



Physiological and pathological roles of the amyloid precursor protein at the presynapse

Tomàs Jordà Siquier

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
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ÉCOLE DOCTORALE
Sciences de la Vie et de la Santé
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Par Tomàs Jordà Siquier

**Physiological and pathological roles of
the amyloid precursor protein at the presynapse**

Sous la direction de : Gaël BARTHET

Soutenue publiquement le 25 février 2021

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Saramago: "Life is like the paintings; you have to look at them 4 steps back"

Marie curie: "Be less curious about people and more curious about ideas"

Toni Nadal: "Never an excuse made us win a match"

Isabel Coixet: "What I love is to tell stories, wherever it is. You leave me in Mongolia and I will make you a movie"

Leonard Cohen: "If I knew where the good songs came from, I'd go there more often"

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“And you can tell everybody this is your song It may be quite simple but now that it's done I hope you don't mind, I hope you don't mind that I put down in words, how wonderful life is while you're in the world”.

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RESUME OF THESIS

Physiological and pathological roles of the amyloid precursor protein at the presynapse

Alzheimer's disease (AD) is a progressive neurodegenerative disease which affects 47 million people worldwide, being the most prominent type of dementia. The etiology of the disease is unknown but genetic evidence from the familial form of the disease indicates that the amyloid precursor protein (APP) plays a key role in the pathology. Importantly, APP is the substrate in the proteolytic reaction producing A β peptides which compose the amyloid plaques, one of the main pathological hallmarks in AD brain. In addition, APP is ubiquitously expressed by neurons where it interacts with multiple presynaptic proteins but the role of these interactions is elusive.

The aim of my thesis was to study the physiological and pathological functions of APP related to its location at the presynapse. First, we studied the consequences on presynaptic mechanisms of the genetic deletion of presenilin, the catalytic subunit of γ -secretase, the intramembrane protease which cleaves APP. We observed that in absence of presenilin, APP accumulates in axons. By combining optogenetic to electrophysiology, we assessed synaptic transmission and plasticity in the CA3 region of the hippocampus. The presynaptic facilitation, the increase in synaptic vesicle release during repetitive stimulation, was altered whereas the basal neurotransmission was not. The impairment of presynaptic mechanisms was due to the accumulation of APP Cter, which decreases the abundance of synaptotagmin-7, a calcium sensor essential for facilitation. Using a similar approach, we investigated the consequences of the genetic deletion of APP itself and observed again an impairment of presynaptic facilitation. Together, these results demonstrate the importance of APP homeostasis in presynaptic plasticity.

I then investigated possible alterations of APP, other than the amyloid peptides, in the AD brain. I discovered that APP dramatically accumulates together with presynaptic proteins around dense-core amyloid plaques in human AD brain. In addition, the Nter domain, but not the Cter domain of APP is enriched in the core of amyloid plaques uncovering a potential pathological role of the secreted APP Nter in dense-core plaques. Ultrastructural analysis of APP accumulations reveals abundant multivesicular bodies containing presynaptic vesicle proteins and autophagosomal built-up of APP. Finally, we observed that outside the APP accumulations, presynaptic proteins were downregulated, in the neuropil area of the outer molecular layer of the dentate gyrus. Altogether, the data I collected during my thesis supports a role of presynaptic APP in physiology and in AD pathology and highlights APP accumulations as a pathological site where presynaptic proteins are mis-distributed.

Rôles physiologiques et pathologiques de la protéine précurseur amyloïde à la présynapse

La maladie d'Alzheimer (MA) est une maladie neurodégénérative qui touche 47 millions de personnes dans le monde et représente le type de démence le plus répandu. L'étiologie de la maladie est inconnue mais les preuves génétiques de la forme familiale de la maladie indiquent que la protéine précurseur amyloïde (APP) joue un rôle clé dans la pathologie. L'APP est le substrat de la réaction protéolytique produisant des peptides A β qui composent les plaques amyloïdes, l'une des principales caractéristiques pathologiques du cerveau atteint de la MA. De plus, l'APP est exprimée de manière ubiquitaire par les neurones où elle interagit avec plusieurs protéines présynaptiques, mais le rôle de ces interactions est inconnu.

