggressively acidic environment of the lumen.

Endosome-to-lysosome maturation is the gradual decrease of internal pH to  $\approx 5$ , which is required for several physiological processes such as ligand-receptor uncoupling, and lysosomal enzyme activity. This acidification needs the presence of a proton-pumping V-type ATPase that uses the energy released by ATP hydrolysis to pump protons into the lysosomal lumen.

Soluble hydrolases carry out the degradation process in lysosomes; in fact, the lysosomal lumen contains 60 different soluble hydrolases. These enzymes include sulphatases, glycosidases, phosphatases, lipases, nucleases and proteases; with cathepsins being the most abundant lysosomal proteases.

To sum up, hydrolases, the acidification machinery, and membrane proteins must all work together to allow lysosome-mediated cellular clearance activities (Gruenberg, 2019; Settembre et al., 2013).

Interestingly, recent studies demonstrated that ILVs can also be found in lysosomes, where they are preserved from degradation and released as exosomes via a mechanism known as lysosomal exocytosis.

In fact, lysosomal exocytosis is a process in which lysosomes merge with the plasma membrane and secrete their stored material, and it is highly regulated by the transcription factor EB (TFEB). This mechanism is important not just for cellular clearance, but also for plasma membrane repair, as lysosomes migrate and reseal damaged membrane regions (Settembre et al., 2013; Tancini et al., 2019).

In addition, increases in released EVs were observed when cells were treated with bafilomycin A (BafA1) or chloroquine, two treatments that alkalinize lysosomal pH and hence impair

lysosomal activity. As a result, studies have confirmed the hypothesis stating that the production of EVs is a response to lysosomal dysfunction induced by an excess of unneeded or damaged molecules (Buratta et al., 2020).

## 2.2. Autophagic pathway

---

Autophagy is considered as a "self-eating" mechanism that is completely reliant on lysosomal activity. Unlike the endo-lysosomal system, which recycles or degrades extracellular molecules or cellular proteins trafficking along the endolysosomal pathway, the lysosomal-autophagic pathway digests and recycles unneeded or harmful intracellular components and damaged cytosolic organelles (mitochondria, ribosomes, ER fragments, etc.).

The three types of autophagy that have been described are microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Microautophagy is identified by the direct invagination of the lysosomal membrane to integrate the cytosolic elements to be degraded, whereas CMA is characterized by the internalization of these materials mediated by receptors or chaperone proteins. The last type is macroautophagy, which involves encapsulating cytoplasmic particles and/or damaged portion of cytosolic organelles in autophagosomes intended to fuse with lysosomes. Since macroautophagy is the most common form, it is usually referred to as autophagy.

The autophagic process begins with cytosolic nucleation that progress to an open structure known as a phagophore. Phagophores engulf cytoplasmic proteins and organelles before sealing their membranes to create autophagosomes. Autophagosomes later fuse with lysosomes to generate autolysosomes where engulfed contents are degraded (**Figure 11**).

Interestingly, in addition to lysosomal exocytosis, research has demonstrated that the autophagic pathway can also be implicated in the secretory pathway termed as "autophagy-dependent secretory pathway," which is a process for discarding waste material and permitting the extracellular release of certain signaling molecules.

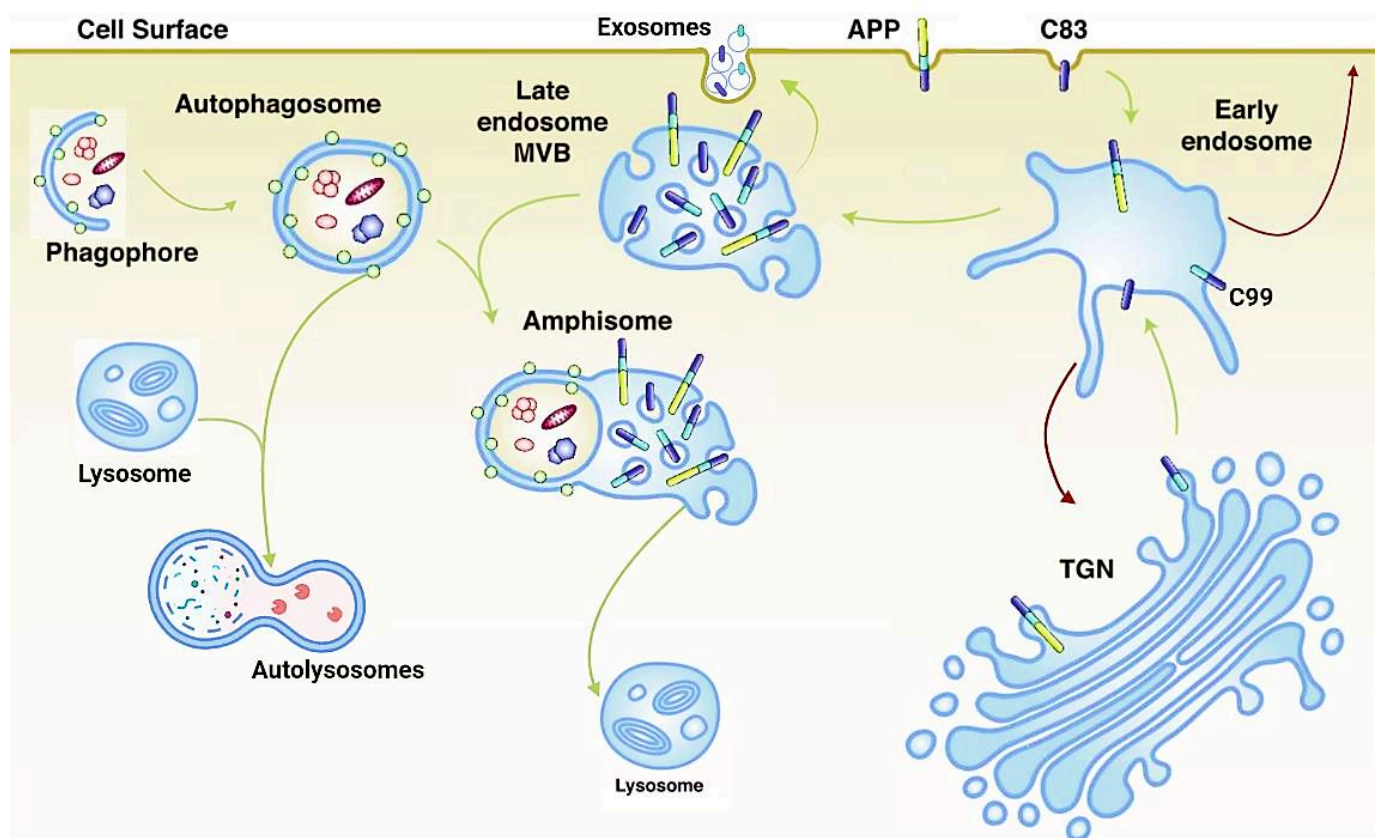
At the organelle level, autophagosomes can merge with MVBs to generate amphisomes, which represent an intersection of the exosome and autophagy pathways (**Figure 11**). Amphisomes are thought to fuse with lysosomes, which destroy their components; nonetheless, these components can also end up being released extracellularly.

Furthermore, both exosomes and autophagy are important in maintaining cellular homeostasis, and one route may compensate for a defect in the other. Defective MVBs and their contents,

for example, may be prone to autophagic degradation, and inhibiting autophagy may save exosome release from MVBs that would otherwise be degraded.

(Gruenberg, 2019; Settembre et al., 2013; Xu et al., 2018).

Numerous studies have demonstrated that the endosomal-lysosomal-autophagic pathway is responsible for the degradation of APP, APP-CTFs, and A $\beta$  (Asai et al., 2011; Boland et al., 2010; Gallwitz et al., 2022; Tian et al., 2011; Vingtdeux et al., 2007) (**Figure 11**). This route has a large number of cellular and molecular actors. Therefore, an alteration in one of the actors of this quite complicated route may result in the dysfunction of this pathway and consequently lead to the accumulation of toxic players in AD.



**Figure 11: Turnover of APP and CTFs via Endosomal Exosomal-Lysosomal-Autophagic network.** APP, C99, and C83 can reach early endosome either via trans-golgi network (TGN), or via cell surface. Early endosome matures to late endosome, also called MVB. APP, C99, and C83 are incorporated into ILVs of MVBs. In parallel, phagophore matures to form autophagosome, which can fuse with endolysosomal compartments, such as MVB. Fusion of autophagosome with MVB results in formation of hybrid organelle called amphisome. Amphisome can fuse with lysosome, promoting final degradation of APP, C99, and C83. Autophagosome can also fuse with lysosome resulting in formation of autolysosomes. Adapted from (Gonzalez et al., 2017).

## 2.3. Dysfunctions of ELN and Autophagy in AD

---

The detection of accumulating immature autophagic vacuoles in AD brains led to the discovery of links between autophagy and AD (Nixon et al., 2005). In the early stages, neurons exhibit an increase in the expression of autophagy activating transcription factors, which leads to the hyperactivation of genes involved in autophagy induction, autophagosome formation, and autophagosome fusion with lysosomes. However, this process in these neurons appears to be gradually reduced as disease progresses, demonstrated by increased autolysosome size and number at advanced stages. These abnormalities are caused by a serious lysosomal clearance deficiency, which results in the accumulation of autophagic vacuoles (Bordi et al., 2016). The accumulation of amphisomes and autophagic vesicles can possibly be explained by a defect in axonal transport, which is required for autophagosome maturation and degradation (Morel et al., 2012).

Since it has been demonstrated that autophagy is also implicated in A $\beta$  degradation (Tian et al., 2011), defects in the autophagic pathway therefore cause an accumulation of A $\beta$  and contribute to the intracellular toxicity of the peptide. However, as another early AD phenotype, it is challenging to determine if autophagic defects lead to A $\beta$  accumulation or the opposite.

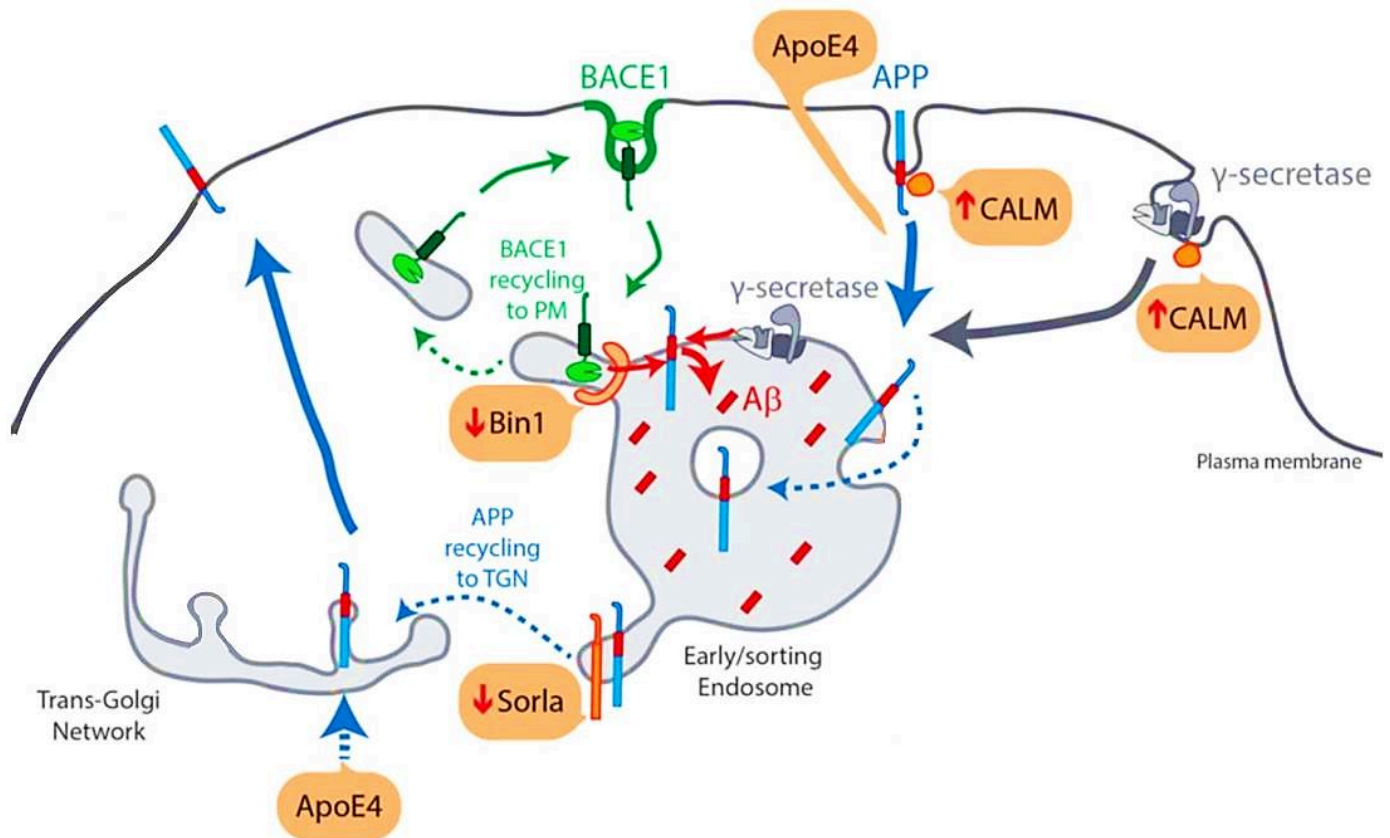
In fact, the increased number of autophagosomes observed in AD due to the enhanced autophagy leads to increased A $\beta$  secretion and consequently has a direct impact on A $\beta$  plaque formation. On the other hand, increased intracellular A $\beta$  levels result from impaired autophagosome clearance (Nilsson et al., 2013).

### 2.3.1. PS1 mutations and AD-risk factors contribute to ELN dysfunctions

The endosomal-lysosomal network and autophagy collaborate to play critical roles in maintaining neuronal homeostasis. Growing evidence suggests that changes to cell systems involved in protein sorting and trafficking may have an impact on neurodegeneration particularly in AD brains by limiting clearance of disease-associated pathologic proteins. Indeed, FAD-associated presenilin mutations and many SAD-associated risk factors are associated to endosomal-lysosomal network disruption and have an effect on the cellular dysfunction seen in AD (Szabo et al., 2022).

PS1 for example, was found to play crucial role in the maturation and trafficking of the v-ATPase, which is in charge of acidifying the lysosomes. Besides from leading to enhanced

cleavage of APP and A $\beta$  production causing early onset FAD, mutations of PS1 were found responsible for disrupting lysosomal and autophagic function, and PS1-FAD mediated increases in A $\beta$ . Importantly PS1 deletion that is PS1-loss of function leads to a similar lysosomal and autophagic dysfunction (Lee et al., 2010). Additionally, a number of GWAS - confirmed risk genes for AD, including APOE4, Sorla, Bin1, and CALM (or PICALM), have been reported as being involved in this degradation pathway (**Figure 12**).



**Figure 12: Scheme of the increased endocytic production of A $\beta$  due to LOAD genetic risk factors.** ApoE4-mediated increase in APP endocytosis and/or via increased APP in the secretory pathway due to increased APP transcription; Bin1 loss-of-function impedes BACE1 to recycle out of sorting endosomes; Sorla loss-of-function decreases APP recycling out of endosomes to the TGN; CALM loss-of-function increases APP and  $\gamma$ -secretase endocytosis and delivery to sorting endosomes. The red arrows indicate the alterations (increase or decrease) of the subcellular mechanisms associated with each predisposition gene. Adapted from (Guimas Almeida et al., 2018).

For instance, the endocytosis of APP and the  $\gamma$ -secretase complex is increased by CALM loss of function, although these observations are controverted (Kanatsu et al., 2014; Xiao et al., 2012). BACE1 is retained in endosomes as a result of BIN-1's loss of function, which impairs membrane recycling of BACE1 and reduces their degradation through lysosomes (Miyagawa et al., 2016; Ubelmann et al., 2017). And the loss of Sorla function reduces retrograde transport of APP to TGN, causing it to accumulate in endosomes (Willnow and Andersen, 2013). Overall, the loss of function of Bin1, CALM, and Sorla cause dysregulation in intracellular trafficking

of APP and/or secretases, as well as an increase in their retention in sorting endosomes, which potentiate A $\beta$ 42 synthesis. Other pathways, however, including inefficient clearance of A $\beta$ 42 via the BBB due to impaired endocytosis/transcytosis via sorting endosomes and defective A $\beta$ 42 lysosomal clearance, have been identified as being affected by the loss of function of the genetic risk factors (Guimas Almeida et al., 2018). Regarding APOE4, it enhances both, the endocytosis of APP and the amount of APP in the secretory pathway due to increased APP transcription. It has also been linked to endosome enlargement in neurons and endosomal-lysosomal network disruption that is unrelated to A $\beta$  and tau pathology (Cataldo et al., 2000; Peng et al., 2019).

### 2.3.2. C99 accumulation contribute to ELN and autophagic dysfunctions

As explained previously, the pH of the endosomes, as well as the presence of APP,  $\beta$  and  $\gamma$ -secretases, allow for the production of C99 and A $\beta$  in this compartment. In fact, enlarged endosomes, increased endocytic activity, and greater ability to produce A $\beta$  under pathological circumstances were observed in sporadic AD patients (Cataldo et al., 1997).

It was later discovered that also elevated levels of C99 in AD patients, independent of A $\beta$ , cause a pathological overactivation of Rab5, resulting as well in accelerated endocytosis, and endosome enlargement (Kim et al., 2016). Two other studies using iPSC that express FAD mutations have confirmed the accumulation of C99 in human AD cell models without any overexpression, and the involvement of C99 in early endosomal dysfunction, and lysosomal-autophagic pathology (Hung and Livesey, 2018; Kwart et al., 2019).

Similar to those seen in endosomes, dysfunctions of the lysosomal degradation pathway are also described to be one of the earliest clinical phenotypes of AD to be identified.

It is believed that there is a bidirectional link between APP, its cleavage products A $\beta$  and C99, and lysosomal dysfunction. APP, C99 and A $\beta$  are degraded in lysosomes under normal circumstances, but in AD, lysosomal dysfunctions enable the formation of misfolded aggregates of APP products, which in turn impair lysosomal activity (Colacurcio et al., 2018).

A $\beta$ , for example, has been shown to permeabilize lysosomal membranes, allowing the release of lysosomal enzymes and calcium into the cytoplasm, inducing apoptosis, and explain the mislocalisation of APP and A $\beta$  to the cytosolic compartment (Guicciardi et al., 2004; Yang et al., 1998). In addition, our laboratory has reported that, C99 is found accumulated in the

subiculum of 3xTgAD mice earlier than A $\beta$  (Lauritzen et al., 2012) and this C99 accumulation contributes to lysosomal pathology independently of A $\beta$  (Lauritzen et al., 2016).

### 3. Secretion of exosomes and their dysfunction in AD

---

#### 3.1. Secretion of exosomes

---

As described previously (see chapter [Endo-Lysosomal Network \(ELN\) – Generalities](#)) ILVs and their cargos that are not destined for degradation can be released extracellularly as exosomes following MVBs' fusion with the plasma membrane. Although the exact mechanisms governing whether MVBs are directed towards the plasma membrane or lysosomes have not yet been identified, studies have observed that MVBs with high cholesterol levels are more likely to fuse with the plasma membrane, releasing exosomes (Mobius et al., 2002).

With EVs being a term used to point to all membrane-limited vesicles released from cells, exosomes in particular are a subset of EVs with a diameter ranging from 30 to 150 nm and distinguished by a set of specific molecular markers. Microvesicles, apoptotic bodies, and ectosomes that originate from the plasma membrane can also be classified as EVs but ranging in size from 200 to 2000 nm in diameter.

The production of ILVs is the first event in the biogenesis of exosomes, and the size, number, and cargos of exosomes are all reliant on this initial step. As previously stated, ILV synthesis is mostly dependent on the ESCRT machinery, which consists of four protein complexes (ESCRT 0, I, II, III) in addition to accessory proteins (ALIX, Tsg101, VSP4). The coordinated and sequential association of ESCRT machinery's proteins with the endosomal membrane regulates the creation of ILVs and the recruitment of exosomal cargo components through the binding to ubiquitinated proteins.

ILVs may also be formed via ESCRT-independent pathways that involve membrane microdomains rich in tetraspanins. In fact, tetraspanin-enriched membrane microdomains can contribute to the entry of proteins and oligonucleotides into exosomes. So in addition to proteins, exosomal cargos can include oligonucleotides such as DNA, mRNAs and species of small RNAs that could also enter exosomes by interacting with RNA-binding proteins or lipid rafts in MVBs.

The molecular composition of exosomes is influenced by the tetraspanins such as, CD63 and Tspan8. Furthermore, the levels of expression of CD63, together with levels of CD9 and CD81, regulate the total amount of exosomes released (Chairoungdua et al., 2010; Escola et al., 1998;

Hurwitz et al., 2016; Kumar et al., 2015). Unfortunately, CD9, CD63, and CD81 were formerly thought to be exosome-specific indicators; however, these proteins have then been suggested to be present in apoptotic bodies and microvesicles (Crescitelli et al., 2013).

Tetraspanins are not the only factor associated to exosome synthesis; depending on the cell type, lipids and lipid-related enzymes are also involved. For instance, ILVs that are intended to be secreted as exosomes include large amounts of ceramide. Moreover, lysobisphosphatidic acid (LBPA)-rich endosomal membrane domains may potentially regulate the biogenesis of exosomes; and ALIX, an LBPA partner, is also present in exosomes and participates in their biogenesis.

After being released into the extracellular space, exosomes can be internalized by the secreted cell or by other cells. Surface integrins are known to facilitate exosome attachment and entry into their intended recipient cells. The uptake of exosomes by the acceptor cell has been reported to occur via various mechanisms: the cargo can be released into the cytosol after the EVs either fuse to the plasma membrane, or internalized by endocytosis. Cargos may then be recycled and re-secreted or routed to the endo-lysosomal pathway for degradation (Buratta et al., 2020; Gruenberg, 2019; Tancini et al., 2019; Xu et al., 2018).

For a long time, it was assumed that exosomes' sole function was to maintain cellular homeostasis by clearing the cell of toxic waste. Nonetheless, exosomes are now considered to be a new participant in cell communication, transferring biological molecules from one cell to another. Furthermore, it is well established that exosomes play an important role in the transfer of mRNA and microRNA (miR) across cells, allowing genetic exchange (Valadi et al., 2007), and the attachment of these exosomes to a recipient cell allows for cell-specific signaling. Exosomes can be secreted by all cell types in the CNS, and these exosomes play a role in physiological processes such synaptic activity, vascularization, and neuronal regeneration (Rajendran et al., 2014). A neuronal exosomal miR-mediated genetic regulation of astrocyte functions has also been suggested by the discovery of exosomal miR-mediated neuron to glial transmission (Men et al., 2019).

### 3.2. Exosomal dysfunction in AD

A 2006 study initially identified A $\beta$  as being present in exosomes (Rajendran et al., 2006). In reality, APP itself, APP-CTFs, and AICD are also present in exosomes and discharged into the extracellular space in addition to A $\beta$  (Perez-Gonzalez et al., 2012; Sharples et al., 2008; Vingtdeux et al., 2007; Yuyama and Igarashi, 2017). In fact, according to Dr. Vingtdeux's



research, lysosome alkalinization enhances AICD enrichment in exosomal vesicles released by APP wild-type (APPwt)-overexpressing neuroblastomas while  $\gamma$ -secretase inhibition promotes the secretion of of exosome-associated APP-CTFs (Sharpley et al., 2008; Vingtdeux et al., 2007). The results of Dr. Laulagnier's most recent study show that exosomes release APP and APP-CTFs; the exosomes isolated from N2a cells overexpressing APPwt are concentrated in C83 and  $\eta$ CTF, whereas those from N2a cells overexpressing APP<sup>swe</sup> are enriched in C83 and C99 (Laulagnier et al., 2018). The identification of A $\beta$  and APP-CTFs in exosomes has led to several hypotheses regarding their role in the disease. Recent findings indicate that exosomes have a dual function in AD pathogenesis, assisting in A $\beta$  clearance while simultaneously playing potentially harmful roles that worsen and spread the pathogenesis (Xiao et al., 2017).

Homeostasis is maintained by a concerted effort of the ELN, autophagy, and exosomes. It was proposed for this reason that activating exosome secretion in AD patients with deficiencies in autophagy or lysosomal function would be advantageous by sending endosomal-lysosomal material into the extracellular space, where macrophages may assist in the degradation of neuronal waste (Mathews and Levy, 2019). As a matter of fact, the decrease in exosome secretion reported in APOE4 carriers, together with changes in the neuronal endosomal-lysosomal pathway, may be consistent with the concept that activating exosome secretion may reduce neuronal vulnerability and the risk of AD (Peng et al., 2019). Very interestingly, a study recently found a strong correlation between endosomal dysfunction and the release of exosomes enriched in APP-CTFs (Miranda and Di Paolo, 2018). In fact, it has been established that AD brains lack PI3P, a master regulator of endolysosomal and autophagic functions (Morel et al., 2013a). Indeed, the research team led by Dr. Di Paolo has demonstrated that endolysosomal-autophagic dysfunction induced by genetic invalidation or pharmacological inhibition of PI3 kinase VSP34 (the enzyme that synthesizes phosphatidylinositol-3-phosphate (PIP3)) results in the secretion of exosomes abnormally enriched in APP-CTFs, as well as in autophagic substrates, such as polyubiquitinated proteins and substrates that were ineffectively degraded. Furthermore, exosomes released following Vsp34 inhibition have a distinct lipid composition; they are rich in ceramides and Bis-Monoacylglycero-Phosphate (BMP) phospholipids, which are normally lacking in exosomes but present in late endosome membranes (Miranda et al., 2018).

Greater levels of phosphorylated Tau, toxic oligomeric A $\beta$ , and A $\beta$ 1-42 have been observed in the exosomes of AD patients even up to 10 years before the onset of clinical symptoms (Fiandaca et al., 2015). Exosomes also contained APP, its metabolites (C99, C83,

and AICD), and members of the secretase family of proteases (BACE, PS1, PS2, and ADAM10) (Perez-Gonzalez et al., 2012; Sharples et al., 2008; Vingtdeux et al., 2007). For example, in Dr. Di Paolo's study, which established that cells tend to discharge undesired and undigested endolysosomal cargoes in exosomes in an attempt to eliminate them, C99 was found to be abundant in these exosomes, along with other toxic proteins or lipids (Miranda and Di Paolo, 2018). Intriguingly, our laboratory discovered that C99 monomers are mostly found at the trans-Golgi level, whereas oligomeric forms are more abundant in exosomes and exceed monomers in this compartment. Additionally,  $\gamma$ -secretase inhibition (D6 treatment or presenilin invalidation) promotes the formation of not only dimers and oligomers of C99, but also heterodimers with C83, and their presence in exosomes (Lauritzen et al., 2019).

As a result, when exosome secretion attempts to compensate for the dysfunction of other degradation pathways in AD, it ironically promotes the spread and progression of the pathology when nearby neurons collect these exosomes (Xu et al., 2018).

## D) THERAPEUTIC APPROACHES AND CLINICAL TRIALS FOR AD

---

### 1. Targeting A $\beta$

---

The amyloid cascade theory has been at the center of AD research for many years since it is supported by genetic, biochemical, and anatomical evidence (see chapter [The Amyloid Cascade Hypothesis](#)). With this theory proposing that the aggregation of A $\beta$  in oligomers, fibrils and deposition into amyloid plaques are the cause of the pathological phenotypes of AD, significant effort is placed into finding therapeutic strategies to reduce A $\beta$  levels, either by preventing its production or by neutralizing it once it has been formed.

#### 1.1. Neutralizing the formed A $\beta$

---

Immunotherapy has been the most extensively studied A $\beta$ -targeted therapy for removing pre-existing amyloid plaque loads. Active immunotherapy involves injecting a vaccine containing the A $\beta$ -antigen into the patient in order to trigger an immune response against the peptide, whereas passive immunotherapy consists of directly administering monoclonal A $\beta$ -specific antibodies to stimulate A $\beta$  clearance.

### 1.1.1. Active immunotherapy

The first vaccine to undergo clinical testing was AN-1792, which consists of a synthetic A $\beta$ 1–42 with QS-21 adjuvant and conducted in 2002. Despite the promising results in amyloid plaque clearance, it was discovered that A $\beta$ 42 of AN-1792 contains a domain responsible for T-cell activation, which is why a percentage of the treated patients developed T-cell-mediated meningoencephalitis. Other A $\beta$  vaccines that target more specific epitopes and lack the portion responsible for inflammatory T-cell activation were developed to avoid T-cell activation, such as CAD106, which is composed of multiple copies of A $\beta$ 1-6 and failed due to unexpected changes in cognitive function, brain volume loss, and body weight loss. Another A $\beta$ -vaccine that was created, ACC-001, which contained A $\beta$ 1-7, similarly failed due to statistically insignificant results. There were several further active vaccines that were investigated afterwards and failed, including ACI-24, affitope AD02, V950, and UB-311. ABvac40 was one of the first vaccines to target the A $\beta$ 40 peptide, and it consists of numerous repeats of A $\beta$ 33-40. In clinical trials, this vaccine displayed excellent safety and elicited a significant immune response characterized by an increase in anti-A $\beta$ 40 antibody levels; however, secondary exploratory efficacy endpoints are still being investigated (Se Thoe et al., 2021; Song et al., 2022).

### 1.1.2. Passive immunotherapy

Although monoclonal antibodies have shown a lack of clinical efficacy, passive immunotherapy has been the most investigated in clinical trials to date. Five antibodies: bapineuzumab, solanezumab, crenezumab, gantenerumab, and BAN2401, reached phase III clinical trials and showed considerable promise in lowering CSF biomarker levels and A $\beta$  plaques. Unfortunately, none of these antibodies have exhibited clinical benefits and failed to slow cognitive or functional impairment in patients. Aducanumab, one of the most potent medications for AD ever developed, had its phase III clinical trials discontinued for ineffectiveness in March 2019 following an interim futility analysis. However, in early 2020, Aducanumab was approved by the FDA in United States with much controversy, after demonstrating slowing of cognitive and functional decline in patients receiving treatment, however, phase III clinical trials will continue until 2023. The FDA is currently evaluating donanemab, an antibody that targets A $\beta$ (p3-42), the pyroglutamate form of A $\beta$ . Finally, after significantly slowing the progression of cognitive decline in patients with very mild to mild AD, the FDA is reviewing Lecanemab, an antibody binding to soluble A $\beta$  protofibrils, for accelerated approval (Se Thoe et al., 2021; Song et al., 2022).

Prevention of A $\beta$  aggregation: The most neurotoxic type of A $\beta$  is soluble oligomers, and these are more closely associated with the severity of AD than are amyloid plaques. Anti-aggregation drugs are thus a reasonable approach in disease-modifying therapy. They bind to soluble A $\beta$  peptides, preventing oligomerization and consequent neurotoxicity. While some of the studies on these molecules are still in progress, the majority have been discontinued due to their ineffectiveness or toxicity, as in the cases of scyllo-inositol and tramiprosate (Panza et al., 2019; Vaz and Silvestre, 2020).

## 1.2. Preventing A $\beta$ production – targeting $\beta$ and $\gamma$ -secretases

---

Inhibiting  $\beta$  and  $\gamma$ -secretases to reduce A $\beta$  production is another extremely well researched approach.

### 1.2.1. BACE1 inhibitors

Although the inhibition of BACE1 is thought to have therapeutic potential for AD, the effectiveness of its inhibitors is greatly limited. In addition to the fact that BACE1's physiological functions could be disrupted by its inhibition, BACE1's amino acid sequence shares 64% similarity with that of BACE2, which has distinct biological roles that could be impacted if BACE1's inhibitors are not highly selective. Furthermore, designing a small molecule that can occupy and inhibit the relatively large active site of BACE1 while be able to cross the BBB is a further challenge in the development of BACE1 inhibitors. Over the past few years, several BACE1 inhibitors have been developed, but only five reached phase III of clinical trials. All five, however, were discontinued due to liver damage in the case of atabecestat, ineffectiveness in the case of verubecestat and lanabecestat, an unfavorable risk-benefit ratio in the case of elenbecestat, or even more cognitive decline following umibecestat treatment (Vaz and Silvestre, 2020).

### 1.2.2. $\gamma$ -secretase inhibitors/modulators

Similar to the  $\beta$ -secretase, reducing the activity of  $\gamma$ -secretase also offers a potential therapeutic treatment. The diversity of  $\gamma$ -secretase substrates, including Notch, has made therapeutically effective inhibitor development difficult. All clinical trials based on these inhibitors, such as on semagacestat and avagacestat, were suspended due to adverse effects such as an increased risk of skin cancer and infection, gastrointestinal bleeding, and even worsening of cognitive function. To avoid such side effects, therapeutic development changed to  $\gamma$ -secretase modulators, which were expected to be safer because they do not block the enzyme's activity, but instead shift the cleavage of C99 toward shorter A $\beta$  species such as A $\beta$ 37 and A $\beta$ 38, thus

selectively inhibiting the aggregation-prone A $\beta$ 42 production while sparing Notch processing and avoiding the accumulation of APP-CTFs. Despite the fact that clinical trials of these modulators, particularly Tarenflurbil, the most well-known, indicated a favorable safety profile, they did not slow cognitive or functional decline due to the drugs' inability to cross the BBB (Vaz and Silvestre, 2020).

Since no cognitive improvements were observed in any of these trials, even when A $\beta$  levels were greatly reduced, it appears evident that decreasing total A $\beta$  alone was insufficient to restore cognitive function. More intriguingly, findings from preclinical studies revealed a link between the accumulation of APP-CTFs brought on by  $\gamma$ -secretase inhibition and the lack of improvement or even worsening of cognitive function in these trials.

In light of the toxic role C99 may play in AD (see chapter C99), clinical studies for  $\gamma$ -secretase inhibitors may have failed in part because  $\gamma$ -secretase can be regarded of as a beneficial C99-inactivating enzyme that prevents the accumulation of C99. Inhibiting  $\gamma$ -secretase may therefore, independently of A $\beta$ , contribute to the pathology of AD carried on by the accumulation of C99. We discussed this important point of view in a review in which I participated and published in the Journal of Biological Chemistry. This review will be presented in Annex.

“Is  $\gamma$ -secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99?”

Frédéric Checler, **Elissa Afram**, Raphaëlle Pardossi-Piquard, and Inger Lauritzen.

## 2. Modulation of the ELN

---

Every treatment strategy for AD has as its major goal reducing toxic accumulating proteins. Besides the most advanced strategy targeting A $\beta$ , one of the potential therapeutic approaches to treat AD would be to restore the function of the endosomal-lysosomal-autophagic degradation pathway as a part of the accumulation is brought on by a dysfunction of this system (see chapters [Dysfunctions of ELN and Autophagy in AD](#) and [Exosomal dysfunction in AD](#)).

As noted previously, lysosomal activity proteolysis is impaired in AD due to a lack of acidity of the lysosomes, which prevents hydrolases activity. This highlights the importance of finding possible therapies that enhance the activity of these hydrolases or can maintain lysosomes' pH.

## 2.1. Activating hydrolases

---

The first strategies were to either genetically or pharmacologically activate cathepsins. Both of these techniques have been found to be effective in numerous AD models. In the first study, lentivirus-mediated expression of cathepsin B in aged mice with neuronal expression of FAD mutant human APP, reduced amyloid plaques in the brain, demonstrating the potential of this technique in clearing pre-existing amyloid plaques (Mueller-Steiner et al., 2006). These findings were confirmed by injecting an adeno-associated virus expressing cathepsin B into the hippocampus of another AD model, which revealed not only a reduction in intracellular and extracellular levels of A $\beta$ , but also an improvement in AD-related memory deficits (Embury et al., 2017). Other research took a different strategy, consisting of invalidating endogenous inhibitors of cysteine protease inhibitor, such as cathepsin B; these inhibitors are known as cystatin B or C. Likewise, this indirect cathepsin B activation decreased A $\beta$  levels, and improved synaptic, and cognitive functions in various FAD models (Sun et al., 2008; Yang et al., 2011; Yang et al., 2014). Interestingly, this approach leads to the same effects on A $\beta$  in mice overexpressing the human APPwt (Wang et al., 2012). Unfortunately, these genetic techniques are difficult to envision in humans, and given the non-permeability of the BBB, a pharmacological approach based on enzyme supplementation does not appear suitable either. However, pharmacological methods based on lysosomal modulators have been investigated since the BBB of AD patients would be altered as the disease advances. A systemic injection of the modulator Z-Phe-Ala-diazomethylketone (PADK) in AD mice models, for example, drastically elevated cathepsin B activity in the brain, resulting in clearance of intracellular A $\beta$  peptide, a reduction in extracellular deposits, and an improvement in cognitive abilities (Bahr et al., 2012; Butler et al., 2011). It also seems more strategic to target cathepsin B, rather than cathepsin D for example because its action pH is higher than that of cat D and therefore it would be more easily activated in neurons where there is a V-ATPase dysfunction.

## 2.2. Normalization and restoration of lysosomal activity

---

Because lysosomal enzymes require an acidic pH, an increase in enzyme levels in the absence of pH correction seems unnecessary. One method for directly restoring lysosomal pH has been studied mostly in animal models of Parkinson's disease and has exhibited promising results. In this approach, Poly(lactic-co-glycolic acid) (PLGA) acidic nanoparticles (aNP) are internalized into cells and trafficked to lysosomes, where they re-acidify and rescue defective lysosomes (Bourdenx et al., 2016). Since researchers are seeking to find non-invasive therapies procedures where treatments can cross the BBB, PLGA nanoparticles (PLGA NPs) are being intensively

studied in the interest of treating neurodegenerative illnesses. PLGA NPs provided numerous advantages, including improved drug solubility, resistance to enzymatic digestion, higher targeting efficiency, and improved cellular internalization (Cunha et al., 2021). This approach of using acidic nanoparticles was also performed in PS1 KO cells (Lee et al., 2015) and in cell models of DS patients (Jiang et al., 2019), improving lysosomal deficiencies and restoring normal lysosomal proteolysis.

Another method for restoring the acidic pH of lysosomes is to increase cyclic-AMP (cAMP) levels by either blocking phosphodiesterases (PDEs; the enzymes that degrade cAMP) or activating adenylyl cyclases (the enzymes that produce cAMP). As cAMP levels rise, protein kinase A (PKA) is activated, increasing the amount of free Zn<sup>2+</sup> in lysosomes. Lysosomes will become acidified once again due to the increased levels of free Zn<sup>2+</sup>. Hence, another potential therapeutic strategy to re-acidify lysosomes is increasing the levels of free Zn<sup>2+</sup> intracellularly or in the lysosomes (Koh et al., 2019; Lai et al., 2021).

The “molecular tweezer” CLR01, is an inhibitor of abnormal protein self-assembly that selectively binds lysine residues, disrupting molecular interactions that are necessary for the toxic aggregation of multiple amyloidogenic proteins. These molecules are being researched as potential therapeutics for amyloidosis and many other proteinopathies (Attar et al., 2014; Shahpasand-Kroner et al., 2022). In AD in particular, CLR01 has been demonstrated to inhibit A $\beta$  and tau aggregation, in vitro and in in vivo models, and to improve behavioral deficits (Attar et al., 2012; Di et al., 2021; Zheng et al., 2015). More impressively, the CLR01 compounds were shown to be mostly localized in lysosomes after being internalized in cells, where they unfold aggregated proteins, reducing lysosomal enlargement and reactivating the autophagy-lysosomal pathway. This, in turn, promotes the enzymatic degradation of misfolded proteins by lysosomal cathepsins (Li et al., 2021; Monaco et al., 2022).

### 2.3. Autophagy activation

---

Autophagy is one of the primary methods through which the cell eliminates aberrant proteins and protein aggregates, both of which have been linked to AD (see chapter [Autophagic pathway](#)). Thus, inducing autophagy could be used to eliminate such protein aggregates. Several autophagy-stimulating treatments have already shown significant therapeutic potential for AD in clinical trials. The majority of the identified drugs promote autophagy by blocking the mammalian target of rapamycin (mTOR) and/or activating AMP-activating protein kinase (AMPK).

Rapamycin, a selective inhibitor of target-of-rapamycin complex 1 (TORC1) and hence regulator of mTOR pathway activity, is the most well-known mTOR inhibitor. When rapamycin is administered to AD mouse models, neurofibrillary tangles and A $\beta$  protein aggregation are significantly reduced, and cognitive abilities including learning and memory are enhanced (Ozcelik et al., 2013; Spilman et al., 2010). Beneficial effects of mTOR pathway inhibition have also been demonstrated in studies with temsirolimus, a rapamycin prodrug. In a mouse model of AD, temsirolimus was demonstrated to enhance autophagy-dependent A $\beta$  clearance and to improve learning and memory (Jiang et al., 2014). Additionally, SMER28, a small molecule rapamycin enhancer, reduces levels of A $\beta$  peptide and APP-CTFs by activating autophagy through an Atg5-dependent route (Tian et al., 2011).

The anti-psychiatric medicine lithium has been shown to promote autophagy in an AMPK-dependent manner (Bao et al., 2019), enhancing cognition in AD patients and those with MCI (Matsunaga et al., 2015). Also via AMPK activation, resveratrol, a deacetylase activator and anti-aging drug from the sirtuin family SIRT1, has been shown to activate autophagy, decreasing A $\beta$  accumulation (Vingtdeux et al., 2011).

Metformin, which is extensively used to treat type 2 diabetes, stimulates autophagy by activating AMPK and/or inhibiting mTORC1, lowers amyloid plaques and tauopathies, and has shown promising outcomes in clinical trials in enhancing cognitive abilities (Koenig et al., 2017). A multicenter, Phase 2/3 prevention trial is slated to begin in August 2020 on early or late MCI overweight or obese without diabetes and will run through 2024 (Metformin in Alzheimer's Dementia Prevention (MAP) study, Alzforum).

Memantine, an FDA-approved AD therapy medicine, and carbamazepine, an FDA-approved antiepileptic drug, have both been demonstrated to promote autophagy. Carbamazepine has intriguing therapeutic potential for AD by activating the autophagy, along with a number of other drugs, such as trehalose, which activates autophagy via the transcription factor TFEB; however, these treatments are not yet in human clinical trial phases. All of these medications are discussed in detail in the review (Zhang et al., 2021).

### 3. Exosomes as drug carriers and biomarkers

---

#### 3.1. Exosomes as drug carriers

---

Despite their multiples roles in inter-cellular communications, clearing the cell of toxic waste or spreading toxic molecules responsible for the propagation of disease (see chapter [Secretion of exosomes](#)), exosomes can serve as effective carriers for therapeutic drugs. As was highlighted for a range variety of treatments, crossing the BBB is a significant obstacle for drug



development. Exosomes, which are biocompatible, self-derived, and less prone to elicit immunological responses, can cross the BBB, making them an attractive choice for drug delivery into the CNS. In addition, because of their RNA transport potential, studies are trying to employ exosomes as carriers to transfer nucleic acid fragments such as miRNA and siRNA for the treatment of AD. For example, miR-124a delivered via exosomes is internalized by astrocytes, regulating synaptic activity, increasing glutamate uptake, as well as decreasing neuronal apoptosis in AD through increased expression of excitatory amino acid transporter 2 (GLT1) (Morel et al., 2013b). In AD mice, exosomes containing siRNA were tested as a potential therapy, and they significantly reduced BACE1 levels and A $\beta$  deposition (Alvarez-Erviti et al., 2011). This supported the use of exosomes as gene therapy carriers for diffuse distribution to the brain in the treatment of neurodegenerative diseases. Furthermore, a study from Chen and colleagues demonstrated the therapeutic performance of exosomes derived from mesenchymal stem cells (MSC-exosomes) in the treatment of AD, proving that these exosomes encapsulating a variety of MSC factors reduced A $\beta$  expression, cleared brain glucose metabolism, and improved neuronal plasticity, memory, and cognitive function (Chen et al., 2021). Conforming to this study, neprilysin was discovered to be highly concentrated in exosomes isolated from adipose tissue-derived mesenchymal stem cells (ADSCs), and internalization of these exosomes resulted in a decrease in extracellular and intracellular A $\beta$  levels (Katsuda et al., 2013). Exosomes enriched in a neprilysin variant with improved specificity and efficacy in degrading A $\beta$ , reduced A $\beta$ 40 levels in vitro with greater potency than exosomes derived from ADSCs, and were found to selectively target the hippocampus in vivo, lowering proinflammatory gene expression (Yu et al., 2021).

### 3.2. Exosomes as biomarkers

---

Another major challenge in AD today is the discovery of an early biomarker that would allow patients to be treated as early as possible, ideally preventing or significantly slowing the disease's progression. As already mentioned, the analysis of t-Tau, p-Tau and A $\beta$ 1-42 in the CSF has widely been studied as diagnostic biomarkers (see chapter [Biomarkers](#)). However, other AD biomarker candidates are being investigated due to the invasiveness of the CSF sampling method. Blood-based biomarkers could be a promising alternative to CSF testing in the diagnosis of AD because they are far less invasive. Exosomes are being evaluated for use as a biomarker to track the pathological development of AD since it has been established that they are produced, released from the brain, and found in peripheral blood (Zhuang et al., 2011). Interestingly, a recent study revealed that exosomal A $\beta$ 1-42, T-Tau, and p-Tau levels were higher in AD patients and correlated with those in CSF, suggesting that these exosomal proteins

have the same diagnostic utility as those in CSF (Jia et al., 2019). What's more intriguing is that a different study recently used the amplified plasma exosomes (APEX) technology to measure different populations of blood-circulating A $\beta$ . This study revealed that the exosome-bound A $\beta$  population can strongly reflect brain plaque deposition and correlate with PET imaging more effectively (Lim et al., 2019). Neuroinflammation-related proteins in astrocyte-derived blood exosome extracts have been found to be substantially greater in AD patients (Winston et al., 2019), and a large number of small miRNAs found in exosomes are being investigated as a potential diagnostic method in AD (Manna et al., 2020). Furthermore, several additional proteins known to be affected in AD, such as lysosomal, autolysosomal, presynaptic, and postsynaptic proteins, are detectable in neuron-derived exosomes. All these mentioned studies provide evidence for the use of exosomes as AD biomarkers.

## E) NON-CANONICAL APP-PROCESSING PATHWAYS

---

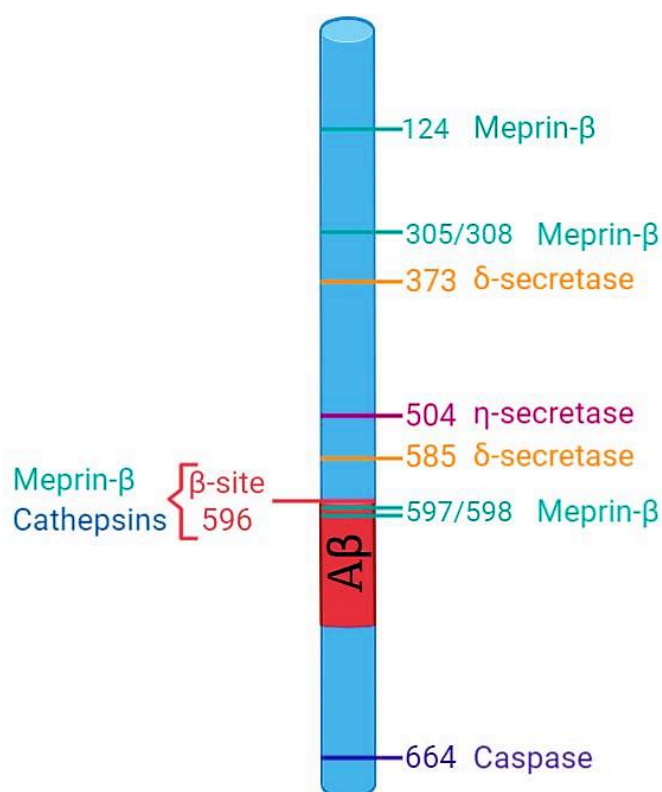
Although cleavages of APP by  $\alpha$ ,  $\beta$  and  $\gamma$ -secretases are complicated enough, additional APP cleavages have been described in recent years, making the comprehension of APP processing even further complex. As an alternative to the classical amyloidogenic enzymes, new proteases have been identified as APP-processing enzymes. Among them are  $\delta$ -secretase, meprin- $\beta$ , cathepsins, caspases, and  $\eta$ -secretase.

### 1. $\delta$ -secretase

---

This APP cleavage by  $\delta$ -secretase was initially described when a cleavage activity 12 residues N-terminal to the  $\beta$ -site was discovered (Simons et al., 1996). The cleavage's responsible enzyme was later revealed to be asparagine endopeptidase (AEP or legumain), a lysosomal cysteine protease cleaving APP695 at N585 ( $\delta$ -site) (Zhang et al., 2015). The same study also discovered a second cleavage site for APP695 at N373, mediated by AEP. These cleavages produce two C-terminal fragments (CTF374-695 and CTF586-695) that can both be processed by  $\alpha$ ,  $\beta$ , and  $\gamma$ -secretases. However, CTF374-695 was observed to increase A $\beta$  levels by acting as a substrate for  $\beta$ -secretase more effectively than APP-FL, indicating that  $\delta$ -secretase may be involved in AD pathogenesis. Additionally, the toxicity and neurodegeneration brought on by the N-terminal ectodomain sAPP $\delta$ 18-373, as well as the accumulation of AEP and its enhanced cleavage of APP with aging, were both found to support the pro-amyloidogenic feature of AEP. AEP may serve as a possible target for therapeutic intervention given that the KO of AEP in the mouse AD model repaired synaptic loss, A $\beta$  buildup, and behavioral impairments (Zhang

et al., 2015; Zhang et al., 2016). Recent research supports this notion by demonstrating that CTF586-695 interacts with CCAAT/enhancer binding protein beta CEBPB, an inflammatory transcription factor, and increases its transcriptional activity on APP, MAPT,  $\delta$ -secretase, and inflammatory cytokines, inducing AD pathology and cognitive deficits that were reversible after elimination of APP C586-695 (Yao et al., 2021). In addition to APP, AEP has also been described to cleave tau protein inducing microtubule destabilization and neurofibrillary tangles formation (Zhang et al., 2014b).



**Figure 13: Non-canonical APP-cleavages.**

$\delta$ -secretase cleaves in two sites of extracellular part of APP. Meprin- $\beta$  cleaves APP in six sites: 3 on the N-terminal part of APP, 1 on  $\beta$ -site and 2 within A $\beta$  sequence. Cathepsins can cleave on  $\beta$ -site of APP and caspases within the intracytoplasmic domain. Cleavage by  $\eta$ -secretase occurs 92 residues above the  $\beta$ -site. All the residues are termed using APP695 numbering. Figure created with BioRender.com free software.

## 2. Meprin- $\beta$

Meprin- $\beta$  is a type I transmembrane zinc metalloprotease that cleaves APP at several sites in both N-terminal ectodomain and around  $\beta$ -site. First, the Meprin- $\beta$  cleavages described on APP695 N-terminal ectodomain (Ala124/Asp125, Glu380/Thr381, and Gly383/Asp384) generate soluble non-toxic fragments with a molecular mass of 11 kDa to 20kDa that are detected in both healthy and AD brains (Jefferson et al., 2011). Although the enzyme is

primarily membrane-bound, these APP N-terminal cleavages do occur when meprin- $\beta$  is in its soluble form. In contrast, membrane-anchored meprin- $\beta$  cleaves APP more close to its transmembrane domain, at the Met596-Asp597 site ( $\beta$ -site), as well as the proximal Asp597-Ala598 and Ala598-Glu599 sites (APP695) (Bien et al., 2012). Unlike BACE1, Meprin- $\beta$  cleaves APP at plasma membrane before endocytosis and these cleavages are reliant on subsequent  $\gamma$ -secretase cleavage producing A $\beta$  peptides with different size. When compared to non-truncated A $\beta$  species, A $\beta$ 2-X and A $\beta$ 3-X originating from meprin- $\beta$  and  $\gamma$ -secretase activity are more prone to aggregate and are commonly found in AD brains (Schonherr et al., 2016; Wiltfang et al., 2001). Of particular interest, the glutamate residue at position 3 of A $\beta$ 3-X generated by meprin- $\beta$  can be converted into pyroglutamate, producing the neurotoxic pGlu-3A $\beta$  (Bayer, 2021).

Furthermore, the direct interaction with ADAM10 promotes the shedding of meprin- $\beta$ , which in its soluble form loses its  $\beta$ -secretase function (Marengo et al., 2022), and it was recently discovered that meprin- $\beta$  deletion decreased brain A $\beta$  levels and enhanced learning and memory in an AD mice model (Scharfenberg et al., 2019).

### 3. Caspases

---

Caspases are cysteine aspartyl-specific proteases that are essential for the initiation and execution of apoptosis. Caspases are classified into three functional groups: initiator caspases (caspases 2, 8, 9, and 10) that initiate the apoptosis signal, executioner caspases (caspases 3, 6, and 7) that carry out the mass proteolysis that leads to apoptosis, and inflammatory caspases (caspases 1, 4, 5, 11, and 12) that are involved in inflammatory cytokine signaling but do not function in apoptosis (Chang and Yang, 2000).

Caspases (predominantly caspase-3) have been shown to cleave mature APP695 at Asp664, behind the VEVD sequence, similar to the consensus caspase-3 “DEVD” motif. This cleavage occurs in the AICD domain, resulting in the intracellular peptide C31. This cleavage is considered to occur in apoptotic neuronal cells (Gervais et al., 1999; Weidemann et al., 1999). Moreover, C31 has been demonstrated to exhibit cytotoxicity, to be linked to A $\beta$ -induced synaptic damage, and to form APP/31 complexes that activate cellular toxicity signals (Galvan et al., 2006; Lu et al., 2003; Park et al., 2020; Park et al., 2009). JCasp fragment which is produced following  $\gamma$ -secretase and caspase cleavages, has also been proved to inhibit APP, thus blocking the release of excitatory synaptic vesicles (Fanutza et al., 2015).

## 4. Cathepsins

---

Cathepsins are the most abundant hydrolases in lysosomes, and their proteolytic activity is responsible for the degradation of proteins in these compartments. As described before, lysosomes are involved in APP degradation (see chapter [Lysosomal and autophagic pathway and their dysfunctions in AD](#)), and it has also been shown that cathepsin D is primarily engaged in A $\beta$  clearance (Hamazaki, 1996; McDermott and Gibson, 1996; Nixon, 2017).

Cysteine (Cathepsin B) and aspartyl (Cathepsin D) cathepsins are also thought to have the ability to cleave APP at  $\beta$ -site, in addition to their critical role in the pathogenesis of AD and in the degradation of APP, APP-CTF and A $\beta$  (Haque et al., 2008; Hook et al., 2008a).

Cathepsin D has been discovered to be approximately 280-fold more expressed in human brains than BACE 1, and exhibiting BACE 1-like  $\beta$ -secretase specificity, but with better activity towards the  $\beta$ -secretase site on APP with the Swedish mutation than APPwt (Schechter and Ziv, 2008). Cathepsin B, on the other hand, prefers to cleave the wild-type  $\beta$ -secretase site over the Swedish mutant  $\beta$ -secretase site (Hook et al., 2008b).

Furthermore, in AD brains, cathepsin B and cathepsin D were detected in high concentrations in early endosomes, where APP is primarily trafficked to be cleaved by the amyloidogenic pathway, in contrast to normal brains, where both cathepsins were present in only a minimal proportion of endosomes (Cataldo et al., 1997).

Cathepsin B and D present a dual role in AD pathology: cleaving APP at  $\beta$ -site, they contribute to A $\beta$  production but on the contrary, they could have a neuroprotective function through A $\beta$  and C99 degradation.

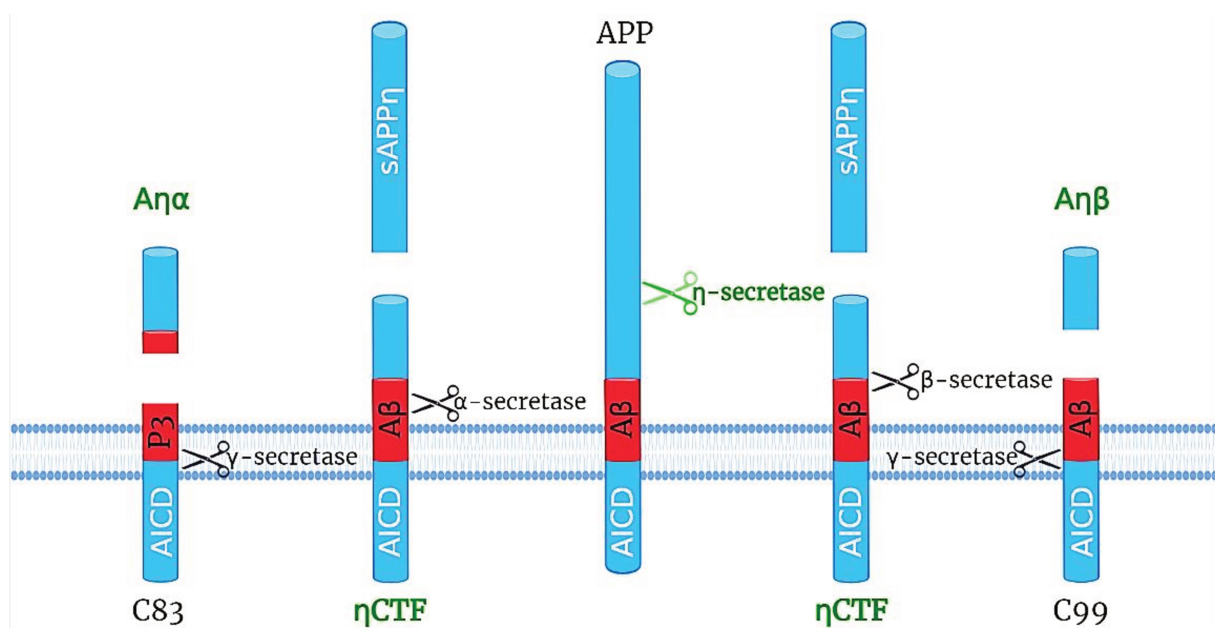
## 5. $\eta$ -secretase

---

$\eta$ -secretase cleaves APP between residues Asn504 and Met505 (APP695 numbering), to generate a soluble N-terminal fragment, sAPP $\eta$ , and  $\eta$ CTF, a fragment of approximately 30 kDa (Willem et al., 2015).  $\eta$ CTF undergoes subsequent cleavages by  $\alpha$ , and  $\beta$ -secretases, producing A $\eta\alpha$  and A $\eta\beta$  fragments, respectively (**Figure 14**) The activity of this  $\eta$ -secretase is ensured by the matrix metalloproteinases (MMPs): MT1-MMP (MMP14), MT3-MMP (MMP16) and MT5-MMP (MMP24) have been described to be able to cleave APP at this  $\eta$ -site (detailed below) (Ahmad et al., 2006; Baranger et al., 2016; Higashi and Miyazaki, 2003; Willem et al., 2015).