Le but de ma thèse était d'étudier les fonctions physiologiques et pathologiques de l'APP liées à sa localisation présynaptique. D'abord, nous avons étudié les conséquences sur les mécanismes présynaptiques de la délétion génétique de la préséniline, la sous-unité catalytique de la γ -sécrétase, la protéase intramembranaire qui clive l'APP. Nous avons observé qu'en l'absence de préséniline, l'APP s'accumule dans les axones. En combinant l'optogénétique à l'électrophysiologie, nous avons évalué la transmission synaptique et la plasticité dans la région CA3 de l'hippocampe. La facilitation présynaptique, l'augmentation de la libération de vésicules synaptiques pendant la stimulation répétitive était réduite alors que la neurotransmission basale ne l'était pas. L'altération des mécanismes présynaptiques était due à l'accumulation d'APP Cter qui diminue l'abondance de synaptotagmine-7, une protéine essentielle à la facilitation. En utilisant une approche similaire, nous avons étudié les conséquences de la suppression génétique de l'APP elle-même et observé à nouveau une altération de la facilitation présynaptique. Dans leur ensemble, ces résultats démontrent l'importance de l'homéostasie de l'APP dans la plasticité présynaptique.

J'ai ensuite étudié les altérations possibles de l'APP, en plus des peptides amyloïdes, dans le cerveau de la MA. J'ai découvert que l'APP s'accumule abondamment avec des protéines présynaptiques autour des plaques amyloïdes à noyau dense dans le cerveau humain atteint de la MA. De plus, le domaine Nter, mais pas le domaine Cter de l'APP, est enrichi dans le noyau des plaques amyloïdes révélant un rôle pathologique potentiel de l'APP Nter sécrété dans les plaques à noyau dense. L'analyse ultrastructurale des accumulations de l'APP révèle d'abondants corps multivesiculaires contenant des protéines des vésicules présynaptiques et une accumulation d'APP dans les autophagosomes. Enfin, nous avons observé qu'en dehors des accumulations APP, l'abondance des protéines présynaptiques étaient réduite, dans le neuropile de la couche moléculaire externe du gyrus denté. Dans l'ensemble, les données que j'ai collectées au cours de ma thèse soutiennent un rôle présynaptique de l'APP en physiologie et en pathologie dans la MA et mettent en évidence les accumulations d'APP comme un site pathologique où les protéines présynaptiques sont mal distribuées.

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ABBREVIATIONS

AD - Alzheimer disease

ADAM10 or α -secretase - A Disintegrin and metalloproteinase domain-containing protein 10

AICD - APP intracellular C-terminal domain

α 7-nAChRs - Alpha 7 nicotinic receptor

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APH-1 - Anterior pharynx-defective 1