However, unlike MT3, MT1 and MT5-MMP have been described to play important role in A $\beta$  production favoring the APP amyloidogenic pathway (Baranger et al., 2017; Baranger et al., 2016; Paumier et al., 2019).

Independently of its protease activity, MT1 and MT5-MMP interacts with APP and C99, promoting their endosomal localization and hence the amyloidogenic process (Garcia-Gonzalez et al., 2021; Paumier et al., 2019). Furthermore,  $\eta$ CTF could be consider as the precursor to toxic C99, A $\beta$ , as well as A $\eta\alpha$  and A $\eta\beta$ , which have recently been identified as having the ability to influence synaptic activity (Mensch et al., 2021; Willem et al., 2015). These findings make this enzyme and its pathway highly intriguing. Considering that our team is interested in investigating the implication of many fragments, such as C99 and AICD, and their implication in AD, we observed that  $\eta$ CTF is a novel interesting fragment worthy of serious research and attention, especially since no study has yet concentrated on this fragment.



**Figure 14: The  $\eta$ -secretase pathway.**

$\eta$ -secretase cleavage of APP releases the soluble fragment sAPP $\eta$  and the membrane-embedded  $\eta$ CTF, which is processed by  $\alpha$ - or  $\beta$ -secretase, thus generating A $\eta\alpha$  or A $\eta\beta$  peptides, respectively. Figure created with BioRender.com free software.

## F) BIOLOGY OF MATRIX METALLOPROTEINASES

---

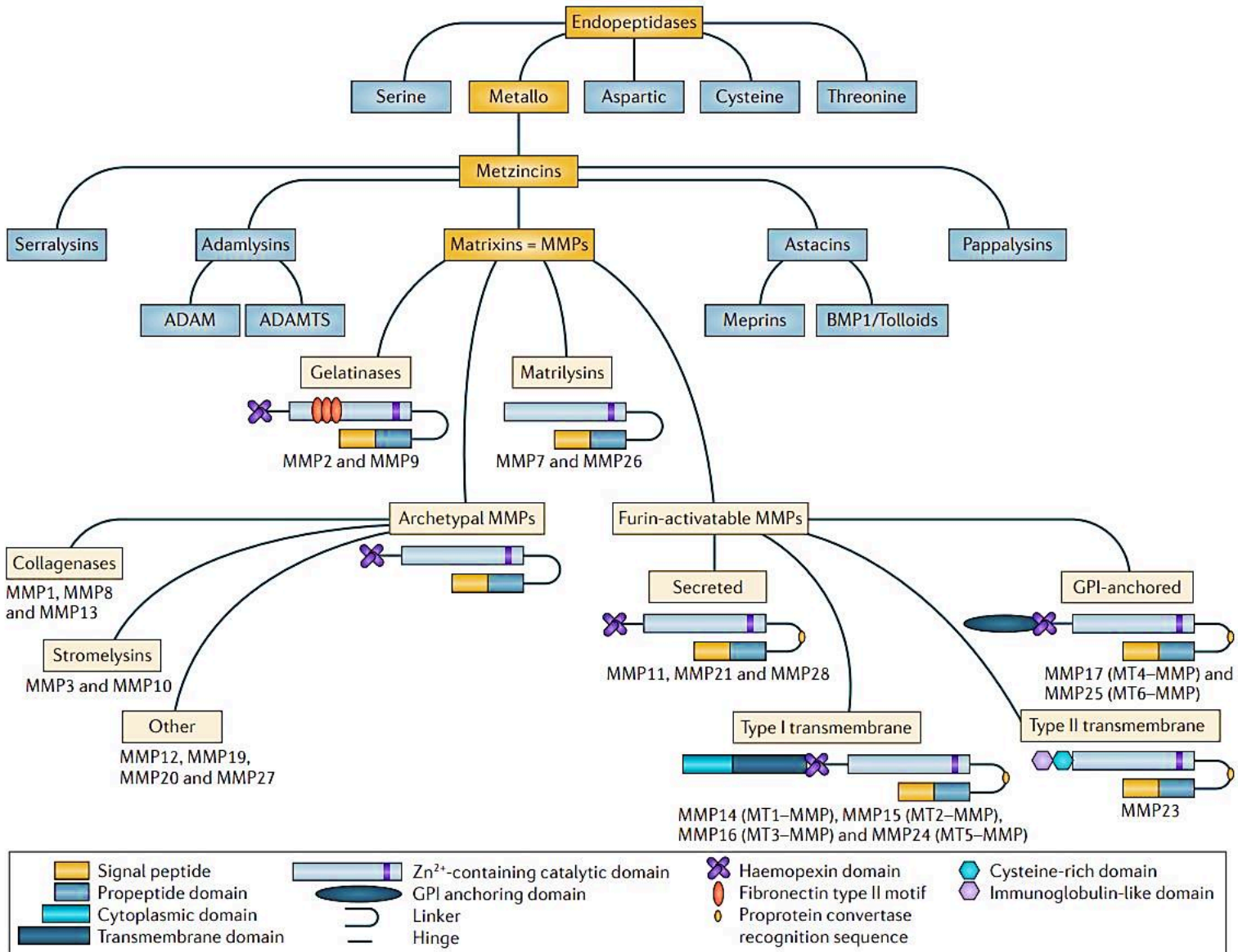
### A) MATRIX METALLOPROTEINASES (MMPS) OVERVIEW

---

The metalloproteinases are the largest of the five primary families into which all proteases are commonly classified. Metzincins, in particular, are a metalloproteinase superfamily with a conserved methionine and 3 histidines residues in their catalytic site and whose activity is reliant on a zinc ion.

MMPs and ADAMs, which play multiple roles in pathophysiology of AD, along with ADAMs with thrombospondin motifs (ADAMTSs) are all members of this metzincin subfamily. In humans, twenty-four MMPs have been identified and divided into five groups based on structure and substrate specificity: gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), collagenases (MMP-1, -8 and -13), membrane-type MMPs (MT-MMPs, MMP-14 or MT1-MMP, MMP-15, MMP-16 or MT3-MMP, MMP -17, MMP -24 or MT5-MMP and MMP-25) and the other MMPs (e.g., MMP-7, MMP-12 or MMP-28) (Rivera et al., 2019; Rivera et al., 2010)( **Figure 15**).

MMPs were formerly thought to only degrade ECM proteins, but this belief is now changing as non-matrix substrates of MMPs, such as signaling molecules, trophic factors, receptors, cell adhesion molecules, and even nuclear proteins, have been discovered. Numerous studies have highlighted MMPs' important roles in development, post-injury plasticity, learning, and memory. The functional diversity of MMPs and, more crucially, their role in neuroinflammation, and in neurodegenerative diseases such as AD were reviewed by Santiago Rivera's team (Rivera et al., 2019; Rivera et al., 2010).



**Figure 15: Metzincins superfamily members and classification of MMPs based on their domain arrangement.**

Matrix metalloproteinases (MMPs) share a common domain structure: the pre-domain that contains a signal peptide responsible for secretion; the pro-domain that keeps the enzyme inactive by an interaction between a cysteine residue and the Zn<sup>2+</sup> ion group from the catalytic domain; and the haemopexin-like carboxy-terminal domain, which is linked to the catalytic domain by a flexible hinge region. Membrane-type MMPs (MT-MMPs) have a transmembrane domain or a glycosylphosphatidylinositol (GPI) anchor at the C terminus. ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motifs; BMP1, bone morphogenetic protein 1. (Vandenbroucke and Libert, 2014).



Due to their ability to degrade A $\beta$ , some MMPs, particularly MMP-2,7,9 and MT1-MMP, have been linked to AD. MMP-9 can cleave A $\beta$ 40, whereas MMP-2 was found to be more effective at cleaving A $\beta$ 42; MMP-7 and MT1-MMP were also reported to be able to cleave both, preventing the formation of neurotoxic  $\beta$ -sheet and thus amyloid plaques (Backstrom et al., 1996; Hernandez-Guillamon et al., 2015; Liao and Van Nostrand, 2010; Taniguchi et al., 2017). In addition to being a substrate for MMPs, A $\beta$  also modulates their expression, perhaps indicating the presence of regulatory feedback mechanisms. A $\beta$  was reported to enhance the expression of MMP-2, -3, and -9 in neuronal and astrocyte cultures, and to increase the levels of MMP-1, -3, -9, -10, -12, and -19 in human postmortem brain microglia (Deb and Gottschall, 1996; Walker et al., 2006). Similar to these in vitro findings, MMP-2, -9, and MT1-MMP expression were elevated in mouse AD models, particularly in A $\beta$ -enriched areas (Py et al., 2014; Yin et al., 2006). Human studies have mostly observed an increase in MMP levels, such as MT1-MMP and MMP-1, -3, and 14, in AD patients as when compared to healthy controls (Langenfurth et al., 2014; Leake et al., 2000; Mroczko et al., 2014). MMP-9 levels, on the other hand, have been found to be reduced in the CSF and plasma of AD patients in certain studies (Horstmann et al., 2010; Mroczko et al., 2014) and increased in others (Lorenzl et al., 2003; Lorenzl et al., 2008; Stomrud et al., 2010).

While an increase in MMPs may be considered to be beneficial due to their A $\beta$ -degrading activity, MMPs are also dependent on certain substrates that may be involved in pathogenic processes in AD. For example, MMP-2 and MMP-9 have been related to the disrupted blood-brain barrier (BBB) found in AD patients and mice, which contributes to inflammatory and neurodegenerative processes (Hernandez-Guillamon et al., 2010; Spampinato et al., 2017). MMP-9 has been linked to neuroinflammatory processes and the death of hippocampal neurons (Jourquin et al., 2003), and MMP-3 has been linked to the disruption of the blood-CSF barrier (BCSFB), which limits the restriction of the passage of unwanted molecules and pathogens into the brain (Brkic et al., 2015). Aside from its link with A $\beta$ , MMP-9 can cleave Tau, resulting in fragments that are susceptible to the formation of toxic tau oligomers (Nubling et al., 2012).

## B) MT-MMP AND APP CLEAVAGE

---

MT-MMPs in particular, have also been demonstrated to have A $\beta$ -degrading and APP cleaving activity, implying that they play key roles in the regulation of APP functions in tissues such as the CNS (García-González et al., 2019).

MT1-MMP, like MMP-2, -3, -7 and -9, was identified as an A $\beta$ -degrading enzyme. First, Higashi and colleagues described the MT1-MMP cleavage site on APP770 (Asn579-Met580) in a fibrosarcoma cell line corresponding to the latter called  $\eta$ -site (Higashi and Miyazaki, 2003). Further research revealed that this cleaving site, along with the cleaving sites Ala463-Met464, Asn579-Met580, His622-Ser623, and His685-Gln686, are a target for MT3-MMP also. The His685-Gln686 cleavage occurs within the A $\beta$  sequence, but unlike MT1-MMP, MT3-MMP does not change A $\beta$  levels in HEK293, implying that MT3-MMP processes APP in cell compartments that do not affect amyloidogenesis (Ahmad et al., 2006). The same study on HEK293 cells revealed that MT5-MMP may cleave APP at the same sites as MT1-MMP and MT3-MMP.

Indeed, it has been demonstrated that MT5-MMP can cleave the shorter neuronal isoform of APP695 at the N504–M505 ( $\eta$ -site), resulting in the production of a soluble APP fragment of sAPP95 in HEKswe with the release of a complementary transmembrane fragment of ~30 kDa (CTF-30) (Baranger et al., 2016).

Interestingly, in both Rivera's and Willem's study, MT5-MMP, was identified as  $\eta$ -secretase in vivo, cleaving at this N504–M505/ $\eta$ -site of APP695 (Baranger et al., 2016; Willem et al., 2015). In addition, among all MMP, MT5-MMP is the most abundant MMP expressed in the brain with important function in brain development and synaptic plasticity.

## C)MT5-MMP

---

### 1. Overview

---

The MMP24 gene, which is located on chromosome 20q11.22, encodes MT5-MMP (also known as MMP-24) ([https://www.proteinatlas.org/ENSG00000125966-MMP24#gene\\_information](https://www.proteinatlas.org/ENSG00000125966-MMP24#gene_information)).

MT5-MMP can be present in the kidneys, and pancreas, although the neurons of the PNS and CNS have the highest expression levels (Komori et al., 2004). During embryonic and early postnatal development, MT5-MMP is highly expressed in the nervous system (Komori et al., 2004). Adults maintain this expression primarily in the cerebellum, with a reduced levels of expression in the hippocampus, dentate gyrus, and olfactory bulb (Jaworski, 2000).

## 2. Structure

---

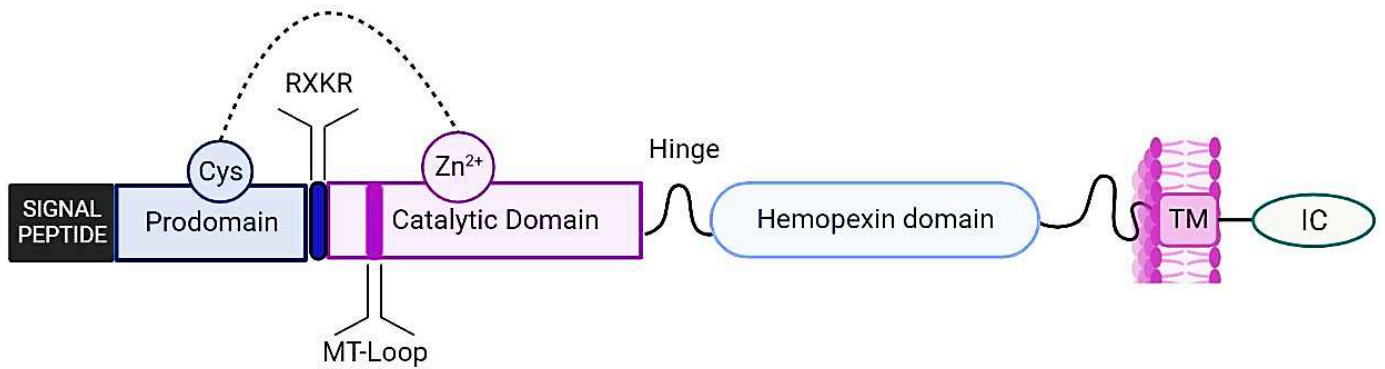
MT5-MMP's domain structure is similar to that of other MT-MMPs, consisting of a signal peptide, prodomain, RXKR activation motif, catalytic domain, hinge region hemopexin-like domain. MT5-MMP is referred to as a membrane-type MMP with a transmembrane domain and a short cytosolic tail (Pei, 1999) (**Figure 16**).

The signal peptide at the N-terminus directs the MT-MMP to the secretory pathway and is followed by a cysteine-containing prodomain. This cysteine residue of the prodomain, binds to the Zn<sup>2+</sup> of the catalytic domain, causing the prodomain to fold to the catalytic site, inhibiting the proteolytic activity of the MT-MMPs and leaving them in an inactive form known as "zymogens." Therefore, until activated by enzymes that cleave the prodomain or release the cysteine bond, these metzincin proteases remain in a latent condition. MT5-MMP belonging to Furin-activable MMPs is activated in the trans-Golgi network where furin, the proprotein convertase, cleaves its RXKR motif (Rivera et al., 2010). The ability of furin to activate and shed MT5-MMP provides cells with a mechanism to balance the ratio of activated/membrane-bound and activated/shed MT5-MMP by modulating the concentration of the convertases in the TGN (Wang and Pei, 2001).

The catalytic domain is highly conserved and binds and provides stability to Zn<sup>2+</sup> via a consensus motif with three histidine residues (HExxHxxGxxH). The catalytic domain of all MT-MMP contains also the MT-loop, an 8 amino acid loop structure that allows the enzyme to be localized to focal adhesion locations and effectively degrading the underlying matrices (Itoh, 2015).

A small hinge region downstream of the catalytic site marks a shift to the hemopexin domain, the latter being important for their specificity for substrates and inhibitors (Rivera et al., 2010).

MT5-MMP has a type I transmembrane domain that anchors it to the plasma membrane and hence controls, together with an intracytoplasmic domain, its trafficking and proteolytic activity (Wang and Pei, 2001)



**Figure 16 Domain organization in MT5-MMP.**

MT5-MM's structure contains a signal peptide, a prodomain, a RXKR activation motif, a catalytic domain, a hinge region hemopexin-like domain a transmembrane domain and a short cytosolic tail. Figure created with BioRender.com free software.

### 3. Physiological Role

The fact that MT5-MMP's expression is highest during embryonic development and is mostly restricted to the cerebellum in adults, with a lower level in the hippocampus, dentate gyrus, and olfactory bulb, which are known to be neuronal plasticity regions, supports MT5-MMP's involvement in brain development and synaptic plasticity (Jaworski, 2000).

Proteoglycans, including chondroitin sulfate proteoglycans, are primary ECM components in the brain that have been shown to inhibit neurite growth and axonal regeneration (Jin et al, 2017). These proteoglycans were also discovered to be the preferred substrates of MT5-MMP (Jaworski et al, 2000), implying that this metalloproteinase enhances neurite outgrowth. Another study found that MT5-MMP, along with ADAM10, has a function in post-lesion synaptic structure modification following traumatic brain injury (Warren et al., 2012).

N-cadherin is a non-ECM substrate of MT5-MMP that promotes neural stem cells (NSCs) quiescence and adherence to their niches. MT5-MMP mediates the release and activation of NSCs in tissue renewal and repair by shedding N-cadherin (Porlan et al., 2014).

N-cadherin is also a synaptic protein that is involved in synapse remodeling and maintenance. It was discovered that MT5-MMP and  $\gamma$ -secretase work together to provide synaptic autoregulation by cleaving N-cadherin on both sides of the synapse (Restituito et al., 2011).

Despite MT5-MMP's multiple roles and predicted developmental importance, MT5-MMP deficient mice are viable. However, when these mice were subjected to sciatic nerve injury, no

sprouting nor invasion of A $\beta$ -fibers were observed, along with the absence of mechanical allodynia caused by such lesions. This favors the fact that of MT5-MMP plays critical role in neuronal plasticity and remodeling (Komori et al., 2004).

#### 4. MT5-MMP in AD

---

First, Sekine-Aizawa and colleagues found MT5-MMP colocalized with amyloid plaques in post-mortem brain tissue from AD patients (Sekine-Aizawa et al., 2001). To further understand the role of MT5-MMP in AD, Rivera's group developed the 5xFAD/MT5<sup>-/-</sup> model, which was created by crossing 5xFAD mice with familial AD mutations in the human APP and Psen1 genes with MT5-MMP<sup>-/-</sup> mice. In the cortical and hippocampus regions, 5xFAD/MT5<sup>-/-</sup> mice showed a significant decrease in soluble and oligomeric A $\beta$ 40 and A $\beta$ 42, as well as C99. This intriguing reduced amyloidosis is accompanied by a decline in pro-inflammatory cytokines, interleukine-1 $\beta$  (IL-1 $\beta$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), as well as a decrease in glial reactivity, all while preserving LTP, spatial learning, and memory (Baranger et al., 2016).

The same team then discovered a second mechanism through which MT5-MMP exerts proamyloidogenic action. They demonstrated that MT5-MMP can be internalized in endosomes while interacting with APP, increasing APP sorting in endosomes, a major site of A $\beta$  production (Baranger et al., 2017; Vassar et al., 1999).

#### 5. MT5-MMP as $\eta$ -secretase

---

Independent studies from two teams have described the processing of APP by MT5-MMP through its  $\eta$ -secretase activity

In 2015, Willem and colleagues identified the cleavage site N504–M505 of APP695 as  $\eta$ -site, and MT5-MMP, responsible for this cleavage in vivo, as  $\eta$ -secretase (Willem et al., 2015).

They characterized the fragments produced by this processing as " $\eta$ -CTF," a new CTF with an estimated molecular weight of 30 kDa, and "sAPP $\eta$ , a soluble N-terminal APP fragment of 80 kDa. These two fragments match with the descriptions of sAPP95 and CTF-30 reported in the same time by Baranger and collaborators (Baranger et al., 2017; Baranger et al., 2016).

The implication of sAPP $\eta$  in AD is unknown; however, a recent study found that sAPP, including sAPP $\alpha$ , sAPP $\beta$  and sAPP $\eta$ , can bind to GABABR1a, leading to decreased presynaptic release and therefore decreasing hippocampal synaptic plasticity and

neurotransmission in vivo This could represent a negative-feedback mechanism to maintain proper homeostatic control of neural activity and prevent any hyperexcitability (Rice et al., 2019).

$\eta$ CTF was discovered to be accumulated in dystrophic neurites of APP-PS1 mice brains and human AD brains (Willem et al., 2015). Another study found increased levels of  $\eta$ CTF in the CSF of patients with PSEN1 familial mutation, sporadic AD, and Down Syndrome compared to age-matched controls, implying that this fragment could be a possible diagnostic biomarker of AD (Garcia-Ayllon et al., 2017). Additionally, a CTF of 26 kDa was found in exosomes from rat cortical neurons and was characterized in this work to most likely correspond to  $\eta$ CTF, implying that this fragment may contribute to the disease after being endocytosed by neurons (Laulagnier et al., 2018). They also observed that exosomes released by N2a cells expressing APP<sup>wt</sup> are enriched in both  $\alpha$ -CTF (C83) and  $\eta$ CTF while those purified from N2a cells expressing APP<sup>sw</sup> are rather enriched in both  $\alpha$ -CTF (C83) and  $\alpha$ -CTF (C99); however, inhibition of BACE1 in the latter model results in increased exosomal levels of  $\eta$ CTF (Laulagnier et al., 2018). This could be explained by the Swedish mutation, which enhances APP cleavage by  $\beta$ -secretase in amyloidogenic processing, which may compete with  $\eta$ -secretase cleavage, or possibly explained by extensive cleavage of  $\eta$ CTF at C99 when this mutated form of APP is expressed. Willem and colleagues further stated that the transmembrane  $\eta$ -CTF undergoes subsequent cleavages by  $\alpha$  and  $\beta$ -secretases generating A $\eta\alpha$  and A $\eta\beta$  respectively and also investigated the impact of these new soluble fragments in AD. In their first study, they exposed hippocampal slices to 100 nM of synthetic A $\eta\alpha$  and A $\eta\beta$  and discovered that only A $\eta\alpha$ , not A $\eta\beta$ , displays neurotoxic properties and impairs LTP at the CA3-CA1 synapse (Willem et al., 2015). However, the same team published contradicting results years later, reporting that both A $\eta\alpha$  and A $\eta\beta$ , even at low nanomolar concentrations of 5 nM, impair LTP ex vivo (Mensch et al., 2021). Further studies are needed to elucidate synaptic function and pathological contribution of each  $\eta$ -secretase-derived fragment in AD pathology.

## II. OBJECTIVES

---

AD is by far the most common cause of dementia and a major global health concern. In the absence of effective treatments, AD has significant financial, physical, and mental health consequences on individuals and society. This explains why searching for therapeutic strategies is therefore one of the greatest challenges that scientists have been facing for decades. The A $\beta$  peptide, according to the amyloid cascade hypothesis, is the origin of AD. However, the failure of A $\beta$ -targeted therapies highlights the multifactorial nature of the disease. Indeed, several studies, including research from my team, have shown for example that independently of A $\beta$ , the C99 fragment accumulates very early in AD models causing several impairments associated with AD. Due to the fact that AD is a complex disease, my team is interested in investigating APP-derived fragments other than A $\beta$  that may be implicated in the pathology and may contribute in the discovery of new therapeutic targets in the future.

With all the focus of the research in the field of AD aiming to better understand the APP processing pathways; the recent discovery of  $\eta$ -secretase, as new APP cleaving enzyme has revealed the existence of novel fragments that may have toxic potential, complicating understanding of the AD pathophysiology.

With just approximately ten papers describing this cleavage and some of the resulting fragments, more eyes must be opened and greater focus should be placed on this pathway. As a consequence, the objectives of my PhD research were to principally concentrate on the  $\eta$ CTF fragment: the  $\eta$ -secretase-derived APP membrane embedded fragment; especially given how little is known about this fragment. My first priority was to characterize it, and define its role in the pathophysiology associated with AD, which will serve to better understand the complexity of the APP metabolism.

Importantly, my laboratory had developed all the tools, plasmids, and methodological approaches to express these fragments not only in cellular models but also ex vivo and in vivo using Adeno-Associated Virus10 (AAV10). The purpose of my study consisted in characterizing the newly discovered  $\eta$ CTF fragment using biochemical, pharmacological, and immunohistochemical approaches, and then thoroughly evaluating its functional influence on AD by electrophysiological and behavioral experiments. For this goal, I used various study models with their strengths and weaknesses described below.

#### ❖ *In vitro*:

First, in order to study the expression, fate, processing and cellular localization of  $\eta$ CTF, I start to express it in the SH-SY5Y neuroblastoma cells; the advantage of these cells is that they are



immortalized human neuronal-like cell line, but not differentiated into mature neuronal cells. Human neuroblastoma SH-SY5Y stably overexpressing human wild-type APP (SH-SY5Y APPwt) were also used to attempt the detection of the APPwt-derived  $\eta$ CTF, as endogenous  $\eta$ CTF is difficult to detect. In addition, mouse embryonic fibroblasts naturally devoid of APLP1 and knocked out for APP and APLP2 (MEF APP KO) served also as important cell line in my study, although they are not a human nor neuronal-like cell line, they were a crucial model for identifying fragments that are genuinely derived from  $\eta$ CTF transiently transfected in these cells, eliminating the possibility of them being mistaken for endogenous APP-derived fragments. MEF expressing endogenous wild-type APP (MEF-APPwt) served as controls. Finally, due to the small size of SH-SY5Y cells and in order to better characterizing the specific intracellular localization of  $\eta$ CTF, we decided to use the human cervical cancer cells (HeLa), for immunocytochemistry, despite the fact that they are non-neuronal cells.

❖ *Ex-vivo:*

My team recently developed a study model that consists of the culture of wild-type mouse (C57BL/6) organotypic hippocampal slices which have advantages over the in vitro models, because it conserves the composition, structure, and synaptic organization of the hippocampus while avoiding cell isolation and lack of contact (Valverde et al., 2021b). This model enabled us to infect the slices with virus expressing  $\eta$ CTF (AAV-CTF), which allows expressing and analyzing the localization of this fragment of interest in a more integrated model.

❖ *In vivo:*

- *Mouse models:*

To investigate the functional impact of  $\eta$ CTF expression in vivo models, we developed the "AAV-CTF" mouse model that was obtained by intraventricular injection of 1-day-old WT mice (C57BL/6) with AAV-CTF virus, which allows the expression in mouse brain of the human  $\eta$ CTF fragment under the control of the neuronal promoter synapsin-1.

In order to determine if  $\eta$ CTF is present in AD models, we chose the 3xTgAD triple transgenic mouse model (APP<sup>swe</sup>, TauP301L and PS1M146V). These mice overexpress the human APP with the Swedish familial mutation (APP<sup>swe</sup>) and Tau protein with the P301L mutation. These mice also express an endogenous level of PS1 with the M146V familial mutation (knock-in). It is a widely used mouse model in the field of AD since these mice replicate the majority of the pathological phenotypes, including late senile plaque formation and neurofibrillary degeneration, as well as cognitive and synaptic impairments. Interestingly, 3xTgAD mice

accumulate C99 earlier than A $\beta$ , which correlates with endolysosomal dysfunction (Lauritzen et al., 2016), mitochondrial alterations (Vaillant-Beuchot et al., 2021), apathetic behavior (Pardossi-Piquard et al., 2016) and contributes to AD-like cognitive dysfunction (Bourgeois et al., 2018).

- *Human models:*

To examine whether  $\eta$ CTF is present in AD patients, human brain samples were also tested. Only information on the patients' age, sex, and neuropathology were provided, while samples were anonymized. Brain samples from AD patients were isolated from post-mortem patients who had been diagnosed with the disease, and they were categorized according to their Braak and Braaks's NFT stage (I to VI), while samples from controls were taken from individuals who had a negative diagnosis for several AD-unrelated neuropathologies (Braak and Braak's NFT stage -).

Thus my results are divided into two parts:

- ❖ First, study of expression, processing, fate, and subcellular localization of  $\eta$ CTF in different in vitro, ex vivo, and in vivo models.
- ❖ Second, preliminary investigations on the detection of  $\eta$ CTF in human AD patients, and functional implication of  $\eta$ CTF and related fragments (A $\eta\alpha$  and A $\eta\beta$ ) on synaptic activity.

### III. RESULTS

---

## 1) The study of expression, processing, fate, and subcellular localization of $\eta$ CTF in different in vitro, ex vivo, and in vivo models.

---

This study gave rise to my first-author article currently under review to the journal, Cellular and Molecular Life Sciences (CMLS), and the preprint is available online on Research Square (<https://www.researchsquare.com/article/rs-2285279/v1>).

### « The $\eta$ -secretase-derived APP fragment $\eta$ CTF is localized in Golgi, endosomes and extracellular vesicles and contributes to $A\beta$ production »

**Elissa Afram**, Inger Lauritzen, Alexandre Bourgeois, Wejdane El Manaa, Eric Duplan, Mounia Chami, Audrey Valverde, Bauer Charlotte, Raphaëlle Pardossi-Piquard and Frederic Checler.

Since the discovery of the  $\eta$ -secretase cleavage and the resulting fragments, dates only a few years, very little is known about  $\eta$ CTF and, decades of research are certainly required to properly define its contribution to the pathology associated with AD. For this reason, the objective of my first-author article was to start by characterizing the  $\eta$ CTF fragment by studying its expression, processing, fate, and subcellular localization in different in vitro, ex vivo, and in vivo models, aiming that these results will serve as a starting point of the research towards the understanding of the toxic potential of this fragment. It's interesting to note that we developed a new antibody named  $\eta$ CTF-Nter that exclusively recognizes  $\eta$ CTF,  $A\eta\alpha$ , and  $A\eta\beta$ . This antibody proved useful for in situ and biochemical analyses of  $\eta$ CTF in both mice and human models. We confirmed that  $\eta$ CTF is processed by  $\alpha$ ,  $\beta$ , and  $\gamma$ -secretases using a pharmacological approach, demonstrating for the first time that  $\eta$ CTF is a genuine precursor of  $A\beta$ . Furthermore, we showed that  $\eta$ CTF is degraded by both proteasomal and autophagic pathways. We then performed immunostaining experiments in different study models to identify the localization of  $\eta$ CTF in the trans-golgi compartment and endosomes. In an interesting matter, and in agreement with this endosomal localization, we detected  $\eta$ CTF in exosomes. Lastly,  $\eta$ CTF fragments were found surrounding amyloid plaques in brain slices of 3xTgAD mice.

Overall, these findings suggest that  $\eta$ CTF could be a potential AD player, and further investigation will be required to evaluate whether it can serve as a possible therapeutic target.

## The $\eta$ -secretase-derived APP fragment $\eta$ CTF is localized in Golgi, endosomes and extracellular vesicles and contributes to A $\beta$ production.

Elissa Afram<sup>1</sup>, Inger Lauritzen<sup>1</sup>, Alexandre Bourgeois<sup>1</sup>, Wejdane El Manaa<sup>1</sup>, Eric Duplan<sup>1</sup>, Mounia Chami<sup>1</sup>, Audrey Valverde<sup>1,2</sup>, Bauer Charlotte<sup>1</sup>, Raphaëlle Pardossi-Piquard<sup>1-3</sup> and Frederic Checler<sup>1,3</sup>.

<sup>1</sup>Université Côte d'Azur, INSERM, CNRS, IPMC, UMR7275, team labeled « Laboratory of Excellence (Labex) DISTALZ », 660 route des Lucioles, 06560, Sophia-Antipolis, Valbonne, France.

<sup>2</sup>Present address : Fonds de dotation CLINATEC, 17 rue des Martyrs, Bat 43, 38054 Grenoble

<sup>3</sup>Correspondence should be addressed to Dr. Raphaëlle Pardossi-Piquard, ([pardossi@ipmc.cnrs.fr](mailto:pardossi@ipmc.cnrs.fr)) or to Dr. Frédéric Checler ([checler@ipmc.cnrs.fr](mailto:checler@ipmc.cnrs.fr)), IPMC, CNRS, UMR7275, 660 route des Lucioles, 06560 Valbonne, France, Tel: (33) 493953458; Fax: (33) 493957708.

### Abstract

The processing of the amyloid precursor protein (APP) is one of the key events contributing to Alzheimer's disease (AD) etiology. Canonical cleavages by  $\beta$ - and  $\gamma$ -secretases lead to A $\beta$  production which accumulate in amyloid plaques. Recently, the matrix metalloprotease MT5-MMP, referred to as  $\eta$ -secretase, has been identified as a novel APP cleaving enzyme producing a transmembrane fragment,  $\eta$ CTF that undergoes subsequent cleavages by  $\alpha$ - and  $\beta$ -secretases yielding the A $\eta\alpha$  and A $\eta\beta$  peptides, respectively. The functions and contributions of  $\eta$ CTF and its related fragments to AD pathology are poorly understood. In this study, we designed a novel immunological probe referred to as  $\eta$ CTF-Nter antibody that specifically interacts with the N-terminal part of  $\eta$ CTF targeting  $\eta$ CTF, A $\eta\alpha$ , A $\eta\beta$  but not C99, C83 and A $\beta$ . We examined the fate and localization of  $\eta$ CTF fragment in various cell models and in mice. We found that overexpressed  $\eta$ CTF undergoes degradation in the proteasomal and autophagic pathways and accumulates mainly in the Golgi and in endosomes. Moreover, we observed the presence of  $\eta$ CTF in small extracellular vesicles (sEVs) purified from neuroblastoma cells or from mouse brains expressing  $\eta$ CTF. Importantly, the expression of  $\eta$ CTF in fibroblasts devoid on APP leads to A $\beta$  production demonstrating its contribution to the amyloidogenic pathway. Finally, we observed an  $\eta$ CTF-like immunoreactivity around amyloid plaques and an age-dependent accumulation of  $\eta$ CTF in the triple-transgenic mouse AD model. Thus, our study suggests that the  $\eta$ CTF fragment likely contributes to AD pathology by its exosomal spreading and involvement in A $\beta$  production.

**Keywords:** Alzheimer's disease,  $\eta$ CTF, endosomes, extracellular vesicles, autophagic degradation, A $\beta$ -production

### Introduction

Among all hypotheses proposed to explain the etiology of Alzheimer's disease (AD), the amyloid hypothesis represents the most widely accepted one since it is supported by a large number of biochemical and histopathological evidences. This amyloid cascade hypothesis postulates that accumulation and aggregation of hydrophobic amyloid  $\beta$  peptides ( $A\beta$ ) trigger synaptic dysfunction and neurodegeneration in Alzheimer's disease <sup>1,2</sup>. Genetic evidences also provided strong support to this amyloid cascade. Thus, most of APP mutations responsible for aggressive autosomal dominant familial forms of AD (FAD) lead to exacerbated production of  $A\beta$  or yield aggregation-prone  $A\beta$  species <sup>3</sup>. Conversely the "Icelandic" APP mutation (APP Ala673Thr) decreases  $A\beta$  levels by about 40% and protects against the risk of developing AD <sup>4</sup>.

How APP is proteolytically processed has been the matter of numerous studies. APP undergoes a complex set of proteolytic events by enzymatic activities called secretases. Two main proteolytic pathways referred to as amyloidogenic and non-amyloidogenic pathways have been extensively examined. In the amyloidogenic pathway, APP is first cleaved by the  $\beta$ -secretase to release a C-terminal fragment, C99, which then undergoes subsequent hydrolysis by the  $\gamma$ -secretase to yield not only  $A\beta$  but also the cytosolic APP Intracellular Domain (AICD) that controls the transcription of several genes <sup>5</sup>. However, this amyloidogenic pathway responsible for  $A\beta$  production does not represent the major part of APP metabolism. The non-amyloidogenic pathway that precludes  $A\beta$  formation is the major APP processing route. It involves  $\alpha$ -secretase, yielding a C83 fragment that is further cleaved by  $\gamma$ -secretase, thereby producing AICD and a small N-terminally truncated fragment named p3.

In addition to these two canonical and well described pathways, a newly  $\eta$ -secretase pathway has emerged. First Wang and collaborators reported in HEK293 cells an accumulation of two novel APP-CTF fragments at 15 and 25kDa unraveled upon cathepsins inhibition <sup>6</sup>. They were the first to name the 25kDa APP-CTF fragment as the new APP-CTF $\eta$  or  $\eta$ CTF fragment <sup>6</sup>. Using various antibodies and mass spectroscopy analyses, Willem and colleagues subsequently demonstrated that this  $\eta$ CTF fragment can be cleaved by  $\alpha$ - and  $\beta$ -secretases to generate  $A\eta\alpha$  and  $A\eta\beta$  peptides respectively <sup>7</sup>. They proposed the membrane-type matrix metalloprotease MT5-MMP as a new  $\eta$ -secretase since they observed a reduction of  $A\eta\alpha$  production in brains from MT5-MMP-knockout mice <sup>7</sup>. By distinct approaches, concomitant studies also demonstrated the cleavage of APP by MT5-MMP and showed its contribution to AD pathology. Thus, MT5-MMP deficiency reduced amyloid pathology, neuroinflammation and cognitive decline in 5xFAD mice <sup>8,9</sup>. However, few data were available concerning the fate, biology and cellular localization of this fragment.

In this current study, we observed that overexpressed  $\eta$ CTF undergoes proteasomal and autophagic degradations in human neuroblastoma cells (SH-SY5Y) and for the first time, we show that  $\eta$ CTF behaves as a genuine precursor of  $A\beta$ . Since, the immunological toolbox available to specifically detect  $\eta$ CTF, particularly in *in situ* approach fell short, we designed a novel immunological probe referred to as  $\eta$ CTF-NTer antibody that interacts selectively with  $\eta$ CTF N-terminus thus targeting  $\eta$ CTF,  $A\eta\alpha$ ,  $A\eta\beta$  but importantly, not C83, C99 or AICD. By using this antibody in HeLa transfected cells, we were able to establish a subcellular localization of  $\eta$ CTF mainly in trans-Golgi network but also, to a smaller extent, in endosomes. This endosomal localization was also observed in organotypic hippocampal slices infected with an adeno-associated-virus (AAV) expressing  $\eta$ CTF. In agreement, we detected the presence of  $\eta$ CTF in small extracellular vesicles (sEVs) prepared from culture media of SH-SY5Y expressing APP or from brain tissues of AAV- $\eta$ CTF-injected mice. Of interest, microscopic analysis of  $\eta$ CTF-NTer-like immunoreactivity revealed an  $\eta$ CTF staining surrounding amyloid plaques associated to an age-dependent accumulation of  $\eta$ CTF in cortex and hippocampus of triple transgenic mice (3xTgAD).

## Materials and Methods

### Design of plasmid constructs and viruses:

**pcDNA3 plasmid expressing A $\eta$  $\alpha$  or A $\eta$  $\beta$ :** A $\eta$  $\alpha$  and A $\eta$  $\beta$  constructs were obtained by PCR amplification using APP695 cDNA as template and the forward primer 5'-GATAAGCTTGCCACCATGATTAGTGAACCAAGGATCAGTTAC-3' for both A $\eta$  $\alpha$  and A $\eta$  $\beta$ . This primer contains a Hind III restriction site and a Kozak sequence. We used the reverse primer 5'-GATCTCGAGCTATTTTTGATGATGAACTTCATATCCTGAGTC-3' for A $\eta$  $\alpha$  and the reverse primer 5'-GATCTCGAGCTACATCTTCACTTCAGAGATCTCTCC-3' for A $\eta$  $\beta$ . Both reverse primers contain the XhoI enzyme restriction site. The amplicon was then digested by Hind III and XhoI enzymes and subcloned in the pcDNA3 vector.

**pcDNA3 plasmid expressing  $\eta$ CTF:** the  $\eta$ CTF plasmid construction contains the APP signal sequence and two additional residues (Leu-Glu) from APP695 cDNA in frame with the 5' end of the  $\eta$ CTF sequence. As previously described for SPA4CT<sup>10</sup>, SP-C99 and SP-C83 constructs<sup>11</sup>, the APP signal sequence is required for a correct membrane insertion of the  $\eta$ CTF fragment. In a first PCR reaction, the APP signal sequence was amplified using the primers 5'-GATAAGCTTATGCTGCCCGTTTGGCACT-3' that contains an HindIII restriction site and 5'-TTCATAATCATCTCCAGCGCCCGAGCC-3' containing the first nucleotides of the 5' end of  $\eta$ CTF sequence. In an additional PCR,  $\eta$ CTF sequence was amplified using the primer 5'-GCGCTGGAGATGATTAGTGAACCAAGGATCAGTTA-3' that contains the last nucleotides of the 3' end of the signal peptide sequence, and the primer 5'-GATCTCGAGCTAGTTCTGCATCTGCTCAAAGAA-3' containing XhoI restriction site. Finally, the amplicons resulting from the first two PCR were incubated together in a third PCR reaction to obtain a final DNA fragment containing the APP signal peptide in frame with  $\eta$ CTF. This amplicon digested with HindIII and XhoI enzyme was then subcloned in pcDNA3 vector.

**AAV-10 plasmid expressing  $\eta$ CTF under the control of synapsin-1 promoter:** For viral plasmid construction, the pcDNA3 plasmid expressing  $\eta$ CTF described above was used as a template with the primers 5'-GATGCTAGCCCACCATGCTGCCCGTTTGGCACTGCTCCT-3' and 5'-GATGCTAGCCTAGTTCTGCATCTGCTCAAAGAACTTGTAAGTT-3' that both contain a NheI restriction enzyme site. The PCR product was then digested with NheI and sub-cloned into AAV10 plasmid.

**pGEX plasmid for expression of recombinant  $\eta$ CTF:** the DNA sequence encoding the  $\eta$ CTF was amplified by PCR, using the forward primer 5'-GATGGATCCATGATTAGTGAACCAAGGATCAGTTA-3', which has a BamHI restriction site and the reverse primer 5'-GATCTCGAGCTAGTTCTGCATCTGCTCAAAGAA-3', containing a XhoI restriction site. The PCR product was then sub-cloned between BamHI and XhoI restriction enzymes sites of pGEX-4T-1 vector.

All above constructs were verified by full sequencing.

### Recombinant $\eta$ CTF production:

Recombinant  $\eta$ CTF fragment was obtained as previously described for the production of recombinant PrP<sup>Sc</sup> fragment N1<sup>12</sup>, with some minor changes. Briefly, pGEX-4T-1 vector containing  $\eta$ CTF sequence was transformed into BL21 gold strain of Escherichia coli. After induction with Isopropyl 1-thio-D-galactopyranoside (200  $\mu$ M),

the medium was centrifuged. Cells pellets were resuspended with PBS supplemented with a protease inhibitor mixture, DTT (10mM) and lysozyme (0.2mg/ml), then proteins were solubilized by the addition of Triton X-100 (10%), MgCl<sub>2</sub> (1M), and DNase (1u/μl). After centrifugation, glutathione-Sepharose beads (GE Healthcare) were added to the supernatant, pelleted, and resuspended in PBS (1 ml). Peptides were cleaved with thrombin (5 units/ml; GE Healthcare), and thrombin was removed using Sepharose benzamidine beads (GE Healthcare).

#### **Production of the ηCTF-Nter antibody**

The new ηCTF-Nter antibody is a rabbit polyclonal antibody made following the Covalab's immunization protocol. As immunogen epitope, we designed peptide corresponding to the first 16 N-terminal residues of ηCTF fragment, the C-terminal end of which was blocked by a cystein residue in order to preserve a free N-terminal part of the peptide and to obtain antibodies directed towards the N-terminal moiety (sequence: MISEPRISYGNLALM-C). Rabbit's immunoreactivity and titer were controlled by ELISA then the ηCTF-Nter antibody was purified by antigen-specific affinity with the same peptide used for immunization.

#### **Viral production and mice ICV injection:**

Virus production was performed following a protocol previously described<sup>13</sup>. Briefly, HEK293 cells were transfected with the adenovirus helper plasmid (pXX6), the AAV packaging plasmid (rAAV2-rh10), and the AAV10 plasmid empty vector or encoding human ηCTF under control of the synapsin-1 promoter (AAV-empty, and AAV- ηCTF). Viruses were produced, purified and vector titers were determined by real-time PCR and expressed as viral genomes per ml (vg/ml). Four μl of AAV virus (5.5x10<sup>12</sup>vg/ml) were administrated in 1-day-old C57Bl6JRj mice (Janvier Labs, France) through intra-cerebroventricular (ICV) injection as described previously<sup>13</sup> then mice brains were analyzed at 3-months of age by western-blot and immunochemistry.

#### **Animals:**

Pregnant C57Bl6JRj females (Janvier Labs, France) were ordered for new born mice viral injection (see above). In addition wild-type and 3xTg (APP<sub>swe</sub>; Tau<sub>p301L</sub>; PS1<sub>M146V</sub>) mice were maintained from breeding pairs provided by Dr F. LaFerla<sup>14</sup>. All mice were kept on the original 129/C57Bl6 background strain, backcrossed every 10 generations and genotyped. Animals were housed with 12h/12h light/dark cycle and were given free access to food and water. All experimental procedures were in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and approved by the French Ministry of Higher Education and Research (project number APAFIS#9766-201704261624789.v3).

#### **Cell Culture and treatments**

Human neuroblastoma (SH-SY5Y, ATCC), human epitheloid cervix carcinoma (HeLa, ATCC) and mouse embryonic fibroblasts either wildtype (MEF-APPwt), or naturally devoid of APLP1 and knocked out for APP and APLP2<sup>15</sup> (referred to as MEF APP KO) were cultured in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), penicillin (100 U/ml) and streptomycin (50 μg/ml), and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. Human SH-SY5Y cells stably expressing wild type full-length APP (SH-SY5Y-APPWT) were generated as already described<sup>16</sup>, and maintained in the presence of G-418 (400 μg/ml). Transient transfections of cells were carried out using Lipofectamine 2000 (Life Technologies) for SH-SY5Y and MEFs and JetPrime (Polyplus transfection) for HeLa cells, according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with pharmacological agents: Lactacystin (5 μM; Sigma-Aldrich), Epoxomicin (1 μM; Enzo), MG132 (5 μM), Bafilomycin A1 (100 nM), Smer28 (50 μM), or with secretases inhibitors: β-secretase inhibitor, Bi (30 μM, Elan Pharmaceuticals), α-secretase inhibitor, Gi (GI254023X; 10 μM; Sigma-Aldrich) or γ-secretase inhibitor D6 (1 μM; Elan Pharm/Imago). Cells were analyzed 48 h post-transfection.



### **Western-blot analysis**

Cells were lysed in RIPA buffer (Tris (50 mM); pH 7,4; NaCl (150 mM); EDTA (1mM); Triton X100 (1%); deoxycholate (0,5%); SDS (0,1%)) supplemented with a cocktail of protease inhibitors (Roche diagnostics). Cell homogenate proteins (50 µg) were separated on 16% tris-tricine gels, and sEVs proteins were separated on 16% tris-tricine gels or Bio-Rad 12% stain-free TM TGX FastCast™ acrylamide gels. It should be noted that in few cases, when protein of interests and loading control proteins have similar molecular weights, in order to achieve accurate normalizations, gels were run separately in strictly identical conditions. All full gels are provided as indicated in the legends of figures showing gels. Tris-tricine gels were directly transferred to nitrocellulose membranes using a conventional transfer system and boiled in PBS before saturation. Bio-Rad gels were photoactivated for the visualization of proteins before being electrophoretically transferred to nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo™ Transfer System. Membranes were saturated with skimmed milk, and incubated overnight with the following primary antibodies: APPCter rabbit polyclonal (1:5000, gift from Paul Fraser); WO2 mouse monoclonal (1:5000, Sigma Aldrich); ηCTF-Nter rabbit polyconal (1:1000, homemade with Covalab, see above); α-HSC70 mouse monoclonal (1:1000, Santa Cruz) or α-GAPDH mouse monoclonal (1:5000, EMD Millipore). After probing with primary antibodies, immunological complexes were revealed with anti-mouse or anti-rabbit HRP-conjugated antibodies (1:5000, Jackson ImmunoResearch) followed by electrochemiluminescence (Westernbright™ Sirius™ and Quantum™ chemiluminescent HRP substrate, Advansta, France). Peak height of signal intensities from protein bands were quantified with MultiGauge software. For western-blot analysis of mice brains, following intracardiac perfusion with PBS, hemibrains were extracted then enzymatically and mechanically lysed using “the adult brain dissociation kit” and a GentleMACS Dissociator (Miltenyi Biotec). PBS was added to the cell suspension, which was then filtered through a 70 µm Smartstrainer and centrifuged at 300 x g for 10 min. The cell pellets were lysed in RIPA buffer supplemented with protease inhibitors and then loaded on Bio-Rad 12% stain-free™ TGX FastCast™ acrylamide gels.

### **Sandwich ELISA analysis**

MEF APP KO cells were transiently transfected with pcDNA.3 or ηCTF constructs using Lipofectamine 2000 and treated or not with secretases inhibitors Bi, Gi and D6. The concentrations of human secreted Aβ40 were measured in the culture medium by using the ELISA kit (Invitrogen) following the manufacturer’s instructions.

### **Ana Immunoprecipitation**

MEF APP KO cells were plated on 6 wells plates, transiently transfected with pcDNA.3 or ηCTF constructs using Lipofectamine 2000 and treated or not with secretases inhibitors Bi, Gi and D6, as described above. Culture media were collected, centrifuged at 14000 x g for 5 min to remove cell debris then supernatants were supplemented with RIPA buffer 5X and precleared with 10 µl of Protein A Sepharose (GE Healthcare) for 1h at 4 °C. Resulting supernatants were incubated for 1 h with WO2 mouse monoclonal antibody (1 µl) then 20 µl of Protein A Sepharose beads were added and left for overnight incubation. After three washes with RIPA buffer (and 1 time in PBS), the beads were resuspended with 20 µl of loading buffer, loaded on a tris-tricine gel (16%) and subjected to western blot analysis as described above.

### **Cells immunostaining:**

Hela cells grown on coverslips were fixed in paraformaldehyde 4% solution for 10 min, permeabilized with Triton-X 100 (0.1%) for 10 min, saturated in BSA (5%)/Tween20 (0.1%), and probed for 1h with appropriate primary antibodies: APPCter rabbit polyclonal (1:5000), APPCter, mouse monoclonal (1:5000, Biologend), WO2 mouse monoclonal (1:5000), or ηCTF-Nter rabbit polyconal (1:1000) for ηCTF detection, α-Lamp2 mouse monoclonal

(1:1000, Santa Cruz),  $\alpha$ -EEA1 rabbit monoclonal (1:200, Cell Signaling Technology),  $\alpha$ -CD63 mouse monoclonal (1:1000, Santa Cruz) and  $\alpha$ -TGN46 (Serotec, AHP500G, 1:1000) for lysosomes, early and late endosomes and golgi specific markers, respectively. After washes, coverslips were incubated for 1 h with Alexa Fluor-488 and Alexa Fluor-590 conjugated antibodies (Molecular Probes, 1:1000) and DAPI (1:20,000, Roche) staining. Finally, the sections were washed with PBS, then mounted onto glass slides and cover-slipped. The stained slices were kept at 4°C before analysis with confocal microscopy (Zeiss LSM 780 with 63X Objective).

#### **DAB and Immunofluorescence staining of tissue**

For immunohistochemistry, mice were anesthetized by an intraperitoneal injection of a combination of ketamine (150 mg/kg), xylazine (10 mg/kg) and acepromazine (10 mg/kg) then perfused intra-cardiacally with PBS followed by paraformaldehyde (4%) before collecting the brains. Paraformaldehyde-fixed brains were embedded in paraffin and cut on a microtome in 8  $\mu$ m thick sections (Thermoscientific, France). Brain sections were deparaffined in xylen bath and rehydrated by successive 5 min baths of EtOH (100%, twice), 90%, and then 70%. Antigens were unmasked in a 90% formic acid bath for 5 min and then in citric acid solution at 90-100°C for 20 min (Pressure cooker, Vector Laboratories). For DAB staining, sections were treated with H<sub>2</sub>O<sub>2</sub> 3% for 15 mins. Non-specific binding sites were blocked for 1h in BSA (5%)/Tween20 (0.1%) and brain sections were then incubated at 4 °C overnight with primary antibodies  $\eta$ CTF-Nter rabbit polyclonal (1: 800) or WO2 mouse monoclonal (1:1000). Sections were incubated for 1h with Alexa Fluor-488 and Alexa Fluor-594 conjugated antibodies (1:1000) and DAPI (1:20,000) for immunofluorescence, or with horseradish peroxidase (HRP)-conjugated antibodies (Jackson ImmunoResearch, 1:1000) then revealed with the DAB-ImmPACT system (Vector Laboratories) for DAB-staining. Images were taken with a confocal Leica TCS SP5 microscope (Immunofluorescence) or with DMD108 Leica microsystem (DAB) and processed using ImageJ software. For  $\eta$ CTF-Nter staining, signal was amplified with Avidin Biotin Complex (ABC) method (VECTASTAIN Original ABC Kit, Vector Laboratories).

#### **Purification of exosomes (sEVs)**

SH-APPWT cells were cultivated in DMEM for 24h, rinsed twice with PBS, and treated or not with the  $\beta$ -secretase inhibitor Bi or Bafilomycin A1 as described above, for another 24 h in OptiMEM to allow vesicle secretion. When overexpressing  $\eta$ CTF, cells were transiently transfected 1 day before secretion in OptiMEM. sEVs were purified by ultracentrifugation as previously described<sup>17</sup>. Briefly, media were first harvested and centrifuged at 2000 x g for 20 min, then filtered through a 0.22  $\mu$ m filter. The supernatant was then sequentially centrifuged at 10000 x g for 30 min and 100,000 x g for 125 min. The obtained pellet containing sEVs and contaminating proteins was washed in ice-cold PBS and centrifuged for another 120 min. The final sEVs pellet was resuspended in RIPA buffer and sonicated in an ultrasonic bath for 15 min before western-blot analysis.

sEVs isolated from the extracellular space of AAV- $\eta$ CTF mice brain were purified according to the protocol previously described<sup>17</sup>. Briefly, intracardiac perfusion with PBS was performed then hemibrains were extracted and enzymatically and mechanically lysed using “the adult brain dissociation kit” and a GentleMACS Dissociator (Miltenyi Biotec). PBS was added to the homogenate suspension, which was then filtered through a 70  $\mu$ m Smartstrainer and centrifuged at 300 x g for 10 min. The supernatant was used for sEVs purification and the cell pellet was kept for further analysis by western blot as described above. For sEVs purification, the supernatant was sequentially centrifuged several times as previously described<sup>16</sup>, and the last pellet was loaded on a sucrose gradient and centrifuged at 200000 x g for 16h. The fractions containing the sEVs were collected, diluted with PBS and centrifuged at 100000 x g for 90 min. The final sEVs pellet was resuspended in PBS, lysed in RIPA buffer complemented with protease inhibitors and ultrasonicated before western-blot analysis. Before loading,

nanoparticle analysis was performed on each fraction using the ZetaView instrument (Particle-Metrix) in order to determine particle size distribution and concentration.

### **Membrane Fraction Preparation**

Dissected hippocampi of 3xTgAD (AD) and wild-type (WT) mice were homogenized in hypotonic buffer (HEPES (5mM), pH 7.4, EDTA (1 mM), sucrose (0.25 M) containing a proteases inhibitor cocktail). The homogenate was centrifuged at 1000 x g for 5 min at 4°C and the supernatant was centrifuged at 100000 x g for 1h. Membrane pellets were then solubilized in RIPA buffer complemented with protease inhibitor, and centrifuged at 100000 x g for 20 min. Supernatants were recovered as the soluble membrane fractions and loaded on 16% tris-tricine gels.

### **Statistical Analyses**

Statistical analyses were performed using Prism software (GraphPad Prism 7). Quantitative data are represented as means  $\pm$  SEM and subjected to non-parametric tests such as the Mann Whitney test for single comparisons and the Tukey one-way ANOVA test for multiple comparisons. Statistical significance code is: \*\*\*\*p<0.0001 and \* p<0.05.

## **Results**

### **Proteasomal and autophagic degradation of $\eta$ CTF in human SH-SY5Y neuroblastoma cells.**

A newly described  $\eta$ -secretase cleavage occurs on APP<sub>695</sub> between residues 504 and 505, yielding a 95kDa soluble fragment (sAPP $\eta$  or sAPP95) and a 25-30kDa transmembrane fragment called  $\eta$ CTF, which is subsequently cleaved by  $\alpha$ - or  $\beta$ -secretases to generate A $\eta\alpha$  and A $\eta\beta$  peptides, respectively (Fig. 1a). In order to understand the biology and fate of the  $\eta$ CTF fragment, we expressed it in human neuroblastoma cells (SH-SY5Y) and analyzed its expression by western-blot using APP-Cter and the WO2 antibodies targeting, the carboxy terminal end of APP and the amino-terminal part of A $\beta$  peptides, respectively (Fig. 1a). In SH-SY5Y cells transiently transfected with the  $\eta$ CTF-pcDNA coding vector, a specific band around 30kDa is detected by both APP-Cter and WO2 that displays a similar migrating pathway than recombinant  $\eta$ CTF (Fig. 1b, c), thus firmly supporting the identity of  $\eta$ CTF. As expected, in C99-transfected SH-SY5Y cells, C99 is also recognized by these two antibodies (Fig. 1b, c) while APP-Cter antibody, but not WO2, detects endogenous C83 (Fig. 1b). In both C99 and  $\eta$ CTF overexpressing cells, the C83 levels are increased as compared to control cells, indicating that both of these fragments undergo an  $\alpha$ -secretase-mediated cleavage (Fig. 1b). Similarly, in  $\eta$ CTF overexpressing cells, the C99 levels are increased as compared to control cells indicating that  $\eta$ CTF also behaved as a  $\beta$ -secretase substrate (Fig. 1b). Of note, as expected, WO2 but not APP-Cter recognizes the A $\eta\alpha$  peptide (Fig. 1c). This set of experiments that aimed at characterizing  $\eta$ CTF expression in SH-SY5Y cells indicates that  $\eta$ CTF mostly undergoes  $\alpha$ - and  $\beta$ -secretase cleavages giving rise to C99 and C83 as previously described<sup>7</sup> while the  $\eta$ CTF-derived production of A $\eta\alpha$  remains poorly detectable in cell homogenates.

To uncover the cellular pathways by which  $\eta$ CTF is degraded, we used a pharmacological approach and treated  $\eta$ CTF expressing SH-SY5Y cells with several well characterized inhibitors targeting either proteasomal or autophagic degradative processes. All proteasome inhibitors namely, lactacystin, epoxomicin or MG132 lead to significant increases of  $\eta$ CTF expression (Fig. 2a, b), thus demonstrating the involvement of the proteasomal machinery in  $\eta$ CTF degradation in SH-SY5Y cells. In addition, two lines of evidences indicated significant additional contribution of the autophagic pathway. First,  $\eta$ CTF immunoreactivity is increased by the potent and selective autophagy blocker bafilomycin A1 (Fig. 2c, d). Second,  $\eta$ CTF expression is decreased by the activator

of autophagy SMER28 (Fig. 2c, d). Inhibition of both proteasome and autophagy also leads to an accumulation of endogenous and  $\eta$ CTF-derived C99 and C83 fragments (Fig. 2a, c), which have already been described to be degraded by these two protein degradation systems<sup>13,18-21</sup>. Overall, both proteasomal and autophagic pathways contribute to  $\eta$ CTF degradation overexpressed in human neuronal cells. However, since the increase in endogenous C83 expression is more importantly obtained by bafilomycin A1 treatment (Fig. 2c), this suggests that the autophagic process likely remains the main degradative process of endogenous APP-CTF. Accordingly, C83, C99 and a band corresponding to  $\eta$ CTF are also increased following bafilomycin A1 treatment of SH-SY5Y cells stably expressing wild-type APP protein (SH-APPWT, Sup Fig. 1a).

We aimed at characterizing the influence of secretases inhibitors on  $\eta$ CTF processing but since in native SH-SY5Y cells, it is difficult to detect endogenous  $\eta$ CTF immunoreactivity even after blockade of the proteasomal and autophagic pathways (Fig. 2a, c), we used SH-APPWT cells and treated them with  $\alpha$ -,  $\beta$ - or  $\gamma$ -secretase inhibitors. We analyzed all APP-Cter fragments produced using APP-Cter, WO2 and 82E1 antibodies, the latter targeting specifically the free N-terminal of C99, and thus, is unable to detect  $\eta$ CTF. As expected, the levels of C83 and C99 are reduced upon  $\alpha$ - and  $\beta$ -secretase inhibitions by Gi and Bi, respectively, while both C83 and C99 immunoreactivities were increased when  $\gamma$ -secretase is blocked by its selective and potent inhibitor D6 (Sup Fig. 1b). Interestingly, a band migrating around 30kDa detected by WO2 but not 82E1 antibody is noticeably increased following  $\beta$ -secretase inhibition in pcDNA<sub>3</sub>-transfected cells (Sup Fig. 1b, pcDNA3 lane Bi, see long exposures) while, as expected,  $\beta$ -secretase inhibition fully abolishes APPWT-derived C99 formation detected by both antibodies. This 30kDa band increased by Bi and bafilomycin A1 treatments in pcDNA<sub>3</sub> mock-transfected cells appears with a similar migrating pathway than specific  $\eta$ CTF-like immunoreactivity obtained in  $\eta$ CTF-expressing SH-APPWT cells and thus might correspond to the  $\eta$ CTF fragment derived from the stably expressed wild-type APP protein (Sup Fig. 1a, b). Of note, none of the secretase inhibitors significantly affected the level of  $\eta$ CTF in  $\eta$ CTF-transfected SH-APPWT-cells (Sup Fig. 1b, c).

#### **Expression of $\eta$ CTF in fibroblasts devoid of APP leads to A $\eta$ and A $\beta$ productions.**

APP-CTF fragments (C99 and C83) may theoretically derive from both APP-full length or  $\eta$ CTF in SH-SY5Y cells (see above). In order to delineate those genuinely derived from  $\eta$ CTF, we expressed this fragment in mouse embryonic fibroblasts devoid of endogenous APP and its family members APLP1 and APLP2 (MEF APP KO, Fig. 3a). As expected,  $\eta$ CTF is readily expressed in fibroblasts and detected by both the APP-Cter and the WO2 antibodies (Fig. 3a). We treated the  $\eta$ CTF expressing APP KO fibroblasts with secretase inhibitors and examined the A $\eta$  and A $\beta$  productions. Although  $\alpha$ -,  $\beta$ - or  $\gamma$ -secretase inhibitions do not significantly modulate  $\eta$ CTF expression in MEF APP KO (Fig. 3b), we provide evidence that  $\eta$ CTF is indeed processed by  $\alpha$ -  $\beta$ - and  $\gamma$ -secretase activities. First,  $\eta$ CTF expression in MEF APP KO leads to the production of an APP-Cter- but not WO2-immunoreactive fragment corresponding to C83 (compare lower panels in Fig. 3a). Second, we specifically immunoprecipitate a secreted fragment labeled by WO2 which is fully sensitive to the  $\alpha$ -secretase inhibitor Gi, thus corresponding to A $\eta$  (Fig. 3c). Third, for the first time, we were able to measure by ELISA an increased level of A $\beta$ 40 in  $\eta$ CTF expressing APP KO fibroblasts compared to pcDNA3 control fibroblasts, and as expected, we observed a drastic decrease of A $\beta$ 40 upon cells treatment with either  $\beta$ - or  $\gamma$ -secretase inhibitors (Fig. 3d). Although A $\beta$  production indicates that  $\eta$ CTF is processed by  $\beta$ -secretase, in our conditions, we were unable to detect C99, suggesting that this fragment is either poorly generated or, alternatively, rapidly degraded or further processed into C83 as was demonstrated in other cell models<sup>11,17</sup>. Taken together, our results demonstrate that

$\eta$ CTF expressed in APP KO fibroblasts is indeed cleaved by  $\alpha$ -  $\beta$ - and  $\gamma$ -secretases to yield C83, A $\eta\alpha$  and A $\eta\beta$  peptides as major detectable catabolites.

#### **Subcellular localization of $\eta$ CTF in trans-Golgi network and in endosomes.**

Although the immunological tools available, combined to migration pattern and molecular weight analysis, allow us to firmly identify  $\eta$ CTF *in vitro* by western blot, these antibodies do not allow a definitive identification of the fragment *in situ* and thus, have strong limitations for immunohistochemical analysis. In this context, we aimed at designing a new antibody referred to as  $\eta$ CTF-Nter that would specifically label the N-terminus of  $\eta$ CTF (Fig 4 a). By western blot analysis, we show that this new antibody recognizes not only  $\eta$ CTF, but also, as expected, the  $\eta$ CTF N-terminal sequences corresponding to A $\eta\alpha$  and A $\eta\beta$  peptides (Fig. 4b). In addition, similar increase in  $\eta$ CTF-Nter-like immunoreactivity was obtained in SH-SY5Y treated with proteasomal/autophagic inhibitor (Sup Fig 2) validating the specificity of this band as well as the *in vitro* usefulness of  $\eta$ CTF-Nter. Interestingly, C83 and C99 fragments are not recognized by  $\eta$ CTF-Nter (Fig. 4b) allowing us to get rid of these CTFs and to analyze specifically the subcellular localization of  $\eta$ CTF fragment in HeLa cells transiently transfected with  $\eta$ CTF cDNA. The  $\eta$ CTF-Nter-like immunoreactivity obtained using the new  $\eta$ CTF-Nter antibody is found mainly intracellular (Fig. 4c). and as expected, this specific  $\eta$ CTF staining co-localizes with a part of the APP-Cter-like immunoreactivity that likely corresponds to  $\eta$ -CTF, C83 and C99 (Fig. 4d). Similarly, the  $\eta$ CTF-Nter labeling partly overlaps with WO2-like immunoreactivity (Sup Fig. 3).

To further identify the intracellular compartments in which  $\eta$ CTF-Nter-like immunoreactivity is detectable, we performed co-immunostaining using antibodies labeling different organelles. We found that  $\eta$ CTF co-localizes with TGN-46, which is a marker of the Golgi apparatus and trans-Golgi network in HeLa cells (Fig. 5a). In addition, a small but significant amount of  $\eta$ CTF-immunoreactivity is observed in EEA1- and CD63-positive structures corresponding to early and late endosomal compartments, respectively (Fig. 5b) while little if any co-staining was observed with the lysosomal marker Lamp2 (Fig. 5c, d).

A similar localization of  $\eta$ CTF was observed in wild-type mouse organotypic hippocampal slices infected with an adeno-associated-virus (AAV) expressing  $\eta$ CTF (Sup Fig. 4). We confirmed the presence of  $\eta$ CTF-Nter-like immunoreactivity in EEA1-positive endosomal compartments and observed a characteristic perinuclear trans-Golgi staining and a strong overlap with APP-Cter labeling (Sup. Fig. 4). It is of note that the antibody does not raise significant background even *in situ*.

Overall, the above set of data demonstrates the usefulness of our novel immunological probe to follow  $\eta$ CTF expression in various models expressing  $\eta$ CTF fragment in both *in vitro* (HeLa cells) and *ex vivo* (organotypic mouse hippocampal slices) and leads to the consistent conclusion of a main Golgi and endosomal localizations of the  $\eta$ CTF fragment.

The specific  $\eta$ CTF-staining was also examined *in vivo*, in brain slices of mice expressing  $\eta$ CTF through a previously described AAV-viral strategy<sup>13</sup>. Western-blot analysis of  $\eta$ CTF expression (Fig. 6a) and immunohistochemical assessment with  $\eta$ -CTF-Nter (Fig. 6b c) revealed that  $\eta$ CTF is highly expressed after viral infection in brain homogenate (Fig. 6a) and is specifically detected *in situ* in cortex and hippocampus of AAV- $\eta$ CTF mice (Fig. 6b c).

#### **Detection of $\eta$ CTF fragment in small extracellular vesicles (sEVs).**

The presence of the  $\eta$ CTF fragment in endosomes led us to investigate whether  $\eta$ CTF fragment could be found in exosomes which originate from endosomes. These small extracellular vesicles (sEVs) are currently envisioned as

organelles mediating toxic spreading in various neurodegenerative diseases<sup>22-24</sup>. Our data show that  $\eta$ CTF fragment is detectable in sEVs isolated from  $\eta$ -CTF-expressing cells and mice. Indeed, we detected  $\eta$ CTF in sEVs purified from both media of  $\eta$ -CTF-expressing SH-APPWT and of  $\beta$ -secretase inhibitor-treated pcDNA<sub>3</sub> SH-APPWT cells (Fig. 7a). As expected, we also observed high recoveries of  $\eta$ CTF in sEVs following bafilomycin A1 treatment which is known to increase exosomal secretion (BafA1 Fig. 7b). Since exosomes contain high levels of  $\alpha$ -secretases (ADAM10 and ADAM17)<sup>25,26</sup> and since  $\eta$ CTF was found to undergo  $\alpha$ -secretase cleavage, sEVs were purified from mouse brains in the presence (or not) of the  $\alpha$ -secretase inhibitor (Gi) in order to enhance  $\eta$ CTF recovery. Our data show that  $\eta$ CTF can be detected in sEVs (with a medium size around 140 nm) purified from mouse brains overexpressing  $\eta$ CTF (Fig. 7c, d). This agrees well with Laulagnier and collaborators who reported the presence of  $\eta$ CTF in exosomes purified from primary cortical neurons or neuroblastoma N2a cells overexpressing APP wild-type<sup>27</sup>.

#### **$\eta$ CTF surrounds amyloid plaques and accumulates in an age-dependent manner in 3xTgAD mouse brains.**

Using the  $\eta$ CTF-Nter antibody, we observed  $\eta$ CTF-like immunoreactivity in the hippocampus of 3xTgAD mice, a widely used AD-mice model<sup>14</sup>. The labeling increased progressively from 3 to 20 months of age (Fig. 8a, lower panels) while only a very faint labeling was observed in the cortex of 20-month-old wild-type mice (Fig. 8a, upper panels). This age-related accumulation of  $\eta$ CTF fragment was confirmed by western-blot using the APP-Cter antibody (Fig. 8b). Interestingly, in the subiculum of 20-month-old 3xTgAD mice, the  $\eta$ -CTF-Nter staining obtained by peroxidase/DAB development appears more punctiform suggesting an aggregated state and is mainly surrounding plaques (Fig. 8a, lower panel). These findings were confirmed by immunofluorescence in double immunostaining experiments with  $\eta$ CTF-Nter and WO2 antibodies. WO2 targeting the N-terminal part of A $\beta$  labeled the core of the extracellular plaque while  $\eta$ CTF-Nter staining surrounded WO2-associated labeling (Fig. 8c). This set of experiments confirms the usefulness of the  $\eta$ CTF-Nter antibody to detect  $\eta$ CTF *in vivo* and indicates that  $\eta$ CTF accumulates at late stage of the pathology around plaques in 3xTg-AD mice.

## **Discussion**

The amyloid hypothesis is strongly supported by a bulk of genetic, anatomical and biochemical evidences but an increasing number of studies suggest that A $\beta$  could likely not account for all cellular and behavioral dysfunctions taking place in AD<sup>28</sup>. The limited progress of A $\beta$ -directed clinical trials suggested that other triggers could well be involved in AD etiology. In this context, in order to reconcile genetic grounds and clinical observations, one could envision additional APP-related fragments, distinct from canonical A $\beta$ , as putative contributors to disease onset and/or progression. Along this line of reasoning, several tracks have been followed including numerous truncated forms of A $\beta$  peptides<sup>29-31</sup> as well as the AICD transcription factor<sup>5</sup>. Further, our laboratory demonstrated that the  $\beta$ -secretase-derived APP fragment C99 could account for early dysfunctions observed in AD pathology<sup>32,33</sup>. More recently, the disintegrin MT5-MMP was identified as a novel secretase named  $\eta$ -secretase<sup>7,8</sup>. This metalloprotease triggers a cleavage of APP upstream to the one due to  $\beta$ -secretase, thereby yielding a fragment referred to as  $\eta$ CTF, the biology of which has been poorly examined yet. In this study, we show data on the processing, fate, subcellular localization and exosomal secretion of  $\eta$ CTF.

In neuronal cells, as is the case for APP,  $\eta$ CTF undergoes same  $\alpha$ - and  $\beta$ - secretases cleavages yielding the C-terminal fragments C83 and C99 and their N-terminal counterparts A $\eta\alpha$  and A $\eta\beta$ , respectively. Of interest, we report for the first time that  $\eta$ CTF also undergoes  $\gamma$ -secretase-mediated cleavage giving rise to A $\beta$  peptides. This

was definitively demonstrated by our data obtained in APP KO fibroblasts where the expression of  $\eta$ CTF indeed leads to A $\beta$ 40 production. In the latter cellular model,  $\eta$ CTF undergoes proteolysis by  $\alpha$ -secretase as illustrated by C83 accumulation and A $\eta\alpha$  secretion. This  $\alpha$ -secretase can occur at the cellular plasma membrane as previously extensively described<sup>34</sup> but also inside exosomes where the sheddases ADAM10 and ADAM17 (TACE) are enriched and active<sup>25,26</sup>. Indeed, exosomes have been proposed to serve as a platform for ectodomain shedding of a variety of transmembrane proteins such as TNFR1, CD46, CD44 and the L1 adhesion molecule<sup>35</sup>. It should be noted that although  $\beta$ -secretase cleavage takes place on  $\eta$ CTF (since A $\beta$  is produced), C99 remained poorly detectable. This could be explained by different ways that would not be necessarily exclusive: 1)  $\alpha$ -secretase activity is much higher than  $\beta$ -secretase in fibroblasts and thus, C83 is preferentially produced; 2)  $\alpha$ -secretase displays a much higher affinity for  $\eta$ CTF than  $\beta$ -secretase; 3) a small proportion of whole  $\eta$ CTF meets active  $\beta$ -secretase in endosomes, 4) C99 is more rapidly degraded than C83 in fibroblasts; 5) C99 is produced but itself rapidly converted into C83 as we previously showed<sup>11,17</sup>. Overall, the above set of data indicates that  $\eta$ CTF behaves as a direct precursor of both C99 and A $\beta$  peptide and thus, displays a theoretical toxic potential. This is in agreement with Baranger and colleague's studies that proposed the  $\eta$ -secretase as a new pro-amyloidogenic proteinase<sup>8,9</sup>.

Very little is known about the fate of  $\eta$ CTF and the mechanisms by which this fragment is cleared off the cells. By means of a pharmacological approach, we established that  $\eta$ CTF degradation involves both proteasomal and autophagic pathways. However, the increase of endogenous C83 expression was more importantly triggered by bafilomycin A1 (Figure 2 C) than by proteasome blockers, suggesting that the autophagic process likely remains the main degradative process of endogenous APP-CTF as previously reported<sup>13,19-21,36</sup>. The degradation of  $\eta$ CTF in the autophagic pathway is in agreement with previous report by Wang and collaborators who described  $\eta$ CTF fragment as a new APP-Cter fragment detected in naïves HEK293 cells following cathepsins inhibition<sup>6</sup>. In addition to autophagy, our result demonstrated also a significant degradation of  $\eta$ CTF by the proteasome. However, it should be noted that transient or constitutive ectopic protein expression may lead to mis-localizations and/or artifactual protein aggregation. Thus, when misfolded proteins accumulate in the ER, the unfolded protein response (UPR) and ER-associated degradation (ERAD) mechanism are induced to avoid cellular damages. In line with this warning, Evrard and collaborators have shown that endogenous APP-CTFs are mainly processed by the endosomal/lysosomal pathway while overexpressed C99 was mainly degraded by the proteasome<sup>37</sup>. It could be the reason why in our experimental conditions, overexpressed  $\eta$ CTF is readily degraded by the proteasome. In addition, our results indicate that the  $\eta$ CTF overexpressed either in MEF APP KO fibroblasts or SH-APPWT neuronal cells, is not protected by the inhibition of BACE-1 activity unlike was previously described for the endogenous  $\eta$ CTF<sup>7</sup>. This discrepancy could be also explained by the source of the  $\eta$ CTF fragment (overexpressed vs endogenous)

Although molecular weight estimation and immunological characterization allows clear biochemical identification of  $\eta$ CTF by western-blot, this toolbox falls short when one aims at studying  $\eta$ CTF *in situ*. In this context, we envisioned the design of a novel immunological probe that would discriminate between the plethora of APP-CTFs. Thus, we designed an antibody referred to as  $\eta$ CTF-Nter that labels  $\eta$ CTF N-terminus moiety and thus, would not interact with C83 and C99 or A $\beta$ . This was successfully achieved as illustrated in Figure 4. Thanks to this new  $\eta$ CTF-Nter antibody, immunocytochemical analysis indicated that  $\eta$ CTF is mostly localized in Golgi apparatus and trans-Golgi network in HeLa cells. However, a lower amount of  $\eta$ CTF immunoreactivity is also observed in

endosomes in both  $\eta$ CTF transfected HeLa cells and hippocampal organotypic slices prepared from newborn mice expressing  $\eta$ CTF.

We took advantage of our know-how in the preparation of fully characterized exosomes<sup>17</sup> to examine whether  $\eta$ CTF could be detected in these small extracellular vesicles (sEVs). Indeed, we show the presence of  $\eta$ CTF in sEVs purified from secretates of human neuroblastoma cells overexpressing  $\eta$ CTF. This agrees well with the study by Laulagnier and collaborators who detected  $\eta$ CTF in exosomes secreted by rat cortical neurons expressing endogenous APP protein as well as by mouse N2a cell line overexpressing wild-type APP protein<sup>27</sup>. Of note, we were also able to detect the  $\eta$ CTF fragment derived from wild-type APP protein in sEVs purified from medium of SH-APPWT cells treated with  $\beta$ -secretase inhibitor. Thus, our immunohistochemical analysis of  $\eta$ CTF localization and biochemical analysis of exosomal content in cells all suggest that at least a part of  $\eta$ CTF is transported through endosomes and accumulate in exosomes. Exosomes can be secreted by all cell types such as neurons, oligodendrocytes, astrocytes or microglial cells and have been described to play important physiological and pathological roles in cellular communication as well as in protein aggregates spreading<sup>38</sup>. In AD pathogenesis, Tau protein<sup>39</sup>, A $\beta$  peptides<sup>40,41</sup> and C99 fragment<sup>36,42,43</sup> have been found in multivesicular bodies and exosomes. Our laboratory previously established the presence of C99/C83 oligomers in exosomes and their accumulation is enhanced upon  $\gamma$ -secretase inhibition<sup>17</sup>. Thus, both C99 and its precursor  $\eta$ CTF accumulate similarly in exosomes. Finally, we also documented the usefulness of our novel immunological probe for *in vivo* approaches and demonstrated the presence of  $\eta$ CTF in exosomes purified from brains of mice expressing  $\eta$ CTF. Moreover, we were able to show a high  $\eta$ CTF expression surrounding the core of abundant senile plaques in triple transgenic mice as was also shown in APP/PS1 transgenic mice and in AD brain<sup>7</sup>.

A question arises as to whether  $\eta$ CTF could be considered as a fully toxic trigger in AD or could govern more balanced physiopathological functions. In this regard, similarly to A $\beta$  peptides, C99 or phosphorylated Tau proteins, the presence of  $\eta$ CTF fragments in exosomes could have both beneficial or deleterious consequences. Exosomes carrying  $\eta$ CTF could contribute 1) to eliminate excess of  $\eta$ CTF from the cells; 2) to transport physiological intercellular signals or 3) to be responsible for neuropathological spreading<sup>38</sup>. Nonetheless, the presence of  $\eta$ CTF in exosomes as well as its accumulation and detection around amyloid plaques may support its potential contribution to AD pathology. This postulate is strengthened by the fact that  $\eta$ CTF acts as a direct precursor of C99 that is considered as an early trigger of AD pathology<sup>44</sup>. Therefore  $\eta$ CTF could be a source of intracellular C99/A $\beta$  production but also an indirect way to promote the exosomal C99/A $\beta$  spreading resulting in an extracellular A $\beta$  deposits. In addition, exosomes released from neurons have been described to regulate synaptic activity<sup>45</sup>. Further,  $\eta$ CTF yields A $\eta\alpha$  and A $\eta\beta$ , the influence of which on hippocampal long-term potentiation has led to discrepant conclusions<sup>7,46</sup>. Apparently, A $\eta\alpha$  and A $\eta\beta$  peptides could impair synaptic activity<sup>46</sup>. This adds support to the conclusion that  $\eta$ CTF directly *per se*, or indirectly, as a precursor, could well contribute to AD pathology. Thus preventing  $\eta$ CTF production could be of therapeutic relevance to act at very early stages of AD. This newcomer on disease stage could reconcile, at least to some extent, the numerous failures of clinical trials targeting A $\beta$  with the well supported genetic hypothesis centered on APP.

## **Declarations**

## **Ethics approval:**



All animal experimental procedures were in accordance with the ethical standards of the European Communities Council Directive of 24 November 1986 and approved by the local Institutional Ethics Committee for the Laboratory Animal (CIEPAL-azur) from Nice, the National Council on animal care of the Ministry of Health (APAFIS#9766-201704261624789v3) and the local committee for animal welfare of IPMC institute.

#### **Consent for publication**

All authors read the final version of the article and consent to its publication

#### **Availability of data and materials**

Not applicable. All full gels are provided in Supplementary figure 5

#### **Competing Interests:**

The authors have nothing to disclose and declare no financial conflict of interest.

#### **Fundings**

This work was supported by University hospital Federation (FHU) OncoAge and the LabEx (excellence laboratory) DISTALZ (Development of Innovative Strategies for a Transdisciplinary approach to Alzheimer's disease) which fully provided the PhD funding for EA.

#### **Author's contributions**

FC and RPP designed and supervised the study, analyzed data, and wrote the manuscript. FC supported the project through DISTALZ funding. EA performed most part of experiments. IL supervised exosomal experiments. AB, WEM and ED performed the biological molecular experiment to obtain plasmid constructs and viruses. RPR, AB and WEM participated to virus productions. MC and AV supervised experiments on organotypic hippocampal slices. CB maintained mice colonies. All authors read and approved the final manuscript

#### **Acknowledgements:**

We would like to thank Dr Frank LaFerla for providing the 3xTgAD mice, Paul Fraser for the APP-Ct antibody and Mathilde Cohen-Tannoudji Maria-Grazia Biferi and Martine Barkats for their help in producing AAVs.

#### **References**

- 1 Hardy, J. & Allsop, D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* **12**, 383-388 (1991).
- 2 Selkoe, D. J. & Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* **8**, 595-608, doi:10.15252/emmm.201606210 (2016).
- 3 Cacace, R., Sleegers, K. & Van Broeckhoven, C. Molecular genetics of early-onset Alzheimer's disease revisited. *Alzheimers Dement* **12**, 733-748, doi:10.1016/j.jalz.2016.01.012 (2016).
- 4 Jonsson, T. *et al.* A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* **488**, 96-99, doi:nature11283 [pii]10.1038/nature11283 (2012).
- 5 Pardossi-Piquard, R. & Checler, F. The physiology of the beta-amyloid precursor protein intracellular domain AICD. *J Neurochem* **120 Suppl 1**, 109-124, doi:10.1111/j.1471-4159.2011.07475.x (2012).
- 6 Wang, H. *et al.* Cathepsin L Mediates the Degradation of Novel APP C-Terminal Fragments. *Biochemistry* **54**, 2806-2816, doi:10.1021/acs.biochem.5b00329 (2015).

- 7 Willem, M. *et al.* eta-Secretase processing of APP inhibits neuronal activity in the hippocampus. *Nature*, doi:nature14864 [pii]10.1038/nature14864 (2015).
- 8 Baranger, K. *et al.* MT5-MMP is a new pro-amyloidogenic proteinase that promotes amyloid pathology and cognitive decline in a transgenic mouse model of Alzheimer's disease. *Cell Mol Life Sci* **73**, 217-236, doi:10.1007/s00018-015-1992-1 (2016).
- 9 Baranger, K. *et al.* MT5-MMP Promotes Alzheimer's Pathogenesis in the Frontal Cortex of 5xFAD Mice and APP Trafficking in vitro. *Front Mol Neurosci* **9**, 163, doi:10.3389/fnmol.2016.00163 (2016).
- 10 Dyrks, T. *et al.* Generation of beta A4 from the amyloid protein precursor and fragments thereof. *FEBS Lett* **335**, 89-93, doi:0014-5793(93)80446-2 [pii] (1993).
- 11 Flammang, B. *et al.* Evidence that the Amyloid-beta Protein Precursor Intracellular Domain, AICD, Derives From beta-Secretase-Generated C-Terminal Fragment. *J Alzheimers Dis* **30**, 145-153, doi:571918L966111X8P [pii]10.3233/JAD-2012-112186 (2012).
- 12 Guillot-Sestier, M. V., Sunyach, C., Druon, C., Scarzello, S. & Checler, F. The alpha-secretase-derived N-terminal product of cellular prion, N1, displays neuroprotective function in vitro and in vivo. *J Biol Chem* **284**, 35973-35986, doi:10.1074/jbc.M109.051086 (2009).
- 13 Lauritzen, I. *et al.* Intraneuronal aggregation of the beta-CTF fragment of APP (C99) induces Abeta-independent lysosomal-autophagic pathology. *Acta Neuropathol* **132**, 257-276, doi:10.1007/s00401-016-1577-6 (2016).
- 14 Oddo, S. *et al.* Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron* **39**, 409-421 (2003).
- 15 Heber, S. *et al.* Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. *J Neurosci* **20**, 7951-7963 (2000).
- 16 Oules, B. *et al.* Ryanodine receptor blockade reduces amyloid-beta load and memory impairments in Tg2576 mouse model of Alzheimer disease. *J Neurosci* **32**, 11820-11834, doi:10.1523/JNEUROSCI.0875-12.2012 (2012).
- 17 Lauritzen, I. *et al.* Targeting gamma-secretase triggers the selective enrichment of oligomeric APP-CTFs in brain extracellular vesicles from Alzheimer cell and mouse models. *Transl Neurodegener* **8**, 35, doi:10.1186/s40035-019-0176-6 (2019).
- 18 Nunan, J. *et al.* The C-terminal fragment of the Alzheimer's disease amyloid protein precursor is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur J Biochem* **268**, 5329-5336, doi:ejb2465 [pii] (2001).
- 19 Vingtdeux, V. *et al.* Intracellular pH regulates amyloid precursor protein intracellular domain accumulation. *Neurobiol Dis* (2007).
- 20 Asai, M. *et al.* An alternative metabolic pathway of amyloid precursor protein C-terminal fragments via cathepsin B in a human neuroglioma model. *FASEB J* **25**, 3720-3730, doi:fj.11-182154 [pii]10.1096/fj.11-182154 (2011).
- 21 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* **25**, 1934-1942, doi:fj.10-175158 [pii]10.1096/fj.10-175158 (2011).
- 22 Zhang, T. *et al.* The emerging role of exosomes in Alzheimer's disease. *Ageing Res Rev* **68**, 101321, doi:10.1016/j.arr.2021.101321 (2021).
- 23 Chivet, M. *et al.* Exosomes as a novel way of interneuronal communication. *Biochem Soc Trans* **41**, 241-244, doi:10.1042/BST20120266 (2013).
- 24 Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science* **367**, doi:10.1126/science.aau6977 (2020).
- 25 Kowal, J. *et al.* Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* **113**, E968-977, doi:10.1073/pnas.1521230113 (2016).
- 26 Stoeck, A. *et al.* A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44. *Biochem J* **393**, 609-618, doi:10.1042/BJ20051013 (2006).
- 27 Laulagnier, K. *et al.* Amyloid precursor protein products concentrate in a subset of exosomes specifically endocytosed by neurons. *Cell Mol Life Sci* **75**, 757-773, doi:10.1007/s00018-017-2664-0 (2018).

- 28 Pimplikar, S. W., Nixon, R. A., Robakis, N. K., Shen, J. & Tsai, L. H. Amyloid-independent mechanisms in Alzheimer's disease pathogenesis. *J Neurosci* **30**, 14946-14954, doi:10.1523/JNEUROSCI.4305-10.2010 (2010).
- 29 Dunys, J., Valverde, A. & Checler, F. Are N- and C-terminally truncated Abeta species key pathological triggers in Alzheimer's disease? *J Biol Chem* **293**, 15419-15428, doi:10.1074/jbc.R118.003999 (2018).
- 30 Valverde, A. *et al.* Dipeptidyl peptidase 4 contributes to Alzheimer's disease-like defects in a mouse model and is increased in sporadic Alzheimer's disease brains. *J Biol Chem* **297**, 100963, doi:10.1016/j.jbc.2021.100963 (2021).
- 31 Valverde, A. *et al.* Aminopeptidase A contributes to biochemical, anatomical and cognitive defects in Alzheimer's disease (AD) mouse model and is increased at early stage in sporadic AD brain. *Acta Neuropathol* **141**, 823-839, doi:10.1007/s00401-021-02308-0 (2021).
- 32 Lauritzen, I., Pardossi-Piquard, R., Bourgeois, A., Becot, A. & Checler, F. Does Intraneuronal Accumulation of Carboxyl-terminal Fragments of the Amyloid Precursor Protein Trigger Early Neurotoxicity in Alzheimer's Disease? *Curr Alzheimer Res* **16**, 453-457, doi:10.2174/1567205016666190325092841 (2019).
- 33 Checler, F., Afram, E., Pardossi-Piquard, R. & Lauritzen, I. Is gamma-secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99? *J Biol Chem* **296**, 100489, doi:10.1016/j.jbc.2021.100489 (2021).
- 34 Huovila, A. P., Turner, A. J., Pelto-Huikko, M., Karkkainen, I. & Ortiz, R. M. Shedding light on ADAM metalloproteinases. *Trends Biochem Sci* **30**, 413-422 (2005).
- 35 Keller, S., Sanderson, M. P., Stoeck, A. & Altevogt, P. Exosomes: from biogenesis and secretion to biological function. *Immunol Lett* **107**, 102-108, doi:10.1016/j.imlet.2006.09.005 (2006).
- 36 Vingtdoux, V. *et al.* Alkalinizing drugs induce accumulation of amyloid precursor protein by-products in luminal vesicles of multivesicular bodies. *J Biol Chem* **282**, 18197-18205, doi:10.1074/jbc.M609475200 (2007).
- 37 Evrard, C. *et al.* Contribution of the Endosomal-Lysosomal and Proteasomal Systems in Amyloid-beta Precursor Protein Derived Fragments Processing. *Front Cell Neurosci* **12**, 435, doi:10.3389/fncel.2018.00435 (2018).
- 38 Soliman, H. M. *et al.* Exosomes in Alzheimer's Disease: From Being Pathological Players to Potential Diagnostics and Therapeutics. *Int J Mol Sci* **22**, doi:10.3390/ijms221910794 (2021).
- 39 Saman, S. *et al.* Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J Biol Chem* **287**, 3842-3849, doi:10.1074/jbc.M111.277061 (2012).
- 40 Rajendran, L. *et al.* Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* **103**, 11172-11177, doi:10.1073/pnas.0603838103 (2006).
- 41 Takahashi, R. H. *et al.* Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* **161**, 1869-1879, doi:10.1016/s0002-9440(10)64463-x (2002).
- 42 Sharples, R. A. *et al.* Inhibition of gamma-secretase causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes. *FASEB J* **22**, 1469-1478, doi:10.1096/fj.07-9357com (2008).
- 43 Miranda, A. M. *et al.* Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures. *Nat Commun* **9**, 291, doi:10.1038/s41467-017-02533-w (2018).
- 44 Lauritzen, I. *et al.* The beta-secretase-derived C-terminal fragment of betaAPP, C99, but not Abeta, is a key contributor to early intraneuronal lesions in triple-transgenic mouse hippocampus. *J Neurosci* **32**, 16243-16255a, doi:10.1523/JNEUROSCI.2775-12.2012 (2012).
- 45 Xia, X., Wang, Y., Qin, Y., Zhao, S. & Zheng, J. C. Exosome: A novel neurotransmission modulator or non-canonical neurotransmitter? *Ageing Res Rev* **74**, 101558, doi:10.1016/j.arr.2021.101558 (2022).
- 46 Mensch, M. *et al.* Aeta-alpha and Aeta-beta peptides impair LTP ex vivo within the low nanomolar range and impact neuronal activity in vivo. *Alzheimers Res Ther* **13**, 125, doi:10.1186/s13195-021-00860-1 (2021).

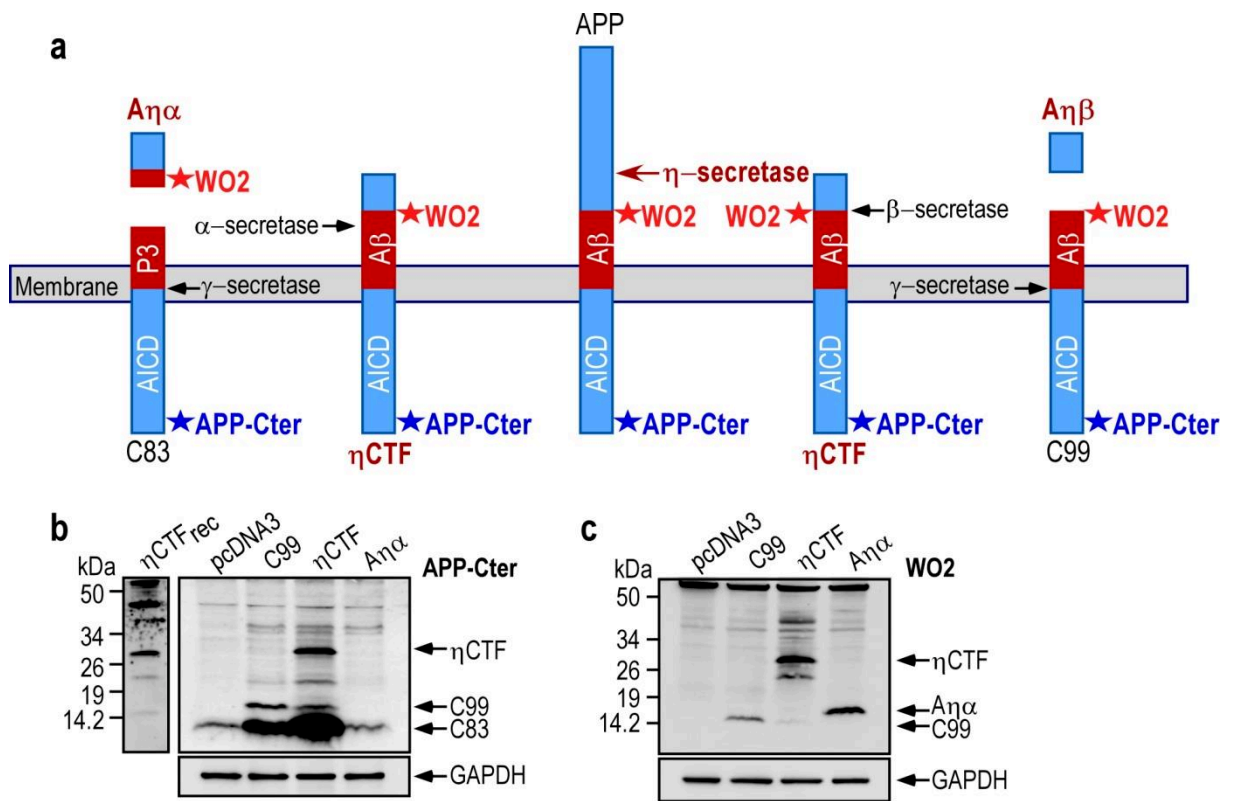


Fig 1

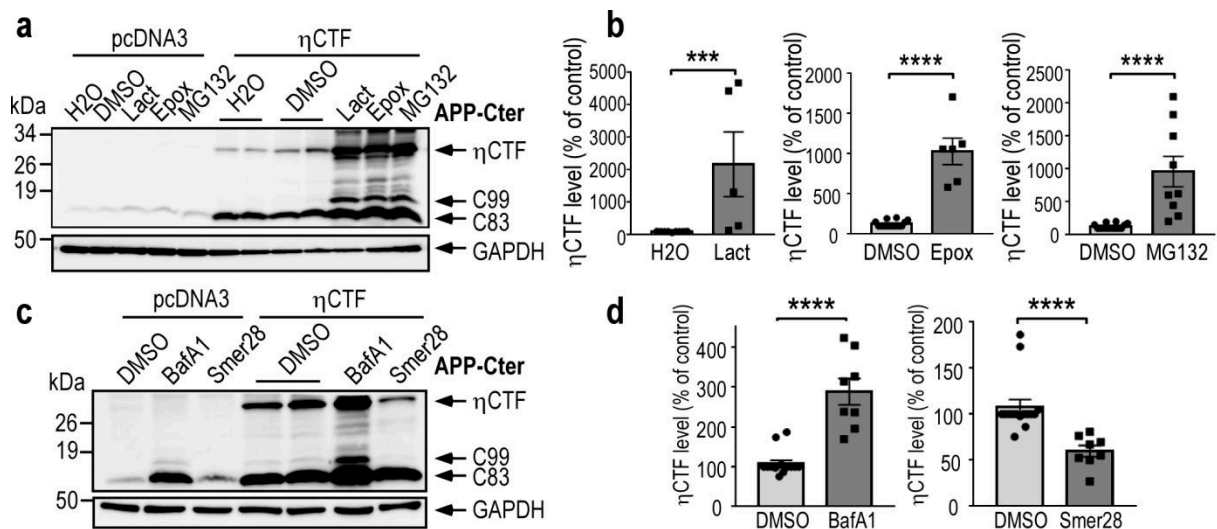


Fig 2

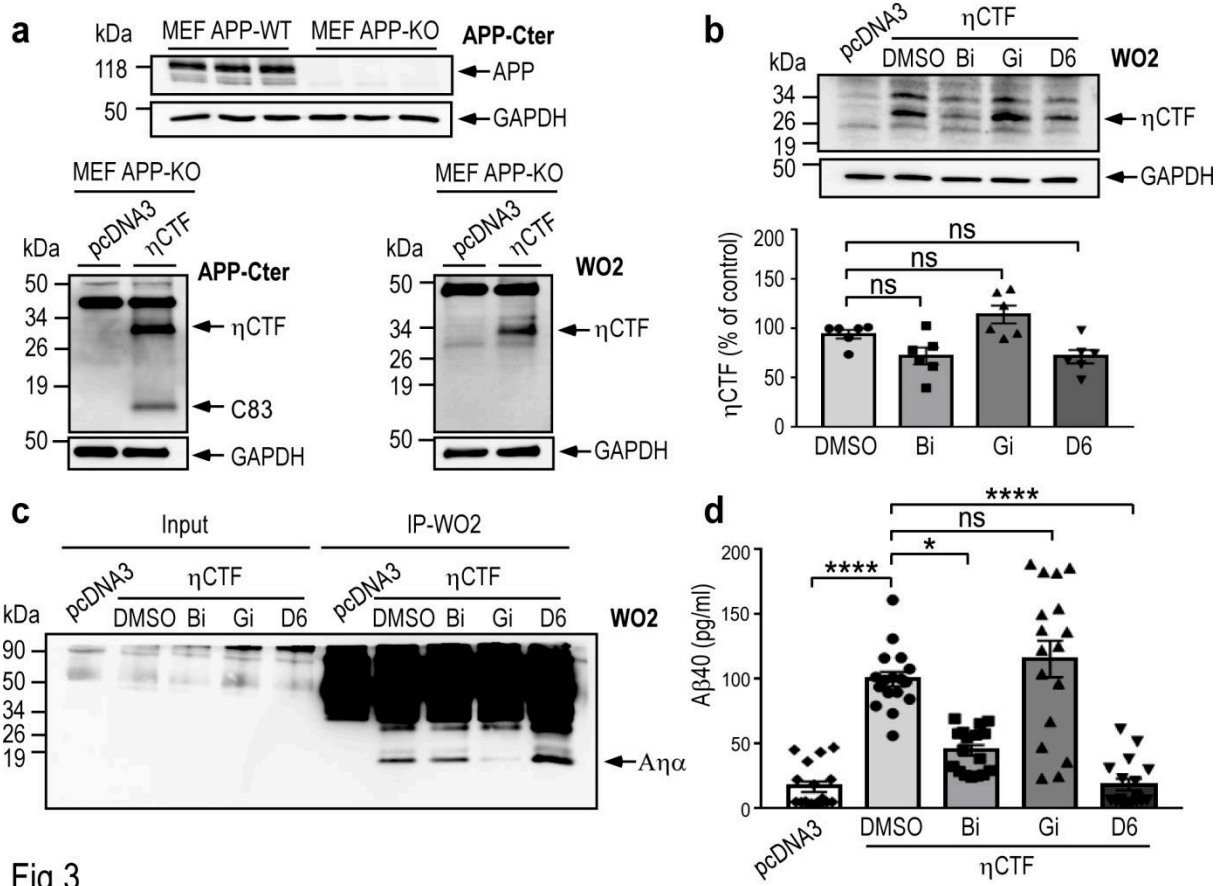


Fig 3

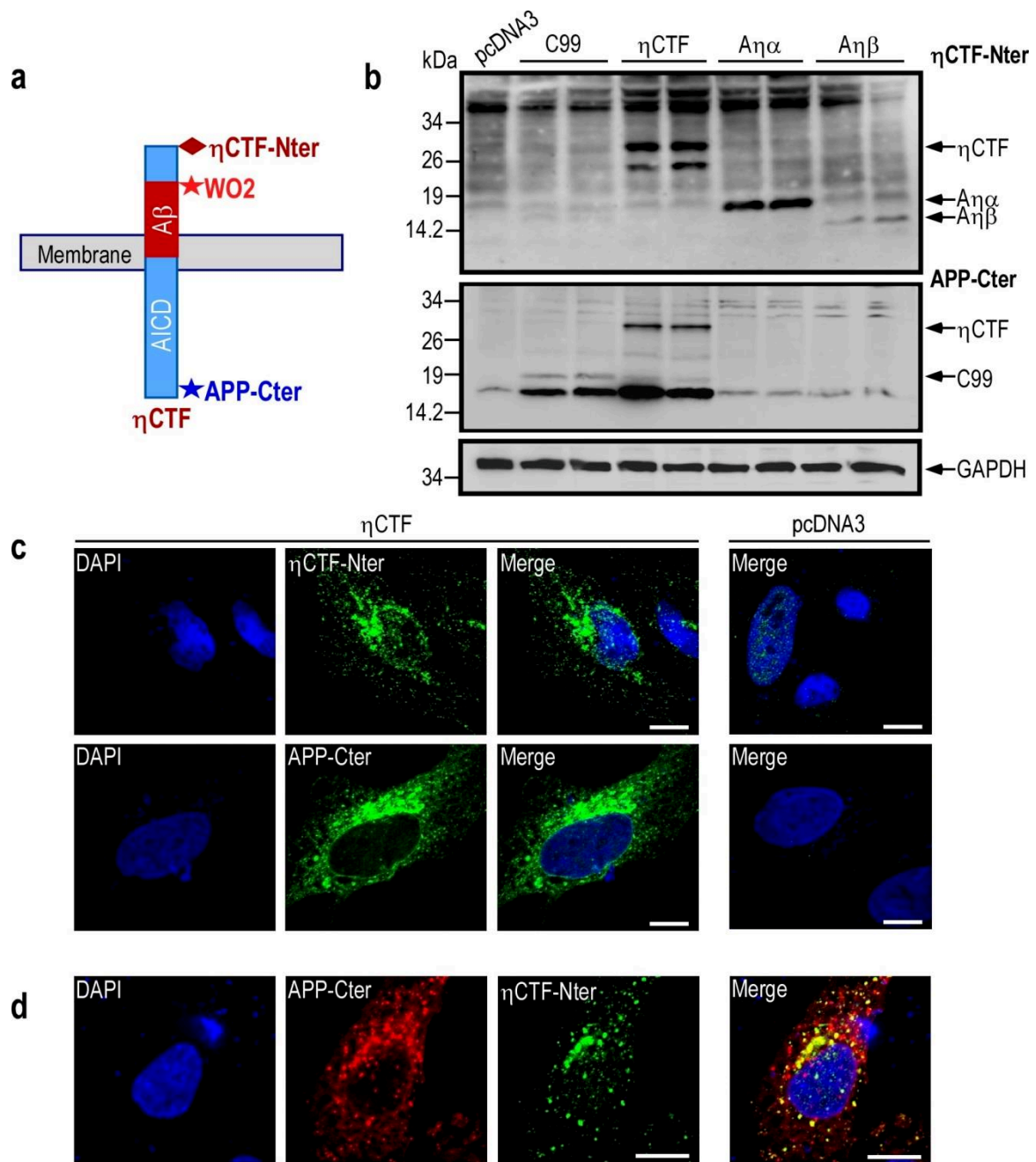


Fig 4

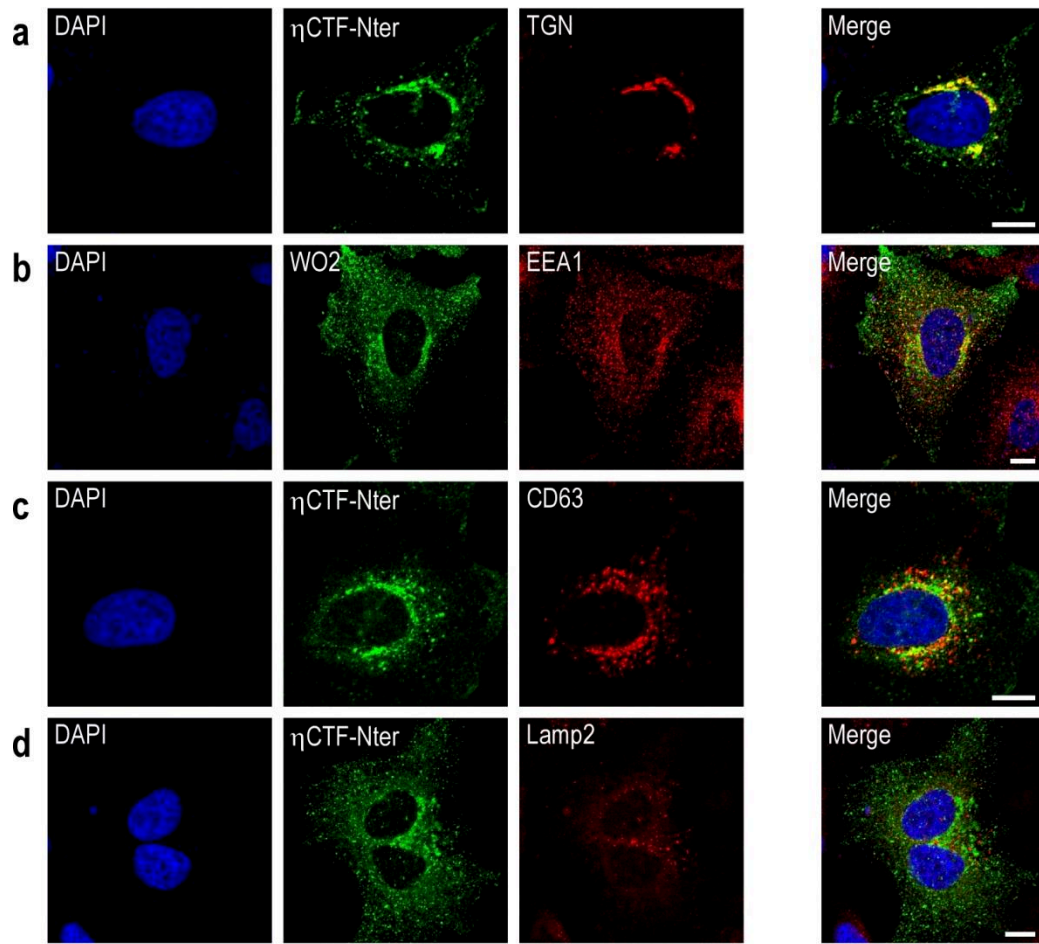
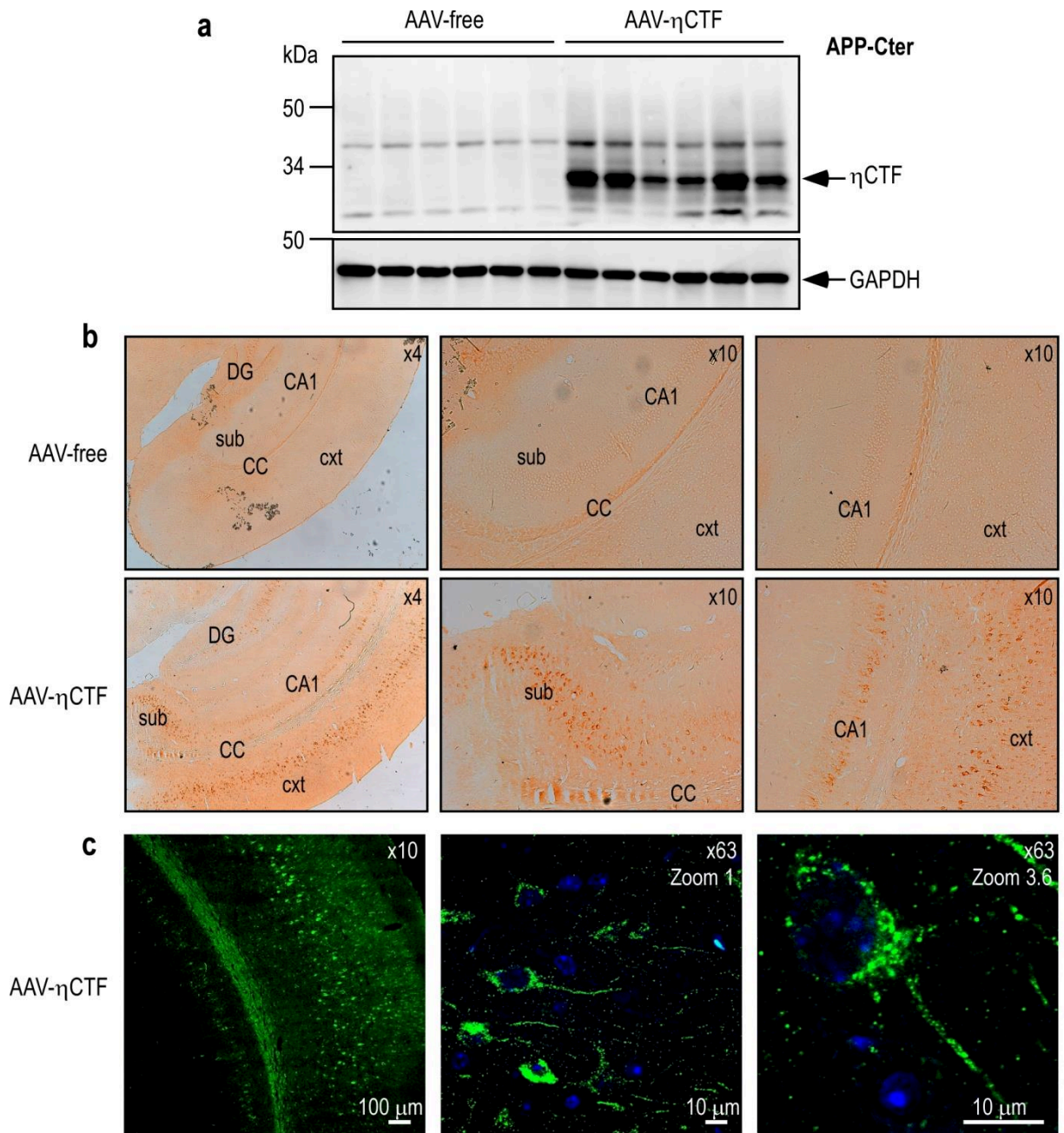
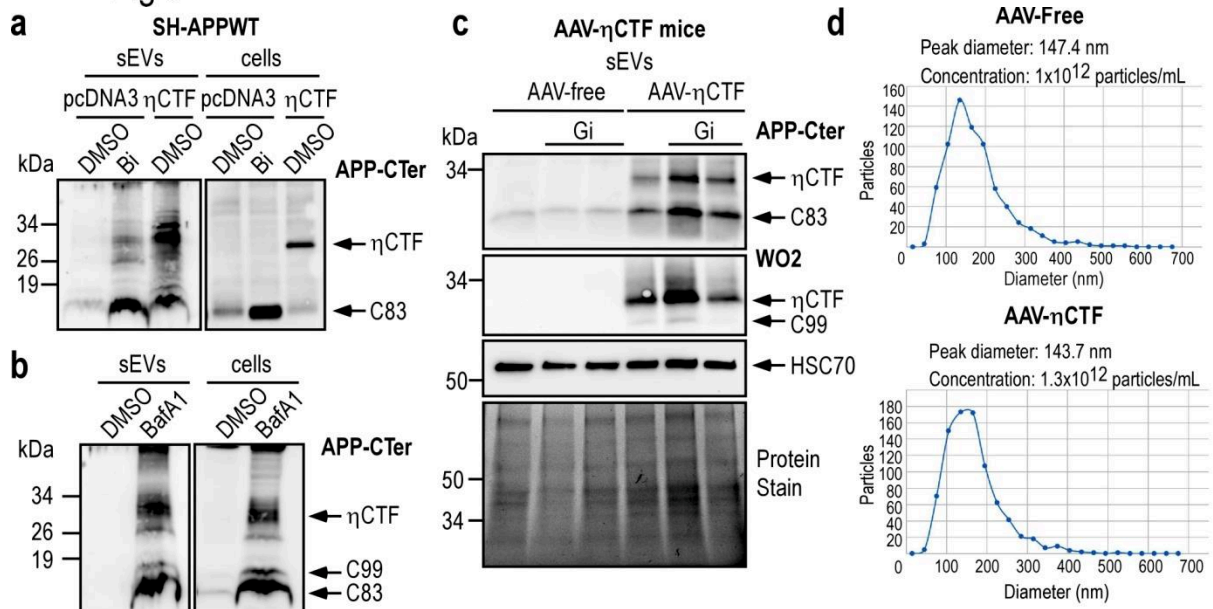


Fig 5



**Fig 6**



**Fig 7**



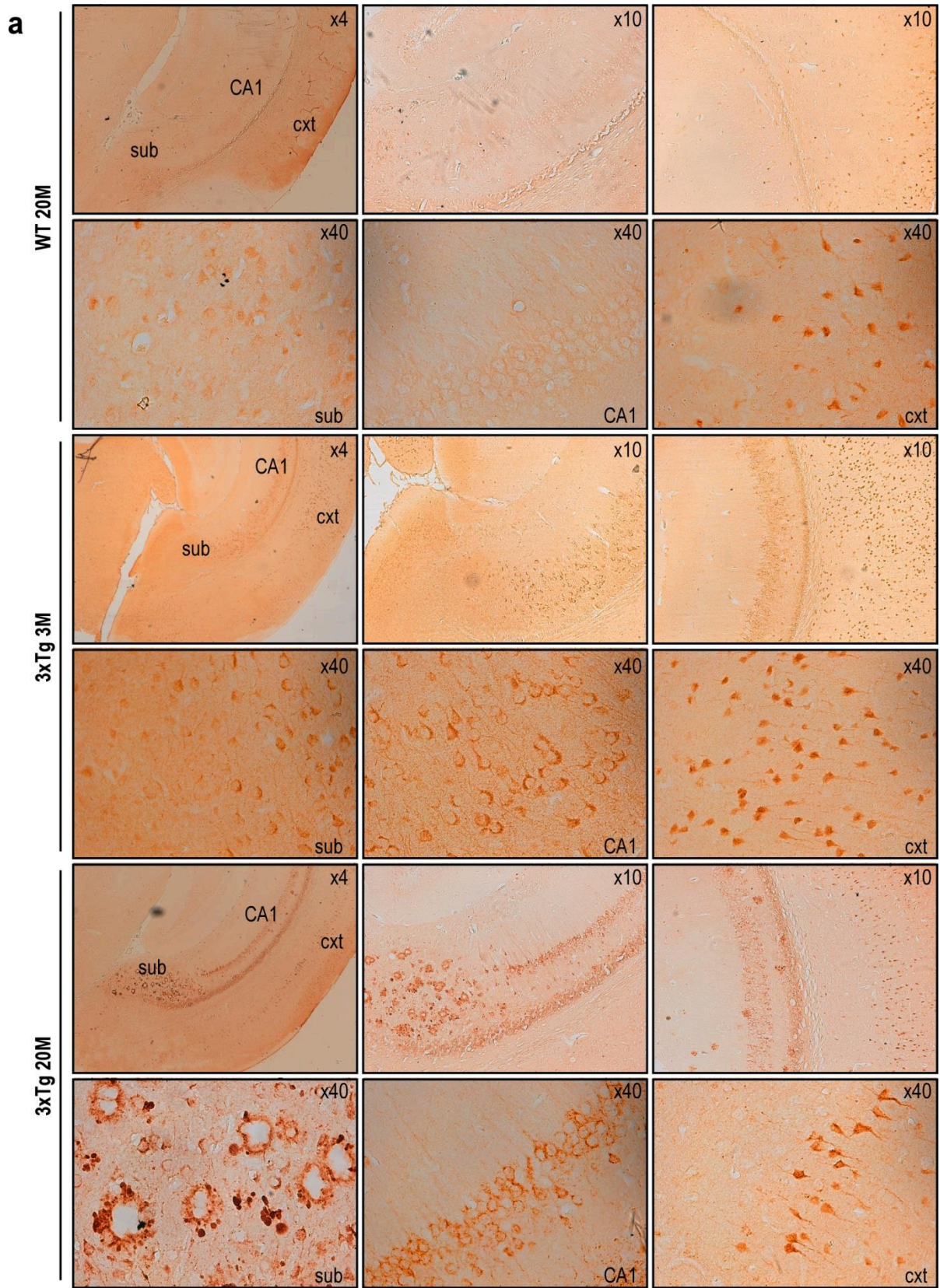


Fig 8

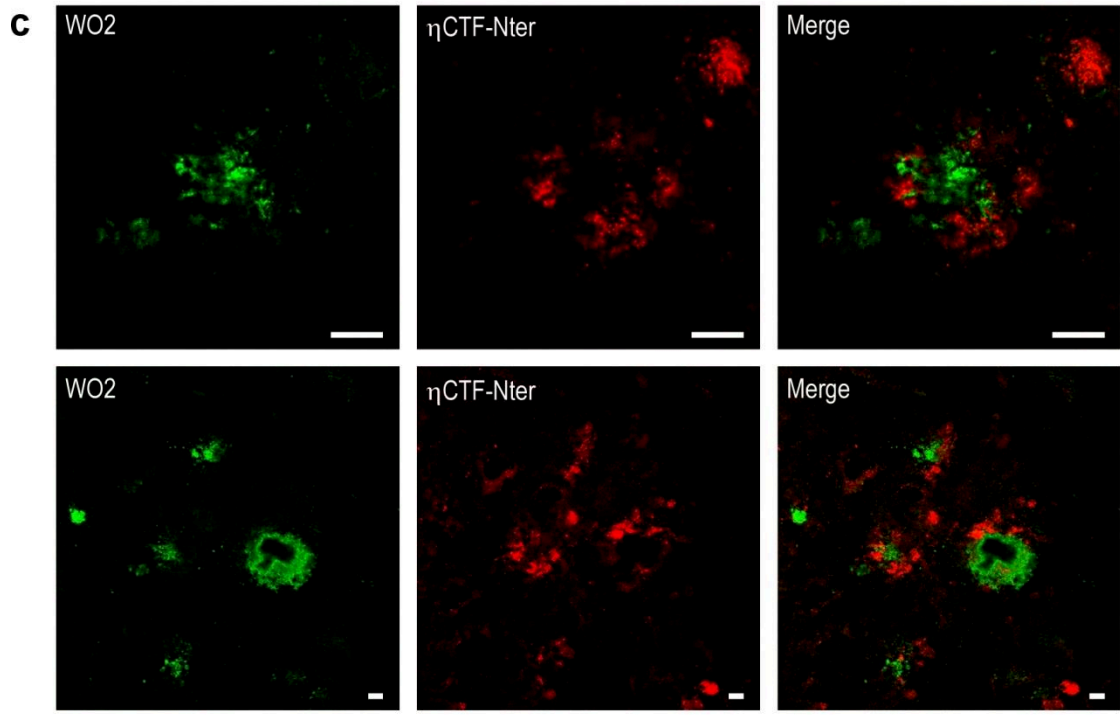
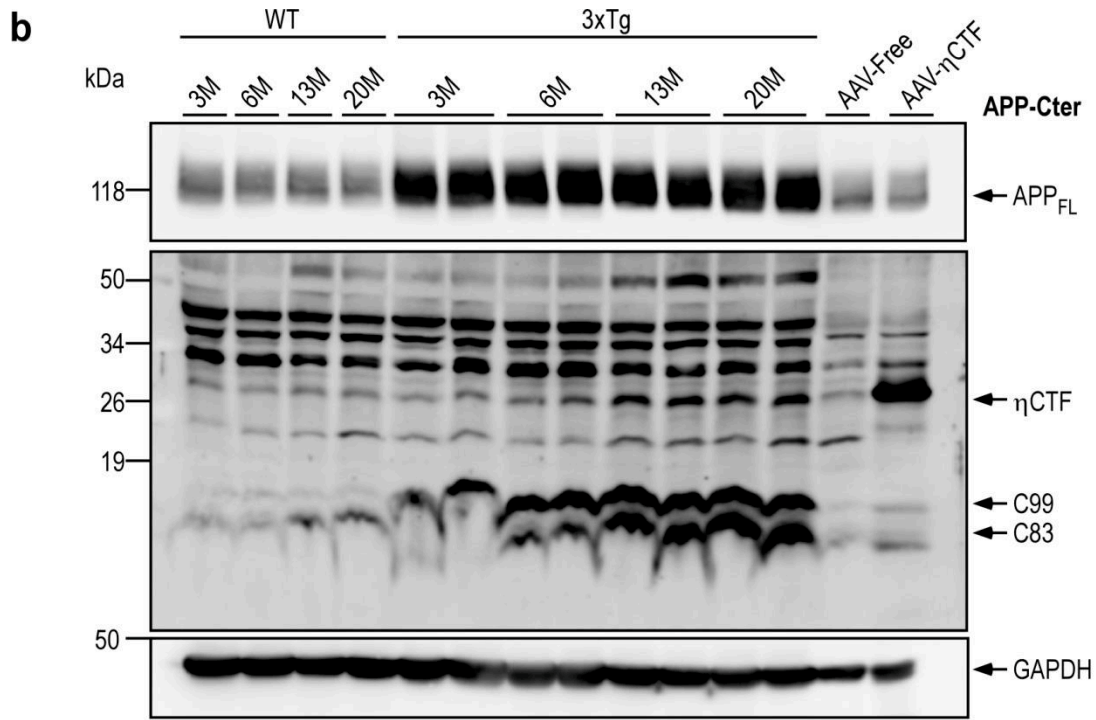


Fig 8 continued

## Legends to Figures

### Fig. 1: Expression and detection of $\eta$ CTF in SH-SY5Y cells.

**a.** Schematic illustration of APP-derived  $\eta$ -secretase-mediated production of  $\eta$ CTF and its subsequent cleavages by indicated secretases. Stars indicate the sites of recognition of proteolytic fragments or peptides by WO2 and APP-Cter antibodies. The  $\eta$ -secretase cleaves APP protein to produce a transmembrane fragment called  $\eta$ CTF that undergoes subsequent cleavages by  $\beta$  and/or  $\alpha$ -secretases yielding  $A\eta\beta$  and  $A\eta\alpha$  respectively. The WO2 antibody targets the amino acid residues 4-10 of  $A\beta$  peptide and recognizes full-length APP,  $\eta$ CTF, C99,  $A\eta\alpha$  but neither C83 nor  $A\eta\beta$ . The APP-Cter antibody is directed toward the last residues of APP protein and recognizes full-length APP,  $\eta$ CTF, C99, C83 but neither  $A\eta\alpha$  nor  $A\eta\beta$ .