APLP1 - Amyloid precursor-like protein 1

APLP2 - Amyloid precursor-like protein 2

APOE - Allele of the apolipoprotein E

APP - Amyloid precursor protein

A β - Amyloid beta peptide

BACE1 or β -secretase - Beta-site APP cleaving enzyme 1

BACE2 - Beta-site APP cleaving enzyme 2

BBB - Blood brain barrier

BRI2 - Broncos domain containing 2

CA - Cornu Ammonis

CASK - Calcium/calmodulin-dependent serine protein kinase

ChIEF - a chimera and point mutant of ChR1 and ChR2 Channelrhodopsins

CNS - Central Nervous system

CSF - Cerebrospinal fluid

Cter - C terminal domain

CTF α - C terminal fragment alpha

CTF β - C terminal fragment beta

DG - Dentate Gyrus

DNA - Deoxyribonucleic acid

DREADDs - Designer receptor exclusively activated by designer drugs

EEG - Electroencephalogram

EM - Electron microscope

ER - Endoplasmic reticulum

FAD - Familial Alzheimer's disease

GABAbR - Gamma-Aminobutyric acid b receptor

GSI - Gamma secretase inhibitors

GWAS - Genome wide association studies

HSV1 - Herpes simplex virus
IPSCs - Induced pluripotent stem cells
KI - Knock-in
KO - Knock-out
LRP1 - Low density lipoprotein receptor-related protein 1
LTD - Long term depression
LTP - Long term potentiation
MCI - Mild cognitive impaired
mEPSCs - Miniature excitatory postsynaptic currents
MF - Mossy fiber
Mint - Munc18 interacting protein
ML - Molecular layer
MRI - Magnetic resonance imaging
MS - Multiple sclerosis
Munc-13 - Mammalian uncoordinated-13
Munc-18 - Mammalian uncoordinated-18
NFT- Neurofibrillary tangles
NMDAR - N-Methyl-D-aspartate receptor
NMJ - Neuromuscular junction
Nter - N terminal domain
PALM - Photo-activated localization microscopy
PD - Parkinson's disease
PEN-2 - Presenilin enhancer 2
PET - Positron emission tomography
PPF - Paired pulse facilitation
PS1 or PSEN1 - Presenilin1
PS2 or PSEN2 - Presenilin 2
PSD95 - Post-synaptic density-95
RIMs - Regulating synaptic membrane exocytosis protein
ROS - Radical oxygen species
SAD - Sporadic Alzheimer's disease
sAPP - Soluble amyloid precursor protein
SNAP25 - Synaptosomal-Associated Protein, 25kDa
SNARE - Soluble NSF Attachment Protein

SNPs - Single nucleotide polymorphism
STED - Stimulated emission depletion
STORM - Stochastic optical reconstruction microscopy
STP - Short term plasticity
TBI - Traumatic brain injury
Tg - Transgenic
TMS - Transcranial magnetic stimulation
VAMP2 - Vesicle-associated membrane protein 2

INTRODUCTION

1. Discovery and early research in AD

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a gradual cognitive decline. AD is the most prominent form of dementia worldwide affecting 47 million people and having severe social and economic consequences globally. The lack of treatments to prevent or reverse the disease progression together with the increased aging of the population has turned this disease into one of the nearest future challenges for population health¹. A summary of the history of AD discovery and posterior characterization allows to appreciate the progresses achieved in the field since the early XX century, when Alois Alzheimer and Oskar Fisher discovered and described for the first time the clinical symptoms and hallmarks of AD^{2,3}.

AD was discovered in 1907, when Alois Alzheimer, a German psychiatrist and neurologist, described the symptoms of a 51-year-old woman named Auguste Deter. She was admitted in the Frankfurt mental hospital in 1901 at the age of 51 and therefore Alzheimer supervised her care, giving a detailed description of the clinical history and progression of the patient. Among the many symptoms reported, some of the most prominent were the progressive loss of memory and cognitive abilities. When Auguste Deter died, Alzheimer conducted a biopsy combined with the Bielschowsky silver staining technique in which he reported the thinned cortical tissue together with amyloid angiopathy, and the presence of two brain hallmarks: neuritic plaques and neurofibrillary tangles (NFT) (Fig. 1). Although Alzheimer never claimed the discovery of the disease, his mentor Emil Krapelin gave him the credit by naming the disease in his own *Handbook of Psychiatry* as 'presenile dementia', nowadays known as familial AD (FAD)⁴. Although multiple efforts to present and report the results in different conferences were done, the academic field did not embrace with excitement this discovery from the beginning. However, after 1911 the medical community was already using Alzheimer's criteria for the diagnosis of the disease.

In 1907, in parallel to Alois Alzheimer, Oskar Fisher, a Czech psychiatrist and neuropathologist reported and published a study of 16 patients with senile dementia, 12 of which had neuritic plaques⁵ (Fig. 1B). Remarkably, plaques were already reported in 1892 by Paul Blocq, Georges Marinesco or Emil Redlich in epileptic and senile demented patients⁶. However, while these neuropathologists believed that plaques were a modified type of glial cell, Fisher and Alzheimer, both suggested that plaques were inclusions of unknown origin and diverse sizes. In addition, Fisher published two more manuscripts in 1910 and 1912 in which he provided a clear

pathological and clinical description of different types of plaques and NFT in different brain regions^{7,8}.