**b-c.** SH-SY5Y cells were transiently transfected with C99-,  $\eta$ CTF-,  $A\eta\alpha$ -bearing vectors or empty pcDNA3 vector and analyzed by western-blot using APP-Cter (**b**) or WO2 (**c**) antibodies. A specific band corresponding to  $\eta$ CTF is detected around 30kDa by both APP-Cter and WO2 antibodies. Recombinant  $\eta$ CTF protein ( $\eta$ CTFrec) is used as molecular weight control and GAPDH as loading control.

### Fig. 2: $\eta$ CTF fragment is degraded by both proteasome and autophagic pathways.

**a-d.** SH-SY5Y cells were transiently transfected with  $\eta$ CTF or pcDNA3 vectors and treated for 24h with proteasome inhibitors (**a-b**, lactacystine (Lact, 5 $\mu$ M), epoxomicin (EpoX, 1 $\mu$ M), MG132 (5 $\mu$ M)) or with bafilomycin A1 (BafA1, 100nM) or Smer28 (50 $\mu$ M) that blocks or activates autophagy respectively (**c-d**) then analyzed by western-blot using APP-Cter antibody. Histograms in **b** and **d** correspond to the quantification of  $\eta$ CTF immunoreactivity obtained in **a** and **c** and are expressed as percent of controls (H<sub>2</sub>O or DMSO-treated cells) taken as 100. Bars are the means  $\pm$  SEM of 5 to 9 independent determinations. \*\*\*\*p<0.0001 according to Mann Whitney test.

### Fig. 3: $\eta$ CTF fragment yields both $A\eta\alpha$ and $A\beta$ peptides.

**a.** Wild-type (MEF APPwt) and APP/APLPs-deficient mouse embryonic fibroblasts (MEF APPKO) were transiently transfected with  $\eta$ CTF or pcDNA3 vectors and analyzed by western-blot using APP-Cter and WO2 antibodies. GAPDH is used as loading control.

**b-d.**  $\eta$ CTF transfected MEF APPKO cells were treated for 24h with  $\alpha$ -  $\beta$ - or  $\gamma$ -secretase inhibitors (Gi:10 $\mu$ M, Bi:30 $\mu$ M, D6:1 $\mu$ M) then analyzed by western-blot using WO2 antibody (**b**). GAPDH is used as loading control. Bars correspond to the quantification of  $\eta$ CTF immunoreactivity expressed as percent of controls (DMSO-treated cells) taken as 100 and are the means  $\pm$  S.E.M of 6 independent determinations. Ns, not statistically significant according to the Tukey one-way ANOVA test (**b**).  $A\eta\alpha$  peptides were immunoprecipitated (IP) using WO2 antibody from conditioned medium of MEF APP KO cells expressing or not  $\eta$ CTF and treated with  $\alpha$ -  $\beta$ - or  $\gamma$ -secretase inhibitors. Note that  $A\eta\alpha$  was not detectable in secretates before immunoprecipitation (Input) (**c**).  $A\beta$ 40 levels were measured by ELISA in the conditioned medium of MEF APPKO cells expressing or not  $\eta$ CTF and treated with  $\alpha$ -  $\beta$ - or  $\gamma$ -secretase inhibitors. Bars indicate the concentration of  $A\beta$  in pg/ml and are the means  $\pm$  S.E.M of 17 independent determinations. \*\*\*\*p<0.0001, \* p<0.05, ns: not statistically significant according to the Tukey one-way ANOVA test (**d**).

### Fig. 4: Characterization of a new $\eta$ -CTF-Nter antibody.

- a.** Schematic illustration of antibody epitopes on  $\eta$ CTF fragment. The new  $\eta$ CTF-Nter antibody is directed towards the free N-terminal epitope of  $\eta$ CTF.
- b.** SH-SY5Y cells were transiently transfected with C99,  $\eta$ CTF, A $\eta\alpha$ , A $\eta\beta$  or empty pcDNA3 vectors and analyzed by western-blot using  $\eta$ CTF-Nter and APP-Cter antibodies. GAPDH is used as loading control. Note that as expected,  $\eta$ CTF-Nter antibody recognizes  $\eta$ CTF, A $\eta\alpha$  and A $\eta\beta$  but neither C99 nor C83 while APP-Cter antibody recognizes  $\eta$ CTF, C99 and C83 but neither A $\eta\alpha$  nor A $\eta\beta$ .
- c.** HeLa cells were transiently transfected with  $\eta$ CTF or empty pcDNA3 vector and analyzed by immunofluorescence using  $\eta$ CTF-Nter or APP-Cter antibodies as described in Methods. Note that both staining are mostly perinuclear with punctuate intracellular staining.
- d.**  $\eta$ CTF-transfected HeLa cells were immunostained with APP-Cter (red) and  $\eta$ CTF-Nter (green) antibodies. As expected, a part of the APP-Cter staining co-localized with  $\eta$ CTF-Nter staining (yellow). Nuclei were stained with DAPI. Scale bar are 10 $\mu$ m

**Fig. 5:  $\eta$ CTF fragment is localized in Golgi and endosomes.**

**a-d.** HeLa cells were transiently transfected with  $\eta$ CTF and immunostained with  $\eta$ CTF-Nter or WO2 (green) antibodies for  $\eta$ CTF detection and antibodies directed towards TGN-46 (trans-Golgi apparatus, red, **a**), EEA1 (early endosomes, red, **b**), CD63 (late endosomes, red, **c**) or lamp2 (lysosomes, red, **d**). Note that staining corresponding to  $\eta$ CTF colocalized mostly with TGN-46 and partially with EEA1 and CD63 antibodies (yellow in merge, **d**). Nuclei were stained with DAPI. Scale bar are 10 $\mu$ m

**Fig. 6:  $\eta$ CTF expression and *in situ* localization in AAV- $\eta$ CTF mouse brains.**

**a-c.** Wild-type newborn mice were infected with adeno-associated virus expressing  $\eta$ CTF (AAV- $\eta$ -CTF) or control empty vector (AAV-free) by intra-cerebro-ventricular (ICV) injection then sacrificed at 3-month-old. Brains were dissected and homogenized for membrane protein purification then analyzed in western-blot using APP-Cter antibody. A specific band corresponding to  $\eta$ CTF is detected around 30kDa. GAPDH is used as loading control (**a**).

Brain sections were immunostained with  $\eta$ CTF-Nter antibody and revealed by horseradish peroxidase DAB (**b**) or by immunofluorescence (**c**). Brain regions are depicted as cortex (cxt), corpus callosum (CC), subiculum (sub), hippocampal CA1 region (CA1) and dentate gyrus (DG). Specific  $\eta$ CTF-Nter immunostaining occurs in cortex, subiculum and hippocampus. Confocal images obtained with  $\eta$ CTF-Nter antibody showed a perinuclear with punctuate intracellular staining (**e**). Nuclei were stained with DAPI.

**Fig. 7:  $\eta$ CTF fragment is detected in sEVs purified from cells and mouse brains.**

**a-b.** SH-APPWT cells were transiently transfected with  $\eta$ CTF or empty pcDNA3 vector and treated for 24h with  $\beta$ -secretase inhibitor (**a**, Bi, 30 $\mu$ M), or bafilomycin A1 (**b**, BafA1, 100nM). Cell lysates and sEVs were purified from culture media as described in methods and analyzed by western-blot using APP-Cter antibody.

**c.** sEVs were purified from brain homogenates of 3-month-old AAV-free and AAV- $\eta$ CTF mice in the presence or not of the  $\alpha$ -secretase inhibitor (Gi :10 $\mu$ M) and analyzed by western-blot using APP-Cter antibody. HSC70 is used as an exosomal marker. Whole loaded proteins were stained by photoactivation using Bio-Rad prestain method (Protein Stain) as loading control.

**d.** Concentration and particles size of each brain mouse exosomal purified samples were analyzed in ZetaView instrument (Particle-Metrix) before loading on gels.

**Fig. 8:  $\eta$ CTF fragment accumulates in 3xTgAD brains.**

**a-c.** Brains of wild-type (WT) and triple transgenic (3xTg) females were analyzed at 3-, 6, 13- and 20-month- old by immunohistology (**a**), western-blot (**b**) or immunofluorescence (**c**). DAB-immunohistochemical staining is obtained using  $\eta$ CTF-Nter antibody as described in the Methods. Higher magnification reveals an intracellular labeling clearly observed in cortex, subiculum and hippocampus of 3xTg mice while a weak staining was detected only in the cortex of wild-type mice. In subiculum of 20-month-old 3xTg mice, an extracellular staining is observed around amyloid plaques (**a**). Brains of wild-type (WT), 3xTg, AAV-free and AAV- $\eta$ CTF were homogenized for membrane protein preparation then analyzed in western-blot using  $\alpha$ -APP-Cter antibody. A specific band corresponding to  $\eta$ CTF is detected around 30kDa in wild-type (WT) mice and accumulates in 3xTg mice. GAPDH is used as loading control (**b**). Confocal images obtained with WO2 (green) and  $\eta$ -CTF-Nter (red) antibodies and merged images from 20-month-old 3xTg hippocampus revealed WO2-positive core plaques surrounded by an  $\eta$ CTF-Nter-like immunoreactivity (**c**).

## Supplementary Materials and Methods

### Organotypic hippocampal slices preparation, culture and immunostaining

Organotypic hippocampal slices were obtained from postnatal day 5-7 C57BL6JRj mice as previously described (1). Briefly, hippocampi were sliced into 400  $\mu\text{m}$  sections and carefully transferred on sterile hydrophilic membrane millicell discs (Millipore, FHLCO1300) placed in semiporous cell culture inserts (Millipore, 0.4  $\mu\text{m}$ ) within a 6-well plate, balanced with the warm culture medium (Minimum Essential Medium Eagle (MEM)+Glutamax-1 (50%), EBSS (18%), EBSS (13%)/D-glucose (5%), penicillin–streptomycin (5000U/ml, 1%), Horse serum (25%) and Nystatin 10000U/ml (0.06%). Slices were infected 2 h after plating with adeno-associated viruses AAV-free or AAV- $\eta\text{CTF}$ :  $5 \times 10^{12}$  vg/ml). Slices were incubated at 37°C, 5% CO<sub>2</sub> and half of the medium was replaced every 3 days. After 9 days in culture, slices were fixed for 20 min in paraformaldehyde 4% then stored at 4°C in PBS and kept for immunostaining.

For immunostaining, organotypic hippocampal slices were permeabilized overnight in PBS Triton (0.4%), blocked with BSA (5%)/Tween20 (0.1%), then incubated with primary antibodies:  $\alpha\text{-APPct}$ , mouse monoclonal (1:1000, Biolegend) or  $\eta\text{CTF-Nter}$  (1:800) for  $\eta\text{CTF}$  detection or/and  $\alpha\text{-EEA1}$  goat polyclonal (1:1000) for early endosomes labelling. After washes, coverslips were incubated with Alexa Fluor-488, Alexa Fluor-590 and Alexa Fluor-647 conjugated antibodies (1:1000) and DAPI (1: 20,000) staining. Finally, the sections were washed with PBS, then mounted onto glass slides and cover-slipped. The stained slices were kept at 4°C before analysis with confocal microscopy (Zeiss LSM 780 with 63X Objective).

### Supplementary legends to figures

#### **Sup Fig 1: Autophagy and $\beta$ -secretase inhibitions enhance $\eta\text{CTF}$ level in SH-APPWT cells.**

**a-c.** SH-APPWT cells were transiently transfected with  $\eta\text{CTF-pcDNA}_3$  or empty  $\text{pcDNA}_3$  vector and treated for 24h with bafilomycin A1 (BafA1, 100nM) or Smer28 (50 $\mu\text{M}$ ) or with  $\alpha$ -  $\beta$ - or  $\gamma$ -secretase inhibitors (Gi:10 $\mu\text{M}$ , Bi:30 $\mu\text{M}$ , D6:1 $\mu\text{M}$ ), then analyzed by western-blot using APP-Cter, WO2 or 82E1 antibodies where indicated. GAPDH is used as loading control. Histograms in **c** correspond to the quantification of  $\eta\text{CTF}$  immunoreactivity obtained in **b** in  $\eta\text{CTF}$ -expressing SH-APPWT. Values are expressed as percentage of DMSO-treated cells (control) taken as 100, and are the means  $\pm$  S.E.M of 11 independent determinations. ns: not statistically significant according to the Tukey one-way ANOVA test. When probed with WO2 and 82E1, the same blots are revealed following short and long exposure (exp). Note that in mock SH-APPWT cells, a band migrating around 30kDa was detected with APP-Cter and WO2 antibodies following bafilomycin A1 treatment (**a**) and  $\beta$ -secretase inhibition (**b**) following long exposure. All full gels are provided in Sup Fig 5.

#### **Sup Fig. 2: $\eta\text{CTF-Nter}$ -like immunoreactivity is recovered upon both proteasome and autophagy inhibitions.**

**a-b.** SH-SY5Y cells were transiently transfected with  $\eta\text{CTF}$  or  $\text{pcDNA}_3$  vector and treated for 24h with proteasome inhibitors (**a**, lactacystine (Lact, 5 $\mu\text{M}$ ), epoxomicin (Epox, 1 $\mu\text{M}$ ), MG132 (5 $\mu\text{M}$ )), with a lysosomal inhibitor (E-64D) or with bafilomycin A1 (BafA1, 100nM) or Smer28 (50 $\mu\text{M}$ ) that blocks or activates autophagy respectively (**b**) then analyzed by western-blot using  $\eta\text{CTF-Nter}$  antibody.

**Sup Fig 3:  $\eta$ CTF Staining obtained with  $\eta$ CTF-Nter antibody colocalized with WO2 staining.**

**a-b.** HeLa cells were transiently transfected with  $\eta$ CTF-pcDNA3 and analyzed by immunofluorescence using WO2 alone (**a**) or in combination with  $\eta$ -CTF-Nter (**b**) as described in the Methods. Merges (**a-b**, right panels) show no overlap between WO2 and DAPI (**a**) while, as expected, part of the WO2 staining co-localized with  $\eta$ -CTF-Nter staining (**b**). Nuclei were stained with DAPI.

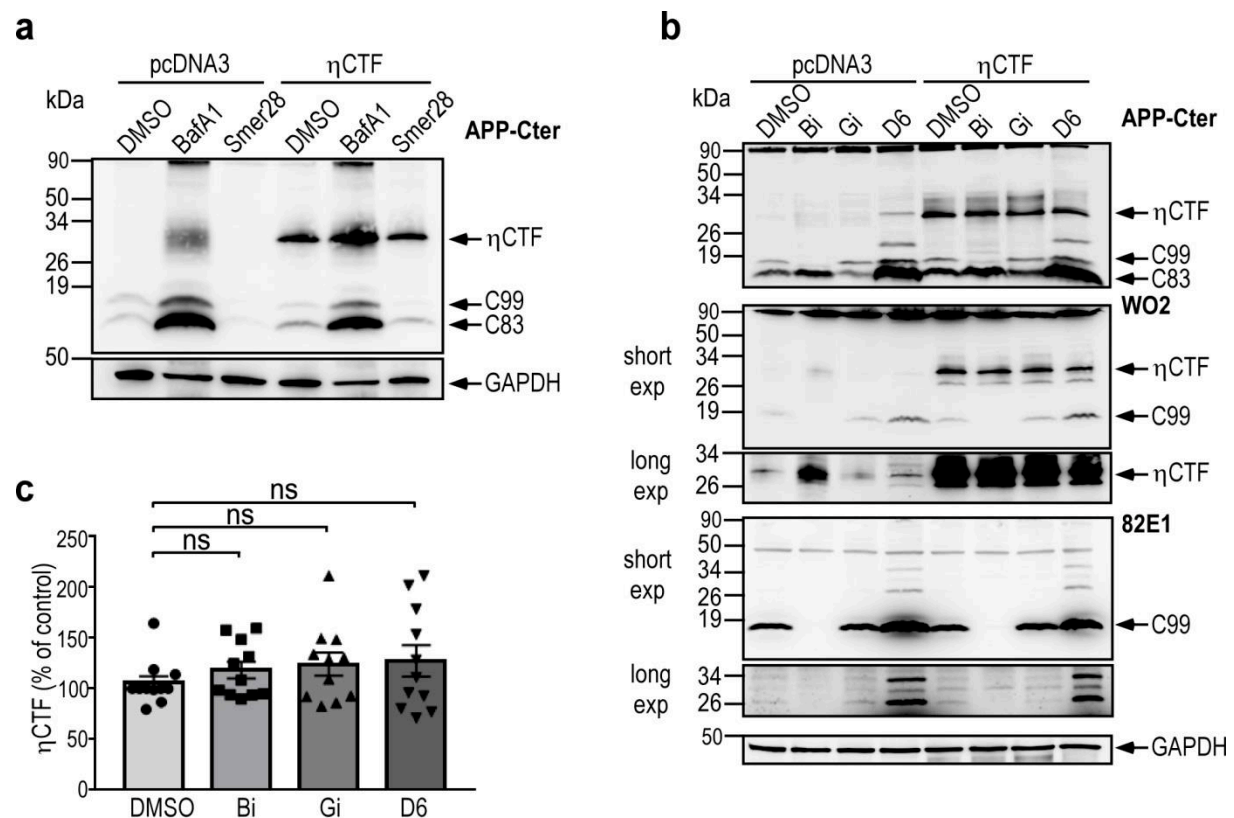
**Sup Fig 4:  $\eta$ -CTF fragment is localized in early-endosomes in organotypic hippocampal slices.**

**a-b.** Organotypic hippocampal slices prepared from wild-type mice as described in supplementary Methods, were infected with AAV-free or AAV- $\eta$ CTF viruses ( $5 \times 10^{12}$  vg/ml) and analyzed by immunofluorescence using  $\eta$ CTF-Nter (green) in combination with APP-Cter (red in **a**) or  $\alpha$ -EEA1 (red in **b**) antibodies as an early endosomal compartment marker. Merge images indicate that  $\eta$ CTF-Nter staining colocalized with APP-Cter staining (**a**, right panel) as well as with EEA1 staining (**b**, right panel).

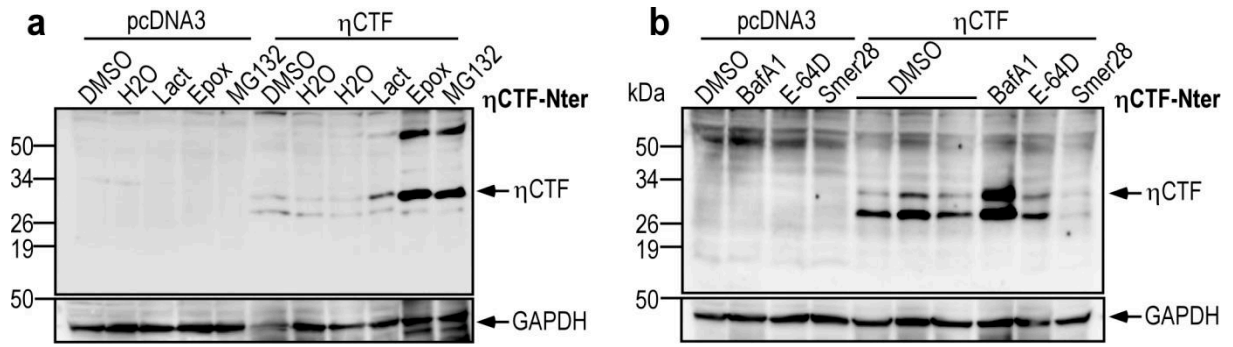
**Sup Fig 5: Full gels of each western-blot from all figures are provided**

**Supplementary Reference**

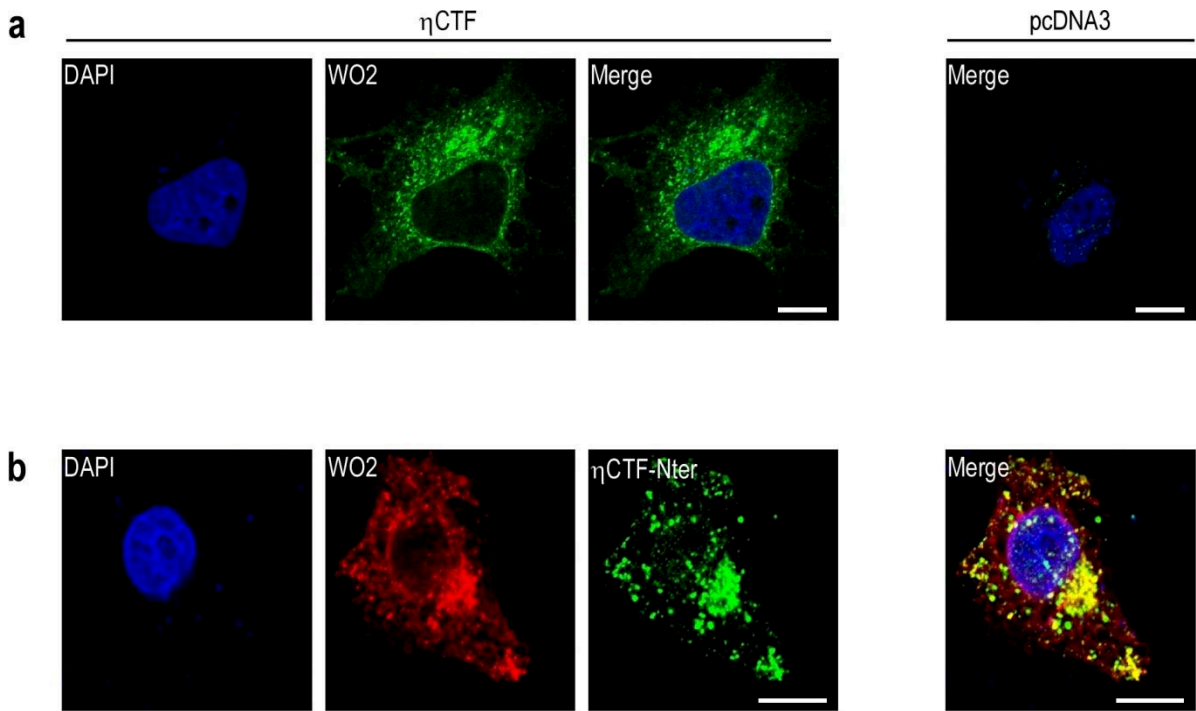
1. Valverde, A., Dunys, J., Lorivel, T., Debayle, D., Gay, A. S., Caillava, C., Chami, M., and Checler, F. (2021) Dipeptidyl peptidase 4 contributes to Alzheimer's disease-like defects in a mouse model and is increased in sporadic Alzheimer's disease brains. *J Biol Chem* **297**, 100963



Sup Fig 1

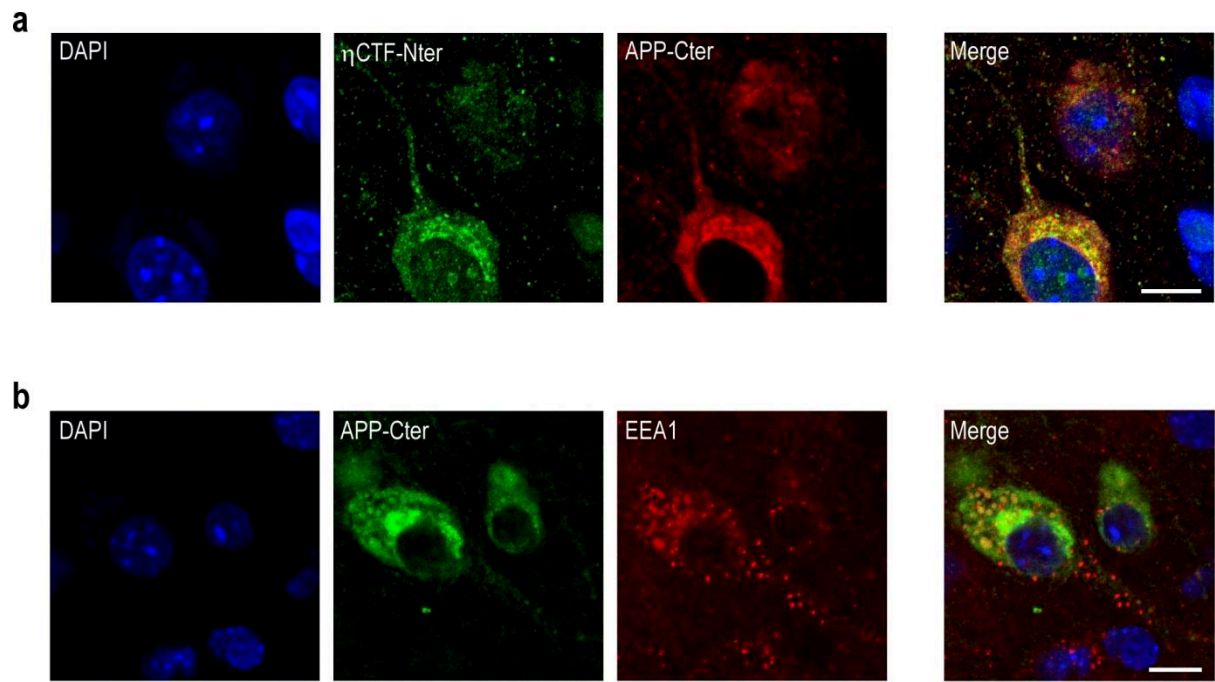


Sup Fig 2



Sup Fig 3





Sup Fig 4

## 2) Additional investigations on the detection of $\eta$ CTF in human AD patients and functional implication of $A\eta\alpha$ and $A\eta\beta$ on synaptic activity

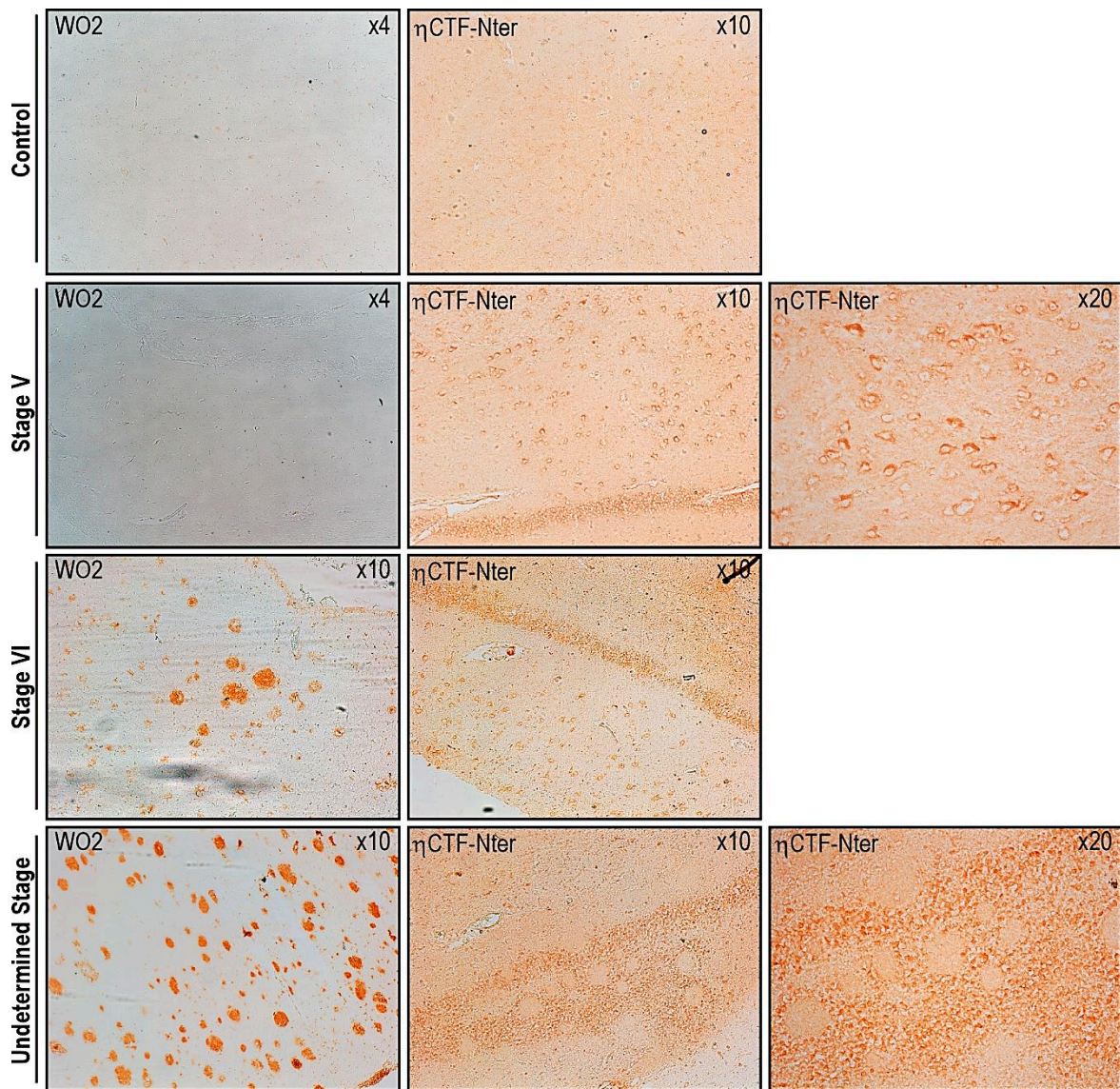
---

### 2.1. Investigations on the detection of $\eta$ CTF in human AD patients

---

After developing and characterizing the  $\eta$ CTF-NTer antibody and providing convincing findings in AD mouse models, it was time to try to detect  $\eta$ CTF in AD patients. Therefore, in the aim of detecting  $\eta$ CTF in humans, Western-blot analyses from patient samples and an immunostaining experiment on paraffin-embedded brain slices of AD patients were performed. Although the results from the human samples could have been included in the paper, we preferred to test more samples first, in order to reduce variability and ensure the reliability of the findings.

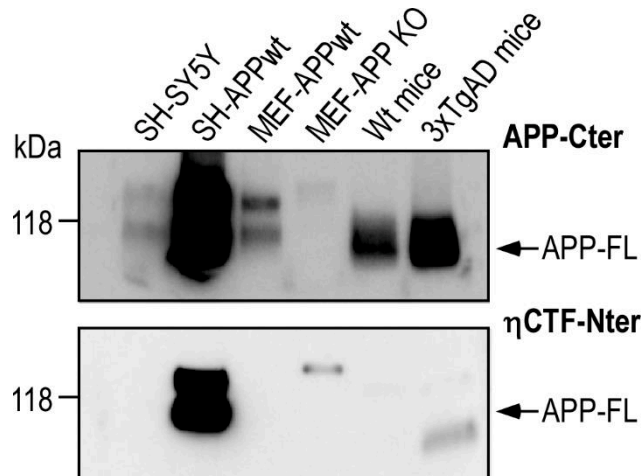
Using the  $\eta$ CTF-NTer antibody for peroxidase/DAB immunostaining, we observe an intracellular  $\eta$ CTF-like immunoreactivity in the hippocampus of AD patients, whereas no labeling is observed in the control patient (**Figure 17**). I examined three controls and six AD patients for this experiment, and only the most representative images are presented in the figure.  $\eta$ CTF fragment doesn't seem to accumulate neither in a stage-dependent nor in a plaque-dependent manner (**Figure 17**, second and third lane of panels). Interestingly, in one patient (CHE in **TABLE 2**) with an undetermined stage of AD,  $\eta$ CTF-NTer antibody appears to label cells that are surrounding the plaques, but not the core of the plaques (**Figure 17**, lower panels). This patient appears to be at an advanced stage of AD; however, the  $\eta$ CTF-NTer staining does not appear to be aggregated around plaques as seen in the AD mouse model (Figure 8 of the article), and the labeling looks more likely to be similar to an APP-full length (APP-FL) staining.



**Figure 17:  $\eta$ CTF-Nter immunostaining in human AD hippocampi.**

The hippocampi of control and AD patients at different braak NFT stages (patients' information in **Table 2**) were analyzed by DAB-immunohistochemical staining and obtained using  $\eta$ CTF-Nter antibody to detect  $\eta$ CTF, or WO2 for the detection of amyloid plaques.

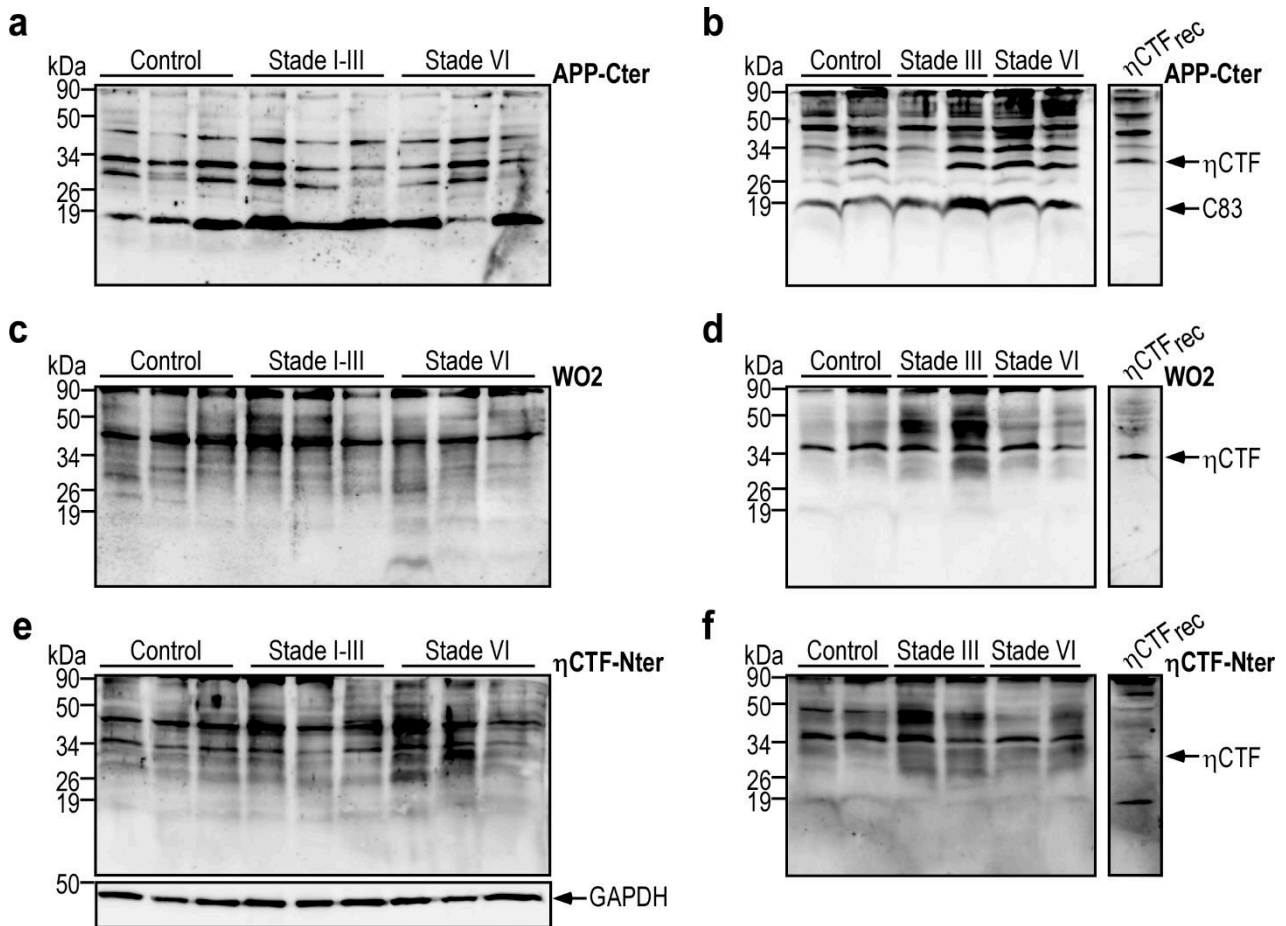
In order to determine whether the  $\eta$ CTF-Nter antibody could recognize APP-FL, we compared the detection of APP-FL in different models by western-blot using APP-Cter and the  $\eta$ CTF-Nter antibodies. As expected, APP-Cter antibody recognizes the endogenous APP-FL in SH-SY5Y and MEF-APPwt cells and in the wild-type mouse model, as well as overexpressed human APP-FL in SH-APPwt, and in 3xTgAD mouse models; while no signal is observed in MEF APP KO since APP is knocked out (**Figure 18**). Strikingly, the  $\eta$ CTF-Nter antibody does also recognize the human APP, but only when its overexpressed, as in the SH-APPwt human cell model and the 3xTgAD mouse model (**Figure 18**). This antibody, on the other hand, does not detect endogenous APP due to its lower sensitivity compared to APP-Cter, nor does it detect murine APP.



**Figure 18 : The detection of human APP-FL in different cell and mouse models, using ηCTF-Nter antibody.**

Brains of wild-type (WT), and 3xTg mice were homogenized for membrane protein preparation then analyzed in western-blot along with SH-SY5Y, SH APPwt, MEF APPwt and MEF APP KO cells for the detection of APP full length (APP-FL), using APP-Cter and ηCTF-Nter antibodies.

By western-blot, we aimed at detecting the ηCTF fragment in brains of AD patients at various Braak NFT stages, compared to control patients, in two separate experiments with samples from different patients. Using the APP-Cter antibody, a band at approximately 30kDa is identified, but with a slightly different migratory pathway than the ηCTFrec (**Figure 19-a, b**). While this band does not appear to accumulate in a stage-related manner (**Figure 19-a, b**), it also does not correspond to the ηCTF fragment, as it is not detected by WO2 (**Figure 19-c, d**) or the ηCTF-Nter antibody (**Figure 19-e, f**). Moreover, the fragment migrating below 19kDa corresponds to C83 since it is recognized by APP-Cter (**Figure 19-a, b**), but not by WO2 (**Figure 19-c, d**) nor by ηCTF-Nter (**Figure 19-e, f**); and as expected, its expression appears to be more important in AD patients in comparison with the controls (**Figure 19-a, b**). Therefore, in contrast to data from Willem and colleagues, in our preliminary experiments, ηCTF seems to remain undetectable in human brains, and this could be explained by different ways: 1) ηCTF is not abundantly produced; 2) ηCTF is directly degraded by the proteasomal and autophagic pathways; or 3) ηCTF is rapidly cleaved by the secretases giving rise to its derived fragments, which is in accordance with the presence and the accumulation of C83 in AD brains (**Figure 19-a, b**).



**Figure 19:  $\eta$ CTF is not detected in AD brains.** Brains of control and AD patients at different Braak NFT stages (patients' information in **TABLE 2**) were analyzed by western blot using APP-Cter (a,b), WO2 (c,d), or  $\eta$ CTF-Nter (e,f) antibodies. GAPDH is used as loading control.

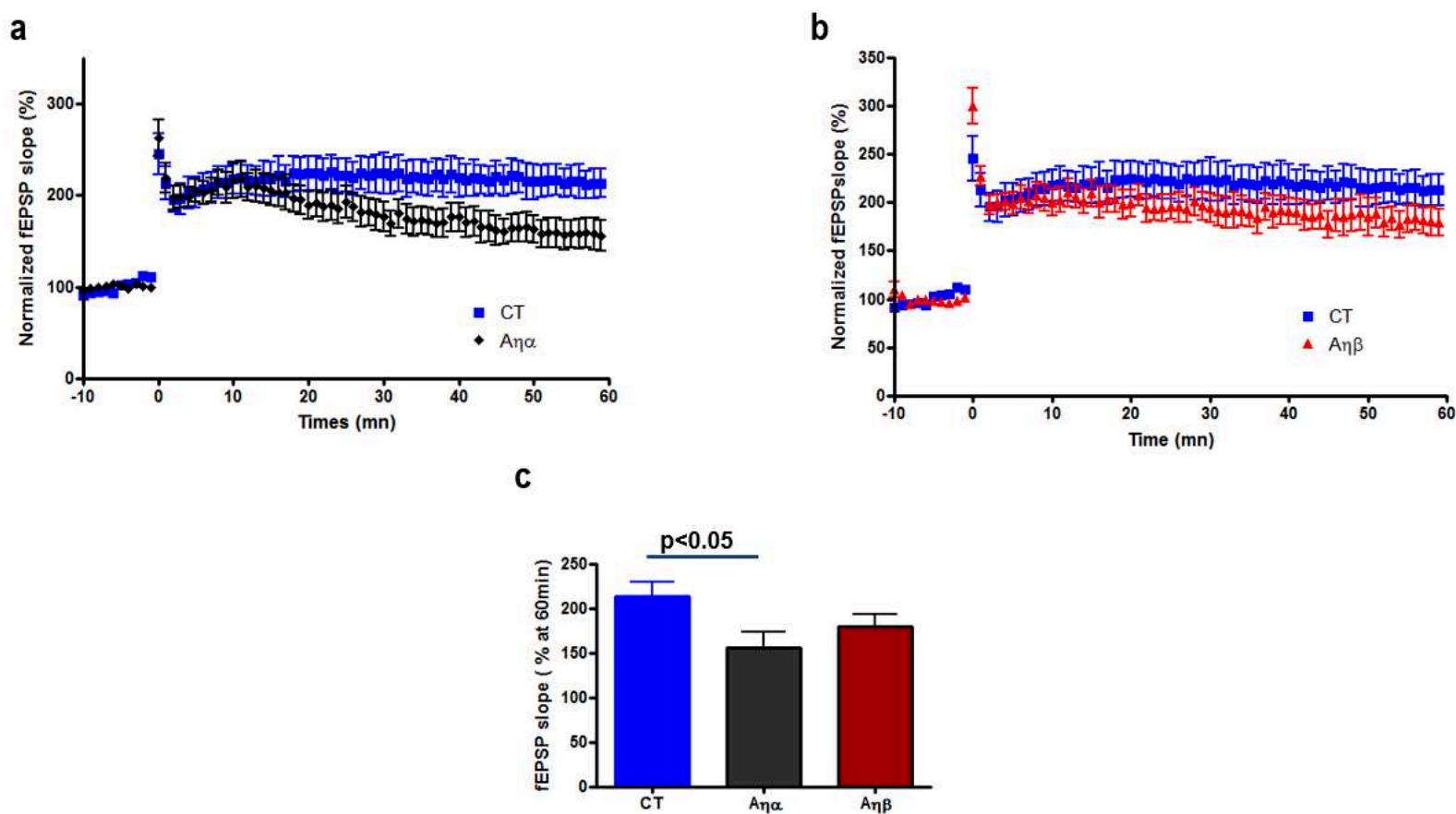
## 2.2. The functional implication of $A\eta\alpha$ and $A\eta\beta$ on synaptic activity

After determining the fate, biology, and localization of  $\eta$ CTF, it was time to move on to examining the role of the fragments released from the cleavage of APP by  $\eta$ -secretase as a first step toward the understanding of the implications of this pathway in AD. Therefore, we were interested in performing preliminary LTP experiments on wild-type mice hippocampal slices incubated with  $A\eta\alpha$  and  $A\eta\beta$ .

$A\eta\alpha$ , but not  $A\eta\beta$ , was the first to be proven to suppress LTP after acute exposure of hippocampal slices to 100 nM this peptide (Willem et al., 2015). However, a more recent study conducted by the same team reported that both synthetic  $A\eta\alpha$  and  $A\eta\beta$  impair LTP *ex vivo*, even at low nanomolar concentrations of 5 nM (Mensch et al., 2021). While these findings are contradictory, we demonstrate in our preliminary tests that the recombinant  $A\eta\alpha$  significantly reduce LTP when incubated to hippocampal slices of C57Bl/6 mice at 100nM (**Figure 20-a, c**).

Recombinant A $\eta$  $\beta$ , on the other hand, exhibits a decreasing tendency of the LTP when incubated with hippocampal slices, but additional number of experiments are required to reach statistically significant results (**Figure 20-b, c**).

Electrophysiological experiments with conditions similar to those done with recombinant peptides are being prepared with synthetic peptides. More importantly, and in an original manner, electrophysiological experiments comparing LTP in hippocampal slices of AAV-free and  $\eta$ CTF-overexpressing mice are intended.



### Figure 20: A $\eta$ $\alpha$ and A $\eta$ $\beta$ impair hippocampal LTP.

LTP was analyzed ex vivo in hippocampal slices of wild-type mice. fEPSP, field excitatory postsynaptic potential.

a,b, fEPSP slope (% baseline) pre- and post-LTP induction (time 0) in control (N=6, n=8) or in presence of a, 100nM A $\eta$  $\alpha$  (N=4, n=9), or b, 100 nM A $\eta$  $\beta$  (N=4, n=8). c, fEPSP magnitude 60 min after LTP induction from graphs in a,b, with statistical analysis \*p< 0.05; one-way analysis of variance (ANOVA); Bars are the means +/- SEM

## 2.3. Materials and Methods

---

### **Ex vivo electrophysiology:**

These experiments were conducted by Dr. Julie Dunys.

Three to five weeks old wild-type mice were anesthetized with isoflurane and then decapitated. Brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid containing (in mM) 124 NaCl, 3.75 KCl, 1.25 NaPO<sub>4</sub>, 2 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, 26.5 NaHCO<sub>3</sub>, 10 glucose (pH, ~ 7.4, gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>). Coronal slices (350 μm thick) were cut with a vibratome (7000 smz2; Campden Instruments) and transferred for recovery to a holding chamber containing oxygenated artificial cerebrospinal fluid as described above (gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>, pH ~ 7.4) at 28–30 °C for 30min before adding of 10μM picrotoxin. Hippocampal slices recovered at least for 60 min before recording. For recording process, individual slices were transferred to a submerged recording chamber, where they were still maintained at 29-30 °C and perfused with artificial cerebrospinal fluid containing or not Aη $\alpha$  or Aη $\beta$  recombinant fragments (100nM), at a rate of 2 ml per min. To record fEPSPs, a monopolar electrode was placed in the Schaffer collaterals, and stimulation was applied at 0.066 Hz (every 20 s) with stimulus intensity ranging from 10μA to 100μA. The recording electrode was placed in the stratum radiatum and fEPSPs recorded with a borosilicate micropipette filled with artificial cerebrospinal fluid. Baseline was recorded for a minimum of 10 min or until stable. LTP was induced by stimulation with 100Hz with three trains of a 1s tetanus separated by 20s. Signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA) digitized by a Digidata 1550B interface (Axon Instruments, Molecular Devices) and sampled at 10 kHz. Recordings were acquired using Clampex 11.0 software (Molecular Devices) and analyzed with Clampfit 11.0 software (Molecular Devices). The initial fESPS slope in mV per ms was determined by the software.

### **Human brain samples:**

All procedures performed in studies involving human brains were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical studies. Informed consent for tissue donation for research is obtained by the Brain Bank NeuroCEB and has been declared at the Ministry of Higher Education and Research (agreement AC-2013-1887) under their approval procedures. We studied control and SAD post-mortem human brains. Cases were anonymized, but information was provided regarding sex, age at death, and neuropathology.

### **Western-blot analysis:**

Human brain samples were homogenized in RIPA buffer (Tris 50 mM; pH 7.4 containing NaCl (150 mM), EDTA (1 mM), Triton X100 (1%), deoxycholate (0.5%), sodium dodecyl sulfate (SDS, 0,1%) and complete protease inhibitor mixture (Sigma). After homogenization with a teflon glass potter, homogenates were centrifuged (100,000 g, 1 h, 4 °C), and supernatants were kept as soluble fractions that were used for western-blot analysis. 80 µg of brain samples proteins were separated on 16% tris-tricine gels.

Cells were lysed in RIPA buffer and supplemented with a cocktail of protease inhibitors (Roche diagnostics). For western-blot analysis of mice brains, following intracardiac perfusion with PBS, hemibrains were extracted then enzymatically and mechanically lysed using “the adult brain dissociation kit” and a GentleMACS Dissociator (Miltenyi Biotec). PBS was added to the cell suspension, which was then filtered through a 70 µm Smartstrainer and centrifuged at 300 x g for 10 mins. The cell pellets were lysed in RIPA buffer supplemented with protease inhibitors. 50 µg of proteins are then loaded on Bio-Rad 7.5% tris-tricine gels.

Tris-tricine gels were directly transferred to nitrocellulose membranes using a conventional transfer system and boiled in PBS before saturation. Membranes were saturated with skimmed milk, and incubated overnight with the following primary antibodies: APPCter rabbit polyclonal (1:5000, gift from Paul Fraser); WO-2 mouse monoclonal (1:5000, Sigma Aldrich); ηCTF-Nter (1:1000, homemade with Covalab). After probing with primary antibodies, immunological complexes were revealed with anti-mouse or anti-rabbit HRP-conjugated antibodies (Jackson ImmunoResearch, 1:5000) followed by electrochemiluminescence (Westernbright™ Sirius™ and Quantum™ chemiluminescent HRP substrate, Advansta, France).

### **DAB and Immunofluorescence staining of tissue:**

Human brain sections were deparaffined in xylen bath and rehydrated by successive 5 min baths of EtOH (100%, twice), 90%, and then 70%. Antigens were unmasked in a 90% formic acid bath for 5 min and then in citric acid solution at 90-100°C for 20 min (Pressure cooker, Vector Laboratories). Brain sections were treated with H<sub>2</sub>O<sub>2</sub> 3% for 15 mins. Non-specific binding sites were blocked for 1h in BSA (5%)/Tween20 (0.1%) and brain sections were then incubated at 4 °C overnight with primary antibodies ηCTF-Nter (1: 800) or WO2 (1:1000). Sections were incubated for 1h with horseradish peroxidase (HRP)-conjugated antibodies (Jackson ImmunoResearch, 1:1000) then revealed with the DAB-ImmPACT system (Vector Laboratories). Images were taken with DMD108 Leica microsystem (DAB) and processed



using ImageJ software. For  $\eta$ CTF-Nter staining, signal was amplified with Avidin Biotin Complex (ABC) method (VECTASTAIN Original ABC Kit, Vector Laboratories).

	Age (years)	Gender	PMD (h)	Braak's NFT stage#
<b>Brain samples used in SDS-PAGE analyses</b>				
Control	52	Female	29	-
Control	71	Male	26	-
Control	84	Male	32	-
Control	71	Female	15	-
Control	60	Female	28	-
AD	68	Female	51	I-II
AD	68	Female	10	I-II
AD	70	Male	31	III
AD	78	Female	18	III
AD	92	Female	NA	III
AD	81	Male	19	VI
AD	91	Female	34	VI
AD	81	Female	NA	VI
AD	82	Female	NA	VI
<b>Brain-derived slices used in immunohistochemistry analyses</b>				
Control	55	Male	NA	-
AD	65	Male	NA	V
AD	86	Male	NA	VI
AD	55	Male	NA	NA

**Table 2 : Demographic data and neuropathological findings related to human brain samples used in SDS-PAGE analyses, and brain-derived slices used in immunohistochemistry analyses.**

PMD: Post mortem delay, h: hours, NA: not available. Controls are brain samples isolated from post-mortem patients diagnosed as negative for several neuropathologies. # Braak and Braak's NFT (neurofibrillary tangle: tau-related pathology) stage, (-) means no NFT detection.



## IV. DISCUSSION

---

## 1) Can $\eta$ CTF be produced under pathophysiological conditions?

In our study models, we mainly used overexpression approaches in order to study the localization of  $\eta$ CTF, its processing and its degradation. In the models we tested,  $\eta$ CTF was mostly localized in the trans-Golgi network, and to a lesser extent, in endosomes. When it came to  $\eta$ CTF's degradation, the proteasomal and autophagic pathways were both implicated. But what exactly occurs under pathophysiological circumstances? Since MT5-MMP is actually located on the plasma membrane (Llano et al, 1999, Wang et al, 2004)  $\eta$ -secretase can potentially cleave APP at this level, producing endogenous  $\eta$ CTF that may be subsequently either cleaved by  $\alpha$ -secretase at the plasma membrane or internalized into early endosomes. As is generally known, the location of a fragment has a significant impact on its fate. Endosomes, for example, are a favored subcellular site of the pro-amyloidogenic pathway, where  $\eta$ CTF can be selectively cleaved by  $\beta$ -secretase, producing C99, and  $\gamma$ -secretase generating  $A\beta$ , as we highlighted, revealing a second pathway that gives rise to C99 and  $A\beta$ . Therefore,  $\eta$ CTF is produced in physiological and pathological settings and is indeed partially internalized into the endo-lysosomal network, where it definitely plays a role as a precursor of fragments that are key actors in the pathology of AD, and potentially as an AD contributor itself.  $\eta$ CTF is eventually either degraded by the autophagic pathway or eliminated via exosomes.

## 2) Can $\eta$ CTF be compared to C99?

As researchers started to give their attention to APP-derived fragments other than  $A\beta$ , C99 was one of the important fragments that were focused on since they are the direct precursors of  $A\beta$  peptides. Our findings on the recently discovered  $\eta$ CTF revealed some quite remarkable similarities between it and the better-defined C99. For instance, some studies have suggested that C99 can be degraded by the UPS (Nunan et al., 2001; Nunan et al., 2003); however, these in vitro studies are mostly based on the overexpression of C99, which will be localized primarily in the ER and the Golgi apparatus, whilst amyloidogenic production occurs endogenously in the endosomal compartments. Accordingly, it has been observed that C99 produced by endogenous or overexpressed APP is primarily processed via the ELN, whereas overexpressed C99 is degraded by the proteasome (Evrard et al., 2018). Concerning  $\eta$ CTF, we determined that both proteasomal and autophagic pathways contribute to the degradation of the overexpressed  $\eta$ CTF in vitro, and more importantly though, that the autophagic pathway is most likely still the primary mechanism by which the  $\eta$ CTF produced by overexpressed wild-type APP is degraded.

We also detected  $\eta$ CTF in endosomes, the pro-amyloidogenic compartments where C99 and A $\beta$  are generated, which is consistent with our other findings, which provided assurance that  $\eta$ CTF is truly their precursor. Furthermore, and in keeping with its presence in endosomes, we located the  $\eta$ CTF fragment in exosomes, as previously observed for C99 (Miranda et al., 2018; Sharples et al., 2008; Vingtdeux et al., 2007).

Overall,  $\eta$ CTF, aside from being its direct precursor, exhibits many similarities with C99 in terms of localisation, degradation, and trafficking.

### 3) $\eta$ CTF: a new AD player?

---

The first indication of  $\eta$ -secretase's involvement in the disease was the presence of  $\eta$ -secretase-derived fragment in the amyloid plaques of AD patients reported by Willem and colleagues (Willem et al., 2015). In contrast, in my experiment using the new characterized antibody targeting  $\eta$ CTF-Nter, I couldn't detect any  $\eta$ CTF-Nter-like immunoreactivity in brain samples of AD patients assessed by both western-blot analysis and immunohistochemistry while  $\eta$ -secretase-derived fragments were easily detected around plaques in old 3xTgAD mice. That being said, it appears that  $\eta$ CTF is present at reduced levels. This can be attributed to a variety of factors, including limited production, direct cleavage by secretases that results in APP-CTFs, or direct processing by distinct degradation pathways.

When Dr. Rivera's team discovered that 5xFAD mice with MT5-MMP deficiency displayed a decrease in C99 as well as a decrease in A $\beta$ , along with the number and size of amyloid plaques in cortical and hippocampal regions, they established the enzyme's pro-amyloidogenic role. Additionally, the MT5-MMP deficiency in these AD mouse models reduced neuroinflammation while maintaining LTP, spatial learning, and memory (Baranger et al., 2017). They then revealed that the pro-amyloidogenic properties of MT5-MMP are based, at least in part, on the enzyme's ability to bind with APP and C99 and enhance their trafficking into endosomes, which are pro-amyloidogenic cellular compartments (Baranger et al., 2017). More interestingly, they found that the fate of C99 can also be controlled by the non-catalytic C-terminal domains of the enzyme, and that even inactive MT5-MMP promotes A $\beta$  accumulation (Garcia-Gonzalez et al., 2021). Given that MT5-MMP may turn into a new therapeutic target for AD. However, inhibiting an enzyme with a wide range of physiological functions could have serious consequences, therefore acting on inhibiting its generated fragments may be a preferable strategy.

Besides the pro-amyloidogenic role of  $\eta$ -secretase, does  $\eta$ CTF itself as a fragment, contribute to AD?

In addition to promoting the amyloidogenic pathway by enhancing the trafficking of APP into endosomes, as reported by Dr. Rivera, MT5-MMP also generates  $\eta$ CTF, and throughout the course of my thesis, I gathered a weight of evidence pointing to its toxic potential. In fact, I highlighted that  $\eta$ CTF produced at the plasma membrane, can be internalized into the endo-lysosomal pathway where it can play a role as a genuine direct precursor of the neurotoxic C99 and  $A\beta$ , hence shedding the light to a new pathway responsible for their production. Therefore, I identified a new route that generates major AD players that may represent a new, promising therapeutic target and an exciting venue for future research.

$\eta$ CTF is not only the precursor of the very well-known C99 and  $A\beta$ , but also of  $A\eta\alpha$  and  $A\eta\beta$  that have recently been described as being able to affect synaptic activity (Mensch et al., 2021; Willem et al., 2015). Since these results were highly controverted by the same team, in our first experiments on synaptic activity, we validated the reduction of LTP and the impairment of the synaptic activity in the presence of recombinant  $A\eta\alpha$  and  $A\eta\beta$  peptides.

#### 4) What does the presence of $\eta$ CTF in exosomes lead us to believe?

Exosomes are believed to be involved in the clearance of amyloidogenic and toxic material, acting as a neuroprotective by attempting to eliminate neurotoxic molecules. However, recent evidence has gradually changed this perception since exosomes are now thought to also transmit these molecules between cells, potentially contributing to neurodegenerative diseases. Dr. Rajendran's team conducted the first study revealing the existence of  $A\beta$  in exosomes in 2006. Indeed, this study, which initially intended to describe the intracellular  $\beta$ -cleavage of APP in early endosomes, discovered the presence of  $A\beta$  in exosomes and detected that exosomal proteins accumulate in amyloid plaques in the brains of AD patients; thereby suggesting for the first time that exosomes are implicated in the propagation of amyloid pathology in AD (Rajendran et al., 2006). Similarly, years after the discovery of  $A\beta$  in exosomes, Dr. Saman's 2012 study confirmed the existence of phosphorylated Tau in exosomes (Saman et al., 2012). The exosomal Tau protein is phosphorylated on various epitopes, most notably on threonine 181 in patients' CSF, which is a well-established biomarker. A subsequent study confirmed it by demonstrating that exosomal secretory pathway inhibition reduces the spread of Tau pathology both in vitro and in vivo (Asai et al., 2015). Along with APP, exosomes contain its metabolites also, such as C99, C83 and AICD (Miranda et al., 2018; Sharples et al., 2008;

Vingtdeux et al., 2007). More intriguingly, oligomers of C99 as well as heterodimers with of C99 and C83 were found in exosomes after  $\gamma$ -secretase inhibition (Lauritzen et al., 2019).

Therefore, it appears that cells have a propensity to release undesired and undigested endolysosomal cargoes in exosomes as a result of the ELN dysfunction that develops in AD. When adjacent neurons collect these exosomes, cells ironically promote the progression and spread of the pathology in an attempt to eliminate harmful molecules and compensate for the dysfunction of the ELN.

During my thesis, I demonstrated that  $\eta$ CTF is present in exosomes recovered from SH-APPWT cells and animal brains overexpressing  $\eta$ CTF in vitro and in vivo. This is consistent with the findings of Dr. Sadoul's team, who identified  $\eta$ CTF in exosomes isolated from primary cortical neurons or neuroblastoma N2a cells overexpressing APP wild-type (Laulagnier et al., 2018). Beyond the intriguing discovery of  $\eta$ CTF in exosomes, a key question is whether these  $\eta$ CTF-enriched exosomes are beneficial or harmful in AD. On one hand, these exosomes could provide an alternative pathway for overcoming ELN dysfunction. Indeed, these vesicles may be a means of eliminating  $\eta$ CTF and allowing its clearance, particularly by microglia. However, the capture of exosomes by microglia could have multiple conflicting consequences: 1. promote the clearance of accumulated  $\eta$ CTF in neurons, and/or 2. induce neuroinflammation, as observed for C99 in AAV-C99 mice (Lauritzen et al., 2016), and/or 3. contribute to the spread of pathology. On the other hand, propagation might also occur from neurons to neurons, perhaps triggering alterations in LTP, memory and behavioral defects.

In conclusion, this opens the door to a large number of questions and perspectives. The objective of future work will therefore be to study the toxicity of these exosomes enriched in  $\eta$ CTF and to determine their role in the spread of AD.





# V. CONCLUSION AND PERSPECTIVES

---

Throughout many years, AD was believed to be exclusively caused by the toxicity of A $\beta$ . Yet, the etiology of the disease has been reevaluated in light of accumulating evidence of clinical trial failures targeting this peptide, leading scientists to search for additional causative factors as well as some other targets.

During my thesis, I focused on the  $\eta$ CTF fragment and analyzed if it could be considered as a new trigger involved in AD. First, we have demonstrated that  $\eta$ CTF undergoes same  $\alpha$ ,  $\beta$ , and  $\gamma$ -secretases cleavages, hence  $\eta$ CTF behaves as a direct precursor of C83, but more interestingly of C99 and A $\beta$  peptide. We also established that  $\eta$ CTF degradation involves both proteasomal and autophagic pathways, with the autophagic process likely remains the main degradative process of endogenous APP-CTF including  $\eta$ CTF. Furthermore, we revealed  $\eta$ CTF localisation in endosomes using a new  $\eta$ CTF-Nter antibody that we produced and found beneficial for both biochemical and in situ analyses of  $\eta$ CTF, in mice models. Accordingly, we discovered the presence of  $\eta$ CTF in sEVs in vitro as well as in-vivo. Additionally, we were able to demonstrate that 3xTg mice have significant  $\eta$ CTF expression surrounding several senile plaques. Our preliminary findings proved what had been controverted, which is that A $\eta\alpha$  and A $\eta\beta$  both induce alterations in LTP. Finally, we fail to detected  $\eta$ CTF in the brains of AD patients; however, more research is required to determine the specificity of the staining and whether  $\eta$ CTF accumulates in a stage-dependent manner. As a matter of fact,  $\eta$ CTF appears to be present in relatively low levels, whether this is due to its direct cleavage by secretases, or to its instability and direct processing by various degradation pathways. In addition, the  $\eta$ CTF-Nter antibody doesn't seem to be very sensitive for western-blots, as much as it is for immunostaining, which highlights the need to develop alternative, more sensitive  $\eta$ CTF detection methods, such as the ELISA test.

Is  $\eta$ CTF a new factor that can contribute to AD and another target that should be considered by researchers for AD treatments in the future?

Although our results clearly demonstrate the toxic potential of  $\eta$ CTF, they are probably a characterization and the beginning of decades of research in order to entirely clarify its impact on AD.

First, it would be intriguing to determine whether the presence of  $\eta$ CTF in endosomes can impair the ELN, as was shown for A $\beta$  and C99, since the dysfunction of the ELN may also contribute to the exosomal elimination of  $\eta$ CTF. Furthermore, because exosomes can mediate

cell-to-cell propagation of toxic fragments in AD, the presence of  $\eta$ CTF in exosomes may be associated with pathology propagation; thus, another interesting thing to consider is to culture neurons and astrocytes/microglia in a microfluidic device, where neurons and astrocytes communicate through grooves and their interaction can be analyzed. This will allow us to examine if  $\eta$ CTF spreads between neurons or between neurons and astrocytes/microglia, which could trigger alterations in LTP, microglia-associated neuroinflammation, memory and behavioral defects. As a result, electrophysiological experiments comparing LTP in AAV-free and  $\eta$ CTF-overexpressing mice are important, as are behavioral studies in which mice will should be examined for locomotor activity, spatial learning, and long-term memory in the Morris water maze.

## VI. REFERENCES

---

2022 Alzheimer's disease facts and figures. *Alzheimers Dement* 18, 700-789.

A. Armstrong, R. (2019). Risk factors for Alzheimer's disease. *Folia Neuropathologica* 57, 87-105.

Ahmad, M., Takino, T., Miyamori, H., Yoshizaki, T., Furukawa, M., and Sato, H. (2006). Cleavage of Amyloid- $\beta$  Precursor Protein (APP) by Membrane-Type Matrix Metalloproteinases. *The Journal of Biochemistry* 139, 517-526.

Albert, M.S., DeKosky, S.T., Dickson, D., Dubois, B., Feldman, H.H., Fox, N.C., Gamst, A., Holtzman, D.M., Jagust, W.J., Petersen, R.C., *et al.* (2011). The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia* 7, 270-279.

Allinson, T.M., Parkin, E.T., Turner, A.J., and Hooper, N.M. (2003). ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res* 74, 342-352.

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhali, S., and Wood, M.J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 29, 341-345.

Alves da Costa, C., Sunyach, C., Pardossi-Piquard, R., Sevalle, J., Vincent, B., Boyer, N., Kawarai, T., Girardot, N., St. George-Hyslop, P., and Checler, F. (2006). Presenilin-Dependent -Secretase-Mediated Control of p53-Associated Cell Death in Alzheimer's Disease. *Journal of Neuroscience* 26, 6377-6385.

Alzheimer's Disease Collaborative, G. (1995). The structure of the presenilin 1 (S182) gene and identification of six novel mutations in early onset AD families. *Nat Genet* 11, 219-222.

Andreasen, N., Minthon, L., Vanmechelen, E., Vanderstichele, H., Davidsson, P., Winblad, B., and Blennow, K. (1999). Cerebrospinal fluid tau and Abeta42 as predictors of development of Alzheimer's disease in patients with mild cognitive impairment. *Neurosci Lett* 273, 5-8.

Anoop, A., Singh, P.K., Jacob, R.S., and Maji, S.K. (2010). CSF Biomarkers for Alzheimer's Disease Diagnosis. *Int J Alzheimers Dis* 2010.

Apostolova, L.G., Green, A.E., Babakchian, S., Hwang, K.S., Chou, Y.Y., Toga, A.W., and Thompson, P.M. (2012). Hippocampal atrophy and ventricular enlargement in normal aging, mild cognitive impairment (MCI), and Alzheimer Disease. *Alzheimer Dis Assoc Disord* 26, 17-27.

Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., Wolozin, B., Butovsky, O., Kugler, S., and Ikezu, T. (2015). Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat Neurosci* 18, 1584-1593.

Asai, M., Yagishita, S., Iwata, N., Saido, T.C., Ishiura, S., and Maruyama, K. (2011). An alternative metabolic pathway of amyloid precursor protein C-terminal fragments via cathepsin B in a human neuroglioma model. *FASEB J* 25, 3720-3730.

Attar, A., Chan, W.T., Klärner, F.G., Schrader, T., and Bitan, G. (2014). Safety and pharmacological characterization of the molecular tweezer CLR01 - a broad-spectrum inhibitor of amyloid proteins' toxicity. *BMC Pharmacol Toxicol* 15, 23.

Attar, A., Ripoli, C., Riccardi, E., Maiti, P., Li Puma, D.D., Liu, T., Hayes, J., Jones, M.R., Lichti-Kaiser, K., Yang, F., *et al.* (2012). Protection of primary neurons and mouse brain from Alzheimer's pathology by molecular tweezers. *Brain* 135, 3735-3748.

Backstrom, J.R., Lim, G.P., Cullen, M.J., and Tokes, Z.A. (1996). Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). *J Neurosci* 16, 7910-7919.

Bahr, B.A., Wisniewski, M.L., and Butler, D. (2012). Positive lysosomal modulation as a unique strategy to treat age-related protein accumulation diseases. *Rejuvenation Res* 15, 189-197.

- Bai, X.C., Yan, C., Yang, G., Lu, P., Ma, D., Sun, L., Zhou, R., Scheres, S.H.W., and Shi, Y. (2015). An atomic structure of human gamma-secretase. *Nature* *525*, 212-217.
- Bao, H., Zhang, Q., Liu, X., Song, Y., Li, X., Wang, Z., Li, C., Peng, A., and Gong, R. (2019). Lithium targeting of AMPK protects against cisplatin-induced acute kidney injury by enhancing autophagy in renal proximal tubular epithelial cells. *FASEB J* *33*, 14370-14381.
- Baranger, K., Bonnet, A.E., Girard, S.D., Paumier, J.-M., García-González, L., Elmanaa, W., Bernard, A., Charrat, E., Stephan, D., Bauer, C., *et al.* (2017). MT5-MMP Promotes Alzheimer's Pathogenesis in the Frontal Cortex of 5xFAD Mice and APP Trafficking in vitro. *Frontiers in Molecular Neuroscience* *9*.
- Baranger, K., Khrestchatisky, M., and Rivera, S. (2016a). MT5-MMP, just a new APP processing proteinase in Alzheimer's disease? *J Neuroinflammation* *13*, 167.
- Baranger, K., Marchalant, Y., Bonnet, A.E., Crouzin, N., Carrete, A., Paumier, J.M., Py, N.A., Bernard, A., Bauer, C., Charrat, E., *et al.* (2016b). MT5-MMP is a new pro-amyloidogenic proteinase that promotes amyloid pathology and cognitive decline in a transgenic mouse model of Alzheimer's disease. *Cell Mol Life Sci* *73*, 217-236.
- Barbier, P., Zejneli, O., Martinho, M., Lasorsa, A., Belle, V., Smet-Nocca, C., Tsvetkov, P.O., Devred, F., and Landrieu, I. (2019). Role of Tau as a Microtubule-Associated Protein: Structural and Functional Aspects. *Frontiers in Aging Neuroscience* *11*.
- Barger, S.W., and Harmon, A.D. (1997). Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. *Nature* *388*, 878-881.
- Baumkotter, F., Schmidt, N., Vargas, C., Schilling, S., Weber, R., Wagner, K., Fiedler, S., Klug, W., Radzimanowski, J., Nickolaus, S., *et al.* (2014). Amyloid precursor protein dimerization and synaptogenic function depend on copper binding to the growth factor-like domain. *J Neurosci* *34*, 11159-11172.
- Bayer, T.A. (2021). N-Truncated Abeta Starting at Position Four-Biochemical Features, Preclinical Models, and Potential as Drug Target in Alzheimer's Disease. *Front Aging Neurosci* *13*, 710579.
- Bellenguez, C., Kucukali, F., Jansen, I.E., Kleindam, L., Moreno-Grau, S., Amin, N., Naj, A.C., Campos-Martin, R., Grenier-Boley, B., Andrade, V., *et al.* (2022). New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat Genet* *54*, 412-436.
- Bien, J., Jefferson, T., Čaušević, M., Jumpertz, T., Munter, L., Multhaup, G., Weggen, S., Becker-Pauly, C., and Pietrzik, C.U. (2012). The Metalloprotease Meprin  $\beta$  Generates Amino Terminal-truncated Amyloid  $\beta$  Peptide Species. *Journal of Biological Chemistry* *287*, 33304-33313.
- Boland, B., Smith, D.A., Mooney, D., Jung, S.S., Walsh, D.M., and Platt, F.M. (2010). Macroautophagy is not directly involved in the metabolism of amyloid precursor protein. *J Biol Chem* *285*, 37415-37426.
- Bordi, M., Berg, M.J., Mohan, P.S., Peterhoff, C.M., Alldred, M.J., Che, S., Ginsberg, S.D., and Nixon, R.A. (2016). Autophagy flux in CA1 neurons of Alzheimer hippocampus: Increased induction overburdens failing lysosomes to propel neuritic dystrophy. *Autophagy* *12*, 2467-2483.
- Bourdenx, M., Daniel, J., Genin, E., Soria, F.N., Blanchard-Desce, M., Bezard, E., and Dehay, B. (2016). Nanoparticles restore lysosomal acidification defects: Implications for Parkinson and other lysosomal-related diseases. *Autophagy* *12*, 472-483.
- Bourgeois, A., Lauritzen, I., Lorivel, T., Bauer, C., Checler, F., and Pardossi-Piquard, R. (2018). Intraneuronal accumulation of C99 contributes to synaptic alterations, apathy-like behavior, and spatial learning deficits in 3xTgAD and 2xTgAD mice. *Neurobiol Aging* *71*, 21-31.

- Braak, H., and Braak, E. (1991). Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* 82, 239-259.
- Breijyeh, Z., and Karaman, R. (2020). Comprehensive Review on Alzheimer's Disease: Causes and Treatment. *Molecules* 25, 5789.
- Brion, J.P., Couck, A.M., Passareiro, E., and Flament-Durand, J. (1985). Neurofibrillary tangles of Alzheimer's disease: an immunohistochemical study. *J Submicrosc Cytol* 17, 89-96.
- Brkic, M., Balusu, S., Van Wonterghem, E., Gorle, N., Benilova, I., Kremer, A., Van Hove, I., Moons, L., De Strooper, B., Kanazir, S., *et al.* (2015). Amyloid beta Oligomers Disrupt Blood-CSF Barrier Integrity by Activating Matrix Metalloproteinases. *J Neurosci* 35, 12766-12778.
- Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., and Müller, T. (2017). Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease. *Progress in Neurobiology* 156, 189-213.
- Buratta, S., Tancini, B., Sagini, K., Delo, F., Chiaradia, E., Urbanelli, L., and Emiliani, C. (2020). Lysosomal Exocytosis, Exosome Release and Secretory Autophagy: The Autophagic- and Endo-Lysosomal Systems Go Extracellular. *International Journal of Molecular Sciences* 21, 2576.
- Bustamante, H.A., Rivera-Dicter, A., Cavieres, V.A., Munoz, V.C., Gonzalez, A., Lin, Y., Mardones, G.A., and Burgos, P.V. (2013). Turnover of C99 is controlled by a crosstalk between ERAD and ubiquitin-independent lysosomal degradation in human neuroglioma cells. *PLoS One* 8, e83096.
- Butler, D., Hwang, J., Estick, C., Nishiyama, A., Kumar, S.S., Baveghems, C., Young-Oxendine, H.B., Wisniewski, M.L., Charalambides, A., and Bahr, B.A. (2011). Protective effects of positive lysosomal modulation in Alzheimer's disease transgenic mouse models. *PLoS One* 6, e20501.
- Buxbaum, J.D., Liu, K.-N., Luo, Y., Slack, J.L., Stocking, K.L., Peschon, J.J., Johnson, R.S., Castner, B.J., Cerretti, D.P., and Black, R.A. (1998). Evidence That Tumor Necrosis Factor  $\alpha$  Converting Enzyme Is Involved in Regulated  $\alpha$ -Secretase Cleavage of the Alzheimer Amyloid Protein Precursor. *Journal of Biological Chemistry* 273, 27765-27767.
- Caccamo, A., Oddo, S., Billings, L.M., Green, K.N., Martinez-Coria, H., Fisher, A., and LaFerla, F.M. (2006). M1 Receptors Play a Central Role in Modulating AD-like Pathology in Transgenic Mice. *Neuron* 49, 671-682.
- Caillava, C., Ranaldi, S., Lauritzen, I., Bauer, C., Fareh, J., Abraham, J.D., and Checler, F. (2014). Study on Abeta34 biology and detection in transgenic mice brains. *Neurobiol Aging* 35, 1570-1581.
- Cao, J., Zhong, M.B., Toro, C.A., Zhang, L., and Cai, D. (2019). Endo-lysosomal pathway and ubiquitin-proteasome system dysfunction in Alzheimer's disease pathogenesis. *Neurosci Lett* 703, 68-78.
- Capell, A., Steiner, H., Willem, M., Kaiser, H., Meyer, C., Walter, J., Lammich, S., Multhaup, G., and Haass, C. (2000). Maturation and pro-peptide cleavage of beta-secretase. *J Biol Chem* 275, 30849-30854.
- Cataldo, A.M., Barnett, J.L., Pieroni, C., and Nixon, R.A. (1997). Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. *J Neurosci* 17, 6142-6151.
- Cataldo, A.M., Peterhoff, C.M., Troncoso, J.C., Gomez-Isla, T., Hyman, B.T., and Nixon, R.A. (2000). Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer's disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. *Am J Pathol* 157, 277-286.

- Chairoungdua, A., Smith, D.L., Pochard, P., Hull, M., and Caplan, M.J. (2010). Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. *J Cell Biol* 190, 1079-1091.
- Chang, H.Y., and Yang, X. (2000). Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 64, 821-846.
- Chasseigneaux, S., and Allinquant, B. (2011). Functions of A $\beta$ , sAPP $\alpha$  and sAPP $\beta$  : similarities and differences. *Journal of Neurochemistry* 120, 99-108.
- Checler, F., Afram, E., Pardossi-Piquard, R., and Lauritzen, I. (2021). Is  $\gamma$ -secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99? *Journal of Biological Chemistry* 296, 100489.
- Chen, Y.A., Lu, C.H., Ke, C.C., Chiu, S.J., Jeng, F.S., Chang, C.W., Yang, B.H., and Liu, R.S. (2021). Mesenchymal Stem Cell-Derived Exosomes Ameliorate Alzheimer's Disease Pathology and Improve Cognitive Deficits. *Biomedicines* 9.
- Chien, D.T., Szardenings, A.K., Bahri, S., Walsh, J.C., Mu, F., Xia, C., Shankle, W.R., Lerner, A.J., Su, M.Y., Elizarov, A., *et al.* (2014). Early clinical PET imaging results with the novel PHF-tau radioligand [F18]-T808. *J Alzheimers Dis* 38, 171-184.
- Cissé, M.A., Sunyach, C., Lefranc-Jullien, S., Postina, R., Vincent, B., and Checler, F. (2005). The Disintegrin ADAM9 Indirectly Contributes to the Physiological Processing of Cellular Prion by Modulating ADAM10 Activity. *Journal of Biological Chemistry* 280, 40624-40631.
- Coburger, I., Hoefgen, S., and Than, M.E. (2014). The structural biology of the amyloid precursor protein APP – a complex puzzle reveals its multi-domain architecture. *Biological Chemistry* 395, 485-498.
- Colacurcio, D.J., Pensalfini, A., Jiang, Y., and Nixon, R.A. (2018). Dysfunction of autophagy and endosomal-lysosomal pathways: Roles in pathogenesis of Down syndrome and Alzheimer's Disease. *Free Radical Biology and Medicine* 114, 40-51.
- Copanaki, E., Chang, S., Vlachos, A., Tschäpe, J.-A., Müller, U.C., Kögel, D., and Deller, T. (2010). sAPP $\alpha$  antagonizes dendritic degeneration and neuron death triggered by proteasomal stress. *Molecular and Cellular Neuroscience* 44, 386-393.
- Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261, 921-923.
- Coulson, E.J., Paliga, K., Beyreuther, K., and Masters, C.L. (2000). What the evolution of the amyloid protein precursor supergene family tells us about its function. *Neurochem Int* 36, 175-184.
- Creemers, J.W., Ines Dominguez, D., Plets, E., Serneels, L., Taylor, N.A., Multhaup, G., Craessaerts, K., Annaert, W., and De Strooper, B. (2001). Processing of beta-secretase by furin and other members of the proprotein convertase family. *J Biol Chem* 276, 4211-4217.
- Crescitelli, R., Lasser, C., Szabo, T.G., Kittel, A., Eldh, M., Dianzani, I., Buzas, E.I., and Lotvall, J. (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles* 2.
- Cummings, J., Lee, G., Nahed, P., Kamar, M.E.Z.N., Zhong, K., Fonseca, J., and Taghva, K. (2022). Alzheimer's disease drug development pipeline: 2022. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* 8.
- Cunha, A., Gaubert, A., Latxague, L., and Dehay, B. (2021). PLGA-Based Nanoparticles for Neuroprotective Drug Delivery in Neurodegenerative Diseases. *Pharmaceutics* 13.



- De Strooper, B., Iwatsubo, T., and Wolfe, M.S. (2012). Presenilins and gamma-secretase: structure, function, and role in Alzheimer Disease. *Cold Spring Harb Perspect Med* 2, a006304.
- Deb, S., and Gottschall, P.E. (1996). Increased production of matrix metalloproteinases in enriched astrocyte and mixed hippocampal cultures treated with beta-amyloid peptides. *J Neurochem* 66, 1641-1647.
- Dederer, V., and Lemberg, M.K. (2021). Transmembrane dislocases: a second chance for protein targeting. *Trends Cell Biol* 31, 898-911.
- Deng, J., Habib, A., Obregon, D.F., Barger, S.W., Giunta, B., Wang, Y.J., Hou, H., Sawmiller, D., and Tan, J. (2015). Soluble amyloid precursor protein alpha inhibits tau phosphorylation through modulation of GSK3beta signaling pathway. *J Neurochem* 135, 630-637.
- Deys, C., Thinakaran, G., and Parent, A.T. (2016). APP Receptor? To Be or Not To Be. *Trends Pharmacol Sci* 37, 390-411.
- Di, J., Siddique, I., Li, Z., Malki, G., Hornung, S., Dutta, S., Hurst, I., Ishaaya, E., Wang, A., Tu, S., *et al.* (2021). The molecular tweezer CLR01 improves behavioral deficits and reduces tau pathology in P301S-tau transgenic mice. *Alzheimers Res Ther* 13, 6.
- Dickerson, B.C., and Sperling, R.A. (2008). Functional abnormalities of the medial temporal lobe memory system in mild cognitive impairment and Alzheimer's disease: insights from functional MRI studies. *Neuropsychologia* 46, 1624-1635.
- Dorard, E., Chasseigneaux, S., Gorisse-Hussonnois, L., Broussard, C., Pillot, T., and Allinquant, B. (2018). Soluble Amyloid Precursor Protein Alpha Interacts with alpha3-Na, K-ATPase to Induce Axonal Outgrowth but Not Neuroprotection: Evidence for Distinct Mechanisms Underlying these Properties. *Mol Neurobiol* 55, 5594-5610.
- Dulin, F., Leveille, F., Ortega, J.B., Moron, J.P., Buisson, A., Callebaut, I., and Colloc'h, N. (2008). P3 peptide, a truncated form of A beta devoid of synaptotoxic effect, does not assemble into soluble oligomers. *FEBS Lett* 582, 1865-1870.
- Dunys, J., Valverde, A., and Checler, F. (2018). Are N- and C-terminally truncated Aβ species key pathological triggers in Alzheimer's disease? *J Biol Chem* 293, 15419-15428.
- Edison, P., Archer, H.A., Hinz, R., Hammers, A., Pavese, N., Tai, Y.F., Hotton, G., Cutler, D., Fox, N., Kennedy, A., *et al.* (2007). Amyloid, hypometabolism, and cognition in Alzheimer disease: an [11C]PIB and [18F]FDG PET study. *Neurology* 68, 501-508.
- Elkin, S.R., Lakoduk, A.M., and Schmid, S.L. (2016). Endocytic pathways and endosomal trafficking: a primer. *Wien Med Wochenschr* 166, 196-204.
- Embury, C.M., Dyavarshetty, B., Lu, Y., Wiederin, J.L., Ciborowski, P., Gendelman, H.E., and Kiyota, T. (2017). Cathepsin B Improves ss-Amyloidosis and Learning and Memory in Models of Alzheimer's Disease. *J Neuroimmune Pharmacol* 12, 340-352.
- Endres, K., Anders, A., Kojro, E., Gilbert, S., Fahrenholz, F., and Postina, R. (2003). Tumor necrosis factor-alpha converting enzyme is processed by proprotein-convertases to its mature form which is degraded upon phorbol ester stimulation. *European Journal of Biochemistry* 270, 2386-2393.
- Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., and Geuze, H.J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 273, 20121-20127.

- Evrard, C., Kienlen-Campard, P., Coevoet, M., Opsomer, R., Tasiaux, B., Melnyk, P., Octave, J.N., Buee, L., Sergeant, N., and Vingtdeux, V. (2018). Contribution of the Endosomal-Lysosomal and Proteasomal Systems in Amyloid-beta Precursor Protein Derived Fragments Processing. *Front Cell Neurosci* 12, 435.
- Fagan, A.M., Roe, C.M., Xiong, C., Mintun, M.A., Morris, J.C., and Holtzman, D.M. (2007). Cerebrospinal fluid tau/beta-amyloid(42) ratio as a prediction of cognitive decline in nondemented older adults. *Arch Neurol* 64, 343-349.
- Fanutza, T., Del Prete, D., Ford, M.J., Castillo, P.E., and D'Adamio, L. (2015). APP and APLP2 interact with the synaptic release machinery and facilitate transmitter release at hippocampal synapses. *Elife* 4, e09743.
- Fiandaca, M.S., Kapogiannis, D., Mapstone, M., Boxer, A., Eitan, E., Schwartz, J.B., Abner, E.L., Petersen, R.C., Federoff, H.J., Miller, B.L., *et al.* (2015). Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimers Dement* 11, 600-607 e601.
- Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 78, 477-513.
- Flammang, B., Pardossi-Piquard, R., Sevalle, J., Debayle, D., Dabert-Gay, A.S., Thevenet, A., Lauritzen, I., and Checler, F. (2012). Evidence that the amyloid-beta protein precursor intracellular domain, AICD, derives from beta-secretase-generated C-terminal fragment. *J Alzheimers Dis* 30, 145-153.
- Flood, F., Murphy, S., Cowburn, R.F., Lannfelt, L., Walker, B., and Johnston, J.A. (2005). Proteasome-mediated effects on amyloid precursor protein processing at the gamma-secretase site. *Biochem J* 385, 545-550.
- Freude, K.K., Penjwini, M., Davis, J.L., LaFerla, F.M., and Blurton-Jones, M. (2011). Soluble Amyloid Precursor Protein Induces Rapid Neural Differentiation of Human Embryonic Stem Cells. *Journal of Biological Chemistry* 286, 24264-24274.
- Furukawa, K., Sopher, B.L., Rydel, R.E., Begley, J.G., Pham, D.G., Martin, G.M., Fox, M., and Mattson, M.P. (1996). Increased activity-regulating and neuroprotective efficacy of alpha-secretase-derived secreted amyloid precursor protein conferred by a C-terminal heparin-binding domain. *J Neurochem* 67, 1882-1896.
- Gadhve, K., Bolshette, N., Ahire, A., Pardeshi, R., Thakur, K., Trandafir, C., Istrate, A., Ahmed, S., Lahkar, M., Muresanu, D.F., *et al.* (2016). The ubiquitin proteasomal system: a potential target for the management of Alzheimer's disease. *J Cell Mol Med* 20, 1392-1407.
- Gale, S.A., Acar, D., and Daffner, K.R. (2018). Dementia. *The American Journal of Medicine* 131, 1161-1169.
- Gallwitz, L., Schmidt, L., Marques, A.R.A., Tholey, A., Cassidy, L., Ulku, I., Multhaup, G., Di Spiezio, A., and Saftig, P. (2022). Cathepsin D: Analysis of its potential role as an amyloid beta degrading protease. *Neurobiol Dis* 175, 105919.
- Galvan, V., Gorostiza, O.F., Banwait, S., Ataie, M., Logvinova, A.V., Sitaraman, S., Carlson, E., Sagi, S.A., Chevallier, N., Jin, K., *et al.* (2006). Reversal of Alzheimer's-like pathology and behavior in human APP transgenic mice by mutation of Asp664. *Proc Natl Acad Sci U S A* 103, 7130-7135.
- Garcia-Ayllon, M.S., Lopez-Font, I., Boix, C.P., Fortea, J., Sanchez-Valle, R., Lleo, A., Molinuevo, J.L., Zetterberg, H., Blennow, K., and Saez-Valero, J. (2017). C-terminal fragments of the amyloid precursor protein in cerebrospinal fluid as potential biomarkers for Alzheimer disease. *Sci Rep* 7, 2477.

García-González, L., Paumier, J.M., Louis, L., Pilat, D., Bernard, A., Stephan, D., Jullien, N., Checler, F., Nivet, E., Khrestchatsky, M., *et al.* (2021). MT5-MMP controls APP and beta-CTF/C99 metabolism through proteolytic-dependent and -independent mechanisms relevant for Alzheimer's disease. *FASEB J* *35*, e21727.

García-González, L., Pilat, D., Baranger, K., and Rivera, S. (2019). Emerging Alternative Proteinases in APP Metabolism and Alzheimer's Disease Pathogenesis: A Focus on MT1-MMP and MT5-MMP. *Frontiers in Aging Neuroscience* *11*.

Gervais, F.G., Xu, D., Robertson, G.S., Vaillancourt, J.P., Zhu, Y., Huang, J., LeBlanc, A., Smith, D., Rigby, M., Shearman, M.S., *et al.* (1999). Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. *Cell* *97*, 395-406.

Ghosal, K., Vogt, D.L., Liang, M., Shen, Y., Lamb, B.T., and Pimplikar, S.W. (2009). Alzheimer's disease-like pathological features in transgenic mice expressing the APP intracellular domain. *Proc Natl Acad Sci U S A* *106*, 18367-18372.

Glennner, G.G., and Wong, C.W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* *120*, 885-890.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., *et al.* (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* *349*, 704-706.

Gonzalez, A.E., Munoz, V.C., Cavieres, V.A., Bustamante, H.A., Cornejo, V.H., Januario, Y.C., Gonzalez, I., Hetz, C., daSilva, L.L., Rojas-Fernandez, A., *et al.* (2017). Autophagosomes cooperate in the degradation of intracellular C-terminal fragments of the amyloid precursor protein via the MVB/lysosomal pathway. *FASEB J* *31*, 2446-2459.

Goodger, Z.V., Rajendran, L., Trutzel, A., Kohli, B.M., Nitsch, R.M., and Konietzko, U. (2009). Nuclear signaling by the APP intracellular domain occurs predominantly through the amyloidogenic processing pathway. *J Cell Sci* *122*, 3703-3714.

Gralle, M., Botelho, M.G., and Wouters, F.S. (2009). Neuroprotective secreted amyloid precursor protein acts by disrupting amyloid precursor protein dimers. *J Biol Chem* *284*, 15016-15025.

Gruenberg, J. (2019). Life in the lumen: The multivesicular endosome. *Traffic* *21*, 76-93.

Grundke-Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., and Binder, L.I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A* *83*, 4913-4917.

Guicciardi, M.E., Leist, M., and Gores, G.J. (2004). Lysosomes in cell death. *Oncogene* *23*, 2881-2890.

Guimas Almeida, C., Sadat Mirfakhar, F., Perdigao, C., and Burrinha, T. (2018). Impact of late-onset Alzheimer's genetic risk factors on beta-amyloid endocytic production. *Cell Mol Life Sci* *75*, 2577-2589.

Guner, G., and Lichtenthaler, S.F. (2020). The substrate repertoire of gamma-secretase/presenilin. *Semin Cell Dev Biol* *105*, 27-42.

Haapasalo, A., Viswanathan, J., Bertram, L., Soininen, H., Tanzi, R.E., and Hiltunen, M. (2010). Emerging role of Alzheimer's disease-associated ubiquitin-1 in protein aggregation. *Biochem Soc Trans* *38*, 150-155.

Haass, C., Kaether, C., Thinakaran, G., and Sisodia, S. (2012). Trafficking and Proteolytic Processing of APP. *Cold Spring Harbor Perspectives in Medicine* *2*, a006270-a006270.

- Haass, C., and Selkoe, D.J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol* 8, 101-112.
- Hamazaki, H. (1996). Cathepsin D is involved in the clearance of Alzheimer's beta-amyloid protein. *FEBS Lett* 396, 139-142.
- Haque, A., Banik, N.L., and Ray, S.K. (2008). New insights into the roles of endolysosomal cathepsins in the pathogenesis of Alzheimer's disease: cathepsin inhibitors as potential therapeutics. *CNS Neurol Disord Drug Targets* 7, 270-277.
- Hardy, J.A., and Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256, 184-185.
- Hernandez-Guillamon, M., Mawhirt, S., Blais, S., Montaner, J., Neubert, T.A., Rostagno, A., and Ghiso, J. (2015). Sequential Amyloid-beta Degradation by the Matrix Metalloproteases MMP-2 and MMP-9. *J Biol Chem* 290, 15078-15091.
- Hernandez-Guillamon, M., Mawhirt, S., Fossati, S., Blais, S., Pares, M., Penalba, A., Boada, M., Couraud, P.O., Neubert, T.A., Montaner, J., *et al.* (2010). Matrix metalloproteinase 2 (MMP-2) degrades soluble vasculotropic amyloid-beta E22Q and L34V mutants, delaying their toxicity for human brain microvascular endothelial cells. *J Biol Chem* 285, 27144-27158.
- Higashi, S., and Miyazaki, K. (2003). Novel processing of beta-amyloid precursor protein catalyzed by membrane type 1 matrix metalloproteinase releases a fragment lacking the inhibitor domain against gelatinase A. *Biochemistry* 42, 6514-6526.
- Higgins, L.S., Murphy, G.M., Jr., Forno, L.S., Catalano, R., and Cordell, B. (1996). P3 beta-amyloid peptide has a unique and potentially pathogenic immunohistochemical profile in Alzheimer's disease brain. *Am J Pathol* 149, 585-596.
- Hiltunen, M., Lu, A., Thomas, A.V., Romano, D.M., Kim, M., Jones, P.B., Xie, Z., Kounnas, M.Z., Wagner, S.L., Berezovska, O., *et al.* (2006). Ubiquitin 1 modulates amyloid precursor protein trafficking and A $\beta$  secretion. *J Biol Chem* 281, 32240-32253.
- Hong, L., Huang, H.C., and Jiang, Z.F. (2014). Relationship between amyloid-beta and the ubiquitin-proteasome system in Alzheimer's disease. *Neurol Res* 36, 276-282.
- Hook, V., Schechter, I., Demuth, H.U., and Hook, G. (2008a). Alternative pathways for production of beta-amyloid peptides of Alzheimer's disease. *Biol Chem* 389, 993-1006.
- Hook, V.Y., Kindy, M., and Hook, G. (2008b). Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. *J Biol Chem* 283, 7745-7753.
- Horstmann, S., Budig, L., Gardner, H., Koziol, J., Deuschle, M., Schilling, C., and Wagner, S. (2010). Matrix metalloproteinases in peripheral blood and cerebrospinal fluid in patients with Alzheimer's disease. *Int Psychogeriatr* 22, 966-972.
- Hsia, H.-E., Tüshaus, J., Brummer, T., Zheng, Y., Scilabra, S.D., and Lichtenthaler, S.F. (2019). Functions of 'A disintegrin and metalloproteases (ADAMs)' in the mammalian nervous system. *Cellular and Molecular Life Sciences* 76, 3055-3081.
- Hung, C.O.Y., and Livesey, F.J. (2018). Altered gamma-Secretase Processing of APP Disrupts Lysosome and Autophagosome Function in Monogenic Alzheimer's Disease. *Cell Rep* 25, 3647-3660 e3642.

- Hurwitz, S.N., Conlon, M.M., Rider, M.A., Brownstein, N.C., and Meckes, D.G., Jr. (2016). Nanoparticle analysis sheds budding insights into genetic drivers of extracellular vesicle biogenesis. *J Extracell Vesicles* 5, 31295.
- Huse, J.T., Pijak, D.S., Leslie, G.J., Lee, V.M., and Doms, R.W. (2000). Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. *J Biol Chem* 275, 33729-33737.
- Itoh, Y. (2015). Membrane-type matrix metalloproteinases: Their functions and regulations. *Matrix Biology* 44-46, 207-223.
- Jack, C.R., Jr., Bennett, D.A., Blennow, K., Carrillo, M.C., Feldman, H.H., Frisoni, G.B., Hampel, H., Jagust, W.J., Johnson, K.A., Knopman, D.S., *et al.* (2016). A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology* 87, 539-547.
- Jack, C.R., Jr., Shiung, M.M., Gunter, J.L., O'Brien, P.C., Weigand, S.D., Knopman, D.S., Boeve, B.F., Ivnik, R.J., Smith, G.E., Cha, R.H., *et al.* (2004). Comparison of different MRI brain atrophy rate measures with clinical disease progression in AD. *Neurology* 62, 591-600.
- Jager, S., Leuchtenberger, S., Martin, A., Czirr, E., Wesselowski, J., Dieckmann, M., Waldron, E., Korth, C., Koo, E.H., Heneka, M., *et al.* (2009). alpha-secretase mediated conversion of the amyloid precursor protein derived membrane stub C99 to C83 limits Abeta generation. *J Neurochem* 111, 1369-1382.
- Jaworski, D.M. (2000). Developmental regulation of membrane type-5 matrix metalloproteinase (MT5-MMP) expression in the rat nervous system. *Brain Res* 860, 174-177.
- Jefferson, T., Čaušević, M., auf dem Keller, U., Schilling, O., Isbert, S., Geyer, R., Maier, W., Tschickardt, S., Jumpertz, T., Weggen, S., *et al.* (2011). Metalloprotease Meprin  $\beta$  Generates Nontoxic N-terminal Amyloid Precursor Protein Fragments in Vivo. *Journal of Biological Chemistry* 286, 27741-27750.
- Jia, L., Qiu, Q., Zhang, H., Chu, L., Du, Y., Zhang, J., Zhou, C., Liang, F., Shi, S., Wang, S., *et al.* (2019). Concordance between the assessment of Abeta42, T-tau, and P-T181-tau in peripheral blood neuronal-derived exosomes and cerebrospinal fluid. *Alzheimers Dement* 15, 1071-1080.
- Jiang, T., Yu, J.T., Zhu, X.C., Tan, M.S., Wang, H.F., Cao, L., Zhang, Q.Q., Shi, J.Q., Gao, L., Qin, H., *et al.* (2014). Temsirolimus promotes autophagic clearance of amyloid-beta and provides protective effects in cellular and animal models of Alzheimer's disease. *Pharmacol Res* 81, 54-63.
- Jiang, Y., Mullaney, K.A., Peterhoff, C.M., Che, S., Schmidt, S.D., Boyer-Boiteau, A., Ginsberg, S.D., Cataldo, A.M., Mathews, P.M., and Nixon, R.A. (2010). Alzheimer's-related endosome dysfunction in Down syndrome is Abeta-independent but requires APP and is reversed by BACE-1 inhibition. *Proc Natl Acad Sci U S A* 107, 1630-1635.
- Jiang, Y., Sato, Y., Im, E., Berg, M., Bordi, M., Darji, S., Kumar, A., Mohan, P.S., Bandyopadhyay, U., Diaz, A., *et al.* (2019). Lysosomal Dysfunction in Down Syndrome Is APP-Dependent and Mediated by APP-betaCTF (C99). *J Neurosci* 39, 5255-5268.
- Johnston, J.A., Cowburn, R.F., Norgren, S., Wiehager, B., Venizelos, N., Winblad, B., Vigo-Pelfrey, C., Schenk, D., Lannfelt, L., and O'Neill, C. (1994). Increased beta-amyloid release and levels of amyloid precursor protein (APP) in fibroblast cell lines from family members with the Swedish Alzheimer's disease APP670/671 mutation. *FEBS Lett* 354, 274-278.
- Jonsson, T., Atwal, J.K., Steinberg, S., Snaedal, J., Jonsson, P.V., Bjornsson, S., Stefansson, H., Sulem, P., Gudbjartsson, D., Maloney, J., *et al.* (2012). A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* 488, 96-99.

- Jourquin, J., Tremblay, E., Decanis, N., Charton, G., Hanessian, S., Chollet, A.M., Le Diguardher, T., Khrestchatisky, M., and Rivera, S. (2003). Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. *Eur J Neurosci* *18*, 1507-1517.
- Jung, E.S., Hong, H., Kim, C., and Mook-Jung, I. (2015). Acute ER stress regulates amyloid precursor protein processing through ubiquitin-dependent degradation. *Sci Rep* *5*, 8805.
- Jurisch-Yaksi, N., Sannerud, R., and Annaert, W. (2013). A fast growing spectrum of biological functions of gamma-secretase in development and disease. *Biochim Biophys Acta* *1828*, 2815-2827.
- Kamino, K., Orr, H.T., Payami, H., Wijsman, E.M., Alonso, M.E., Pulst, S.M., Anderson, L., O'Dahl, S., Nemens, E., White, J.A., *et al.* (1992). Linkage and mutational analysis of familial Alzheimer disease kindreds for the APP gene region. *Am J Hum Genet* *51*, 998-1014.
- Kanatsu, K., Morohashi, Y., Suzuki, M., Kuroda, H., Watanabe, T., Tomita, T., and Iwatsubo, T. (2014). Decreased CALM expression reduces Abeta42 to total Abeta ratio through clathrin-mediated endocytosis of gamma-secretase. *Nat Commun* *5*, 3386.
- Kaneko, M., Koike, H., Saito, R., Kitamura, Y., Okuma, Y., and Nomura, Y. (2010). Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid-beta generation. *J Neurosci* *30*, 3924-3932.
- Katsuda, T., Tsuchiya, R., Kosaka, N., Yoshioka, Y., Takagaki, K., Oki, K., Takeshita, F., Sakai, Y., Kuroda, M., and Ochiya, T. (2013). Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* *3*, 1197.
- Khan, S., Barve, K.H., and Kumar, M.S. (2020). Recent Advancements in Pathogenesis, Diagnostics and Treatment of Alzheimer's Disease. *Curr Neuropharmacol* *18*, 1106-1125.
- Kim, H.S., Kim, E.M., Lee, J.P., Park, C.H., Kim, S., Seo, J.H., Chang, K.A., Yu, E., Jeong, S.J., Chong, Y.H., *et al.* (2003). C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. *FASEB J* *17*, 1951-1953.
- Kim, S., Sato, Y., Mohan, P.S., Peterhoff, C., Pensalfini, A., Rigoglioso, A., Jiang, Y., and Nixon, R.A. (2016). Evidence that the rab5 effector APPL1 mediates APP-betaCTF-induced dysfunction of endosomes in Down syndrome and Alzheimer's disease. *Mol Psychiatry* *21*, 707-716.
- Kimura, A., Hata, S., and Suzuki, T. (2016). Alternative Selection of beta-Site APP-Cleaving Enzyme 1 (BACE1) Cleavage Sites in Amyloid beta-Protein Precursor (APP) Harboring Protective and Pathogenic Mutations within the Abeta Sequence. *J Biol Chem* *291*, 24041-24053.
- Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D.P., Bergstrom, M., Savitcheva, I., Huang, G.F., Estrada, S., *et al.* (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann Neurol* *55*, 306-319.
- Koenig, A.M., Mechanic-Hamilton, D., Xie, S.X., Combs, M.F., Cappola, A.R., Xie, L., Detre, J.A., Wolk, D.A., and Arnold, S.E. (2017). Effects of the Insulin Sensitizer Metformin in Alzheimer Disease: Pilot Data From a Randomized Placebo-controlled Crossover Study. *Alzheimer Dis Assoc Disord* *31*, 107-113.
- Koh, J.Y., Kim, H.N., Hwang, J.J., Kim, Y.H., and Park, S.E. (2019). Lysosomal dysfunction in proteinopathic neurodegenerative disorders: possible therapeutic roles of cAMP and zinc. *Mol Brain* *12*, 18.
- Komori, K., Nonaka, T., Okada, A., Kinoh, H., Hayashita-Kinoh, H., Yoshida, N., Yana, I., and Seiki, M. (2004). Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase. *FEBS Letters* *557*, 125-128.

Kopan, R., and Ilagan, M.X. (2004). Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* 5, 499-504.

Kuhn, A.J., Abrams, B.S., Knowlton, S., and Raskatov, J.A. (2020). Alzheimer's Disease "Non-amyloidogenic" p3 Peptide Revisited: A Case for Amyloid- $\alpha$ . *ACS Chem Neurosci* 11, 1539-1544.

Kuhn, A.J., and Raskatov, J. (2020). Is the p3 Peptide (A $\beta$ 17-40, A $\beta$ 17-42) Relevant to the Pathology of Alzheimer's Disease? *J Alzheimers Dis* 74, 43-53.

Kuhn, P.-H., Wang, H., Dislich, B., Colombo, A., Zeitschel, U., Ellwart, J.W., Kremmer, E., Roßner, S., and Lichtenthaler, S.F. (2010). ADAM10 is the physiologically relevant, constitutive  $\alpha$ -secretase of the amyloid precursor protein in primary neurons. *The EMBO Journal* 29, 3020-3032.

Kumar, D., Gupta, D., Shankar, S., and Srivastava, R.K. (2015). Biomolecular characterization of exosomes released from cancer stem cells: Possible implications for biomarker and treatment of cancer. *Oncotarget* 6, 3280-3291.

Kwart, D., Gregg, A., Scheckel, C., Murphy, E.A., Paquet, D., Duffield, M., Fak, J., Olsen, O., Darnell, R.B., and Tessier-Lavigne, M. (2019). A Large Panel of Isogenic APP and PSEN1 Mutant Human iPSC Neurons Reveals Shared Endosomal Abnormalities Mediated by APP beta-CTFs, Not A $\beta$ . *Neuron* 104, 256-270 e255.

Lai, A., Sisodia, S.S., and Trowbridge, I.S. (1995). Characterization of sorting signals in the beta-amyloid precursor protein cytoplasmic domain. *J Biol Chem* 270, 3565-3573.

Lai, S.S.M., Ng, K.Y., Koh, R.Y., Chok, K.C., and Chye, S.M. (2021). Endosomal-lysosomal dysfunctions in Alzheimer's disease: Pathogenesis and therapeutic interventions. *Metab Brain Dis* 36, 1087-1100.

Langemeyer, L., Fröhlich, F., and Ungermann, C. (2018). Rab GTPase Function in Endosome and Lysosome Biogenesis. *Trends in Cell Biology* 28, 957-970.

Langenfurth, A., Rinnenthal, J.L., Vinnakota, K., Prinz, V., Carlo, A.S., Stadelmann, C., Siffrin, V., Peaschke, S., Endres, M., Heppner, F., *et al.* (2014). Membrane-type 1 metalloproteinase is upregulated in microglia/brain macrophages in neurodegenerative and neuroinflammatory diseases. *J Neurosci Res* 92, 275-286.

Lashley, T., Schott, J.M., Weston, P., Murray, C.E., Wellington, H., Keshavan, A., Foti, S.C., Foiani, M., Toombs, J., Rohrer, J.D., *et al.* (2018). Molecular biomarkers of Alzheimer's disease: progress and prospects. *Dis Model Mech* 11.

Laulagnier, K., Javalet, C., Hemming, F.J., Chivet, M., Lachenal, G., Blot, B., Chatellard, C., and Sadoul, R. (2018). Amyloid precursor protein products concentrate in a subset of exosomes specifically endocytosed by neurons. *Cell Mol Life Sci* 75, 757-773.

Lauritzen, I., Becot, A., Bourgeois, A., Pardossi-Piquard, R., Biferi, M.G., Barkats, M., and Checler, F. (2019). Targeting gamma-secretase triggers the selective enrichment of oligomeric APP-CTFs in brain extracellular vesicles from Alzheimer cell and mouse models. *Transl Neurodegener* 8, 35.

Lauritzen, I., Pardossi-Piquard, R., Bauer, C., Brigham, E., Abraham, J.D., Ranaldi, S., Fraser, P., St-George-Hyslop, P., Le Thuc, O., Espin, V., *et al.* (2012). The beta-secretase-derived C-terminal fragment of betaAPP, C99, but not A $\beta$ , is a key contributor to early intraneuronal lesions in triple-transgenic mouse hippocampus. *J Neurosci* 32, 16243-11655a.

Lauritzen, I., Pardossi-Piquard, R., Bourgeois, A., Pagnotta, S., Biferi, M.G., Barkats, M., Lacor, P., Klein, W., Bauer, C., and Checler, F. (2016). Intraneuronal aggregation of the beta-CTF fragment of APP (C99) induces A $\beta$ -independent lysosomal-autophagic pathology. *Acta Neuropathol* 132, 257-276.

- Leake, A., Morris, C.M., and Whateley, J. (2000). Brain matrix metalloproteinase 1 levels are elevated in Alzheimer's disease. *Neurosci Lett* *291*, 201-203.
- Lee, J.-H., Yu, W.H., Kumar, A., Lee, S., Mohan, P.S., Peterhoff, C.M., Wolfe, D.M., Martinez-Vicente, M., Massey, A.C., Sovak, G., *et al.* (2010). Lysosomal Proteolysis and Autophagy Require Presenilin 1 and Are Disrupted by Alzheimer-Related PS1 Mutations. *Cell* *141*, 1146-1158.
- Lee, J.H., McBrayer, M.K., Wolfe, D.M., Haslett, L.J., Kumar, A., Sato, Y., Lie, P.P., Mohan, P., Coffey, E.E., Kompella, U., *et al.* (2015). Presenilin 1 Maintains Lysosomal Ca(2+) Homeostasis via TRPML1 by Regulating vATPase-Mediated Lysosome Acidification. *Cell Rep* *12*, 1430-1444.
- Li, Z., Siddique, I., Hadrovic, I., Kirupakaran, A., Li, J., Zhang, Y., Klarner, F.G., Schrader, T., and Bitan, G. (2021). Lysine-selective molecular tweezers are cell penetrant and concentrate in lysosomes. *Commun Biol* *4*, 1076.
- Liao, M.C., and Van Nostrand, W.E. (2010). Degradation of soluble and fibrillar amyloid beta-protein by matrix metalloproteinase (MT1-MMP) in vitro. *Biochemistry* *49*, 1127-1136.
- Liebsch, F., Kulic, L., Teunissen, C., Shobo, A., Ulku, I., Engelschalt, V., Hancock, M.A., van der Flier, W.M., Kunach, P., Rosa-Neto, P., *et al.* (2019). Abeta34 is a BACE1-derived degradation intermediate associated with amyloid clearance and Alzheimer's disease progression. *Nat Commun* *10*, 2240.
- Lim, C.Z.J., Zhang, Y., Chen, Y., Zhao, H., Stephenson, M.C., Ho, N.R.Y., Chen, Y., Chung, J., Reilhac, A., Loh, T.P., *et al.* (2019). Subtyping of circulating exosome-bound amyloid beta reflects brain plaque deposition. *Nat Commun* *10*, 1144.
- Liu, T., Zhang, T., Nicolas, M., Boussicault, L., Rice, H., Soldano, A., Claeys, A., Petrova, I., Fradkin, L., De Strooper, B., *et al.* (2021). The amyloid precursor protein is a conserved Wnt receptor. *Elife* *10*.
- Lopez Salon, M., Pasquini, L., Besio Moreno, M., Pasquini, J.M., and Soto, E. (2003). Relationship between beta-amyloid degradation and the 26S proteasome in neural cells. *Exp Neurol* *180*, 131-143.
- Lorenzl, S., Albers, D.S., Relkin, N., Ngyuen, T., Hilgenberg, S.L., Chirichigno, J., Cudkowicz, M.E., and Beal, M.F. (2003). Increased plasma levels of matrix metalloproteinase-9 in patients with Alzheimer's disease. *Neurochem Int* *43*, 191-196.
- Lorenzl, S., Buerger, K., Hampel, H., and Beal, M.F. (2008). Profiles of matrix metalloproteinases and their inhibitors in plasma of patients with dementia. *Int Psychogeriatr* *20*, 67-76.
- Lott, I.T., and Head, E. (2019). Dementia in Down syndrome: unique insights for Alzheimer disease research. *Nat Rev Neurol* *15*, 135-147.
- Lu, D.C., Soriano, S., Bredesen, D.E., and Koo, E.H. (2003). Caspase cleavage of the amyloid precursor protein modulates amyloid beta-protein toxicity. *J Neurochem* *87*, 733-741.
- Lu, P., Bai, X.C., Ma, D., Xie, T., Yan, C., Sun, L., Yang, G., Zhao, Y., Zhou, R., Scheres, S.H.W., *et al.* (2014). Three-dimensional structure of human gamma-secretase. *Nature* *512*, 166-170.
- Manna, I., De Benedittis, S., Quattrone, A., Maisano, D., Iaccino, E., and Quattrone, A. (2020). Exosomal miRNAs as Potential Diagnostic Biomarkers in Alzheimer's Disease. *Pharmaceuticals (Basel)* *13*.
- Marengo, L., Armbrust, F., Schoenherr, C., Storck, S.E., Schmitt, U., Zampar, S., Wirths, O., Altmepfen, H., Glatzel, M., Kaether, C., *et al.* (2022). Mepripin  $\beta$  knockout reduces brain A $\beta$  levels and rescues learning and memory impairments in the APP/Jon mouse model for Alzheimer's disease. *Cellular and Molecular Life Sciences* *79*.



Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A* *82*, 4245-4249.

Mathews, P.M., and Levy, E. (2019). Exosome Production Is Key to Neuronal Endosomal Pathway Integrity in Neurodegenerative Diseases. *Frontiers in Neuroscience* *13*.

Matsunaga, S., Kishi, T., Annas, P., Basun, H., Hampel, H., and Iwata, N. (2015). Lithium as a Treatment for Alzheimer's Disease: A Systematic Review and Meta-Analysis. *J Alzheimers Dis* *48*, 403-410.

McDermott, J.R., and Gibson, A.M. (1996). Degradation of Alzheimer's beta-amyloid protein by human cathepsin D. *Neuroreport* *7*, 2163-2166.

Meakin, P.J., Mezzapesa, A., Benabou, E., Haas, M.E., Bonardo, B., Grino, M., Brunel, J.M., Desbois-Mouthon, C., Biddinger, S.B., Govers, R., *et al.* (2018). The beta secretase BACE1 regulates the expression of insulin receptor in the liver. *Nat Commun* *9*, 1306.

Meckler, X., and Checler, F. (2016). Presenilin 1 and Presenilin 2 Target  $\gamma$ -Secretase Complexes to Distinct Cellular Compartments. *Journal of Biological Chemistry* *291*, 12821-12837.

Men, Y., Yelick, J., Jin, S., Tian, Y., Chiang, M.S.R., Higashimori, H., Brown, E., Jarvis, R., and Yang, Y. (2019). Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. *Nat Commun* *10*, 4136.

Mensch, M., Dunot, J., Yishan, S.M., Harris, S.S., Blistein, A., Avdiu, A., Pousinha, P.A., Giudici, C., Busche, M.A., Jedlicka, P., *et al.* (2021). A $\eta$ - $\alpha$  and A $\eta$ - $\beta$  peptides impair LTP ex vivo within the low nanomolar range and impact neuronal activity in vivo. *Alzheimer's Research & Therapy* *13*.

Milosch, N., Tanriover, G., Kundu, A., Rami, A., Francois, J.C., Baumkotter, F., Weyer, S.W., Samanta, A., Jaschke, A., Brod, F., *et al.* (2014). Holo-APP and G-protein-mediated signaling are required for sAPP $\alpha$ -induced activation of the Akt survival pathway. *Cell Death Dis* *5*, e1391.

Miranda, A.M., and Di Paolo, G. (2018). Endolysosomal dysfunction and exosome secretion: implications for neurodegenerative disorders. *Cell Stress* *2*, 115-118.

Miranda, A.M., Lasiecka, Z.M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., Chan, R.B., Oliveira, T.G., Small, S.A., and Di Paolo, G. (2018). Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures. *Nature Communications* *9*.

Miyagawa, T., Ebinuma, I., Morohashi, Y., Hori, Y., Young Chang, M., Hattori, H., Maehara, T., Yokoshima, S., Fukuyama, T., Tsuji, S., *et al.* (2016). BIN1 regulates BACE1 intracellular trafficking and amyloid-beta production. *Hum Mol Genet* *25*, 2948-2958.

Mobius, W., Ohno-Iwashita, Y., van Donselaar, E.G., Oorschot, V.M., Shimada, Y., Fujimoto, T., Heijnen, H.F., Geuze, H.J., and Slot, J.W. (2002). Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O. *J Histochem Cytochem* *50*, 43-55.

Moller, H.J., and Graeber, M.B. (1998). The case described by Alois Alzheimer in 1911. Historical and conceptual perspectives based on the clinical record and neurohistological sections. *Eur Arch Psychiatry Clin Neurosci* *248*, 111-122.

Monaco, A., Maffia, V., Sorrentino, N.C., Sambri, I., Ezhova, Y., Giuliano, T., Cacace, V., Nusco, E., De Risi, M., De Leonibus, E., *et al.* (2022). The Amyloid Inhibitor CLR01 Relieves Autophagy and Ameliorates Neuropathology in a Severe Lysosomal Storage Disease. *Mol Ther* *30*, 3499.

Morel, E., Chamoun, Z., Lasiecka, Z.M., Chan, R.B., Williamson, R.L., Vetanovetz, C., Dall'Armi, C., Simoes, S., Point Du Jour, K.S., McCabe, B.D., *et al.* (2013a). Phosphatidylinositol-3-phosphate

- regulates sorting and processing of amyloid precursor protein through the endosomal system. *Nat Commun* 4, 2250.
- Morel, L., Regan, M., Higashimori, H., Ng, S.K., Esau, C., Vidensky, S., Rothstein, J., and Yang, Y. (2013b). Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. *J Biol Chem* 288, 7105-7116.
- Morel, M., Heraud, C., Nicaise, C., Suain, V., and Brion, J.P. (2012). Levels of kinesin light chain and dynein intermediate chain are reduced in the frontal cortex in Alzheimer's disease: implications for axoplasmic transport. *Acta Neuropathol* 123, 71-84.
- Morley, J.E., Farr, S.A., Banks, W.A., Johnson, S.N., Yamada, K.A., and Xu, L. (2010). A Physiological Role for Amyloid- $\beta$  Protein: Enhancement of Learning and Memory. *Journal of Alzheimer's Disease* 19, 441-449.
- Morley, J.E., Farr, S.A., Nguyen, A.D., and Xu, F. (2019). What is the Physiological Function of Amyloid-Beta Protein? *The journal of nutrition, health & aging* 23, 225-226.
- Mosconi, L., Berti, V., Quinn, C., McHugh, P., Petrongolo, G., Osorio, R.S., Connaughty, C., Pupi, A., Vallabhajosula, S., Isaacson, R.S., *et al.* (2017). Perimenopause and emergence of an Alzheimer's bioenergetic phenotype in brain and periphery. *PLoS One* 12, e0185926.
- Moss, M.L., Powell, G., Miller, M.A., Edwards, L., Qi, B., Sang, Q.-X.A., De Strooper, B., Tesseur, I., Lichtenthaler, S.F., Taverna, M., *et al.* (2011). ADAM9 Inhibition Increases Membrane Activity of ADAM10 and Controls  $\alpha$ -Secretase Processing of Amyloid Precursor Protein. *Journal of Biological Chemistry* 286, 40443-40451.
- Mroczo, B., Groblewska, M., Zboch, M., Kulczynska, A., Koper, O.M., Szmitkowski, M., Kornhuber, J., and Lewczuk, P. (2014). Concentrations of matrix metalloproteinases and their tissue inhibitors in the cerebrospinal fluid of patients with Alzheimer's disease. *J Alzheimers Dis* 40, 351-357.
- Mueller-Steiner, S., Zhou, Y., Arai, H., Roberson, E.D., Sun, B., Chen, J., Wang, X., Yu, G., Esposito, L., Mucke, L., *et al.* (2006). Anti-amyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer's disease. *Neuron* 51, 703-714.
- Mullan, M. (1992). Familial Alzheimer's disease: second gene locus located. *BMJ* 305, 1108-1109.
- Müller, T., Meyer, H.E., Egensperger, R., and Marcus, K. (2008). The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics—Relevance for Alzheimer's disease. *Progress in Neurobiology* 85, 393-406.
- Müller, U.C., Deller, T., and Korte, M. (2017). Not just amyloid: physiological functions of the amyloid precursor protein family. *Nature Reviews Neuroscience* 18, 281-298.
- Muller, U.C., and Zheng, H. (2012). Physiological functions of APP family proteins. *Cold Spring Harb Perspect Med* 2, a006288.
- Nakatsukasa, K., Hoyer, G., Michaelis, S., and Brodsky, J.L. (2008). Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. *Cell* 132, 101-112.
- Nalivaeva, N.N., and Turner, A.J. (2013). The amyloid precursor protein: a biochemical enigma in brain development, function and disease. *FEBS Lett* 587, 2046-2054.
- Naseri, N.N., Wang, H., Guo, J., Sharma, M., and Luo, W. (2019). The complexity of tau in Alzheimer's disease. *Neuroscience Letters* 705, 183-194.

- Nilsberth, C., Westlind-Danielsson, A., Eckman, C.B., Condron, M.M., Axelman, K., Forsell, C., Sten, C., Luthman, J., Teplow, D.B., Younkin, S.G., *et al.* (2001). The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A $\beta$  protofibril formation. *Nat Neurosci* 4, 887-893.
- Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., Tanaka, M., Iwata, N., Saito, T., and Saido, Takaomi C. (2013). A $\beta$  Secretion and Plaque Formation Depend on Autophagy. *Cell Reports* 5, 61-69.
- Nixon, R.A. (2017). Amyloid precursor protein and endosomal-lysosomal dysfunction in Alzheimer's disease: inseparable partners in a multifactorial disease. *The FASEB Journal* 31, 2729-2743.
- Nixon, R.A., Wegiel, J., Kumar, A., Yu, W.H., Peterhoff, C., Cataldo, A., and Cuervo, A.M. (2005). Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* 64, 113-122.
- Nubling, G., Levin, J., Bader, B., Israel, L., Botzel, K., Lorenzl, S., and Giese, A. (2012). Limited cleavage of tau with matrix-metalloproteinase MMP-9, but not MMP-3, enhances tau oligomer formation. *Exp Neurol* 237, 470-476.
- Nunan, J., Shearman, M.S., Checler, F., Cappai, R., Evin, G., Beyreuther, K., Masters, C.L., and Small, D.H. (2001). The C-terminal fragment of the Alzheimer's disease amyloid protein precursor is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur J Biochem* 268, 5329-5336.
- Nunan, J., Williamson, N.A., Hill, A.F., Sernee, M.F., Masters, C.L., and Small, D.H. (2003). Proteasome-mediated degradation of the C-terminus of the Alzheimer's disease beta-amyloid protein precursor: effect of C-terminal truncation on production of beta-amyloid protein. *J Neurosci Res* 74, 378-385.
- Obregon, D., Hou, H., Deng, J., Giunta, B., Tian, J., Darlington, D., Shahaduzzaman, M., Zhu, Y., Mori, T., Mattson, M.P., *et al.* (2012). Soluble amyloid precursor protein-alpha modulates beta-secretase activity and amyloid-beta generation. *Nat Commun* 3, 777.
- Ohkawara, T., Nagase, H., Koh, C.-S., and Nakayama, K. (2011). The amyloid precursor protein intracellular domain alters gene expression and induces neuron-specific apoptosis. *Gene* 475, 1-9.
- Ozcelik, S., Fraser, G., Castets, P., Schaeffer, V., Skachokova, Z., Breu, K., Clavaguera, F., Sinnreich, M., Kappos, L., Goedert, M., *et al.* (2013). Rapamycin attenuates the progression of tau pathology in P301S tau transgenic mice. *PLoS One* 8, e62459.
- Panza, F., Lozupone, M., Dibello, V., Greco, A., Daniele, A., Seripa, D., Logroscino, G., and Imbimbo, B.P. (2019). Are antibodies directed against amyloid-beta (A $\beta$ ) oligomers the last call for the A $\beta$  hypothesis of Alzheimer's disease? *Immunotherapy* 11, 3-6.
- Pardossi-Piquard, R., and Checler, F. (2012). The physiology of the beta-amyloid precursor protein intracellular domain AICD. *J Neurochem* 120 Suppl 1, 109-124.
- Pardossi-Piquard, R., Lauritzen, I., Bauer, C., Sacco, G., Robert, P., and Checler, F. (2016). Influence of Genetic Background on Apathy-Like Behavior in Triple Transgenic AD Mice. *Curr Alzheimer Res* 13, 942-949.
- Pardossi-Piquard, R., Petit, A., Kawarai, T., Sunyach, C., Alves da Costa, C., Vincent, B., Ring, S., D'Adamio, L., Shen, J., Müller, U., *et al.* (2005). Presenilin-Dependent Transcriptional Control of the A $\beta$ -Degrading Enzyme Nephilysin by Intracellular Domains of  $\beta$ APP and APLP. *Neuron* 46, 541-554.
- Park, G., Nhan, H.S., Tyan, S.H., Kawakatsu, Y., Zhang, C., Navarro, M., and Koo, E.H. (2020). Caspase Activation and Caspase-Mediated Cleavage of APP Is Associated with Amyloid beta-Protein-Induced Synapse Loss in Alzheimer's Disease. *Cell Rep* 31, 107839.

- Park, S.A., Shaked, G.M., Bredesen, D.E., and Koo, E.H. (2009). Mechanism of cytotoxicity mediated by the C31 fragment of the amyloid precursor protein. *Biochem Biophys Res Commun* 388, 450-455.
- Paumier, J.M., Py, N.A., Garcia-Gonzalez, L., Bernard, A., Stephan, D., Louis, L., Checler, F., Khrestchatisky, M., Baranger, K., and Rivera, S. (2019). Proamyloidogenic effects of membrane type 1 matrix metalloproteinase involve MMP-2 and BACE-1 activities, and the modulation of APP trafficking. *FASEB J* 33, 2910-2927.
- Pei, D. (1999). Identification and characterization of the fifth membrane-type matrix metalloproteinase MT5-MMP. *J Biol Chem* 274, 8925-8932.
- Peng, K.Y., Pérez-González, R., Alldred, M.J., Goulbourne, C.N., Morales-Corraliza, J., Saito, M., Saito, M., Ginsberg, S.D., Mathews, P.M., and Levy, E. (2019). Apolipoprotein E4 genotype compromises brain exosome production. *Brain* 142, 163-175.
- Perez-Gonzalez, R., Gauthier, S.A., Kumar, A., and Levy, E. (2012). The Exosome Secretory Pathway Transports Amyloid Precursor Protein Carboxyl-terminal Fragments from the Cell into the Brain Extracellular Space. *Journal of Biological Chemistry* 287, 43108-43115.
- Pimplikar, S.W., Nixon, R.A., Robakis, N.K., Shen, J., and Tsai, L.H. (2010). Amyloid-independent mechanisms in Alzheimer's disease pathogenesis. *J Neurosci* 30, 14946-14954.
- Porlan, E., Martí-Prado, B., Morante-Redolat, J.M., Consiglio, A., Delgado, A.C., Kypta, R., López-Otín, C., Kirstein, M., and Fariñas, I. (2014). MT5-MMP regulates adult neural stem cell functional quiescence through the cleavage of N-cadherin. *Nature Cell Biology* 16, 629-638.
- Pulina, M.V., Hopkins, M., Haroutunian, V., Greengard, P., and Bustos, V. (2020). C99 selectively accumulates in vulnerable neurons in Alzheimer's disease. *Alzheimers Dement* 16, 273-282.
- Puzzo, D., Privitera, L., Fa, M., Staniszewski, A., Hashimoto, G., Aziz, F., Sakurai, M., Ribe, E.M., Troy, C.M., Mercken, M., *et al.* (2011). Endogenous amyloid- $\beta$  is necessary for hippocampal synaptic plasticity and memory. *Annals of Neurology* 69, 819-830.
- Puzzo, D., Privitera, L., Leznik, E., Fa, M., Staniszewski, A., Palmeri, A., and Arancio, O. (2008). Picomolar Amyloid- $\beta$  Positively Modulates Synaptic Plasticity and Memory in Hippocampus. *Journal of Neuroscience* 28, 14537-14545.
- Py, N.A., Bonnet, A.E., Bernard, A., Marchalant, Y., Charrat, E., Checler, F., Khrestchatisky, M., Baranger, K., and Rivera, S. (2014). Differential spatio-temporal regulation of MMPs in the 5xFAD mouse model of Alzheimer's disease: evidence for a pro-amyloidogenic role of MT1-MMP. *Front Aging Neurosci* 6, 247.
- Qing, H., Zhou, W., Christensen, M.A., Sun, X., Tong, Y., and Song, W. (2004). Degradation of BACE by the ubiquitin-proteasome pathway. *FASEB J* 18, 1571-1573.
- Rajendran, L., Honsho, M., Zahn, T.R., Keller, P., Geiger, K.D., Verkade, P., and Simons, K. (2006). Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 103, 11172-11177.
- Ray, W.J., Yao, M., Mumm, J., Schroeter, E.H., Saftig, P., Wolfe, M., Selkoe, D.J., Kopan, R., and Goate, A.M. (1999). Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. *J Biol Chem* 274, 36801-36807.
- Restituto, S., Khatri, L., Ninan, I., Mathews, P.M., Liu, X., Weinberg, R.J., and Ziff, E.B. (2011). Synaptic Autoregulation by Metalloproteases and  $\gamma$ -Secretase. *Journal of Neuroscience* 31, 12083-12093.

- Rice, H.C., de Malmazet, D., Schreurs, A., Frere, S., Van Molle, I., Volkov, A.N., Creemers, E., Vertkin, I., Nys, J., Ranaivoson, F.M., *et al.* (2019). Secreted amyloid- $\beta$  precursor protein functions as a GABABR1a ligand to modulate synaptic transmission. *Science* *363*.
- Riedel, B.C., Thompson, P.M., and Brinton, R.D. (2016). Age, APOE and sex: Triad of risk of Alzheimer's disease. *J Steroid Biochem Mol Biol* *160*, 134-147.
- Rivera, S., García-González, L., Khrestchatisky, M., and Baranger, K. (2019). Metalloproteinases and their tissue inhibitors in Alzheimer's disease and other neurodegenerative disorders. *Cellular and Molecular Life Sciences* *76*, 3167-3191.
- Rivera, S., Khrestchatisky, M., Kaczmarek, L., Rosenberg, G.A., and Jaworski, D.M. (2010). Metzincin Proteases and Their Inhibitors: Foes or Friends in Nervous System Physiology? *Journal of Neuroscience* *30*, 15337-15357.
- Rose, C., Dorard, E., Audrain, M., Gorisse-Hussonnois, L., Cartier, N., Braudeau, J., and Allinquant, B. (2018). Transient increase in sAPP $\alpha$  secretion in response to Abeta1-42 oligomers: an attempt of neuronal self-defense? *Neurobiol Aging* *61*, 23-35.
- Rovelet-Lecrux, A., Hannequin, D., Raux, G., Le Meur, N., Laquerriere, A., Vital, A., Dumanchin, C., Feuillet, S., Brice, A., Vercelletto, M., *et al.* (2006). APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat Genet* *38*, 24-26.
- Ryan, K.A., and Pimplikar, S.W. (2005). Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. *J Cell Biol* *171*, 327-335.
- Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Saman, S., Jackson, B., McKee, A.C., Alvarez, V.E., Lee, N.C., *et al.* (2012). Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J Biol Chem* *287*, 3842-3849.
- Sannerud, R., Esselens, C., Ejsmont, P., Mattera, R., Rochin, L., Tharkeshwar, A.K., De Baets, G., De Wever, V., Habets, R., Baert, V., *et al.* (2016). Restricted Location of PSEN2/gamma-Secretase Determines Substrate Specificity and Generates an Intracellular Abeta Pool. *Cell* *166*, 193-208.
- Scharfenberg, F., Armbrust, F., Marengo, L., Pietrzik, C., and Becker-Pauly, C. (2019). Regulation of the alternative  $\beta$ -secretase meprin  $\beta$  by ADAM-mediated shedding. *Cellular and Molecular Life Sciences* *76*, 3193-3206.
- Schechter, I., and Ziv, E. (2008). Kinetic properties of cathepsin D and BACE 1 indicate the need to search for additional beta-secretase candidate(s). *Biol Chem* *389*, 313-320.
- Scheyer, O., Rahman, A., Hristov, H., Berkowitz, C., Isaacson, R.S., Diaz Brinton, R., and Mosconi, L. (2018). Female Sex and Alzheimer's Risk: The Menopause Connection. *The Journal Of Prevention of Alzheimer's Disease*, 1-6.
- Schönherr, C., Bien, J., Isbert, S., Wichert, R., Prox, J., Altmeyen, H., Kumar, S., Walter, J., Lichtenthaler, S.F., Weggen, S., *et al.* (2016). Generation of aggregation prone N-terminally truncated amyloid beta peptides by meprin beta depends on the sequence specificity at the cleavage site. *Mol Neurodegener* *11*, 19.
- Scott, C.C., Vacca, F., and Gruenberg, J. (2014). Endosome maturation, transport and functions. *Seminars in Cell & Developmental Biology* *31*, 2-10.
- Se Thoe, E., Fauzi, A., Tang, Y.Q., Chamyuang, S., and Chia, A.Y.Y. (2021). A review on advances of treatment modalities for Alzheimer's disease. *Life Sci* *276*, 119129.

- Sekine-Aizawa, Y., Hama, E., Watanabe, K., Tsubuki, S., Kanai-Azuma, M., Kanai, Y., Arai, H., Aizawa, H., Iwata, N., and Saido, T.C. (2001). Matrix metalloproteinase (MMP) system in brain: identification and characterization of brain-specific MMP highly expressed in cerebellum. *Eur J Neurosci* *13*, 935-948.
- Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* *81*, 741-766.
- Selkoe, D.J., and Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* *8*, 595-608.
- Serpell, L.C. (2000). Alzheimer's amyloid fibrils: structure and assembly. *Biochim Biophys Acta* *1502*, 16-30.
- Settembre, C., Fraldi, A., Medina, D.L., and Ballabio, A. (2013). Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nature Reviews Molecular Cell Biology* *14*, 283-296.
- Shahpasand-Kroner, H., Siddique, I., Malik, R., Linares, G., Ivanova, M.I., Ichida, J., Weil, T., Munch, J., Sanchez-Garcia, E., Klarner, F.G., *et al.* (2022). Molecular tweezers - supramolecular hosts with broad-spectrum biological applications. *Pharmacol Rev*.
- Sharples, R.A., Vella, L.J., Nisbet, R.M., Naylor, R., Perez, K., Barnham, K.J., Masters, C.L., and Hill, A.F. (2008). Inhibition of  $\gamma$ -secretase causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes. *The FASEB Journal* *22*, 1469-1478.
- Sisodia, S.S. (1992). Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proc Natl Acad Sci U S A* *89*, 6075-6079.
- Soba, P., Eggert, S., Wagner, K., Zentgraf, H., Siehl, K., Kreger, S., Lower, A., Langer, A., Merdes, G., Paro, R., *et al.* (2005). Homo- and heterodimerization of APP family members promotes intercellular adhesion. *EMBO J* *24*, 3624-3634.
- Song, C., Shi, J., Zhang, P., Zhang, Y., Xu, J., Zhao, L., Zhang, R., Wang, H., and Chen, H. (2022). Immunotherapy for Alzheimer's disease: targeting beta-amyloid and beyond. *Transl Neurodegener* *11*, 18.
- Spampinato, S.F., Merlo, S., Sano, Y., Kanda, T., and Sortino, M.A. (2017). Astrocytes contribute to A $\beta$ -induced blood-brain barrier damage through activation of endothelial MMP9. *J Neurochem* *142*, 464-477.
- Sperling, R.A., Aisen, P.S., Beckett, L.A., Bennett, D.A., Craft, S., Fagan, A.M., Iwatsubo, T., Jack, C.R., Kaye, J., Montine, T.J., *et al.* (2011). Toward defining the preclinical stages of Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia* *7*, 280-292.
- Spilman, P., Podluskaya, N., Hart, M.J., Debnath, J., Gorostiza, O., Bredesen, D., Richardson, A., Strong, R., and Galvan, V. (2010). Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One* *5*, e9979.
- Stomrud, E., Bjorkqvist, M., Janciauskiene, S., Minthon, L., and Hansson, O. (2010). Alterations of matrix metalloproteinases in the healthy elderly with increased risk of prodromal Alzheimer's disease. *Alzheimers Res Ther* *2*, 20.
- Sun, B., Zhou, Y., Halabisky, B., Lo, I., Cho, S.H., Mueller-Steiner, S., Devidze, N., Wang, X., Grubb, A., and Gan, L. (2008). Cystatin C-cathepsin B axis regulates amyloid beta levels and associated neuronal deficits in an animal model of Alzheimer's disease. *Neuron* *60*, 247-257.

- Szabo, M.P., Mishra, S., Knupp, A., and Young, J.E. (2022). The role of Alzheimer's disease risk genes in endolysosomal pathways. *Neurobiology of Disease* *162*, 105576.
- Tackenberg, C., and Nitsch, R.M. (2019). The secreted APP ectodomain sAPPalpha, but not sAPPbeta, protects neurons against Abeta oligomer-induced dendritic spine loss and increased tau phosphorylation. *Mol Brain* *12*, 27.
- Tai, H.C., Serrano-Pozo, A., Hashimoto, T., Frosch, M.P., Spires-Jones, T.L., and Hyman, B.T. (2012). The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated with dysfunction of the ubiquitin-proteasome system. *Am J Pathol* *181*, 1426-1435.
- Takashima, A., Wolozin, B., and Buee, L. (2019). Tau Biology. In *Advances in Experimental Medicine and Biology*.
- Tan, J.Z.A., and Gleeson, P.A. (2019). The trans-Golgi network is a major site for alpha-secretase processing of amyloid precursor protein in primary neurons. *J Biol Chem* *294*, 1618-1631.
- Tancini, B., Buratta, S., Sagini, K., Costanzi, E., Delo, F., Urbanelli, L., and Emiliani, C. (2019). Insight into the Role of Extracellular Vesicles in Lysosomal Storage Disorders. *Genes* *10*, 510.
- Taniguchi, M., Matsuura, K., Nakamura, R., Kojima, A., Konishi, M., and Akizawa, T. (2017). MMP-7 cleaves amyloid beta fragment peptides and copper ion inhibits the degradation. *Biometals* *30*, 797-807.
- Taylor, C.J., Ireland, D.R., Ballagh, I., Bourne, K., Marechal, N.M., Turner, P.R., Bilkey, D.K., Tate, W.P., and Abraham, W.C. (2008). Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. *Neurobiol Dis* *31*, 250-260.
- Taylor, H.A., Przemyska, L., Clavane, E.M., and Meakin, P.J. (2022). BACE1: More than just a beta-secretase. *Obes Rev* *23*, e13430.
- Thinakaran, G., and Koo, E.H. (2008). Amyloid Precursor Protein Trafficking, Processing, and Function. *Journal of Biological Chemistry* *283*, 29615-29619.
- Tian, Y., Bustos, V., Flajolet, M., and Greengard, P. (2011). A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway. *FASEB J* *25*, 1934-1942.
- Tian, Y., Crump, C.J., and Li, Y.M. (2010). Dual role of alpha-secretase cleavage in the regulation of gamma-secretase activity for amyloid production. *J Biol Chem* *285*, 32549-32556.
- Tseng, B.P., Green, K.N., Chan, J.L., Blurton-Jones, M., and LaFerla, F.M. (2008). Abeta inhibits the proteasome and enhances amyloid and tau accumulation. *Neurobiol Aging* *29*, 1607-1618.
- Ubelmann, F., Burrinha, T., Salavessa, L., Gomes, R., Ferreira, C., Moreno, N., and Guimas Almeida, C. (2017). Bin1 and CD2AP polarise the endocytic generation of beta-amyloid. *EMBO Rep* *18*, 102-122.
- Vaillant-Beuchot, L., Mary, A., Pardossi-Piquard, R., Bourgeois, A., Lauritzen, I., Eysert, F., Kinoshita, P.F., Cazareth, J., Badot, C., Fragaki, K., *et al.* (2021). Accumulation of amyloid precursor protein C-terminal fragments triggers mitochondrial structure, function, and mitophagy defects in Alzheimer's disease models and human brains. *Acta Neuropathol* *141*, 39-65.
- Valverde, A., Dunys, J., Lorivel, T., Debayle, D., Gay, A.S., Caillava, C., Chami, M., and Checler, F. (2021a). Dipeptidyl peptidase 4 contributes to Alzheimer's disease-like defects in a mouse model and is increased in sporadic Alzheimer's disease brains. *J Biol Chem* *297*, 100963.

- Valverde, A., Dunys, J., Lorivel, T., Debayle, D., Gay, A.S., Lacas-Gervais, S., Roques, B.P., Chami, M., and Checler, F. (2021b). Aminopeptidase A contributes to biochemical, anatomical and cognitive defects in Alzheimer's disease (AD) mouse model and is increased at early stage in sporadic AD brain. *Acta Neuropathol* *141*, 823-839.
- Vandenbroucke, R.E., and Libert, C. (2014). Is there new hope for therapeutic matrix metalloproteinase inhibition? *Nat Rev Drug Discov* *13*, 904-927.
- Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., *et al.* (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* *286*, 735-741.
- Vaz, M., and Silvestre, S. (2020). Alzheimer's disease: Recent treatment strategies. *Eur J Pharmacol* *887*, 173554.
- Vergheze, P.B., Castellano, J.M., and Holtzman, D.M. (2011). Apolipoprotein E in Alzheimer's disease and other neurological disorders. *The Lancet Neurology* *10*, 241-252.
- Vincent, B., and Checler, F. (2012). alpha-Secretase in Alzheimer's disease and beyond: mechanistic, regulation and function in the shedding of membrane proteins. *Curr Alzheimer Res* *9*, 140-156.
- Vingtdeux, V., Chandakkar, P., Zhao, H., d'Abramo, C., Davies, P., and Marambaud, P. (2011). Novel synthetic small-molecule activators of AMPK as enhancers of autophagy and amyloid-beta peptide degradation. *FASEB J* *25*, 219-231.
- Vingtdeux, V., Hamdane, M., Loyens, A., Gelé, P., Drobeck, H., Bégard, S., Galas, M.-C., Delacourte, A., Beauvillain, J.-C., Buée, L., *et al.* (2007). Alkalinizing Drugs Induce Accumulation of Amyloid Precursor Protein By-products in Luminal Vesicles of Multivesicular Bodies. *Journal of Biological Chemistry* *282*, 18197-18205.
- von Rotz, R.C., Kohli, B.M., Bosset, J.r.m., Meier, M., Suzuki, T., Nitsch, R.M., and Konietzko, U. (2004). The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. *Journal of Cell Science* *117*, 4435-4448.
- Walker, D.G., Link, J., Lue, L.F., Dalsing-Hernandez, J.E., and Boyes, B.E. (2006). Gene expression changes by amyloid beta peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. *J Leukoc Biol* *79*, 596-610.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* *416*, 535-539.
- Walsh, S., Merrick, R., Milne, R., and Brayne, C. (2021). Aducanumab for Alzheimer's disease? *BMJ* *374*, n1682.
- Wang, C., Sun, B., Zhou, Y., Grubb, A., and Gan, L. (2012). Cathepsin B degrades amyloid-beta in mice expressing wild-type human amyloid precursor protein. *J Biol Chem* *287*, 39834-39841.
- Wang, X., and Pei, D. (2001). Shedding of Membrane Type Matrix Metalloproteinase 5 by a Furin-type Convertase. *Journal of Biological Chemistry* *276*, 35953-35960.
- Wang, X., Wang, Z., Chen, Y., Huang, X., Hu, Y., Zhang, R., Ho, M.S., and Xue, L. (2014). FoxO mediates APP-induced AICD-dependent cell death. *Cell Death & Disease* *5*, e1233-e1233.
- Wang, Z., Xu, Q., Cai, F., Liu, X., Wu, Y., and Song, W. (2019). BACE2, a conditional beta-secretase, contributes to Alzheimer's disease pathogenesis. *JCI Insight* *4*.



- Warren, K.M., Reeves, T.M., and Phillips, L.L. (2012). MT5-MMP, ADAM-10, and N-Cadherin Act in Concert To Facilitate Synapse Reorganization after Traumatic Brain Injury. *Journal of Neurotrauma* 29, 1922-1940.
- Wegmann, S., Biernat, J., and Mandelkow, E. (2021). A current view on Tau protein phosphorylation in Alzheimer's disease. *Current Opinion in Neurobiology* 69, 131-138.
- Weidemann, A., Paliga, K., Durrwang, U., Reinhard, F.B., Schuckert, O., Evin, G., and Masters, C.L. (1999). Proteolytic processing of the Alzheimer's disease amyloid precursor protein within its cytoplasmic domain by caspase-like proteases. *J Biol Chem* 274, 5823-5829.
- Willem, M., Tahirovic, S., Busche, M.A., Ovsepian, S.V., Chafai, M., Kootar, S., Hornburg, D., Evans, L.D.B., Moore, S., Daria, A., *et al.* (2015).  $\eta$ -Secretase processing of APP inhibits neuronal activity in the hippocampus. *Nature* 526, 443-447.
- Willnow, T.E., and Andersen, O.M. (2013). Sorting receptor SORLA--a trafficking path to avoid Alzheimer disease. *J Cell Sci* 126, 2751-2760.
- Wiltfang, J., Esselmann, H., Cupers, P., Neumann, M., Kretschmar, H., Beyermann, M., Schleuder, D., Jahn, H., R  ther, E., Kornhuber, J., *et al.* (2001). Elevation of  $\beta$ -Amyloid Peptide 2–42 in Sporadic and Familial Alzheimer's Disease and Its Generation in PS1 Knockout Cells. *Journal of Biological Chemistry* 276, 42645-42657.
- Wingo, T.S. (2012). Autosomal Recessive Causes Likely in Early-Onset Alzheimer Disease. *Archives of Neurology* 69, 59.
- Winston, C.N., Goetzl, E.J., Schwartz, J.B., Elahi, F.M., and Rissman, R.A. (2019). Complement protein levels in plasma astrocyte-derived exosomes are abnormal in conversion from mild cognitive impairment to Alzheimer's disease dementia. *Alzheimers Dement (Amst)* 11, 61-66.
- Wolfe, M.S. (2020). Unraveling the complexity of  $\gamma$ -secretase. *Seminars in Cell & Developmental Biology* 105, 3-11.
- Xiao, Q., Gil, S.C., Yan, P., Wang, Y., Han, S., Gonzales, E., Perez, R., Cirrito, J.R., and Lee, J.M. (2012). Role of phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia (PICALM) in intracellular amyloid precursor protein (APP) processing and amyloid plaque pathogenesis. *J Biol Chem* 287, 21279-21289.
- Xiao, T., Zhang, W., Jiao, B., Pan, C.Z., Liu, X., and Shen, L. (2017). The role of exosomes in the pathogenesis of Alzheimer' disease. *Transl Neurodegener* 6, 3.
- Xu, J., Camfield, R., and Gorski, S.M. (2018). The interplay between exosomes and autophagy – partners in crime. *Journal of Cell Science* 131.
- Yamazaki, Y., Zhao, N., Caulfield, T.R., Liu, C.-C., and Bu, G. (2019). Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. *Nature Reviews Neurology* 15, 501-518.
- Yan, R. (2017). Physiological Functions of the beta-Site Amyloid Precursor Protein Cleaving Enzyme 1 and 2. *Front Mol Neurosci* 10, 97.
- Yang, A.J., Chandswangbhuvana, D., Margol, L., and Glabe, C.G. (1998). Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Abeta1-42 pathogenesis. *J Neurosci Res* 52, 691-698.
- Yang, D.S., Stavrides, P., Mohan, P.S., Kaushik, S., Kumar, A., Ohno, M., Schmidt, S.D., Wesson, D., Bandyopadhyay, U., Jiang, Y., *et al.* (2011). Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. *Brain* 134, 258-277.

- Yang, D.S., Stavrides, P., Saito, M., Kumar, A., Rodriguez-Navarro, J.A., Pawlik, M., Huo, C., Walkley, S.U., Saito, M., Cuervo, A.M., *et al.* (2014). Defective macroautophagic turnover of brain lipids in the TgCRND8 Alzheimer mouse model: prevention by correcting lysosomal proteolytic deficits. *Brain* *137*, 3300-3318.
- Yao, Y., Kang, S.S., Xia, Y., Wang, Z.-H., Liu, X., Muller, T., Sun, Y.E., and Ye, K. (2021). A delta-secretase-truncated APP fragment activates CEBPB, mediating Alzheimer's disease pathologies. *Brain* *144*, 1833-1852.
- Yin, K.J., Cirrito, J.R., Yan, P., Hu, X., Xiao, Q., Pan, X., Bateman, R., Song, H., Hsu, F.F., Turk, J., *et al.* (2006). Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism. *J Neurosci* *26*, 10939-10948.
- Yu, Y., Li, W., Mao, L., Peng, W., Long, D., Li, D., Zhou, R., and Dang, X. (2021). Genetically engineered exosomes display RVG peptide and selectively enrich a neprilysin variant: a potential formulation for the treatment of Alzheimer's disease. *J Drug Target* *29*, 1128-1138.
- Yuyama, K., and Igarashi, Y. (2017). Exosomes as Carriers of Alzheimer's Amyloid-ss. *Front Neurosci* *11*, 229.
- Zhang, F., Zhong, R.J., Cheng, C., Li, S., and Le, W.D. (2021). New therapeutics beyond amyloid-beta and tau for the treatment of Alzheimer's disease. *Acta Pharmacol Sin* *42*, 1382-1389.
- Zhang, X., Li, Y., Xu, H., and Zhang, Y.-w. (2014a). The  $\beta$ -secretase complex: from structure to function. *Frontiers in Cellular Neuroscience* *8*.
- Zhang, Z., Song, M., Liu, X., Kang, S.S., Kwon, I.S., Duong, D.M., Seyfried, N.T., Hu, W.T., Liu, Z., Wang, J.Z., *et al.* (2014b). Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nat Med* *20*, 1254-1262.
- Zhang, Z., Song, M., Liu, X., Su Kang, S., Duong, D.M., Seyfried, N.T., Cao, X., Cheng, L., Sun, Y.E., Ping Yu, S., *et al.* (2015). Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease. *Nature Communications* *6*.
- Zhang, Z., Xie, M., and Ye, K. (2016). Asparagine endopeptidase is an innovative therapeutic target for neurodegenerative diseases. *Expert Opinion on Therapeutic Targets* *20*, 1237-1245.
- Zhao, X., and Yang, J. (2010). Amyloid-beta peptide is a substrate of the human 20S proteasome. *ACS Chem Neurosci* *1*, 655-660.
- Zheng, X., Liu, D., Klarnar, F.G., Schrader, T., Bitan, G., and Bowers, M.T. (2015). Amyloid beta-protein assembly: The effect of molecular tweezers CLR01 and CLR03. *J Phys Chem B* *119*, 4831-4841.
- Zhuang, X., Xiang, X., Grizzle, W., Sun, D., Zhang, S., Axtell, R.C., Ju, S., Mu, J., Zhang, L., Steinman, L., *et al.* (2011). Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* *19*, 1769-1779.

## VII. ANNEX

---

# Is $\gamma$ -secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99?

Received for publication, October 9, 2020, and in revised form, February 26, 2021 Published, Papers in Press, March 1, 2021,  
<https://doi.org/10.1016/j.jbc.2021.100489>

Frédéric Checler\*, Elissa Afram, Raphaëlle Pardossi-Piquard, and Inger Lauritzen

From the Université Côte d'Azur, Institut de Pharmacologie Moléculaire et Cellulaire UMR7275 CNRS, Laboratoire d'Excellence Distalz, Sophia-Antipolis, Valbonne, France

Edited by Paul Fraser

Genetic, biochemical, and anatomical grounds led to the proposal of the amyloid cascade hypothesis centered on the accumulation of amyloid beta peptides ( $A\beta$ ) to explain Alzheimer's disease (AD) etiology. In this context, a bulk of efforts have aimed at developing therapeutic strategies seeking to reduce  $A\beta$  levels, either by blocking its production ( $\gamma$ - and  $\beta$ -secretase inhibitors) or by neutralizing it once formed ( $A\beta$ -directed immunotherapies). However, so far the vast majority of, if not all, clinical trials based on these strategies have failed, since they have not been able to restore cognitive function in AD patients, and even in many cases, they have worsened the clinical picture. We here propose that AD could be more complex than a simple  $A\beta$ -linked pathology and discuss the possibility that a way to reconcile undoubted genetic evidences linking processing of APP to AD and a consistent failure of  $A\beta$ -based clinical trials could be to envision the pathological contribution of the direct precursor of  $A\beta$ , the  $\beta$ -secretase-derived C-terminal fragment of APP,  $\beta$ CTF, also referred to as C99. In this review, we summarize scientific evidences pointing to C99 as an early contributor to AD and postulate that  $\gamma$ -secretase should be considered as not only an  $A\beta$ -generating protease, but also a beneficial C99-inactivating enzyme. In that sense, we discuss the limitations of molecules targeting  $\gamma$ -secretase and propose alternative strategies seeking to reduce C99 levels by other means and notably by enhancing its lysosomal degradation.

Alzheimer's disease (AD) is the most frequent age-related neurodegenerative disease. After initial clinical characterization, histopathological analysis revealed the presence of two major anatomical lesions signing this pathology: senile plaques that are extracellular protein aggregates and neurofibrillary tangles that are intracellular neuronal lesions (1–3). Senile plaques are mainly composed of the small 4 kDa amyloid beta peptide ( $A\beta$ ) (4) and neurofibrillary tangles of hyperphosphorylated and cleaved forms of the microtubule-associated protein tau (5, 6). The cloning of the  $A\beta$  precursor, the  $\beta$ -amyloid precursor protein (APP), in the late 80s was a key step in the understanding of the pathology. APP was found to be localized on chromosome 21 (7), thus explaining the

development of early-onset dementia and the presence of senile plaques in Down syndrome patients carrying an extra copy of APP due to a duplication of this chromosome (8). Furthermore, the identification of APP mutations responsible for autosomal dominant form of AD (FAD), including the "Dutch" (9) "London" (10) and "Swedish" mutations (11), showed that the functional consequences of these mutations were to augment the load of  $A\beta$  and/or to shift  $A\beta$  production to more aggregating  $A\beta$  peptides (12, 13). The role of  $A\beta$  in AD etiology was further confirmed by the discovery few years later of the first mutations in presenilins that were found to be involved in  $A\beta$  production and, similarly to APP mutations, seemed to exacerbate  $A\beta$  accumulation in cells, animal models as well as in AD-affected human brains (14–16). A last, but very important genetic evidence of a key role of  $A\beta$  in AD etiology was the recent findings of the Icelandic APP mutation that was shown to be protective by reducing cognitive decline and  $A\beta$  load by about 40% (17).

Thus, this set of histopathological, genetic, and biochemical data concurred to support the view that  $A\beta$  accumulation could be the etiological cause of the pathology, as stated in the amyloid cascade hypothesis that was proposed in 1992 by Hardy and Higgs (18). Indeed, mutations in three distinct proteins, namely APP, PS1, and PS2, are all responsible for FAD and have in common to modulate both APP processing and  $A\beta$  production. In this context, one can understand the huge efforts aimed at determining the mechanisms and enzymes involved in  $A\beta$  production and designing potent, specific, and bioavailable inhibitors of these enzymes (19, 20) or  $A\beta$  neutralizing antibodies. However, until so far, the outcomes of these  $A\beta$ -centered strategies have been extremely disappointing in our quest for meaningful treatments (21, 22) (Table 1). This has led to question the validity of the amyloid cascade hypothesis (23) or to discuss in a more cautious and balanced manner the ins, outs, and limitations of the procedures of clinical trials (24). It remains that before "throwing out the baby and the bath water," one should try to reconcile undoubted genetic evidences linking APP processing to AD and failures of  $A\beta$ -based clinical trials. In this context, a way to reconcile these observations could be to envisage the possible contribution of other APP-derived fragments distinct from  $A\beta$  itself to AD pathology. Indeed, growing evidence proposes that the direct precursor of  $A\beta$  (see below), the  $\beta$ -secretase-derived

\* For correspondence: Frédéric Checler, [checler@ipmc.cnrs.fr](mailto:checler@ipmc.cnrs.fr).

fragment, C99, could be an early and main contributor to AD. Thus, in this review, we address the possibility that the failure of A $\beta$ -centric clinical trials could be explained, at least partly, by their lack of effect on C99. To go further, we describe clues and evidences suggesting that  $\gamma$ -secretase should be considered as a beneficial C99-inactivating enzyme and argument against therapeutic strategies targeting this enzyme. We instead propose alternative strategies seeking to circumvent C99 accumulation, which would then have the advantage to reduce both C99 and A $\beta$  levels.

### APP processing and APP mutations

A $\beta$  is derived from APP that undergoes sequential limited proteolysis catalyzed by proteases called “secretases” (Fig. 1A) (25). In the nonamyloidogenic pathway, cleavage by  $\alpha$ -secretase and  $\gamma$ -secretase ends up with the production of three fragments: a large secreted N-terminal fragment (sAPP $\alpha$ ), a small soluble peptide p3, and the APP intracellular domain (AICD) (Fig. 1A). In the amyloidogenic pathway, a first cleavage of APP by the  $\beta$ -secretase is followed by  $\gamma$ -secretase cleavage. Again, the cleavage by  $\beta$ -secretase generates a large soluble extracellular secreted domain (sAPP $\beta$ ) and the remaining membrane stub, the  $\beta$ -secretase-derived fragment, C99, undergoes  $\gamma$ -secretase cleavage, thus liberating A $\beta$  and its C-terminal counterpart AICD (Fig. 1B). Other noncanonical cleavages on APP have also been more recently described (26). Among them, the  $\eta$ -secretase activity, carried by the matrix metalloproteinases (MT1-MMP and MT5-MMP), cleaves APP in its extracellular domain, thus producing a soluble fragment (sAPP $\eta$ ) and a membrane-bound C-terminal fragment,  $\eta$ CTF. This latter fragment can subsequently be processed by  $\alpha$ - or  $\beta$ -secretase, which will generate An $\alpha$  and C83 and An $\beta$  and C99, respectively (Fig. 1C) (27, 28).

Until so far, 28 pathogenic APP mutations have been identified with all, except the Swedish mutation, lying within the sequence of C99 ([www.alzforum.org/mutations](http://www.alzforum.org/mutations)). The Swedish variant (APP<sup>swe</sup>, KM670/671NL), although not located within C99 but two residues upstream, strongly increases the levels of this fragment, as well as those of A $\beta$  peptides, by boosting  $\beta$ -secretase cleavage (12, 13, 29). All pathogenic APP mutations lie either close to the  $\alpha$ -secretase cleavage site (the middle part of the A $\beta$  domain of C99), within the  $\gamma$ -secretase cleavage sites, or near the  $\beta$ -secretase cleavage site (N-terminal part), thus modifying the cleavages of APP by either of these secretases. While the knowledge about the exact effects of the pathogenic APP mutations is still limited, it seems that mutations close to the  $\beta$ -secretase (such as the Swedish and Leuven (E682K) mutations) favor C99 production, those close to the  $\alpha$ -secretase site (such as the Flemish [E693Q] or Arctic [E693G] mutations) lead to the generation of particular aggregation-prone A $\beta$  species, while mutations lying within the  $\gamma$ -secretase cleavage site elevate the A $\beta$ 42/A $\beta$ 40 ratio. In all cases, the development of AD pathology linked to these mutations is believed to be caused by an excessive A $\beta$  deposition (30). Nevertheless, the recent study by Xu *et al.* (31) showed that most (20 out of 28) APP mutations

not only alter A $\beta$  production, but are also less efficiently processed by  $\gamma$ -secretase and thereby also enhance the levels of C99.

### The $\beta$ -secretase

The nature of  $\beta$ -secretase was described in 1999 in four independent works and was referred to as BACE1 ( $\beta$ -site APP cleaving enzyme), memapsin 2 or ASP2 (32–35). BACE1 is an aspartic protease cleaving mainly within the Golgi and endosomes due to its optimal enzymatic activity at acidic pH (36). It cleaves APP at two distinct sites called the  $\beta$  and  $\beta'$  site, respectively. While only the cleavage on the  $\beta$ -site (Asp1) leads to A $\beta$  production (Fig. 1B), BACE1 is considered to cut APP primarily at the  $\beta'$ -site (Glu11) (Fig. 1B), thus generating the C89 fragment and a truncated nonpathological A $\beta$  peptide (A $\beta$  11-x) (36). Until so far, no mutations have been found in  $\beta$ -secretase, but the Swedish and the Leuven APP mutations are known to shift the cleavage from the  $\beta'$ -site to the  $\beta$ -site, thus leading to a much higher C99 and A $\beta$  production (12, 13, 37, 38). Inversely, the Icelandic mutation (A673T) seems to be protective against AD by decreasing  $\beta$ -secretase cleavage and thus reducing by 40% the amyloidogenic fragments sAPP $\beta$  and A $\beta$  (17, 39).

### The $\gamma$ -secretase and role of PS mutations

The first hint of the identity of the protease yielding the C-terminal end of A $\beta$ , the  $\gamma$ -secretase, was the discovery in 1995 of presenilin 1 (PS1) (40–42) and its family member presenilin 2 (PS2) (43, 44) proposed to constitute this enzyme (Fig. 2A). The definitive demonstration that presenilins could be involved in  $\gamma$ -secretase activity came from subsequent functional characterization showing that their depletion fully prevents A $\beta$  production (45, 46). However, later studies revealed that  $\gamma$ -secretase is a multiproteic complex built of not only PS1 or PS2 but also of nicastrin, the anterior pharynx defective 1 (Aph1), and presenilin enhancer 2 (PEN2) (47–49) (Fig. 2B), but the exact molecular structure of this complex was revealed by crystallography only in 2014 (50). Further studies also revealed a very complex cleavage of APP-CTFs by  $\gamma$ -secretase, since it takes place within the membrane and successively at several sites ( $\epsilon$ ,  $\zeta$ ,  $\gamma$ - and  $\gamma'$ ). The first endopeptidase cleavage ( $\epsilon$ -cleavage) can occur at two distinct sites leading to the generation of A $\beta$ 48 and A $\beta$ 49, which is then shortened by sequential carboxypeptidase cleavages (trimming) at  $\zeta$ ,  $\gamma$ - and  $\gamma'$ -sites, occurring at each three or four residues and *via* two distinct product lines (A $\beta$ 49 > A $\beta$ 46 > A $\beta$ 43 > A $\beta$ 40 > A $\beta$ 37) or (A $\beta$ 48 > A $\beta$ 45 > A $\beta$ 42 > A $\beta$ 38) and the final occurrence of peptides of varying lengths but two major species of 40 or 42 amino acid residues, respectively (51). Whereas A $\beta$ 40 is the most abundant peptide, A $\beta$ 42 is considered as the most pathogenic due to its high aggregation propensity.

So far, more than 300 mutations in presenilins are proposed to be responsible for early onset and familial forms of AD (FAD) and are now believed to comprise at least 70% of all FAD cases with the vast majority on PS1 ([www.alzforum.org/mutations](http://www.alzforum.org/mutations)). Although a huge effort has been made to try

**Table 1**  
Principal antiamyloid clinical drugs and strategies

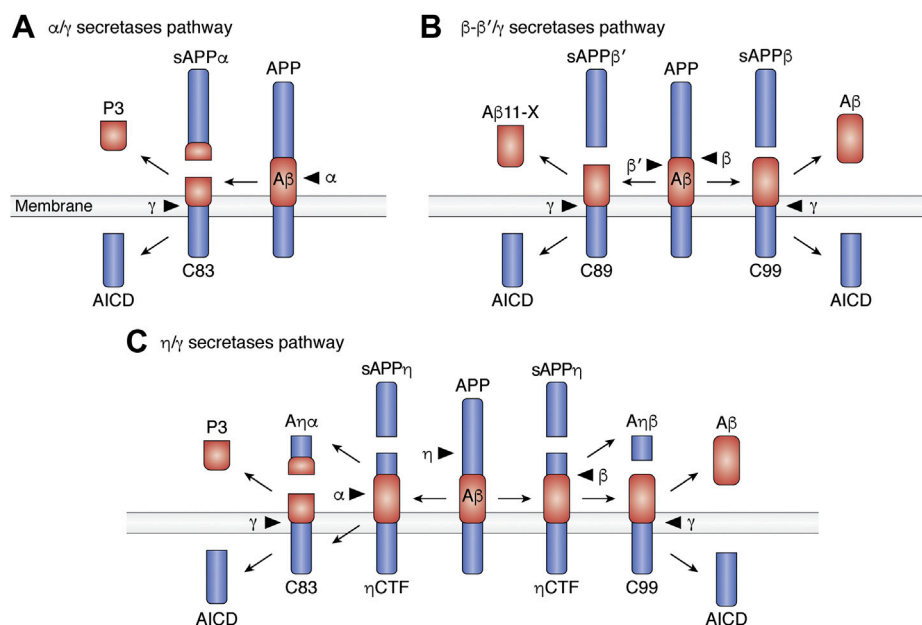
Strategy	Drug/specific target	A $\beta$ modulation in treated AD patients	FDA statute and participants	Side effects/cognitive readout	Reference
Active immunotherapy	AN-1792 (synthetic A $\beta$ 42, Janssen)	$\geq$ 60–70% A $\beta$ load reduction in the brain (post-mortem immunostaining)	Discontinued in 2002 (mild to moderate AD patients)	Meningoencephalitis	(208) (209) (210) (211)
	CAD106 (multiple copies of A $\beta$ 1-6 peptide, Novartis)	1.3% A $\beta$ reduction in brain PET scan (florbetapir) 2–3-fold increase in plasma A $\beta$ 40 at 450 mg	Discontinued in 2019 (asymptomatic carriers of APOE-4)	Worsens cognition, headache, nasopharyngitis, pyrexia, hypertension, back pain...	(212) (213)
Passive immunotherapy	Crenezumab (monomers, oligomers, and fibrils of A $\beta$ , Roche)	$\geq$ 70% A $\beta$ 42 increase in CSF at 15 mg/kg	Phase II ongoing in asymptomatic carriers of PS mutations	Lack of efficacy in mild to moderate AD	(212)
	Solanezumab (monomeric and soluble forms of A $\beta$ Eli Lilly)	170- and 18-fold increase in plasma A $\beta$ 40 and A $\beta$ 42 respectively no A $\beta$ modulation in brain PET scan (florbetapir) at 400 mg	Phase III ongoing in asymptomatic people who have biomarker evidence of brain amyloid deposition	Lack of efficacy in mild to moderate AD and in asymptomatic carriers of APP and PS mutations	(213)
	Aducanumab (oligomers, and fibrils of A $\beta$ , Biogen)	80% A $\beta$ reduction in brain PET scan (florbetapir) at 10 mg/kg	Phase III ongoing in an open-label extension study in mild to moderate AD patients	One of the two trials (EMERGE) was positive with a significant reduction in cognitive decline in mild to moderate AD a biologics license application was submitted to the FDA for approval on July 2020	<a href="http://www.alzforum.org/therapeutics/aducanumab">www.alzforum.org/therapeutics/aducanumab</a>
	Gantenerumab (oligomers, and fibrils of A $\beta$ , Roche)	$\geq$ 15%, 35% and 78% A $\beta$ reduction in brain PET scan (florbetapir) at 60, 200 and 1200 mg respectively	Phase III ongoing in an open-label extension study in mild to moderate AD patients (SCarlet RoAD, Marguerite RoAD, and GRADUATE) and in asymptomatic carriers of APP and PS mutations (Dian-Tu)	A directional trend for slower clinical decline in mild to moderate AD	(214) (215)
$\beta$ -secretase inhibitors	BAN2401 (soluble A $\beta$ protofibrils, Biogen)	$\geq$ 120% A $\beta$ 40 increase in plasma 300 fold A $\beta$ 42 increase in CSF 93% A $\beta$ reduction in brain PET scan (florbetapir) at 10 mg/kg	Phase III ongoing in early symptomatic AD patients (Clarity AD) and in asymptomatic people who have biomarker evidence of brain amyloid deposition (AHEAD 3–45)	47% and 30% reduction in cognitive decline as judged by the ADAS-Cog and the ADCOMS respectively	(216) <a href="http://www.alzforum.org/therapeutics/ban2401">www.alzforum.org/therapeutics/ban2401</a>
	Verubecestat (MK-8931Merck)	$\geq$ 57–84% A $\beta$ reduction in CSF at 12–60 mg	Discontinued in 2018 (prodromal, mild to moderate AD patients)	Worsens cognition, anxiety, depression, and sleep problems	(217) (218)
	Atabecestat (Janssen)	$\geq$ 67–90% A $\beta$ reduction in CSF at 10–50 mg	Discontinued in 2018 (asymptomatic people)	Worsens cognition, elevated liver enzymes, depression, anxiety, and sleep problems	(219) (220)
	Lanabecestat (AZD3293, Eli Lilly)	$\geq$ 64% to 78% A $\beta$ reduction in plasma at 15–50 mg $\geq$ 51% to 76% A $\beta$ reduction in CSF at 15–50 mg	Discontinued in 2018 (prodromal and mild AD patients)	Lack of efficacy on cognition, neuropsychiatric adverse events, weight loss, hair color changes	(221) (222)
	Umibecestat (Novartis)	$\geq$ 90% A $\beta$ reduction in CSF at 85 mg	Discontinued in 2019 (asymptomatic carriers of APOE-4)	Worsens cognition, brain atrophy and weight loss	(223) (187) <a href="http://www.alzforum.org/therapeutics/umibecestat">www.alzforum.org/therapeutics/umibecestat</a>
	Elenbecestat (Biogen)	$\geq$ 5.8% and $\geq$ 13.6% A $\beta$ reduction in brain PET scan (florbetaben and florbetapir respectively) at 50 mg	Discontinued in 2019 (prodromal, mild to moderate AD patients)	Weight loss, skin rashes and neuropsychiatric adverse events	(224) (225)

Table 1—Continued

Strategy	Drug/specific target	A $\beta$ modulation in treated AD patients	FDA statute and participants	Side effects/cognitive readout	Reference
$\gamma$ -Secretase inhibitors	Semagacestat (Eli Lilly)	$\geq 58.2\%$ to $64.6\%$ A $\beta$ reduction in plasma at 100–140 mg $\geq 47$ – $84\%$ newly A $\beta$ reduction in CSF at 100–280 mg $\geq 40\%$ A $\beta$ reduction in CSF at 125 mg	Discontinued in 2011 (AD patients)	<b>Worsens cognition</b> , skin cancer and infections.	(110) (111) (112)
$\gamma$ -Secretase modulators	Avagacestat (Bristol-Myers Squibb)	Not determined	Discontinued in 2012 (prodromal AD)	<b>Worsens cognition</b> , skin cancers, diarrhea, nausea, vomiting, and rash	(116)
	Rofecoxib (Merck)	No A $\beta$ 42 modulation in plasma and CSF	Discontinued in 2004 (mild to moderate AD patients)	<b>Lack of efficacy</b> , cardiovascular damage	(122)
	Tarenfluril (Myriad Genetics)	No A $\beta$ 42 modulations in CSF	Discontinued in 2009 (mild AD patients)	<b>Worsens cognition</b> , dizziness, upper respiratory tract infection and constipation.	(226) (123)
	Naproxen (Procter & Gamble)	No A $\beta$ 42 modulations in CSF	Discontinued in 2019 (asymptomatic carriers of APP and PS mutations)	<b>Lack of efficacy</b> , hypertension, gastrointestinal, and vascular or cardiac problems	(124)

ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADCOMS, Alzheimer's Disease Composite Score; CSF, cerebrospinal fluid; FDA, US Food and Drug Administration; MCI, mild cognitive impairment.

to understand the pathogenic mechanisms linked to these mutations, their exact functional consequences remain enigmatic and are probably not explained by one common mechanism. Two main, but not exclusive, hypotheses have been proposed and are still hotly debated. First, Hardy and Selkoe proposed, accordingly to the amyloid cascade hypothesis, that the effect of PS mutations should be a gain of function of  $\gamma$ -secretase (52). However, later studies revealed that most PS mutations are actually loss-of-function mutants, and a revised version of this hypothesis became focused on the relative levels of A $\beta$ 42 to A $\beta$ 40 (A $\beta$ 42/A $\beta$ 40) rather than absolute increases in A $\beta$ 42 (53). It was suggested that partial loss of PS mutations shifts the cleavage specificity of the mutant enzyme to favor A $\beta$ 42 production and that even a minor increase in A $\beta$ 42 levels could be sufficient for seed formation (54, 55). In that way, loss of function should be considered as gain of “toxic function” because of a relative increase in more aggregation-prone A $\beta$  (56). On the other hand, Shen and Kelleher proposed an alternative hypothesis “the presenilin hypothesis,” stating that loss of general PS function should be the trigger of neurodegeneration and defects in cognitive function (57). They proposed that pathogenic PS mutations behave as dominant-negative mutation due to a mechanism in which mutant PS interferes with the activity of wild-type PS, thereby reducing their physiological functions (58, 59). In agreement with this hypothesis, the extensive work of Sun and colleagues (60), in which 138 pathogenic PS mutations were analyzed, revealed that more than 90% of them led to reduced production of both A $\beta$ 40 and A $\beta$ 42, and this is in a dominant negative manner (60). This conclusion of Sun and colleagues was based on purified  $\gamma$ -secretases harboring PS mutations and assay of C99 cleavage *in vitro* or in a cell-based assay. In contrast, Szaruga and colleagues (61) measured  $\gamma$ -secretase activity in brain extracts from patients carrying PS1 mutations (one healthy and one disease allele) and found a variable effect on endopeptidase cleavage but a consistent reduction in carboxypeptidase activity, seen as A $\beta$ 38/A $\beta$ 42. These data therefore seemed to fit with the postulate that a qualitative shift in A $\beta$  profiles toward the generation of longer aggregation-prone peptides ( $>$ A $\beta$ 42) should be the common denominator of AD (61) and were in agreement with the earlier work from Saito and Saido, demonstrating a particular high toxicity of A $\beta$ 43 (62) and the initial statements of a role of aggregating-prone A $\beta$  in seed formation (63–65). However, arguing against a direct and unique role of the A $\beta$ 42/A $\beta$ 40 ratio was the findings in the work of Sun and colleagues that observed that the age of onset of FAD did not seem to correlate well with the change in this ratio (60) in contradiction to what was previously proposed (66). A recent study indicated that the pathogenicity of PS1 mutations could be related to an intracellular mislocalization of the  $\gamma$ -secretase complex composed of these mutants (67, 68). Indeed, in contrary to wild-type PS1 having a broad cellular distribution, mutated PS1 was found to be restricted to endosomal/lysosomal compartments (67, 68). Hence, it was postulated that mutated PS would favor intraneuronal A $\beta$



**Figure 1. APP metabolism.** *A*, represents the  $\alpha/\gamma$ -secretase pathway in which APP is first cleaved by the  $\alpha$ -secretase producing the soluble fragment sAPP $\alpha$  and the membrane-embedded C-terminal fragment, C83 that is then cleaved by  $\gamma$ -secretase to release the cytosolic AICD fragment and p3 peptide, respectively. *B*, represents the  $\beta$  or  $\beta'/\gamma$ -secretase pathway in which APP is first cleaved by the  $\beta$ -secretase at  $\beta$  or  $\beta'$  site to liberate the soluble fragments sAPP $\beta$  or sAPP $\beta'$  and the membrane-embedded C99 or C89 fragments, respectively. Then  $\gamma$ -secretase cleavage releases the cytosolic AICD fragment and A $\beta$  peptides (A $\beta$ 1-40 or A $\beta$ 1-42) or A $\beta$ 11-40, respectively. Note that in physiological conditions,  $\beta$ -secretase mainly cleaves at the  $\beta'$  site, but some FAD mutations shift the cleavage to the  $\beta$ -site to produce more C99. *C*, represents the  $\eta/\gamma$ -secretase pathway in which  $\eta$ -secretase cleavage releases the soluble fragment sAPP $\eta$  and the membrane-embedded  $\eta$ CTF, which is processed by  $\alpha$ - or  $\beta$ -secretase, thus generating A $\eta\alpha$  or A $\eta\beta$  peptides, respectively.

production, which could be particularly toxic (69–71). Moreover, the acidity of endosomal/lysosomal compartments could be less propitious for carboxypeptidase activity, thus leading to the generation of longer and more aggregating A $\beta$  peptides (67) and the acidic pH and high concentrations of A $\beta$  in these compartments could be particularly propitious for A $\beta$  aggregation and thus toxicity.