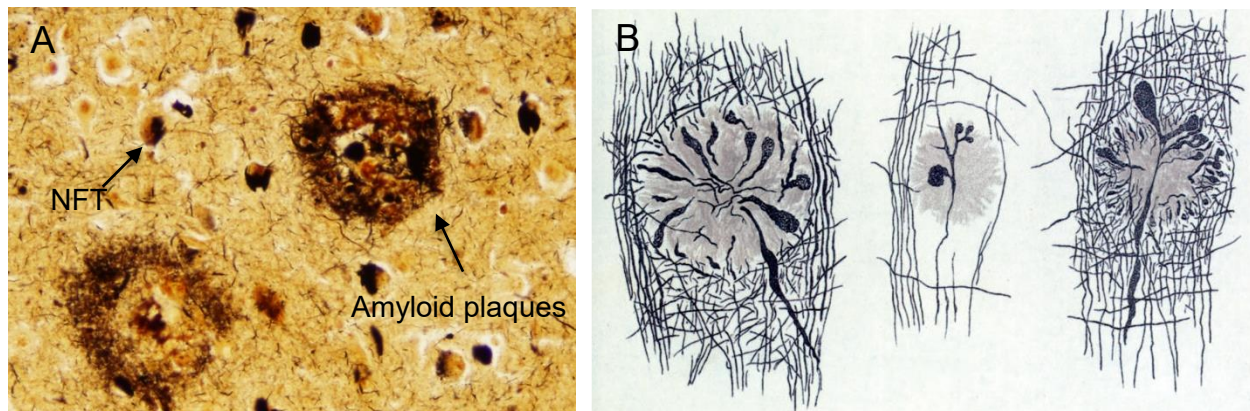


Figure 1 – AD hallmarks. A) Bielschowski silver stain in a cortical section of an AD patient where Amyloid plaques and NFT can be observed. B) Drawings by Oskar Fischer illustrating amyloid plaques and neurites in 1907 paper.

After the discovery and the clinical description of the disease, the first psychological tests were developed and performed between the 60s and 70s, aiming to objectively screen and evaluate the cognitive decline suffered by AD patients. These tests assessed the patient's behavior as well as the correlation between different parameters such as aging and the number of plaques with the demented score^{9,10}. In the early 70s, a series of studies taken by J.A.N Corsellis described the pathological changes affecting the hippocampus of AD patients and compared them with patients who suffer from bilateral temporal lesions affecting memory¹¹. These results were experimentally confirmed by Edgar Miller who demonstrated and defined AD as a memory disorder in which the long and short term storages are affected due to the processing and transfer of information^{12–15}. By that time, most AD studies were performed in patients with an early onset before 65 years and it was considered presenile dementia. Yet, several studies showed that similar clinical symptoms and histological features were also found in elderly people better known as senile dementia^{16,17}. A major step forward in the understanding of the disease was when compiled evidence showed that presenile dementia and senile dementia had identical histopathological features being considered the same disease^{16–18}. Consequently, AD turned from being a rare disease into the fourth leading cause of death in the elderly. Therefore, this caused a major change in the scientific community, which clearly glimpsed a major threatening issue for health. In further years to come, a shift in the research and public attention for the disease was obvious, defining the differences to other types of dementia and establishing better diagnosis criteria².

In the field of neuropsychology, the new criteria to define AD prompted new studies to evaluate the cognitive consequences and deficits of the disease. Several reports in the 80s proved that

episodic memory impairment was one of the earliest symptoms of the disease, corroborating previous results on the affection of medial temporal lobe structures (hippocampus and entorhinal cortex)^{19–21}. Other studies helped in differentiating AD from other types of memory diseases showing the inability to encode and store new information^{22,23}. The fact that the neuropathology of AD is present in other structures like the temporal, parietal or frontal cortices was also studied by other tests proving valuable information in the impairment of semantic knowledge, attention, working memory or visuospatial deficits^{24–28}. As a conclusion, from the early days until now, neuropsychological tests have been an important approach to study and diagnose AD (Fig. 2).