A more recent hypothesis suggests that the pathogenicity of PS mutations could also be linked, or at least partly, to their effect on C99 accumulation. Indeed, although few studies have investigated the effects of FAD mutations on C99 levels, recent studies seem to reveal a consistent increase in C99 in the presence of most pathogenic PS mutants (72–74). This was even the case in neurons derived from pluripotent stem cells (iPSCs) harboring a wide panel of pathogenic PS mutations in the absence of overexpression (73). Whether this effect on C99 is explained by a loss of function (reduced  $\gamma$ -secretase activity) and/or other PS-associated side effects remains unclear. Indeed, accumulating evidence indicates that both PS1 and PS2 are involved in essential cellular processes, independently of their role in  $\gamma$ -secretase, and proposed to be due notably to their function as chaperons and/or control of calcium release from the endoplasmic reticulum (for reviews see (75, 76)). Some of these functions have been suggested to be modified by FAD mutations, although again, many contrasting findings have been reported. Indeed, most studies on noncatalytic functions of PS have been carried out on knockout models, thus in the absence of PS, whereas fewer works have investigated the impact of FAD mutations on these functions. One of the most consistently described noncatalytic functions of PS is

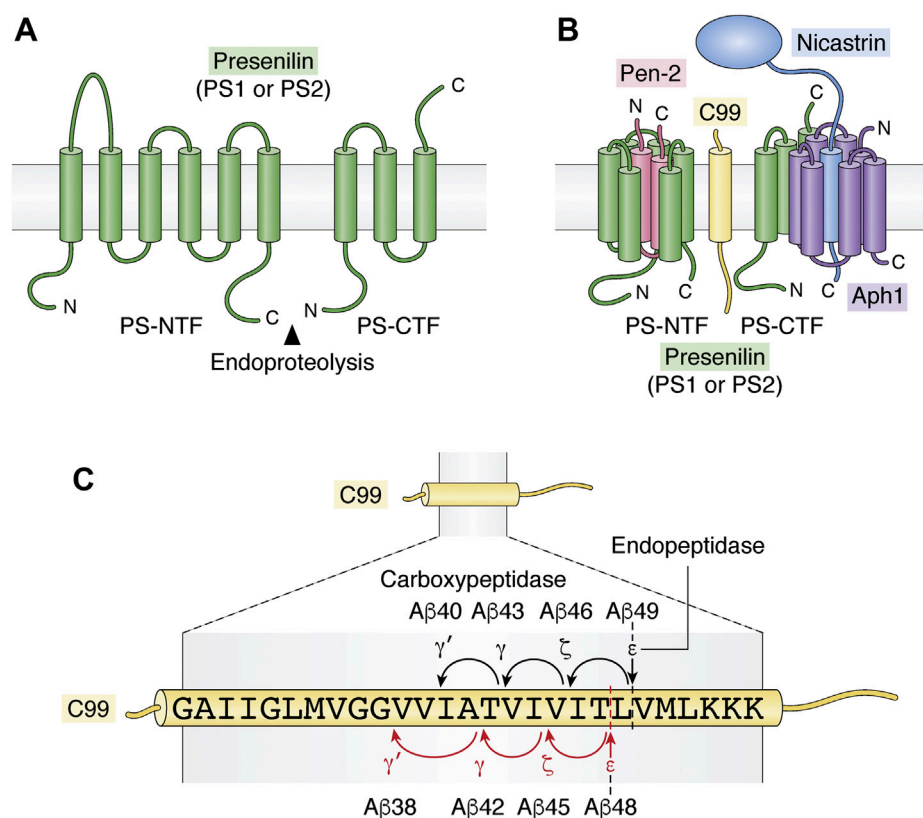
their involvement in the regulation of both autophagy and lysosomal function (75, 76) known also to be involved in C99 degradation (see below). Whether these  $\gamma$ -secretase-independent functions contribute to, or are responsible for, C99 accumulation, in cells expressing PS mutations, remains to be established.

### Physiological function of $\gamma$ -secretase

FAD cases correspond to only a very small subset of patients (about 1%) (77). Thus, both A $\beta$  and C99 accumulation in FAD could reflect an exacerbated process or a dysfunction occurring only in few cases. The situation seems to be different in sporadic AD (SAD) that corresponds to most cases. In SAD, A $\beta$  accumulation is mostly believed to be linked to age-related impairment in A $\beta$  clearance, rather than to an increased A $\beta$  biogenesis. Thus, A $\beta$  should be considered firstly as a physiological product derived from normal processing of APP (78, 79). Although little is still known about the exact physiological role of A $\beta$ , the peptide is known to regulate synaptic activity, to have antimicrobial and tumor suppressing properties (for review see (80)), and to be neuroprotective in some pathological conditions (81–83). Thus, to some extent the failure of clinical trials aimed at either inhibiting A $\beta$  production or neutralizing A $\beta$  once formed could be explained, at least in part, by the abolition of such A $\beta$ -mediated physiological functions.

If considering A $\beta$  as a physiological product, this implies that a main role of  $\gamma$ -secretase should be to generate bioactive molecules involved in important vital processes. Indeed, this point of view fits with genetic evidences indicating that the





**Figure 2.  $\gamma$ -secretase.** *A*, displays a schematic representation of presenilins (PS1 or PS2) composed of nine transmembrane domains and harboring the catalytic core of the complex with two aspartyl residues in the transmembrane domains TM-6 and TM-7. During maturation, presenilin undergoes endoproteolysis, and the resulting N-terminal fragment (PS-NTF) and C-terminal fragment (PS-CTF) remain associated. *B*, represents the multimeric  $\gamma$ -secretase complex composed of four membrane proteins: presenilin (green), nicastrin (blue) composed of a single transmembrane and a large glycosylated N-terminal ectodomain, Aph-1 (purple) composed of seven transmembrane domains, and Pen-2 (pink) composed of two transmembrane domains. The substrate C99 (yellow) interacts with  $\gamma$ -secretase to form an enzyme substrate complex. *C*, represents the successive proteolysis of C99 transmembrane domain by  $\gamma$ -secretase. First, an endoproteolytic cleavage occurs close to the membrane-cytosol interface at two possible  $\epsilon$  sites yielding long A $\beta$  peptides A $\beta$ 48 or A $\beta$ 49, respectively. Then a carboxypeptidase activity of  $\gamma$ -secretase trims A $\beta$ 48 and A $\beta$ 49 each 3 or 4 amino acid at successive  $\zeta$ ,  $\gamma$ , and  $\gamma'$ -sites leading to two A $\beta$  product lines: A $\beta$ 49→A $\beta$ 46→A $\beta$ 43→A $\beta$ 40 and A $\beta$ 48→A $\beta$ 45→A $\beta$ 42→A $\beta$ 38, respectively.

invalidation of  $\gamma$ -secretase in mice is lethal (84) and that the conditional knockout of PS1 leads to neurodegeneration at the adulthood (85). Of course, these observations are probably explained by the numerous, more than 150, other  $\gamma$ -secretase proteolytic targets involved in physiological processes (86). Indeed, similar to APP, most of  $\gamma$ -secretase substrates are type I transmembrane proteins, which undergo “Regulated Intra-membrane proteolysis (RIP), a proteolytic process in which  $\gamma$ -secretase-mediated cleavage occurs inside the membrane ending up with the production of an intracellular fragment that shuttles to the nucleus and acts as a transcription factor (87, 88). The seminal example of RIP was described for Notch that undergoes  $\gamma$ -secretase-mediated cleavage, thereby generating the Notch Intracellular Domain (NICD) (89), a key player of embryonic development (90). For instance, this lack of Notch cleavage (91) is believed to explain the lethal embryologic defects observed in PS-invalidated mouse embryos (84). In addition, numerous other substrates (the list is far from being exhaustive) have been reported to undergo RIP including cadherins (N and E Cadh-ICDs) (92), alcadeins (93), CD44 (94), or more recently TREM2 (95). Generally, the generation of the intracellular domain of these proteins is essential in the transcriptional regulation of physiological

processes such as neurite outgrowth, cell adhesion and migration, or synaptogenesis (96, 97). This phenomenon is also described for APP for which the intracellular domain AICD has been found to form a transcriptional active complex with Fe65 and Tip60 (98, 99) that regulates key proteins involved in the control of cell death (p53) (100), A $\beta$  degradation (Neprilysin) (101, 102), as well as tau phosphorylation (GSK3 $\beta$ ) (103, 104). Thus,  $\gamma$ -secretase cleavage should be considered as a necessary step in the formation of both A $\beta$  and AICD. Besides this role,  $\gamma$ -secretase cleavage has also been proposed to behave as a “membranous proteasome” essential for the inactivation of the buildup of toxic membranous stubs (105). It remains unclear, whether this “proteasome-like activity” is nonselective and inactivates all substrates equally, or if some substrates are cleaved by specific protease complexes. Recent evidences seem to indicate at least some substrate specificity and that not only the exact protein composition of the secretase complex, but also the cellular context, subcellular localization, and the presence of nonessential cofactors (106) can be key determinants for this control. For instance, protease heterogeneity has been shown to be related to the exact protein composition of the protease and alternative splicing of Aph1 leads to further complexity (107, 108). For instance, in

**Table 2**  
C99-associated toxicity in AD mice models and human brain

Mouse models	Mutations	C99 accumulation-associated pathology	References
3xTgAD 2xTgAD	APP <sup>K670N,M671L</sup> , PS1 <sup>M146V</sup> , MAPT <sup>P301L</sup> APP <sup>K670N,M671L</sup> , MAPT <sup>P301L</sup>	Endolysosomal and autophagic dysfunction Inflammation, LTP alterations Mitochondrial pathology	(128, 137, 139, 163, 168)
5xFAD APP/PS1	APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> , APP <sup>V717I</sup> , PS1 <sup>M146L,L286V</sup> APP <sup>751<sub>K670N,M671L</sub></sup> , PS1 <sup>G384A</sup>	Mitochondrial pathology Accumulation in MAMs	(224) (155)
APPE663Q TgCRND8 J20 Knock-in APP <sup>NL</sup> APP <sup>NL-F</sup> and APP <sup>NL-G-F</sup> APP <sup>Hm/Hm</sup>	APP <sup>E663Q</sup> APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> APP <sup>KM670/671NL</sup> , APP <sup>KM670/671NL,L1716F</sup> APP <sup>KM670/671NL,E1716F,E693G</sup> hA $\beta$ sequence (G67/68,F681,Y,R684H) Various APP and PS mutants	Lysosomal dysfunction, inflammation Brain network alterations Brain network alterations Nd Nd	(131) (129, 227) (130) (132–134)
Human iPSCs	Various APP and PS mutants	Lysosomal dysfunction	(143)
Down syndrome fibroblasts	Ts65DN mice or human patients	Endosomal pathology Endosomal pathology lysosomal dysfunction Axonal transport defects	(140, 228–230)
Human brain	FAD SAD	Nd Correlation with neuronal vulnerability	(126, 137, 138, 175)

APP, amyloid precursor protein; FAD, familial Alzheimer disease; IPSC, induced pluripotent stem cells; LTP, long-term potentiation; MAPT, microtubule-associated protein tau; nd, not determined; PS, presenilin; SAD, sporadic Alzheimer's disease.

mice the knockout of the 3 Aph1 subunits led to an important increase in some, but not all, membrane-bound fragments and APP-dependent neurodegeneration (107, 108). Hu and colleagues demonstrated that nicastrin is required for APP processing but not Notch processing, whereas Aph1 is necessary for the processing of both APP and Notch (109). Selectivity also seems to be determined by the subcellular localization of the protease, since, as described above, PS1- or PS2-containing complexes do not have the same cellular localization and do not cleave APP identically (67, 68). Interestingly, mutations in PS1 change the localization toward that of PS2-containing complexes (67).

### Challenges and complications of targeting $\gamma$ -secretase in AD

After the discovery of  $\gamma$ -secretase, a huge amount of research was carried out to develop potent and bioavailable  $\gamma$ -secretase inhibitors (GSIs), but the clinical trials based on these inhibitors all failed (Table 1). Indeed, none of them led to improvement of AD-linked cognitive decline and even sometimes they worsened them. In most of these trials, A $\beta$  levels in plasma or cerebrospinal fluid (CSF) were significantly decreased, thus clearly indicating that a reduction in total A $\beta$  was not sufficient to restore cognitive function. This was the case for Semagacestat, the first GSI tested in late-stage clinical trials for AD, which was reported to reduce A $\beta$  levels by more than 60% in the plasma (110), and new A $\beta$  synthesis was decreased by more than 80% in CSF (111). More alarming, many trials were readily stopped because of increased risk of developing skin cancer and infections, which was supposed to be tightly linked to a defective Notch signaling in the presence of the inhibitors (112). Therefore, several groups started to screen for drugs having a higher affinity for APP than toward Notch, such as Avagacestat, initially reported to be 140-fold more selective for APP than Notch (113), although its Notch sparing ability remains controversial (114, 115). Avagacestat led to a 40% A $\beta$  reduction in CSF (Table 1), but demonstrated similar side effects in clinical trials (116). Therefore, due to the huge number of different  $\gamma$ -secretase substrates, it has appeared difficult to target specifically and exclusively the  $\gamma$ -secretase-mediated APP cleavage.

Furthermore, data from preclinical trials proposed that the lack of efficacy, or even worsening, of cognitive function in these trials was also linked to the progressive accumulation of APP-CTFs in the presence of GSIs (117). To avoid such effects, therapeutic development became focused on  $\gamma$ -secretase modulators (GSMs) that were expected to be safer, since they interact with  $\gamma$ -secretase complex through the allosteric binding site, thereby modifying the enzyme activity but not blocking it (118). The concept of  $\gamma$ -secretase modulation was discovered with some nonsteroidal anti-inflammatory drugs (NSAIDs) such as flurbiprofen, indomethacin, and ibuprofen, which are considered as GSMs, because they induce conformational changes in PS1 and shift the cleavage of C99 toward shorter A $\beta$  species such as A $\beta$ 37 and A $\beta$ 38 (16, 119). In that way, GSMs do not lead to increased APP-CTFs levels. Indeed,

in AD animal models, the chronic treatment with GSMs did not lead to the worsening of cognitive function observed with GSIs (117). Moreover, GSMs are also safer because they reduce the level of A $\beta$ 42 and increase shorter A $\beta$  peptides without affecting Notch signaling (120, 121). Nonetheless, the first GSMs tested in clinical trials, Rofecoxib (122), Tarenflurbil (123) or Naproxen (124), did not show efficacy, but the lack of effects seemed to be related to a very poor blood–brain barrier crossing ability of these drugs (Table 1).

Thus, taken together,  $\gamma$ -secretase-based strategies have so far been unsuccessful, even if GSMs seem to be more promising (125). Still, if considering  $\gamma$ -secretase as a C99-inactivating enzyme, we believe that one should carefully question the validity of targeting this enzyme.

### **C99 is toxic and $\gamma$ -secretase inhibitors potentiate pathogenic phenotypes**

Emerging data propose a pathological role of the intraneuronal accumulation of C99 (Table 2). Indeed, a very recent work showed that the accumulation of C99, rather than that of A $\beta$ , correlates with neuronal vulnerability in AD-affected patients (126). To distinguish between C99 and A $\beta$  *in situ*, which can be very challenging because the two of them have common epitopes, the group of Drs Bustos and Greengard developed a sophisticated technique adapted from the proximity ligation assay (PLA) and using both N- and C-terminal directed antibodies. This link between neuronal vulnerability and C99 in human AD was an important observation previously supported by many animal models. The first evidence of C99 accumulation in AD mouse models came from studies on the widely used 3xTg-AD model (APP<sub>swe</sub>, Tau<sub>p310L</sub>, PS1<sub>M146V</sub>) developed by the group of La Ferla (127). In the original work, the authors used N-terminal A $\beta$  antibodies and claimed that these mice harbor intracellular A $\beta$  accumulation (127). However, years later our own work, using different and complementary approaches, indicated that this accumulated material corresponded to C99 and not to A $\beta$  (128). Indeed, as stated above, it is challenging to discriminate between A $\beta$  and C99 *in situ*. Hence, only a detailed characterization using N- and C-terminal antibodies, pharmacological treatments ( $\beta$ - and  $\gamma$ -secretase inhibitors), and/or genetic approaches (as PS mutations) can allow this discrimination. Most and even recent studies do not provide this detailed characterization, which may explain the lack of information concerning C99 in the literature. In the 3xTgAD model, C99 accumulates early and much before A $\beta$  can be detected (128). Of importance, C99 accumulation was found to also occur in many other transgenic models including Tg-CRND8 (129) and JA20 (130) mice carrying the APP<sub>swe</sub> mutation, known to increase C99 production, but also in mice displaying the APP<sup>E693Q</sup> Dutch mutation (131), in which a C99 overproduction is not expected to occur. Interestingly, and importantly, C99 accumulation also occurs in the knock-in models APP-NL and APP-NL-F, harboring the Swedish (KM670/671NL) mutation alone (APP-NL) or in combination with the I706F mutation (APP-NL-F), respectively (132, 133) (Table 2) indicating that C99

accumulation was not just an artificial phenotype linked to APP overexpression, as earlier described for other events taking place in transgenic mouse models that sometimes express very high levels of APP (132). The presence of the Swedish mutation could indeed, at least to some extent, explain the C99 accumulation in these knock-in mice. However, recent studies clearly showed that humanizing APP (substituting the three amino acids G676R, F681Y, and R684H) in mice or rats is sufficient to increase C99 levels (134–136). In animals displaying rodent APP, the protein was found to be mainly processed by  $\alpha$ -secretase, thus generating almost only C83, whereas the presence of human APP increases  $\beta$ -secretase cleavage and leads to enhanced C99 levels (134). These data thus reveal a different APP processing of human and rodent APP, which could explain the higher levels of C99 in human as compared with rodents (137) and may be one of the reasons for the C99 accumulation observed even in SAD cases (126, 137, 138). Overall, these findings ruled out the possibility that it could be “model specific” but rather suggest that it should be a common and early alteration in AD.

In AD mouse models, such as in 3xTg-AD mice, C99 accumulation was described to be both the cause and the consequence of endosomal perturbations, ultimately leading to failure of lysosomal and autophagic processes (139). A similar link between C99 accumulation and lysosomal dysfunction was described in the mouse bearing the Dutch mutation APP<sup>E693Q</sup> (131). Moreover, in fibroblasts from Down syndrome patients or from the mouse model Ts65Dn, C99 were found to induce enlargement of Rab5 positive early endosomes, aberrant endocytosis, and impaired endosomal transport (140). Interestingly, these alterations were fully A $\beta$ -independent, since they were rescued by  $\beta$ -secretase inhibitors or partial BACE1 genetic depletion and were enhanced by GSIs (139–142). Of utmost interest, C99 accumulation has also been associated to endolysosomal dysfunction in human cellular AD models. Indeed, the earlier mentioned transcriptomic analysis of induced pluripotent stem cells (iPSCs) harboring AD-related APP or PS1 mutations indicated similar changes in endocytosis-associated genes (73). Strikingly, these mutations brought divergent data concerning A $\beta$ , but they all displayed increases in C99 levels as well as endosomal dysfunction (73). The use of notably a BACE inhibitor, GSMs and GSIs, showed that endosomal pathology was linked to C99 and not to the changes in A $\beta$  (73). Resembling data were obtained in another study using iPSCs-derived neurons obtained from AD patients harboring APP or PS1 mutations, which displayed both C99 accumulation and dysfunction of the endosomal–lysosomal network (143). As a difference to the work of Kwarts and colleagues, mutations in APP but not in PS1 led to early endosomal (Rab5 associated) pathology, but all mutations led to enlarged late endosomes and defective lysosomal degradation. These effects were APP-dependent, reversed by BACE inhibition, and exacerbated by GSIs. These results agree with the consistent data linking C99 to a dysfunction in the endolysosomal network occurring even in the presence of physiological APP levels (144). Whether C99 accumulation in these systems is linked to the partial loss of

function of  $\gamma$ -secretase or to other PS but  $\gamma$ -secretase-independent mechanisms, such as impaired lysosomal-autophagic degradation, as discussed earlier in this review, remains to be established. Related to endolysosomal dysfunction, the pathogenicity of C99 could also be due to its propensity to aggregate and to spread through exosomes, small vesicles secreted from cells (145). Indeed, exosomes originate from endosomes and C99 is detected in these vesicles (145–147). Our recent work showed the possible presence of C99 existing as oligomers (C99 homomers and heteromers composed of C99/C83) in exosomes, the levels of which were drastically enhanced upon  $\gamma$ -secretase inhibition (145). The latter statement is of importance due to the proposed role of exosomes in prion-like transmission of neurotoxic molecules (148).

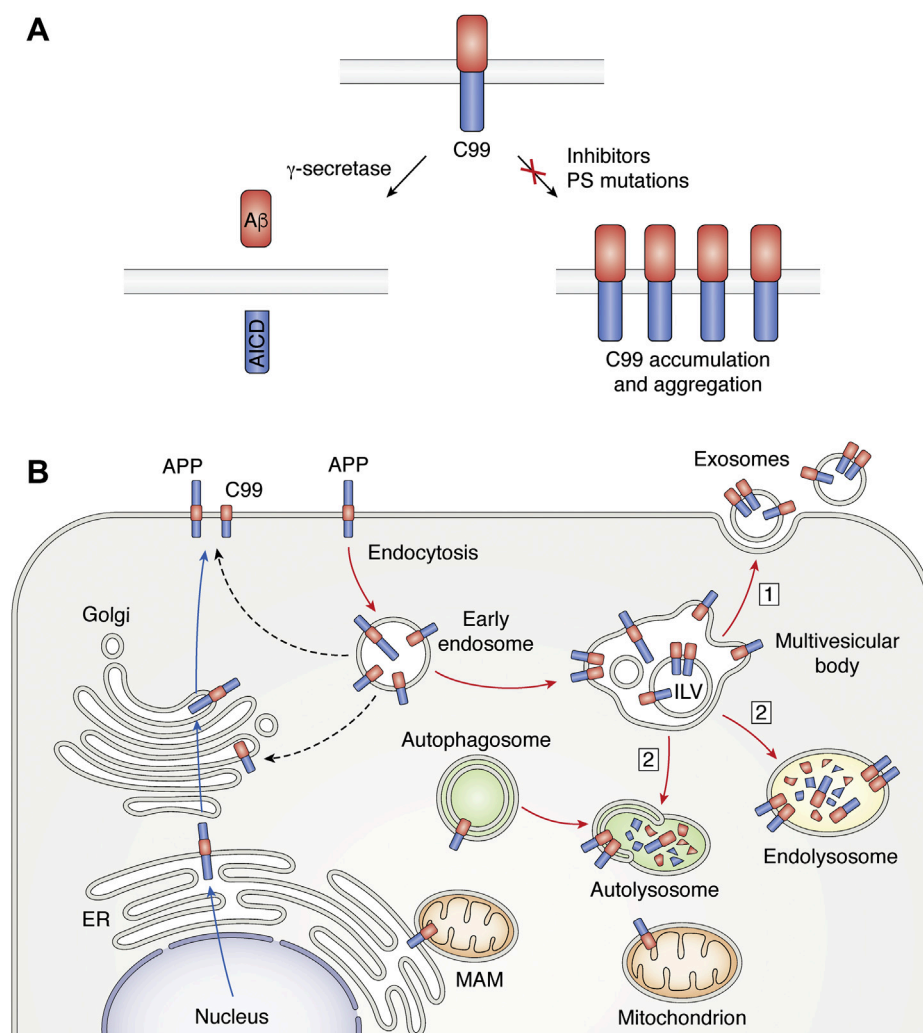
In addition to endosomal and lysosomal alterations, mitochondrial dysfunction is a key feature of AD with altered mitochondrial potential, increased levels of reactive oxygen species (ROS) (149–151), as well as a defect in clearance of abnormal mitochondria (mitophagy) (152, 153). Our recent work proposed that in cellular models, C99 can trigger all these alterations (137). In agreement, mitochondrial structure alterations and mitophagy defects were observed in young preplaque 3xTg-AD mice, as well as in virus-induced C99 expressing mice (AAV10-C99 mice) and notably following GSI treatment (137). Other studies have demonstrated C99 accumulation in mitochondria-associated membranes (MAMs) (154, 155) and have proposed its contribution to neutral lipid accumulation (155) and cholesterol trafficking (156).

Neuroinflammation is also a key feature of AD, but little is known about its molecular triggers. The contribution of C99 to neuroinflammation was proposed as early as in 1998 in mice, in which the intracerebroventricular injection of recombinant C99 (CT105) was found to induce reactive gliosis and neurodegeneration (157). CT105 was also found to interact with inflammatory cytokines and produce a synergistic effect on working memory (158). Two more recent works also proposed a link between intraneuronal accumulation of C99 and astrocytic activation and astrogliosis in 3xTgAD and APP<sup>E693Q</sup> mice, respectively (128, 131). Furthermore, in preplaque TgCRND8 mice, C99 was shown to correlate with a TNF $\alpha$  augmentation and microglial activation (129).

Does C99 accumulation also trigger functional and cognitive alterations? It is well established that AD is characterized by a hippocampal altered long-term potentiation (LTP) and depression (LTD) (159), as well as network activity dysfunctions (160). All of these electrophysiological signatures are linked to memory consolidation and learning ability and appear altered before senile plaques and neurodegeneration in human AD (161) and many mouse models. The first evidence of a neurotoxic effect of C99 on synaptic function was demonstrated years ago in a transgenic mouse model expressing directly the C99 fragment (162). These mice were found to develop both LTP and memory alterations, as well as neuroinflammation and neuronal loss. Our own recent studies in AAV10-C99 mice showed that LTP was significantly reduced in preplaque mice and that GSIs did not reverse LTP alterations (139). Synaptic alterations also seemed to be

temporarily and spatially correlated to C99 accumulation in 3xTgAD mice (163) and in the Danish dementia mouse model, in which a deficiency in the BRI2 protein leads to increased APP levels and in which LTP alterations and memory defects were rescued by the pharmacological inhibition of  $\beta$ -secretase, but not  $\gamma$ -secretase (164). Hippocampal network oscillations, as well as  $\theta$ / $\gamma$  coupling alterations, were reported at early “A $\beta$ -free” stages in the Tg-CRND8 mouse (165). In this study, these early network defects occurred in C99-accumulating regions and were fully rescued by  $\beta$ -secretase inhibition (130). In contrast, another recent study reported an absence of rescue of network alterations by  $\beta$ -secretase inhibition in the J20 model, although the levels of both C99 and A $\beta$  were clearly reduced (133). These data show that synaptic function is governed by complex mechanisms that may be difficult to rescue. First of all, in these studies  $\beta$ -secretase inhibition led to a decrease in C99 and A $\beta$ , but it also led to a concomitant increase in C83 that maybe could contribute to these alterations, although this was not discussed by the authors. Second of all, and maybe more importantly, the lack of rescue by BACE1 inhibitors could be due to the suppression of important cell signaling pathways involved in synaptic function, due to the inactivation of other BACE1 substrates, such as Neuregulin-1 and Sez6 (166, 167). In the same way, studies investigating the role of C99 accumulation in AD-related memory impairment in mouse AD models have reported conflicting data. In 3xTgAD mice, C99 accumulation seems to trigger both electrophysiological hippocampal defects and mild cognitive dysfunction (163, 168). Similar defects were observed in CT105-expressing mice (162). However, the studies from knock-in models seem to argue for an A $\beta$ -rather than a C99-dependent contribution to synaptic alterations. In fact, the two knock-in models APP-NL and APP-NL-F, harboring the Swedish (KM670/671NL) mutation alone (APP-NL), or in combination with the I706F mutation (APP-NL-F), respectively, both accumulate C99 (132), but only the APP-NL-F, displaying higher A $\beta$  levels, develops late-stage cognitive dysfunction (132, 169). Moreover, the knock-in mouse APP-NL-G-F harboring a third mutation, the E693G Arctic mutation, which generates a particular high level of oligomerized A $\beta$ , displays cognitive dysfunction at a much younger age. Thus, taken together, these observations could suggest either that only A $\beta$  is toxic or that A $\beta$  and C99 contribute differently to the pathology. Whereas A $\beta$ , as a soluble peptide, could be expected to act mainly outside cells, the toxic effect of membrane-embedded C99 should be intracellular. It is possible that intraneuronal C99 is a trigger of a more slowly developing pathological process that might be more prominent in transgenic mice that display higher levels of C99 than knock-in mice.

In 3xTgAD mice, these electrophysiological defects are not only accompanied by alterations in spatial learning and memory, but are also associated to a decrease in spontaneous activity that is reminiscent to apathy (168), a phenotype considered as an early neuropsychiatric symptom in AD patients (170, 171). Several observations suggested that this apathy-like behavior could be linked to C99 accumulation.



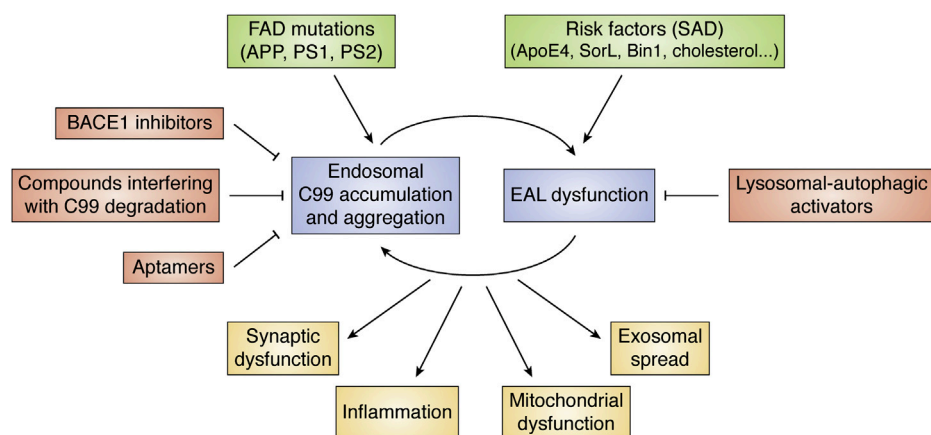
**Figure 3. Schematic models for C99 accumulation.** A, displays a schematic model of C99 fate in the absence or presence of  $\gamma$ -secretase cleavage. In physiological conditions,  $\gamma$ -secretase cleavage of C99 (blue/red sticks) leads to the generation of A $\beta$  and AICD, whereas when  $\gamma$ -secretase activity is impaired (inhibitors or PS mutations), C99 accumulates and aggregates. B, displays a schematic view of C99 generation and accumulation in conditions of blocked  $\gamma$ -secretase. APP (long blue/red bar) matures and traffics through the Golgi to the plasma membrane (blue arrows), where a small part of it is processed by  $\alpha$ -secretase (non-amyloidogenic pathway). APP that escapes  $\alpha$ -secretase-mediated cleavage is endocytosed into early endosomes and some of it is recycled to the plasma membrane, either directly or through the Golgi network (open black arrows). Within early endosomes, some APP is cleaved by  $\beta$ -secretase generating C99 (short blue/red bar), which then can undergo  $\gamma$ -secretase cleavage, thereby releasing the A $\beta$  peptide and AICD. Some C99 escapes from  $\gamma$ -secretase cleavage and is incorporated in ILVs (intra luminal vesicles), which are either released as exosomes after the fusion of multivesicular bodies (MVBs) with the plasma membrane (1) or degraded after their fusion of MVBs with lysosomes or with autophagosomes, which then fuse with lysosomes (2). The presence of APP and PS mutations or  $\gamma$ -secretase inhibitors and/or lowered lysosomal degradation lead to lysosomal, autolysosomal, and exosomal C99 accumulation. C99 has also been found to accumulate in MAMs and mitochondria.

This phenotype not only appears at a stage much before A $\beta$  could be detected, but it similarly occurs in a 2xTg-AD model (harboring APP<sup>Swe</sup> and Tau P30L), which was found to accumulate identical amounts C99 to the 3xTgAD mouse, but which display very low A $\beta$  levels even at late stages (168).

### Is $\beta$ -secretase a good drug target?

BACE1 cleavage of APP represents the rate-limiting step of A $\beta$  production, and targeting this enzyme would provide advantage to prevent production of both A $\beta$  and C99. To date, there is no evidence of AD-linked mutations on BACE1, but a clue of an indirect importance of BACE1 in AD came from genetic studies showing that APP mutations lying close to the  $\beta$ -secretase cleaving site strongly affect the cleavage by this

enzyme and can either cause early-onset AD (the Swedish and Leuven mutations) or be protective (the Icelandic mutation). BACE1 has also been shown to have a role in SAD as its expression was reported to be increased in AD patients. Thus, BACE1 protein was found to be increased 3-fold in cortical areas of AD patients as compared with age-matched controls (172), and these data were confirmed at the mRNA level in the frontal cortex of SAD patients (172, 173). Yang and colleagues also showed a significant elevation of BACE1 in temporal cortex and hippocampal samples from a cohort of clinically diagnosed and neuropathologically confirmed AD patients (174). This study used fluorimetric analysis assays and *in vitro* proteolysis to confirm that this increase in BACE1 expression led to enhanced functional enzyme. At that stage, most of the



**Figure 4. C99 toxicity.** C99 accumulation can be a consequence of either the presence of FAD mutations or a lowered degradation (possibly linked to risk factors). C99 firstly accumulates in endosomes and can itself be a direct cause of a dysfunction of the endosomal–lysosomal–autophagic (EAL) degradation pathway, thus leading to a vicious pathological cycle, in which more C99 accumulates, aggregates, and becomes neurotoxic by causing synaptic dysfunction, inflammation, mitochondrial dysfunction, and exosomal spread. Therapeutic strategies aimed at reducing C99 levels could be through either BACE1 inhibitors and/or BACE1 aptamers, which should be expected to reduce its production or autophagic/lysosomal activators or other drugs interfering with C99 degradation, which should increase its degradation.

studies attempted to correlate BACE1 expression to A $\beta$  load. However, some later studies analyzed C99 levels and reported also an increase in both FAD and SAD brains (137, 138, 175). Of most interest, the recent work from Pulina and colleagues quantifying both C99 and A $\beta$  in either controls, mildly or severely-affected AD brains, showed that C99 accumulates specifically in vulnerable neurons and correlated with the degree of cognitive alterations, whereas A $\beta$  load was increased in both vulnerable and resistant brain regions in AD (126). The latter data are to be put back into context with the consistent former observations that A $\beta$  expression and distribution within brain areas do not fully match neuronal loss, cognitive defects severity, and dementia AD progression (176–178). It should be emphasized that two recent studies documented that BACE may inactivate an intravesicular domain of APP, thereby exacerbating glutamate release (179), a process enhanced in a rat knock-in model bearing the Swedish mutation (135).

As stated above, BACE1 mainly cleaves APP at the  $\beta'$  site to generate C89 and therefore can be considered to be protective. In addition, BACE1 is also able to cleave A $\beta$ 40 and A $\beta$ 42 into the nonamyloidogenic (180) and neuroprotective (181) A $\beta$ 34. A recent study also proposed another neuroprotective role of BACE1, since the inhibition of this enzyme was found to enhance the levels of both  $\eta$ -secretase and A $\eta\alpha$ , the latter was proposed to be neurotoxic (27). Thus, in AD, the shift from a beneficial to a deleterious BACE1 cleavage could be due to the age-dependent augmentation of BACE1 expression occurring in sporadic AD or to the expression of a subset of pathogenic mutations in FAD, thereby enhancing C99 levels above physiological threshold (32).

All clinical trials targeting BACE-1 that have entered in phase III have been reported to decrease significantly and dose-dependently A $\beta$  levels in the brain, plasma, or CSF (Table 1). However, all of them have been terminated mostly due to reported toxicity. Indeed, many studies have

demonstrated serious adverse effects such as an abnormal elevation in liver enzymes and clinical worsening (Table 1). The reasons for these side effects observed in human trials are not well established, but could be related to the high number of BACE1 substrates being involved in vital functions. BACE1 is known to cleave nearly 40 different neuronal membrane proteins (182) and some of these substrates, such as Sez6 or neuron myelination like neuregulin-1, are known to be essential for synaptic function (183). Indeed, several studies reported that BACE1 inhibition triggers synaptic and cognitive dysfunctions (166, 167). BACE1 inhibitors could also cross over with not only its homologue BACE2, but also with cathepsin D, another aspartyl protease (184, 185). Inhibition of cathepsin D would be expected to be highly deleterious because of its role in lysosomal degradation. Of note, cathepsin D is one of the key enzymes involved in C99 degradation (139, 186) and would therefore also be expected to slow down the degradation of C99 (139, 186). It is therefore of high importance to identify inhibitors that target BACE1 but no other acidic proteases. Moreover, ideally these inhibitors should specifically decrease the processing of APP but not that of other BACE1 substrates.

Until yet, one of the main obstacles of BACE1 inhibitors is linked to their limited drug availability due to their peptidic feature and high molecular size. Small molecules may be less efficient than APP to fill the large substrate pocket, but higher sized molecules may display reduced bioavailability. These drugs need to cross the brain barrier, but also the cell membrane to reach endosomes, which are the main sites for  $\beta$ -secretase (36). Great advances in the field have been the recent development of nonpeptidic inhibitors with promising pharmacological properties, but still these drugs have not demonstrated convincing effects on cognitive function. This has been the case for the clinical trial with the small-molecule  $\beta$ -secretase inhibitor Umibecestat, which was administered to preasymptomatic carriers of APOE4 and led to more than 90% reduced A $\beta$  levels

in CSF but was stopped due to deleterious cognitive and side effects (187).

### Alternative strategies to decrease C99 buildup

Besides being processed by  $\gamma$ -secretase, a part of C99 is eliminated by degradation through the autophagic/lysosomal pathway (92, 143) and in some AD models, C99 accumulation is a consequence of a defective lysosomal clearance (139). Endosomal–lysosomal–autophagic dysfunction is indeed one of the earliest and major features of AD and has been proposed to be caused by both FAD mutations (as described above) and risk factors associated with SAD (for review see (188)). Thus, the activation of this degradation pathway could be expected to reduce C99 levels and to be beneficial. Rapamycin, a specific inhibitor of mTOR and a well-known inducer of autophagy, has been reported to ameliorate tau pathology, reduce amyloid- $\beta$  (A $\beta$ ) deposition, and improve cognition in preclinical studies (189, 190). However, rapamycin, as well as other autophagic activators, enhances autophagy without increasing lysosomal function that may be associated with an accumulation of nondigested autophagic vacuoles. Thus, a better way to enhance degradation could be to activate both autophagy and lysosomal function by overexpressing or pharmacologically activating the transcription factor EB (TFEB), the main regulator of the lysosomal/autophagic degradation pathway (191). TFEB activates the different steps of autophagy, from its initiation to the fusion of autophagosomes with lysosomes, but it also stimulates the biogenesis of lysosomal hydrolyses and regulates lysosomal acidification (191). Indeed, in animals, TFEB activation has been found to efficiently decrease C99 levels (192–194) and to ameliorate cognitive function, whereas it does not seem to produce any harmful effects in wild-type animals (143). The recent findings concerning TFEB activation by a curcumin analog are promising, because of the lack so far of specific TFEB-activating molecules. Earlier studies have proposed cyclodextrin, a modifier of cholesterol efflux used for the treatment of Niemann–Pick disease, as a TFEB activating drug. However, a recent work has demonstrated conflicting data concerning this molecule (195). Whereas short-term treatment was found to lead to activated autophagic degradation, long-term treatment had an opposite effect probably caused by the effect of cyclodextrin on membrane lipids (195). In addition, pharmacological treatments such as acidic nanoparticles, PLGA (poly lactic-co-glycolic acid, called acidic nanoparticles (196) could constitute an alternative way to increase lysosomal function. These nanoparticles localize to lysosomes, where they degrade, and subsequently release their acidic components to acidify the local lysosomal environment (197). Acidic nanoparticles have notably been proven efficient in cellular and animal models of Parkinson's disease (197).

Finally, recent works have proposed novel mechanisms involved in C99 degradation and have provided new potential drug targets. For instance, it was demonstrated that the selective phosphorylation of presenilin 1 at ser367 could be a way to increase autophagic degradation of C99. Indeed, this

phosphorylation of PS1 was found to facilitate autophagic–lysosomal fusion, an effect that was mediated through the interaction of phosphorylated PS1 with Annexin 2 and snare proteins. These findings therefore proposed that drugs designed to selectively increase phosphorylation of presenilin1 at ser367 could be a way to circumvent C99 accumulation (198, 199). Other works have shown that lysosomal/autophagic degradation of C99 can be promoted by its binding to the adipocyte differentiation protein AMAP, which could therefore also constitute a new drug target (200, 201). The  $\gamma$ -secretase complex, often *via* the presenilins, binds to many endogenous proteins, which will influence  $\gamma$ -secretase cleavage in distinct ways (202, 203).  $\gamma$ -Secretase has also been found to bind APP-CTFs, thereby stabilizing them for nonspecific degradation (204). A recent study proposed that ILE1, a member of the FAM3 superfamily, interferes with these chaperone properties and thereby enhances C99 degradation (204). ILE1 and molecules having similar properties may therefore also be plausible targets for future therapies.

Besides the strategies aimed at reducing C99 levels by enhancing its degradation, two other alternative strategies are possible. Firstly, the design of aptamers (so-called chemical antibodies) and the successful outcomes of their use in pathological conditions (205) allow to envisage this strategy to preclude side effects of BACE1 inhibitors on its physiological substrates. These chemical probes should be designed to prevent direct physical interaction of BACE1 with its targeted APP sequence in order to selectively hamper BACE1-mediated production of C99. Secondly, C99 levels could be reduced by repressing the transcriptional of APP. Indeed, a recent study has showed such effects of the compound Posiphen, which normalized C99 levels, but also reversed early endosome enlargement in Ts65Dn mice (206). This study not only reaffirms the link between C99 and endosomal perturbation but also indicates that the upstream regulation of its precursor APP could be a potential track to circumscribe C99 accumulation and subsequent cellular alterations.

### Conclusion and perspectives

Taken together, two lines of conclusions can be drawn from the above-described observations. First, studies on a vast number of  $\gamma$ -secretase substrates have mostly if not always shown that their catalytic processing leads to the conversion into bioactive fragments, the generation of which governs a large spectrum of vital functions (96, 97). This is probably also the case of  $\gamma$ -secretase cleavage on APP leading to the generation of physiological levels of A $\beta$  and AICD. Second, a network of consistent evidence suggests that, first, both FAD and SAD are associated with C99 accumulation; second, that C99, independently of A $\beta$ , behaves as an early pathogenic trigger in AD, and third, that all C99-associated biochemical, anatomical, and cognitive alterations are drastically potentiated by  $\gamma$ -secretase inhibitors (Fig. 3). C99 accumulation is believed to begin mainly in endosomes and has been shown to be a direct cause of endolysosomal network dysfunction, thus leading to a vicious pathological cycle, in which C99

accumulates, aggregates and becomes neurotoxic by causing synaptic dysfunction, inflammation, mitochondrial pathology, and exosomal spread (Fig. 4). The causes of this AD-related C99 accumulation could be an increased production (presence of APP mutations in FAD or enhanced BACE expression in SAD), a loss of  $\gamma$ -secretase cleavage (FAD PS and APP mutations) or a decreased degradation due to a defective lysosomal–autophagic degradation (early feature linked to FAD mutations and risk factors in SAD).

Our conclusion reconciles genetic grounds indicating a strong link between  $\beta$ APP and AD pathology and the systematic failure of A $\beta$ -centric clinical assays. So far, evidence points to a particularly important role of C99 at early stages of AD, but these findings do not preclude the undoubted role of A $\beta$  and particularly at later stages. The toxic effect of C99 could also be completely different from that of A $\beta$ . While the membrane-embedded C99 could affect mostly intracellular pathways, A $\beta$  could be expected to act mostly extracellularly. We propose that  $\gamma$ -secretase is an important C99-inactivating enzyme and that the consistent failure, and sometimes the deleterious worsening, of clinical trials based on  $\gamma$ -secretase inhibition could be linked, at least partly, to the impairment of C99 inactivation. GSMs are certainly more promising than GSIs, because they are not expected to increase C99 levels. However, the lack of C99 inactivation in the presence of these molecules still might be problematic. Future studies are needed to clarify this issue.

If one considers the fate of C99, secretases could overall be seen either as direct C99 inactivators, such as  $\gamma$ -secretase as stated above,  $\alpha$ -secretase that cleaves C99 to generate C83 (207), and even  $\beta$ -secretase that mainly cleaves  $\beta$ APP at the  $\beta'$  site, thereby precluding C99 production (Fig. 1B). In that sense, our point of view is that therapeutic strategies targeting secretases would not be the best way to tackle AD. Indeed, besides the complexity and the high number of substrates other than APP, inhibiting these cleavages would also impair C99 degradation.

Thus, in conclusion, we here suggest that  $\gamma$ -secretase is a crucial enzyme involved in not only the production of physiological bioactive fragments but also the inactivation of neurotoxic C99. We propose alternative strategies aiming to reduce the levels of C99, either by interfering with its production or by promoting its degradation (Fig. 4).

**Acknowledgments**—This work was partly supported by the “Fondation Alzheimer,” the “Fondation pour la Recherche Médicale” and the French National Foundation for Alzheimer’s Disease and Related Disorders. This work has been developed and supported through the LABEX (excellence laboratory, program investment for the future) DISTALZ (Development of Innovative Strategies for a Transdisciplinary approach to Alzheimer’s disease) and the University hospital Federation (FHU) OncoAge.

**Author contributions**—All the authors contributed to the writing of this article

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: A $\beta$ , amyloid  $\beta$ ; AD, Alzheimer’s disease; AICD, APP intracellular domain; APH, anterior pharynx defective; BACE,  $\beta$ -site APP cleaving enzyme;  $\beta$ APP, amyloid precursor protein; CSF, cerebrospinal fluid; CTF, C-terminal fragment; EAL, endosomal–lysosomal–autophagy; ER, endoplasmic reticulum; FAD, familial Alzheimer’s disease; GSM,  $\gamma$ -secretase modulators; LTP and LTD, long-term potentiation and depression; MAM, mitochondrial membrane associated; NCT, nicastrin; PEN, PS enhancer; PS, presenilin; ROS, reactive oxygen species; SAD, sporadic Alzheimer’s disease; TFEb, transcriptional factor EB.

## References

- Calderon-Garciduenas, A. L., and Duyckaerts, C. (2017) Alzheimer disease. *Handbook Clin. Neurol.* **145**, 325–337
- DeTure, M. A., and Dickson, D. W. (2019) The neuropathological diagnosis of Alzheimer’s disease. *Mol. Neurodegener.* **14**, 32
- Sengoku, R. (2020) Aging and Alzheimer’s disease pathology. *Neuropathology* **40**, 22–29
- Glennner, G. G., and Wong, C. W. (1984) Alzheimer’s disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *BBRC* **120**, 885–890
- Brion, J. P., Flament-Durand, J., and Dustin, P. (1986) Alzheimer’s disease and tau proteins. *Lancet* **2**, 1098
- Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) Multiple isoforms of human microtubule-associated protein tau: Sequences and localization in neurofibrillary tangles of Alzheimer’s disease. *Neuron* **3**, 519–526
- Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Müller-Hill, B. (1987) The precursor of Alzheimer’s disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733–736
- Head, E., and Lott, I. T. (2004) Down syndrome and beta-amyloid deposition. *Curr. Opin. Neurol.* **17**, 95–100
- Levy, E., Carman, M. D., Fernandez-Madrid, I., Power, M. D., Lieberburg, I., Van Drinen, S. G., Bots, G. T. M., Luyenkijk, W., and Frangione, B. (1990) Mutation of the Alzheimer’s disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* **248**, 1124–1126
- Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidami, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., *et al.* (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer’s disease. *Nature* **349**, 704–706
- Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer’s disease in the APP gene at the N-terminus of b-amyloid. *Nat. Genet.* **1**, 345–347
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) Mutation of the b-amyloid precursor protein in familial Alzheimer’s disease increases b-protein production. *Nature* **360**, 672–674
- Cai, X.-D., Golde, T. E., and Younkin, S. G. (1993) Release of excess amyloid b protein from a mutant amyloid b protein precursor. *Science* **259**, 514–516
- Wolfe, M. S. (2020) Unraveling the complexity of gamma-secretase. *Semin. Cell Dev. Biol.* **105**, 3–11
- Escamilla-Ayala, A., Wouters, R., Sannerud, R., and Annaert, W. (2020) Contribution of the Presenilins in the cell biology, structure and function of gamma-secretase. *Semin. Cell Dev. Biol.* **105**, 12–26
- Lleo, A., Berezovska, O., Growdon, J. H., and Hyman, B. T. (2004) Clinical, pathological, and biochemical spectrum of Alzheimer disease associated with PS-1 mutations. *Am. J. Geriatr. Psychiatry* **12**, 146–156
- Jonsson, T., Atwal, J. K., Steinberg, S., Snaedal, J., Jonsson, P. V., Björnsson, S., Stefansson, H., Sulem, P., Gudbjartsson, D., Maloney, J.,



- Hoyte, K., Gustafson, A., Liu, Y., Lu, Y., Bhargale, T., *et al.* (2012) A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* **488**, 96–99
18. Hardy, J. A., and Higgins, G. A. (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256**, 184–185
  19. Nie, P., Vartak, A., and Li, Y. M. (2020) gamma-Secretase inhibitors and modulators: Mechanistic insights into the function and regulation of gamma-Secretase. *Semin. Cell Dev. Biol.* **105**, 43–53
  20. Moussa-Pacha, N. M., Abidin, S. M., Omar, H. A., Alniss, H., and Al-Tel, T. H. (2020) BACE1 inhibitors: Current status and future directions in treating Alzheimer's disease. *Med. Res. Rev.* **40**, 339–384
  21. Huang, Y. M., Shen, J., and Zhao, H. L. (2019) Major clinical trials failed the amyloid hypothesis of Alzheimer's disease. *J. Am. Geriatr. Soc.* **67**, 841–844
  22. Panza, F., Lozupone, M., Logroscino, G., and Imbimbo, B. P. (2019) A critical appraisal of amyloid-beta-targeting therapies for Alzheimer disease. *Nat. Rev. Neurol.* **15**, 73–88
  23. Herrup, K. (2015) The case for rejecting the amyloid cascade hypothesis. *Nat. Neurosci.* **18**, 794–799
  24. Haass, C., and Levin, J. (2019) [Did Alzheimer research fail entirely? Failure of amyloid-based clinical studies]. *Der. Nervenarzt* **90**, 884–890
  25. Checler, F. (1995) Processing of the b-amyloid precursor protein and its regulation in Alzheimer's disease. *J. Neurochem.* **65**, 1431–1444
  26. Andrew, R. J., Kellett, K. A., Thinakaran, G., and Hooper, N. M. (2016) A Greek tragedy: The growing complexity of Alzheimer amyloid precursor protein proteolysis. *J. Biol. Chem.* **291**, 19235–19244
  27. Willem, M., Tahirovic, S., Busche, M. A., Ovsepian, S. V., Chafai, M., Kootar, S., Hornburg, D., Evans, L. D., Moore, S., Daria, A., Hampel, H., Muller, V., Giudici, C., Nuscher, B., Wenninger-Weinzierl, A., *et al.* (2015) eta-Secretase processing of APP inhibits neuronal activity in the hippocampus. *Nature* **526**, 443–447
  28. Baranger, K., Marchalant, Y., Bonnet, A. E., Crouzin, N., Carrete, A., Paumier, J. M., Py, N. A., Bernard, A., Bauer, C., Charrat, E., Moschke, K., Seiki, M., Vignes, M., Lichtenthaler, S. F., Checler, F., *et al.* (2016) MT5-MMP is a new pro-amyloidogenic proteinase that promotes amyloid pathology and cognitive decline in a transgenic mouse model of Alzheimer's disease. *Cell Mol. Life Sci.* **73**, 217–236
  29. Citron, M., Vigo-Pelfrey, C., Teplow, D. B., Miller, C., D, S., Johnston, J., Winblad, B., Venizelos, N., Lannfelt, L., and Selkoe, D. J. (1994) Excessive production of amyloid  $\beta$ -protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11993–11997
  30. Chavez-Gutierrez, L., and Szaruga, M. (2020) Mechanisms of neurodegeneration - insights from familial Alzheimer's disease. *Semin. Cell Dev. Biol.* **105**, 75–85
  31. Xu, T. H., Yan, Y., Kang, Y., Jiang, Y., Melcher, K., and Xu, H. E. (2016) Alzheimer's disease-associated mutations increase amyloid precursor protein resistance to gamma-secretase cleavage and the Abeta42/Abeta40 ratio. *Cell Discov.* **2**, 16026
  32. Vassar, R., Bennett, B. D., Babu-Khan, S., Khan, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., *et al.* (1999) b-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741
  33. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., *et al.* (1999) Purification and cloning of amyloid precursor protein b-secretase from human brain. *Nature* **402**, 537–540
  34. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashler, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrichson, R. L., and Gurney, M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease b-secretase activity. *Nature* **402**, 533–537
  35. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Identification of a novel aspartic protease (Asp2) as b-secretase. *Mol. Cell. Neurosci.* **14**, 419–427
  36. Cole, S. L., and Vassar, R. (2007) The Alzheimer's disease Beta-secretase enzyme, BACE1. *Mol. Neurodegener.* **2**, 22
  37. Zhou, L., Brouwers, N., Benilova, I., Vandersteen, A., Mercken, M., Van Laere, K., Van Damme, P., Demedts, D., Van Leuven, F., Slegers, K., Broersen, K., Van Broeckhoven, C., Vandenberghe, R., and De Strooper, B. (2011) Amyloid precursor protein mutation E682K at the alternative beta-secretase cleavage beta'-site increases Abeta generation. *EMBO Mol. Med.* **3**, 291–302
  38. Seubert, P., Oltersdorf, T., Lee, M. G., Barbour, R., Blomquist, C., Davis, D. L., Bryant, K., Fritz, L. C., Galasko, D., Thal, L. J., Lieberburg, I., and Schenk, D. B. (1993) Secretion of b-amyloid precursor protein cleaved at the amino terminus of the b-amyloid peptide. *Nature* **361**, 260–263
  39. Maloney, J. A., Bainbridge, T., Gustafson, A., Zhang, S., Kyauk, R., Steiner, P., van der Brug, M., Liu, Y., Ernst, J. A., Watts, R. J., and Atwal, J. K. (2014) Molecular mechanisms of Alzheimer disease protection by the A673T allele of amyloid precursor protein. *J. Biol. Chem.* **289**, 30990–31000
  40. Schellenberg, G. D., Bird, T. D., Wijsman, E. M., Orr, H. T., Anderson, L., Nemens, E., White, J. A., Bonnycastle, L., Weber, J. L., Alonso, M. E., Potter, H., Heston, L. L., and Martin, G. M. (1992) Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science* **258**, 668–671
  41. St. George-Hyslop, P., Haines, J., Rogaev, E., Mortilla, M., Vaula, G., Pericak-Vance, M., Foncin, J. F., Montesi, M., Bruni, A., Sorbi, S., Rainero, I., Pinessi, L., Pollen, D., Polinsky, R., Nee, L., *et al.* (1992) Genetic evidence for a novel familial Alzheimer's disease locus on chromosome 14. *Nat. Genet.* **2**, 330–334
  42. Van Broeckhoven, C., Backhovens, H., Cruts, M., De Winter, G., Bruylant, M., Cras, P., and Martin, J. J. (1992) Mapping of a gene predisposing to early-onset Alzheimer's disease to chromosome 14q24.3. *Nat. Genet.* **2**, 335–339
  43. Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., *et al.* (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature* **376**, 775–778
  44. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J. F., Bruni, A. C., Montesi, M. P., *et al.* (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* **375**, 754–760
  45. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387–390
  46. Herreman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L., and De Strooper, B. (2000) Total inactivation of g-secretase activity in presenilin-deficient embryonic stem cells. *Nat. Cell Biol.* **2**, 461–462
  47. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Reconstitution of g-secretase activity. *Nat. Cell Biol.* **5**, 486–488
  48. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) g-secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1 and pen-2. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6382–6387
  49. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the g-secretase complex. *Nature* **422**, 438–441
  50. Lu, P., Bai, X. C., Ma, D., Xie, T., Yan, C., Sun, L., Yang, G., Zhao, Y., Zhou, R., Scheres, S. H. W., and Shi, Y. (2014) Three-dimensional structure of human gamma-secretase. *Nature* **512**, 166–170
  51. Wolfe, M. S. (2000) Substrate recognition and processing by gamma-secretase. *Biochim. Biophys. Acta Biomembr.* **1862**, 183016

52. Hardy, J., and Selkoe, D. (2002) The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* **297**, 353–356
53. Selkoe, D. J., and Hardy, J. (2016) The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med.* **8**, 595–608
54. Selkoe, D. J., and Wolfe, M. S. (2007) Presenilin: Running with scissors in the membrane. *Cell* **131**, 215–221
55. Steiner, H., Fluhrer, R., and Haass, C. (2008) Intramembrane proteolysis by gamma-secretase. *J. Biol. Chem.* **283**, 29627–29631
56. Wolfe, M. S. (2007) When loss is gain: Reduced presenilin proteolytic function leads to increased Abeta42/Abeta40. Talking point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep.* **8**, 136–140
57. Shen, J., and Kelleher, R. J., 3rd. (2007) The presenilin hypothesis of Alzheimer's disease: Evidence for a loss-of-function pathogenic mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 403–409
58. Heilig, E. A., Gutti, U., Tai, T., Shen, J., and Kelleher, R. J., 3rd. (2013) Trans-dominant negative effects of pathogenic PSEN1 mutations on gamma-secretase activity and Abeta production. *J. Neurosci.* **33**, 11606–11617
59. Xia, D., Watanabe, H., Wu, B., Lee, S. H., Li, Y., Tsvetkov, E., Bolshakov, V. Y., Shen, J., and Kelleher, R. J., 3rd. (2015) Presenilin-1 knockin mice reveal loss-of-function mechanism for familial Alzheimer's disease. *Neuron* **85**, 967–981
60. Sun, L., Zhou, R., Yang, G., and Shi, Y. (2017) Analysis of 138 pathogenic mutations in presenilin-1 on the *in vitro* production of Abeta42 and Abeta40 peptides by gamma-secretase. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E476–E485
61. Szaruga, M., Veugelen, S., Benurwar, M., Lismont, S., Sepulveda-Falla, D., Lleo, A., Ryan, N. S., Lashley, T., Fox, N. C., Murayama, S., Gijssen, H., De Strooper, B., and Chavez-Gutierrez, L. (2015) Qualitative changes in human gamma-secretase underlie familial Alzheimer's disease. *J. Exp. Med.* **212**, 2003–2013
62. Saito, T., Suemoto, T., Brouwers, N., Slegers, K., Funamoto, S., Mihira, N., Matsuba, Y., Yamada, K., Nilsson, P., Takano, J., Nishimura, M., Iwata, N., Van Broeckhoven, C., Ihara, Y., and Saido, T. C. (2011) Potent amyloidogenicity and pathogenicity of Abeta43. *Nat. Neurosci.* **14**, 1023–1032
63. Borchelt, D. R., Thinakaran, G., Eckman, C. B., Lee, M. K., Davenport, F., Ratovitsky, T., Prada, C.-M., Kim, G., Seekins, S., Yager, D., Slunt, H. H., Wang, R., Seeger, M., Levey, A. I., Gandy, S. E., *et al.* (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Ab1-42/1-40 *in vitro* and *in vivo*. *Neuron* **17**, 1005–1013
64. Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.-M., Perez-Tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M. N., Holcomb, L., Refolo, L., Zenk, B., *et al.* (1996) Increased amyloid-b42(43) in brains expressing mutant presenilin 1. *Nature* **383**, 710–713
65. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., *et al.* (1996) Secreted amyloid b-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2**, 864–870
66. Kumar-Singh, S., Theuns, J., Van Broeck, B., Pirici, D., Vennekens, K., Corsmit, E., Cruts, M., Dermaut, B., Wang, R., and Van Broeckhoven, C. (2006) Mean age of onset of familial Alzheimer disease caused by presenilin mutations correlates with both increased Ab42 and decreased Ab40. *Hum. Mut.* **27**, 686–695
67. Sannerud, R., Esselens, C., Ejsmont, P., Mattered, R., Rochin, L., Tharakeswar, A. K., De Baets, G., De Wever, V., Habets, R., Baert, V., Vermeire, W., Michiels, C., Groot, A. J., Wouters, R., Dillen, K., *et al.* (2016) Restricted location of PSEN2/gamma-secretase determines substrate specificity and generates an intracellular Abeta pool. *Cell* **166**, 193–208
68. Meckler, X., and Checler, F. (2016) Presenilin 1 and presenilin 2 target gamma-secretase complexes to distinct cellular compartments. *J. Biol. Chem.* **291**, 12821–12837
69. Gouras, G. K., Tsai, J., Naslund, J., Vincent, B., Edgar, M., Checler, F., Greenfield, J. P., Haroutunian, V., Buxbaum, J. D., Xu, H., Greengard, P., and Relkin, N. R. (2000) Intraneuronal Ab42 accumulation in human brain. *Am. J. Pathol.* **156**, 15–20
70. Chui, D. H., Dobo, E., Makifuchi, T., Akiyama, H., Kawakatsu, S., Petit, A., Checler, F., Araki, W., Takahashi, K., and Tabira, T. (2001) Apoptotic neurons in Alzheimer's disease frequently show intracellular Abeta42 labeling. *J. Alzheimers Dis.* **3**, 231–239
71. Chui, D. H., Tanahashi, H., Ozawa, K., Ikeda, S., Checler, F., Ueda, O., Suzuki, H., Araki, W., Inoue, H., Shirota, K., Takahashi, K., Gallyas, F., and Tabira, T. (1999) Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. *Nat. Med.* **5**, 560–564
72. Li, N., Liu, K., Qiu, Y., Ren, Z., Dai, R., Deng, Y., and Qing, H. (2016) Effect of presenilin mutations on APP cleavage; insights into the pathogenesis of FAD. *Front. Aging Neurosci.* **8**, 51
73. Kwart, D., Gregg, A., Scheckel, C., Murphy, E. A., Paquet, D., Duffield, M., Fak, J., Olsen, O., Darnell, R. B., and Tessier-Lavigne, M. (2019) A large panel of isogenic APP and PSEN1 mutant human iPSC neurons reveals shared endosomal abnormalities mediated by APP beta-CTFs, not Abeta. *Neuron* **104**, 1022
74. Cacquevel, M., Aeschbach, L., Houacine, J., and Fraering, P. C. (2012) Alzheimer's disease-linked mutations in presenilin-1 result in a drastic loss of activity in purified gamma-secretase complexes. *PLoS One* **7**, e35133
75. Duggan, S. P., and McCarthy, J. V. (2016) Beyond gamma-secretase activity: The multifunctional nature of presenilins in cell signalling pathways. *Cell Signal.* **28**, 1–11
76. Oikawa, N., and Walter, J. (2019) Presenilins and gamma-secretase in membrane proteostasis. *Cells* **8**, 209
77. Cacace, R., Slegers, K., and Van Broeckhoven, C. (2016) Molecular genetics of early-onset Alzheimer's disease revisited. *Alzheimers Dement.* **12**, 733–748
78. Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, M., Cai, X. D., McKay, D. M., Tintner, R., Frangione, B., and Younkin, S. G. (1992) Production of the Alzheimer amyloid b protein by normal proteolytic processing. *Science* **258**, 126–129
79. Haass, C., Hung, A. Y., Schlossmacher, M. G., Oltersdorf, T., Teplow, D. B., and Selkoe, D. J. (1993) Normal cellular processing of the b-amyloid precursor protein results in the secretion of the amyloid b peptide and related molecules. *Nature* **695**, 109–116
80. Kent, S. A., Spires-Jones, T. L., and Durrant, C. S. (2020) The physiological roles of tau and Abeta: Implications for Alzheimer's disease pathology and therapeutics. *Acta Neuropathol.* **140**, 417–447
81. Sevalle, J., Amoyel, A., Robert, P., Fournie-Zaluski, M. C., Roques, B., and Checler, F. (2009) Aminopeptidase A contributes to the N-terminal truncation of amyloid beta-peptide. *J. Neurochem.* **109**, 248–256
82. Castellani, R. J., Lee, H. G., Perry, G., and Smith, M. A. (2006) Antioxidant protection and neurodegenerative disease: The role of amyloid-beta and tau. *Am. J. Alzheimers Dis. Other Dement.* **21**, 126–130
83. Brothers, H. M., Gosztyla, M. L., and Robinson, S. R. (2018) The physiological roles of amyloid-beta peptide hint at new ways to treat Alzheimer's disease. *Front. Aging Neurosci.* **10**, 118
84. Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D., and Tonegawa, S. (1997) Skeletal and CNS defects in presenilin-1-deficient mice. *Cell* **89**, 629–639
85. Saura, C. A., Choi, S.-Y., Beglopoulos, V., Malkani, S., Zhang, D., Rao, B. S. S., Chattarji, S., Kelleher, R. J., III, Kandel, E. R., Duff, K., Kirkwood, A., and Shen, J. (2004) Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. *Neuron* **42**, 23–36
86. Guner, G., and Lichtenthaler, S. F. (2020) The substrate repertoire of gamma-secretase/presenilin. *Semin. Cell Dev. Biol.* **105**, 27–42
87. Kuhnle, N., Dederer, V., and Lemberg, M. K. (2019) Intramembrane proteolysis at a glance: From signalling to protein degradation. *J. Cell Sci.* **132**, jcs217745
88. Lichtenthaler, S. F., Haass, C., and Steiner, H. (2011) Regulated intramembrane proteolysis—lessons from amyloid precursor protein processing. *J. Neurochem.* **117**, 779–796

89. Okochi, M., Steiner, H., Fukumori, A., Tani, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M., and Haass, C. (2002) Presenilins mediate a dual intramembranous  $\gamma$ -secretase cleavage of Notch-1. *EMBO J.* **21**, 5408–5416
90. Siebel, C., and Lendahl, U. (2017) Notch signaling in development, tissue homeostasis, and disease. *Physiol. Rev.* **97**, 1235–1294
91. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) A presenilin-1-dependent  $\gamma$ -secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518–522
92. Marambaud, P., Wen, P. H., Dutt, A., Takashima, A., Siman, R., and Robakis, N. (2003) A CPB binding transcriptional repressor produced by the PS1/e-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* **114**, 635–645
93. Araki, Y., Miyagi, N., Kato, N., Yoshida, T., Wada, S., Nishimura, M., Komano, H., Yamamoto, T., De Strooper, B., Yamamoto, K., and Suzuki, T. (2004) Coordinated metabolism of Alcadin and amyloid beta-protein precursor regulates FE65-dependent gene transactivation. *J. Biol. Chem.* **279**, 24343–24354
94. Okamoto, I., Kawano, Y., Murakami, D., Sasayama, T., Araki, N., Miki, T., Wong, A. J., and Saya, H. (2001) Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J. Cell Biol.* **155**, 755–762
95. Glebov, K., Wunderlich, P., Karaca, I., and Walter, J. (2016) Functional involvement of gamma-secretase in signaling of the triggering receptor expressed on myeloid cells-2 (TREM2). *J. Neuroinflammation* **13**, 17
96. Jurisch-Yaksi, N., Sannerud, R., and Annaert, W. (2013) A fast growing spectrum of biological functions of gamma-secretase in development and disease. *Biochim. Biophys. Acta* **1828**, 2815–2827
97. Haapasalo, A., and Kovacs, D. M. (2011) The many substrates of presenilin/gamma-secretase. *J. Alzheimers Dis.* **25**, 3–28
98. Cao, X., and Südhof, T. C. (2001) A transcriptionally active complex of APP with Fe65 and histone acetyltransferase tip60. *Science* **293**, 115–120
99. von Rotz, R. C., Kohli, B. M., Bosset, J., Meier, M., Suzuki, T., Nitsch, R. M., and Konietzko, U. (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. *J. Cell Sci.* **117**, 4435–4448
100. Alves da Costa, C., Sunyach, C., Pardossi-Piquard, R., Sevalle, J., Vincent, B., Boyer, N., Kawarai, T., Girardot, N., St George-Hyslop, P., and Checler, F. (2006) Presenilin-dependent gamma-secretase-mediated control of p53-associated cell death in Alzheimer's disease. *J. Neurosci.* **26**, 6377–6385
101. Pardossi-Piquard, R., Petit, A., Kawarai, T., Sunyach, C., Alves da Costa, C., Vincent, B., Ring, S., D'Adamio, L., Shen, J., Muller, U., St George-Hyslop, P., and Checler, F. (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP. *Neuron* **46**, 541–554
102. Belyaev, N. D., Nalivaeva, N. N., Makova, N. Z., and Turner, A. J. (2009) Neprilysin gene expression requires binding of the amyloid precursor protein intracellular domain to its promoter: Implications for Alzheimer disease. *EMBO Rep.* **10**, 94–100
103. Kim, H.-S., Kim, E.-M., Lee, J.-P., Park, C.-H., Kim, S. J., Seo, J.-H., Chang, K.-A., Yu, E., Jeong, S.-J., Chong, Y. H., and Suh, Y.-H. (2003) C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3 $\beta$  expression. *FASEB J.* **17**, 1951–1953
104. Ryan, K. A., and Pimplikar, S. W. (2005) Activation of GSK3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. *J. Cell Biol.* **171**, 327–335
105. Kopan, R., and Ilagan, M. X. (2004) Gamma-secretase: Proteasome of the membrane? *Nat. Rev. Mol. Cell Biol.* **5**, 499–504
106. Wong, E., Frost, G. R., and Li, Y. M. (2020) Gamma-secretase modulatory proteins: The guiding hand behind the running scissors. *Front. Aging Neurosci.* **12**, 614690
107. Serneels, L., Van Biervliet, J., Craessaerts, K., Dejaegere, T., Horre, K., Van Houtvin, T., Esselmann, H., Paul, S., Schafer, M. K., Berezovska, O., Hyman, B. T., Sprangers, B., Sciot, R., Moons, L., Jucker, M., et al. (2009) gamma-Secretase heterogeneity in the Aph1 subunit: relevance for Alzheimer's disease. *Science* **324**, 639–642
108. Acx, H., Serneels, L., Radaelli, E., Muyldermaans, S., Vincke, C., Pepermans, E., Muller, U., Chavez-Gutierrez, L., and De Strooper, B. (2017) Inactivation of gamma-secretases leads to accumulation of substrates and non-Alzheimer neurodegeneration. *EMBO Mol. Med.* **9**, 1088–1099
109. Hu, C., Zeng, L., Li, T., Meyer, M. A., Cui, M. Z., and Xu, X. (2016) Nicastrin is required for amyloid precursor protein (APP) but not Notch processing, while anterior pharynx-defective 1 is dispensable for processing of both APP and Notch. *J. Neurochem.* **136**, 1246–1258
110. Fleisher, A. S., Raman, R., Siemers, E. R., Becerra, L., Clark, C. M., Dean, R. A., Farlow, M. R., Galvin, J. E., Peskind, E. R., Quinn, J. F., Sherzai, A., Sowell, B. B., Aisen, P. S., and Thal, L. J. (2008) Phase 2 safety trial targeting amyloid beta production with a gamma-secretase inhibitor in Alzheimer disease. *Arch. Neurol.* **65**, 1031–1038
111. Bateman, R. J., Siemers, E. R., Mawuenyega, K. G., Wen, G., Browning, K. R., Sigurdson, W. C., Yarasheski, K. E., Friedrich, S. W., Demattos, R. B., May, P. C., Paul, S. M., and Holtzman, D. M. (2009) A gamma-secretase inhibitor decreases amyloid-beta production in the central nervous system. *Ann. Neurol.* **66**, 48–54
112. Doody, R. S., Raman, R., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., Kieburtz, K., He, F., Sun, X., Thomas, R. G., Aisen, P. S., Alzheimer's Disease Cooperative Study Steering Committee, Siemers, E., Sethuraman, G., Mohs, R., and Semagacestat Study Group (2013) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. *N. Engl. J. Med.* **369**, 341–350
113. Albright, C. F., Dockens, R. C., Meredith, J. E., Jr., Olson, R. E., Slemmon, R., Lentz, K. A., Wang, J. S., Denton, R. R., Pilcher, G., Rhyne, P. W., Raybon, J. J., Barten, D. M., Burton, C., Toyn, J. H., Sankaranarayanan, S., et al. (2013) Pharmacodynamics of selective inhibition of gamma-secretase by avagacestat. *J. Pharmacol. Exp. Ther.* **344**, 686–695
114. Chavez-Gutierrez, L., Bammens, L., Benilova, I., Vanderstee, A., Benurwar, M., Borgers, M., Lismont, S., Zhou, L., Van Cleynenbreugel, S., Esselmann, H., Wiltfang, J., Serneels, L., Karran, E., Gijssen, H., Schymkowitz, J., et al. (2012) The mechanism of gamma-Secretase dysfunction in familial Alzheimer disease. *EMBO J.* **31**, 2261–2274
115. Crump, C. J., Castro, S. V., Wang, F., Pozdnyakov, N., Ballard, T. E., Sisodia, S. S., Bales, K. R., Johnson, D. S., and Li, Y. M. (2012) BMS-708, 163 targets presenilin and lacks notch-sparing activity. *Biochemistry* **51**, 7209–7211
116. Coric, V., van Dyck, C. H., Salloway, S., Andreasen, N., Brody, M., Richter, R. W., Soininen, H., Thein, S., Shiovitz, T., Pilcher, G., Colby, S., Rollin, L., Dockens, R., Pachai, C., Portelius, E., et al. (2012) Safety and tolerability of the gamma-secretase inhibitor avagacestat in a phase 2 study of mild to moderate Alzheimer disease. *Arch. Neurol.* **69**, 1430–1440
117. Mitani, Y., Yarimizu, J., Saita, K., Uchino, H., Akashiba, H., Shitaka, Y., Ni, K., and Matsuoka, N. (2012) Differential effects between gamma-secretase inhibitors and modulators on cognitive function in amyloid precursor protein-transgenic and nontransgenic mice. *J. Neurosci.* **32**, 2037–2050
118. Bursavich, M. G., Harrison, B. A., and Blain, J. F. (2016) Gamma secretase modulators: New Alzheimer's drugs on the horizon? *J. Med. Chem.* **59**, 7389–7409
119. Takeo, K., Tanimura, S., Shinoda, T., Osawa, S., Zahariev, I. K., Takegami, N., Ishizuka-Katsura, Y., Shinya, N., Takagi-Niidome, S., Tomimaga, A., Ohsawa, N., Kimura-Someya, T., Shirouzu, M., Yokoshima, S., Yokoyama, S., et al. (2014) Allosteric regulation of gamma-secretase activity by a phenylimidazole-type gamma-secretase modulator. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 10544–10549
120. Xia, W. (2019) gamma-Secretase and its modulators: Twenty years and beyond. *Neurosci. Lett.* **701**, 162–169
121. Kukar, T. L., Ladd, T. B., Bann, M. A., Fraering, P. C., Narlawar, R., Maharvi, G. M., Healy, B., Chapman, R., Welzel, A. T., Price, R. W., Moore, B., Rangachari, V., Cusack, B., Eriksen, J., Jansen-West, K., et al. (2008) Substrate-targeting gamma-secretase modulators. *Nature* **453**, 925–929

122. Reines, S., Block, G., Morris, J., Liu, G., Nessly, M., Lines, C., Norman, B., and Baranak, C. (2004) Rofecoxib: No effect on Alzheimer's disease in a 1-year, randomized, blinded, controlled study. *Neurology* **62**, 66–71
123. Green, R. C., Schneider, L. S., Amato, D. A., Beelen, A. P., Wilcock, G., Swabb, E. A., Zavitz, K. H., and Tarenfluril Phase 3 Study Group (2009) Effect of tarenfluril on cognitive decline and activities of daily living in patients with mild Alzheimer disease: A randomized controlled trial. *JAMA* **302**, 2557–2564
124. Meyer, P. F., Tremblay-Mercier, J., Leoutsakos, J., Madjar, C., Lafaille-Maignan, M. E., Savard, M., Rosa-Neto, P., Poirier, J., Etienne, P., Breitner, J., and Group, P.-A. R. (2019) INTREPAD: A randomized trial of naproxen to slow progress of presymptomatic Alzheimer disease. *Neurology* **92**, e2070–e2080
125. Mekala, S., Nelson, G., and Li, Y. M. (2020) Recent developments of small molecule gamma-secretase modulators for Alzheimer's disease. *RSC Med. Chem.* **11**, 1003–1022
126. Pulina, M. V., Hopkins, M., Haroutunian, V., Greengard, P., and Bustos, V. (2020) C99 selectively accumulates in vulnerable neurons in Alzheimer's disease. *Alzheimers Dement.* **16**, 273–282
127. Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, G., Golde, T. E., Kaye, R., Metherate, R., Mattson, M. P., Akbari, Y., and LaFerla, F. (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Ab and synaptic dysfunction. *Neuron* **39**, 409–421
128. Lauritzen, I., Pardossi-Piquard, R., Bauer, C., Brigham, E., Abraham, J. D., Ranaldi, S., Fraser, P., St-George-Hyslop, P., Le Thuc, O., Espin, V., Chami, L., Dunys, J., and Checler, F. (2012) The beta-secretase-derived C-terminal fragment of betaAPP, C99, but not Abeta, is a key contributor to early intraneuronal lesions in triple-transgenic mouse hippocampus. *J. Neurosci.* **32**, 16243, 16554
129. Cavanagh, C., Colby-Milley, J., Bouvier, D., Farso, M., Chabot, J. G., Quirion, R., and Krantic, S. (2013) betaCTF-correlated burst of hippocampal TNFalpha occurs at a very early, pre-plaque stage in the TgCRND8 mouse model of Alzheimer's disease. *J. Alzheimers Dis.* **36**, 233–238
130. Mondragon-Rodriguez, S., Gu, N., Manseau, F., and Williams, S. (2018) Alzheimer's transgenic model is characterized by very early brain network alterations and beta-CTF fragment accumulation: Reversal by beta-secretase inhibition. *Front. Cell Neurosci.* **12**, 121
131. Kaur, G., Pawlik, M., Gandy, S. E., Ehrlich, M. E., Smiley, J. F., and Levy, E. (2017) Lysosomal dysfunction in the brain of a mouse model with intraneuronal accumulation of carboxyl terminal fragments of the amyloid precursor protein. *Mol. Psychiatry* **22**, 981–989
132. Sasaguri, H., Nilsson, P., Hashimoto, S., Nagata, K., Saito, T., De Strooper, B., Hardy, J., Vassar, R., Winblad, B., and Saido, T. C. (2017) APP mouse models for Alzheimer's disease preclinical studies. *EMBO J.* **36**, 2473–2487
133. Johnson, E. C. B., Ho, K., Yu, G. Q., Das, M., Sanchez, P. E., Djukic, B., Lopez, I., Yu, X., Gill, M., Zhang, W., Paz, J. T., Palop, J. J., and Mucke, L. (2020) Behavioral and neural network abnormalities in human APP transgenic mice resemble those of App knock-in mice and are modulated by familial Alzheimer's disease mutations but not by inhibition of BACE1. *Mol. Neurodegener.* **15**, 53
134. Serneels, L., T'Syen, D., Perez-Benito, L., Theys, T., Holt, M. G., and De Strooper, B. (2020) Modeling the beta-secretase cleavage site and humanizing amyloid-beta precursor protein in rat and mouse to study Alzheimer's disease. *Mol. Neurodegener.* **15**, 60
135. Tambini, M. D., Yao, W., and D'Adamo, L. (2019) Facilitation of glutamate, but not GABA, release in Familial Alzheimer's APP mutant Knock-in rats with increased beta-cleavage of APP. *Aging Cell* **18**, e13033
136. Tambini, M. D., Norris, K. A., and D'Adamo, L. (2020) Opposite changes in APP processing and human Abeta levels in rats carrying either a protective or a pathogenic APP mutation. *Elife* **9**, e52612
137. Vaillant-Beuchot, L., Mary, A., Pardossi-Piquard, R., Bourgeois, A., Lauritzen, I., Eysert, F., Kinoshita, P. F., Cazareth, J., Badot, C., Fragaki, K., Bussiere, R., Martin, C., Mary, C., Bauer, C., Pagnotta, S., et al. (2021) Amyloid precursor protein C-terminal fragments accumulation triggers mitochondrial structure, function and mitophagy defects in Alzheimer's disease models and human brains. *Acta Neuropathol.* **141**, 39–65
138. Pera, M., Alcolea, D., Sanchez-Valle, R., Guardia-Laguarta, C., Colom-Cadena, M., Badiola, N., Suarez-Calvet, M., Llado, A., Barrera-Ocampo, A. A., Sepulveda-Falla, D., Blesa, R., Molinuevo, J. L., Clarimon, J., Ferrer, I., Gelpi, E., et al. (2013) Distinct patterns of APP processing in the CNS in autosomal-dominant and sporadic Alzheimer disease. *Acta Neuropathol.* **125**, 201–213
139. Lauritzen, I., Pardossi-Piquard, R., Bourgeois, A., Pagnotta, S., Biferi, M. G., Barkats, M., Lacor, P., Klein, W., Bauer, C., and Checler, F. (2016) Intraneuronal aggregation of the beta-CTF fragment of APP (C99) induces Abeta-independent lysosomal-autophagic pathology. *Acta Neuropathol.* **132**, 257–276
140. Jiang, Y., Mullaney, K. A., Peterhoff, C. M., Che, S., Schmidt, S. D., Boyer-Boiteau, A., Ginsberg, S. D., Cataldo, A. M., Mathews, P. M., and Nixon, R. A. (2010) Alzheimer's-related endosome dysfunction in Down syndrome is Abeta-independent but requires APP and is reversed by BACE-1 inhibition. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 1630–1635
141. Jiang, Y., Rigoglioso, A., Peterhoff, C. M., Pawlik, M., Sato, Y., Bleiwas, C., Stavrides, P., Smiley, J. F., Ginsberg, S. D., Mathews, P. M., Levy, E., and Nixon, R. A. (2016) Partial BACE1 reduction in a down syndrome mouse model blocks Alzheimer-related endosomal anomalies and cholinergic neurodegeneration: Role of APP-CTF. *Neurobiol. Aging* **39**, 90–98
142. Nixon, R. A. (2017) Amyloid precursor protein and endosomal-lysosomal dysfunction in Alzheimer's disease: Inseparable partners in a multifactorial disease. *FASEB J.* **31**, 2729–2743
143. Hung, C. O. Y., and Livesey, F. J. (2018) Altered gamma-secretase processing of APP disrupts lysosome and autophagosome function in monogenic Alzheimer's disease. *Cell Rep.* **25**, 3647–3660.e3642
144. Lauritzen, I., Pardossi-Piquard, R., Bourgeois, A., Becot, A., and Checler, F. (2019) Does intraneuronal accumulation of carboxyl-terminal fragments of the amyloid precursor protein trigger early neurotoxicity in Alzheimer's disease? *Curr. Alzheimer Res.* **16**, 453–457
145. Lauritzen, I., Becot, A., Bourgeois, A., Pardossi-Piquard, R., Biferi, M. G., Barkats, M., and Checler, F. (2019) Targeting gamma-secretase triggers the selective enrichment of oligomeric APP-CTFs in brain extracellular vesicles from Alzheimer cell and mouse models. *Transl. Neurodegener.* **8**, 35
146. Vingtdoux, V., Hamdane, M., Loyens, A., Gele, P., Drobeck, H., Begard, S., Galas, M. C., Delacourte, A., Beauvillain, J. C., Buee, L., and Sergeant, N. (2007) Alkalinizing drugs induce accumulation of amyloid precursor protein by-products in luminal vesicles of multivesicular bodies. *J. Biol. Chem.* **282**, 18197–18205
147. Miranda, A. M., Lasiecka, Z. M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., Chan, R. B., Oliveira, T. G., Small, S. A., and Di Paolo, G. (2018) Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures. *Nat. Commun.* **9**, 291
148. Jucker, M., and Walker, L. C. (2013) Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature* **501**, 45–51
149. Wang, X., Wang, W., Li, L., Perry, G., Lee, H. G., and Zhu, X. (2014) Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochim. Biophys. Acta* **1842**, 1240–1247
150. Bussiere, R., Lacampagne, A., Reiken, S., Liu, X., Scheuerman, V., Zalk, R., Martin, C., Checler, F., Marks, A. R., and Chami, M. (2017) Amyloid beta production is regulated by beta2-adrenergic signaling-mediated post-translational modifications of the ryanodine receptor. *J. Biol. Chem.* **292**, 10153–10168
151. Lacampagne, A., Liu, X., Reiken, S., Bussiere, R., Meli, A. C., Lauritzen, I., Teich, A. F., Zalk, R., Saint, N., Arancio, O., Bauer, C., Duprat, F., Briggs, C. A., Chakroborty, S., Stutzmann, G. E., et al. (2017) Post-translational remodeling of ryanodine receptor induces calcium leak leading to Alzheimer's disease-like pathologies and cognitive deficits. *Acta Neuropathol.* **134**, 749–767
152. Manczak, M., Calkins, M. J., and Reddy, P. H. (2011) Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: Implications for neuronal damage. *Hum. Mol. Genet.* **20**, 2495–2509

153. Reddy, P. H., Yin, X., Manczak, M., Kumar, S., Pradeepkiran, J. A., Vijayan, M., and Reddy, A. P. (2018) Mutant APP and amyloid beta-induced defective autophagy, mitophagy, mitochondrial structural and functional changes and synaptic damage in hippocampal neurons from Alzheimer's disease. *Hum. Mol. Genet.* **27**, 2502–2516
154. Pera, M., Larrea, D., Guardia-Laguarta, C., Montesinos, J., Velasco, K. R., Agrawal, R. R., Xu, Y., Chan, R. B., Di Paolo, G., Mehler, M. F., Perumal, G. S., Macaluso, F. P., Freyberg, Z. Z., Acin-Perez, R., Enriquez, J. A., et al. (2017) Increased localization of APP-C99 in mitochondria-associated ER membranes causes mitochondrial dysfunction in Alzheimer disease. *EMBO J.* **36**, 3356–3371
155. Del Prete, D., Suski, J. M., Oules, B., Debayle, D., Gay, A. S., Lacas-Gervais, S., Bussiere, R., Bauer, C., Pinton, P., Paterlini-Brechot, P., Wiecekowski, M. R., Checler, F., and Chami, M. (2017) Localization and processing of the amyloid-beta protein precursor in mitochondria-associated membranes. *J. Alzheimers Dis.* **55**, 1549–1570
156. Montesinos, J., Pera, M., Larrea, D., Guardia-Laguarta, C., Agrawal, R. R., Velasco, K. R., Yun, T. D., Stavrovskaya, I. G., Xu, Y., Koo, S. Y., Snead, A. M., Sproul, A. A., and Area-Gomez, E. (2020) The Alzheimer's disease-associated C99 fragment of APP regulates cellular cholesterol trafficking. *EMBO J.*, e103791
157. Song, D.-K., Won, M.-H., Jung, J.-S., Lee, J.-C., Kang, T.-C., Suh, H.-W., Huh, S.-O., Paek, S.-H., Kim, Y.-H., Kim, S.-H., and Suh, Y.-H. (1998) Behavioral and neuropathological changes induced by central injection of carboxyl-terminal fragment of b-amyloid precursor protein in mice. *J. Neurochem.* **71**, 875–878
158. Matsumoto, Y., Watanabe, S., Suh, Y. H., and Yamamoto, T. (2002) Effects of intrahippocampal CT105, a carboxyl terminal fragment of beta-amyloid precursor protein, alone/with inflammatory cytokines on working memory in rats. *J. Neurochem.* **82**, 234–239
159. Selkoe, D. J. (2002) Alzheimer's disease is a synaptic failure. *Science* **298**, 789–791
160. Palop, J. J., and Mucke, L. (2016) Network abnormalities and interneuron dysfunction in Alzheimer disease. *Nat. Rev. Neurosci.* **17**, 777–792
161. Nistico, R., Pignatelli, M., Piccinin, S., Mercuri, N. B., and Collingridge, G. (2012) Targeting synaptic dysfunction in Alzheimer's disease therapy. *Mol. Neurobiol.* **46**, 572–587
162. Nalbantoglu, J., Tirado-Santiago, G., Lahsainl, A., Poirier, J., Goncalves, O., Verge, G., Momoll, F., Welner, S. A., Massicotte, G., Julien, J.-P., and Shapiro, M. L. (1997) Impaired learning and LTP in mice expressing the carboxy-terminus of the Alzheimer amyloid precursor protein. *Nature* **387**, 500–505
163. Pardossi-Piquard, R., Lauritzen, I., Bauer, C., Sacco, G., Robert, P., and Checler, F. (2016) Influence of genetic background on apathy-like behavior in triple transgenic AD mice. *Curr. Alzheimer Res.* **13**, 942–949
164. Tamayev, R., Matsuda, S., Arancio, O., and D'Adamio, L. (2012) beta- but not gamma-secretase proteolysis of APP causes synaptic and memory deficits in a mouse model of dementia. *EMBO Mol. Med.* **4**, 171–179
165. Goutagny, R., Gu, N., Cavanagh, C., Jackson, J., Chabot, J. G., Quirion, R., Krantic, S., and Williams, S. (2013) Alterations in hippocampal network oscillations and theta-gamma coupling arise before Abeta overproduction in a mouse model of Alzheimer's disease. *Eur. J. Neurosci.* **37**, 1896–1902
166. Filser, S., Ovsepian, S. V., Masana, M., Blazquez-Llorca, L., Brandt Elvang, A., Volbracht, C., Muller, M. B., Jung, C. K., and Herms, J. (2015) Pharmacological inhibition of BACE1 impairs synaptic plasticity and cognitive functions. *Biol. Psychiatry* **77**, 729–739
167. Yan, R., and Vassar, R. (2014) Targeting the beta secretase BACE1 for Alzheimer's disease therapy. *Lancet Neurol.* **13**, 319–329
168. Bourgeois, A., Lauritzen, I., Lorivel, T., Bauer, C., Checler, F., and Pardossi-Piquard, R. (2018) Intraneuronal accumulation of C99 contributes to synaptic alterations, apathy-like behavior, and spatial learning deficits in 3xTgAD and 2xTgAD mice. *Neurobiol. Aging* **71**, 21–31
169. Masuda, A., Kobayashi, Y., Kogo, N., Saito, T., Saido, T. C., and Itohara, S. (2016) Cognitive deficits in single App knock-in mouse models. *Neurobiol. Learn Mem.* **135**, 73–82
170. Robert, P. (2002) Understanding and managing behavioural symptoms in Alzheimer's disease and related dementias: Focus on rivastigmine. *Curr. Med. Res. Opin.* **18**, 156–171
171. Robert, P. H., Berr, C., Volteau, M., Bertogliati-Fileau, C., Benoit, M., Guerin, O., Sarazin, M., Legrain, S., Dubois, B., and Pre, A. L. S. G. (2008) Importance of lack of interest in patients with mild cognitive impairment. *Am. J. Geriatr. Psychiatry* **16**, 770–776
172. Holsinger, R. M. D., McLean, C. A., Beyreuther, K., Masters, C. L., and Evin, G. (2002) Increased expression of the amyloid precursor b-secretase in Alzheimer's disease. *Ann. Neurol.* **51**, 783–786
173. Li, R., Lindholm, K., Yang, L. B., Yue, X., Citron, M., Yan, R., Beach, T., Sue, L., Sabbagh, M., Cai, H., Wong, P., Price, D., and Shen, Y. (2004) Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3632–3637
174. Yang, L.-B., Lindholm, K., Xia, R., Citron, M., Xia, W., Yang, X.-L., Beach, T., Sue, L., Wong, P. C., Price, D. L., Li, R., and Shen, Y. (2003) Elevated b-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med.* **9**, 3–4
175. Hebert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahatoglu, A. N., Kauppinen, S., Delacourte, A., and De Strooper, B. (2008) Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 6415–6420
176. Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R. (1991) Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* **30**, 572–580
177. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* **42**, 631–639
178. Nelson, P. T., Alafuzoff, I., Bigio, E. H., Bouras, C., Braak, H., Cairns, N. J., Castellani, R. J., Crain, B. J., Davies, P., Del Tredici, K., Duyckaerts, C., Frosch, M. P., Haroutunian, V., Hof, P. R., Hulette, C. M., et al. (2012) Correlation of Alzheimer disease neuropathologic changes with cognitive status: A review of the literature. *J. Neuropathol. Exp. Neurol.* **71**, 362–381
179. Yao, W., Tambini, M. D., Liu, X., and D'Adamio, L. (2019) Tuning of glutamate, but not GABA, release by an intrasynaptic vesicle APP domain whose function can be modulated by beta- or alpha-secretase cleavage. *J. Neurosci.* **39**, 6992–7005
180. Shi, X. P., Tugusheva, K., Bruce, J. E., Lucka, A., Wu, G. X., Chen-Dodson, E., Price, E., Li, Y., Xu, M., Huang, Q., Sardana, M. K., and Hazuda, D. J. (2003) Beta-secretase cleavage at amino acid residue 34 in the amyloid beta peptide is dependent upon gamma-secretase activity. *J. Biol. Chem.* **278**, 21286–21294
181. Caillava, C., Ranaldi, S., Lauritzen, I., Bauer, C., Fareh, J., Abraham, J. D., and Checler, F. (2014) Study on Abeta34 biology and detection in transgenic mice brains. *Neurobiol. Aging* **35**, 1570–1581
182. Kuhn, P. H., Koroniak, K., Hogg, S., Colombo, A., Zeitschel, U., Willem, M., Volbracht, C., Schepers, U., Imhof, A., Hoffmeister, A., Haass, C., Rossner, S., Brase, S., and Lichtenthaler, S. F. (2012) Secretome protein enrichment identifies physiological BACE1 protease substrates in neurons. *EMBO J.* **31**, 3157–3168
183. Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D. R., Price, D. L., and Wong, P. C. (2001) BACE1 is the major b-secretase for generation of Ab peptides by neurons. *Nat. Neurosci.* **4**, 233–234
184. Chevallier, N., Vizzavona, J., Marambaud, P., Baur, C. P., Spillantini, M., Fulcrand, P., Martinez, J., Goedert, M., Vincent, J. P., and Checler, F. (1997) Cathepsin D displays *in vitro* b-secretase-like specificity. *Brain Res.* **750**, 11–19
185. Zuhl, A. M., Nolan, C. E., Brodney, M. A., Niessen, S., Atchison, K., Houle, C., Karanian, D. A., Ambroise, C., Brulet, J. W., Beck, E. M., Doran, S. D., O'Neill, B. T., Am Ende, C. W., Chang, C., Geoghegan, K. F., et al. (2016) Chemoproteomic profiling reveals that cathepsin D off-target activity drives ocular toxicity of beta-secretase inhibitors. *Nat. Commun.* **7**, 13042

186. Boland, B., Smith, D. A., Mooney, D., Jung, S. S., Walsh, D. M., and Platt, F. M. (2010) Macroautophagy is not directly involved in the metabolism of amyloid precursor protein. *J. Biol. Chem.* **285**, 37415–37426
187. Lopez Lopez, C., Tariot, P. N., Caputo, A., Langbaum, J. B., Liu, F., Riviere, M. E., Langlois, C., Rouzade-Dominguez, M. L., Zalesak, M., Hendrix, S., Thomas, R. G., Viglietta, V., Lenz, R., Ryan, J. M., Graf, A., et al. (2019) The Alzheimer's Prevention Initiative Generation Program: Study design of two randomized controlled trials for individuals at risk for clinical onset of Alzheimer's disease. *Alzheimers Dement. (N Y)* **5**, 216–227
188. Dourlen, P., Kilinc, D., Malmanche, N., Chapuis, J., and Lambert, J. C. (2019) The new genetic landscape of Alzheimer's disease: From amyloid cascade to genetically driven synaptic failure hypothesis? *Acta Neuropathol.* **138**, 221–236
189. Caccamo, A., Magri, A., Medina, D. X., Wisely, E. V., Lopez-Aranda, M. F., Silva, A. J., and Oddo, S. (2013) mTOR regulates tau phosphorylation and degradation: implications for Alzheimer's disease and other tauopathies. *Aging Cell* **12**, 370–380
190. Spilman, P., Podlitskaya, N., Hart, M. J., Debnath, J., Gorostiza, O., Bredesen, D., Richardson, A., Strong, R., and Galvan, V. (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One* **5**, e9979
191. Sardiello, M., Palmieri, M., di Ronza, A., Medina, D. L., Valenza, M., Gennarino, V. A., Di Malta, C., Donaudy, F., Embrione, V., Polishchuk, R. S., Banfi, S., Parenti, G., Cattaneo, E., and Ballabio, A. (2009) A gene network regulating lysosomal biogenesis and function. *Science* **325**, 473–477
192. Xiao, Q., Yan, P., Ma, X., Liu, H., Perez, R., Zhu, A., Gonzales, E., Tripoli, D. L., Czerniewski, L., Ballabio, A., Cirrito, J. R., Diwan, A., and Lee, J. M. (2015) Neuronal-targeted TFEB accelerates lysosomal degradation of APP, reducing Abeta generation and amyloid plaque pathogenesis. *J. Neurosci.* **35**, 12137–12151
193. Song, J. X., Malampati, S., Zeng, Y., Durairajan, S. S. K., Yang, C. B., Tong, B. C., Iyaswamy, A., Shang, W. B., Sreenivasurthy, S. G., Zhu, Z., Cheung, K. H., Lu, J. H., Tang, C., Xu, N., and Li, M. (2020) A small molecule transcription factor EB activator ameliorates beta-amyloid precursor protein and Tau pathology in Alzheimer's disease models. *Aging Cell* **19**, e13069
194. Becot, A., Pardossi-Piquard, R., Bourgeois, A., Duplan, E., Xiao, Q. L., Diwan, A., Lee, J. M., Lauritzen, I., and Checler, F. (2020) The transcription factor EB reduces the intraneuronal accumulation of the beta-secretase-derived APP fragment C99 in cellular and mouse Alzheimer's disease models. *Cells* **9**, 1204
195. Yang, D. S., Stavrides, P., Kumar, A., Jiang, Y., Mohan, P. S., Ohno, M., Dobrenis, K., Davidson, C. D., Saito, M., Pawlik, M., Huo, C., Walkley, S. U., and Nixon, R. A. (2017) Cyclodextrin has conflicting actions on autophagy flux *in vivo* in brains of normal and Alzheimer model mice. *Hum. Mol. Genet.* **26**, 843–859
196. Lee, J. H., McBrayer, M. K., Wolfe, D. M., Haslett, L. J., Kumar, A., Sato, Y., Lie, P. P., Mohan, P., Coffey, E. E., Kompella, U., Mitchell, C. H., Lloyd-Evans, E., and Nixon, R. A. (2015) Presenilin 1 maintains lysosomal Ca(2+) homeostasis via TRPML1 by regulating vATPase-mediated lysosome acidification. *Cell Rep.* **12**, 1430–1444
197. Bourdenx, M., Daniel, J., Genin, E., Soria, F. N., Blanchard-Desce, M., Bezdard, E., and Dehay, B. (2016) Nanoparticles restore lysosomal acidification defects: Implications for Parkinson and other lysosomal-related diseases. *Autophagy* **12**, 472–483
198. Bustos, V., Pulina, M. V., Bispo, A., Lam, A., Flajolet, M., Gorelick, F. S., and Greengard, P. (2017) Phosphorylated Presenilin 1 decreases beta-amyloid by facilitating autophagosome-lysosome fusion. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 7148–7153
199. Bustos, V., Pulina, M. V., Kelahmetoglu, Y., Sinha, S. C., Gorelick, F. S., Flajolet, M., and Greengard, P. (2017) Bidirectional regulation of Abeta levels by presenilin 1. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 7142–7147
200. Mosser, S., Alattia, J. R., Dimitrov, M., Matz, A., Pascual, J., Schneider, B. L., and Fraering, P. C. (2015) The adipocyte differentiation protein APMAP is an endogenous suppressor of Abeta production in the brain. *Hum. Mol. Genet.* **24**, 371–382
201. Gerber, H., Mosser, S., Boury-Jamot, B., Stumpe, M., Piersigilli, A., Goepfert, C., Dengiel, J., Albrecht, U., Magara, F., and Fraering, P. C. (2019) The APMAP interactome reveals new modulators of APP processing and beta-amyloid production that are altered in Alzheimer's disease. *Acta Neuropathol. Commun.* **7**, 13
202. Wakabayashi, T., Craessaerts, K., Bammens, L., Bentahir, M., Borgions, F., Herdewijn, P., Staes, A., Timmerman, E., Vandekerckhove, J., Rubinstein, E., Boucheix, C., Gevaert, K., and De Strooper, B. (2009) Analysis of the gamma-secretase interactome and validation of its association with tetraspanin-enriched microdomains. *Nat. Cell Biol.* **11**, 1340–1346
203. Jeon, A. H., Bohm, C., Chen, F., Huo, H., Ruan, X., Ren, C. H., Ho, K., Qamar, S., Mathews, P. M., Fraser, P. E., Mount, H. T., St George-Hyslop, P., and Schmitt-Ulms, G. (2013) Interactome analyses of mature gamma-secretase complexes reveal distinct molecular environments of presenilin (PS) paralogs and preferential binding of signal peptide peptidase to PS2. *J. Biol. Chem.* **288**, 15352–15366
204. Hasegawa, H., Liu, L., Tooyama, I., Murayama, S., and Nishimura, M. (2014) The FAM3 superfamily member ILEI ameliorates Alzheimer's disease-like pathology by destabilizing the penultimate amyloid-beta precursor. *Nat. Commun.* **5**, 3917
205. Zhou, J., and Rossi, J. (2017) Aptamers as targeted therapeutics: Current potential and challenges. *Nat. Rev. Drug Discov.* **16**, 181–202
206. Chen, X. Q., Salehi, A., Pearn, M. L., Overk, C., Nguyen, P. D., Kle-shevnikov, A. M., Maccacchini, M., and Mobley, W. C. (2021) Targeting increased levels of APP in Down syndrome: Posiphen-mediated reductions in APP and its products reverse endosomal phenotypes in the Ts65Dn mouse model. *Alzheimers Dement.* **17**, 271–292
207. Flammang, B., Pardossi-Piquard, R., Sevalle, J., Debayle, D., Dabert-Gay, A. S., Thevenet, A., Lauritzen, I., and Checler, F. (2012) Evidence that the amyloid-beta protein precursor intracellular domain, AICD, derives from beta-secretase-generated C-terminal fragment. *J. Alzheimers Dis.* **30**, 145–153
208. Nicoll, J. A. R., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R. O. (2003) Neuropathology of human Alzheimer disease after immunization with amyloid-b peptide: A case report. *Nat. Med.* **9**, 448–452
209. Holmes, C., Boche, D., Wilkinson, D., Yadegarfar, G., Hopkins, V., Bayer, A., Jones, R. W., Bullock, R., Love, S., Neal, J. W., Zotova, E., and Nicoll, J. A. (2008) Long-term effects of Abeta42 immunisation in Alzheimer's disease: Follow-up of a randomised, placebo-controlled phase I trial. *Lancet* **372**, 216–223
210. Boche, D., Donald, J., Love, S., Harris, S., Neal, J. W., Holmes, C., and Nicoll, J. A. (2010) Reduction of aggregated Tau in neuronal processes but not in the cell bodies after Abeta42 immunisation in Alzheimer's disease. *Acta Neuropathol.* **120**, 13–20
211. Vandenbergh, R., Riviere, M. E., Caputo, A., Sovago, J., Maguire, R. P., Farlow, M., Marotta, G., Sanchez-Valle, R., Scheltens, P., Ryan, J. M., and Graf, A. (2017) Active Abeta immunotherapy CAD106 in Alzheimer's disease: A phase 2b study. *Alzheimers Dement. (N. Y.)* **3**, 10–22
212. Cummings, J. L., Cohen, S., van Dyck, C. H., Brody, M., Curtis, C., Cho, W., Ward, M., Friesenhahn, M., Rabe, C., Brunstein, F., Quartino, A., Honigberg, L. A., Fuji, R. N., Clayton, D., Mortensen, D., et al. (2018) Abby: A phase 2 randomized trial of crenezumab in mild to moderate Alzheimer disease. *Neurology* **90**, e1889–e1897
213. Honig, L. S., Vellas, B., Woodward, M., Boada, M., Bullock, R., Borrie, M., Hager, K., Andreasen, N., Scarpini, E., Liu-Seifert, H., Case, M., Dean, R. A., Hake, A., Sundell, K., Poole Hoffmann, V., et al. (2018) Trial of solanezumab for mild dementia due to Alzheimer's disease. *N. Engl. J. Med.* **378**, 321–330
214. Ostrowski, S., Deptula, D., Thurfjell, L., Barkhof, F., Bohrmann, B., Brooks, D. J., Klunk, W. E., Ashford, E., Yoo, K., Xu, Z. X., Loetscher, H., and Santarelli, L. (2012) Mechanism of amyloid removal in patients with

- Alzheimer disease treated with gantenerumab. *Arch. Neurol.* **69**, 198–207
215. Klein, G., Delmar, P., Voyle, N., Rehal, S., Hofmann, C., Abi-Saab, D., Andjelkovic, M., Ristic, S., Wang, G., Bateman, R., Kerchner, G. A., Baudler, M., Fontoura, P., and Doody, R. (2019) Gantenerumab reduces amyloid-beta plaques in patients with prodromal to moderate Alzheimer's disease: A PET substudy interim analysis. *Alzheimers Res. Ther.* **11**, 101
  216. Logovinsky, V., Satlin, A., Lai, R., Swanson, C., Kaplow, J., Osswald, G., Basun, H., and Lannfelt, L. (2016) Safety and tolerability of BAN2401—a clinical study in Alzheimer's disease with a protofibril selective Abeta antibody. *Alzheimers Res. Ther.* **8**, 14
  217. Kennedy, M. E., Stamford, A. W., Chen, X., Cox, K., Cumming, J. N., Dockendorf, M. F., Egan, M., Ereshefsky, L., Hodgson, R. A., Hyde, L. A., Jhee, S., Kleijn, H. J., Kuvelkar, R., Li, W., Mattson, B. A., et al. (2016) The BACE1 inhibitor verubecestat (MK-8931) reduces CNS beta-amyloid in animal models and in Alzheimer's disease patients. *Sci. Transl. Med.* **8**, 363ra150
  218. Egan, M. F., Kost, J., Voss, T., Mukai, Y., Aisen, P. S., Cummings, J. L., Tariot, P. N., Vellas, B., van Dyck, C. H., Boada, M., Zhang, Y., Li, W., Furtek, C., Mahoney, E., Harper Mozley, L., et al. (2019) Randomized trial of verubecestat for prodromal Alzheimer's disease. *N. Engl. J. Med.* **380**, 1408–1420
  219. Timmers, M., Streffer, J. R., Russu, A., Tominaga, Y., Shimizu, H., Shiraishi, A., Tatikola, K., Smekens, P., Borjesson-Hanson, A., Andreasen, N., Matias-Guiu, J., Baquero, M., Boada, M., Tesseur, I., Tritsmans, L., et al. (2018) Pharmacodynamics of atabecestat (JNJ-54861911), an oral BACE1 inhibitor in patients with early Alzheimer's disease: Randomized, double-blind, placebo-controlled study. *Alzheimers Res. Ther.* **10**, 85
  220. Henley, D., Raghavan, N., Sperling, R., Aisen, P., Raman, R., and Romano, G. (2019) Preliminary results of a trial of atabecestat in pre-clinical Alzheimer's disease. *N. Engl. J. Med.* **380**, 1483–1485
  221. Cebers, G., Alexander, R. C., Haerberlein, S. B., Han, D., Goldwater, R., Ereshefsky, L., Olsson, T., Ye, N., Rosen, L., Russell, M., Maltby, J., Eketjall, S., and Kugler, A. R. (2017) AZD3293: Pharmacokinetic and pharmacodynamic effects in healthy subjects and patients with Alzheimer's disease. *J. Alzheimers Dis.* **55**, 1039–1053
  222. Wessels, A. M., Tariot, P. N., Zimmer, J. A., Selzler, K. J., Bragg, S. M., Andersen, S. W., Landry, J., Krull, J. H., Downing, A. M., Willis, B. A., Shcherbinin, S., Mullen, J., Barker, P., Schumi, J., Shering, C., et al. (2020) Efficacy and safety of Lanabecestat for treatment of early and mild Alzheimer disease: The AMARANTH and DAYBREAK-ALZ randomized clinical trials. *JAMA Neurol.* **77**, 199–209
  223. Neumann, U., Ufer, M., Jacobson, L. H., Rouzade-Dominguez, M. L., Huledal, G., Kolly, C., Luond, R. M., Machauer, R., Veenstra, S. J., Hurth, K., Rueeger, H., Tintelnot-Blomley, M., Staufenbiel, M., Shishk, D. R., Perrot, L., et al. (2018) The BACE-1 inhibitor CNP520 for prevention trials in Alzheimer's disease. *EMBO Mol. Med.* **10**
  224. Lynch, S. Y., Kaplow, J., Zhao, J., Dhadda, S., Luthman, J., and Albala, B. (2018) Elenbecestat, E2609, a Bace inhibitor: Results from a phase-2 study in subjects with mild cognitive impairment and mild-to-moderate dementia due to Alzheimer's disease. *Alzheimer's Dement.* **14**(suppl), P1623
  225. Imbimbo, B. P., and Watling, M. (2019) Investigational BACE inhibitors for the treatment of Alzheimer's disease. *Expert Opin. Investig. Drugs* **28**, 967–975
  226. Galasko, D. R., Graff-Radford, N., May, S., Hendrix, S., Cottrell, B. A., Sagi, S. A., Mather, G., Laughlin, M., Zavitz, K. H., Swabb, E., Golde, T. E., Murphy, M. P., and Koo, E. H. (2007) Safety, tolerability, pharmacokinetics, and Abeta levels after short-term administration of R-flur-biprofen in healthy elderly individuals. *Alzheimer Dis. Assoc. Disord.* **21**, 292–299
  227. Hamm, V., Heraud, C., Bott, J. B., Herbeaux, K., Strittmatter, C., Mathis, C., and Goutagny, R. (2017) Differential contribution of APP metabolites to early cognitive deficits in a TgCRND8 mouse model of Alzheimer's disease. *Sci. Adv.* **3**, e1601068
  228. Kim, S., Sato, Y., Mohan, P. S., Peterhoff, C., Pensalfini, A., Rigoglioso, A., Jiang, Y., and Nixon, R. A. (2016) Evidence that the rab5 effector APPL1 mediates APP-betaCTF-induced dysfunction of endosomes in Down syndrome and Alzheimer's disease. *Mol. Psychiatry* **21**, 707–716
  229. Xu, W., Weissmiller, A. M., White, J. A., 2nd, Fang, F., Wang, X., Wu, Y., Pearn, M. L., Zhao, X., Sawa, M., Chen, S., Gunawardena, S., Ding, J., Mobley, W. C., and Wu, C. (2016) Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. *J. Clin. Invest* **126**, 1815–1833
  230. Jiang, Y., Sato, Y., Im, E., Berg, M., Bordi, M., Darji, S., Kumar, A., Mohan, P. S., Bandyopadhyay, U., Diaz, A., Cuervo, A. M., and Nixon, R. A. (2019) Lysosomal dysfunction in down syndrome is APP-dependent and mediated by APP-betaCTF (C99). *J. Neurosci.* **39**, 5255–5268