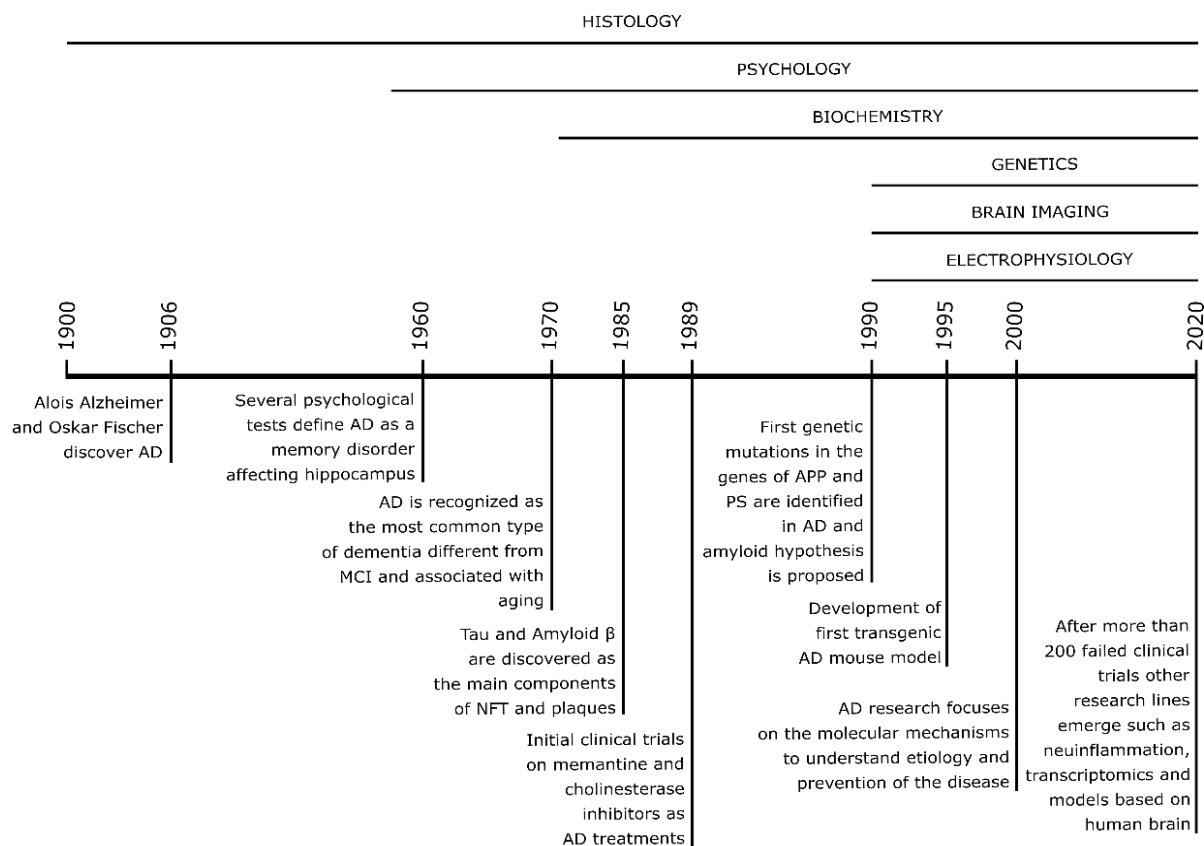


Figure 2 – Timeline of AD research history and the methods used.

2. The initial genetic studies and the proposition of the amyloid cascade hypothesis

The genetics field was also crucial in depicting the etiology of the disease, providing relevant information on the genes and proteins directly involved in AD. One of the first key discoveries was done by Glenner and Wong in 1984, who purified the cerebrovascular amyloid deposits found in Down Syndrome and AD cases, providing evidence of a peptide of 4.2 kDa, between 40 or 42 amino acids²⁹, present in both pathologies. This first biochemical evidence showing the shared composition of the cerebrovascular deposits between Down Syndrome and AD suggested a

common genetic defect in chromosome 21 in both pathologies. The idea was confirmed in following years by the discovery that both cerebral and cerebrovascular amyloid deposits have similar peptide composition³³ and that the gene encoding the amyloid peptide is located on chromosome 21^{30–32}. These results made researchers speculate that the peptide could have its origin in the cleavage of a larger precursor protein. The precursor was discovered in 1987 when Kang and colleagues cloned and sequenced the precursor of the peptide known as the amyloid precursor protein (APP), which was a 695 amino acid protein with cell-surface receptor properties³⁴. The peptide isolated by Glenner and Wong would be referred to as the amyloid β peptide ($A\beta$). Importantly in 1986 researchers also discovered Tau protein as the main component of the tangles, the second hallmark of the disease³⁵.

The decade of the 90s constituted an unprecedented advancement in the identification of AD related genes (Fig. 3). The identification of mutations present in three different genes was the first causative evidence to explain inherited type of AD in large families: familial AD (FAD). In these studies, different mutations present in the genes of APP on chromosome 21, presenilin 1 (PS1) on chromosome 14 (Fig 4A) and presenilin 2 (PS2) on chromosome 1 were characterized by being of autosomal dominant inheritance^{36–39}. These important findings let researchers delineate a complex cascade of events involved in the processing of APP and production of $A\beta$ by the β -site amyloid precursor protein-cleaving enzyme 1 (BACE1) or β -secretase^{34,40} and PS (γ -secretase)⁴¹ leading to the proposition of the amyloid cascade hypothesis by John Hardy and Gerald Higgins⁴². The hypothesis stated that amyloid plaques were the toxic cause of the disease and the trigger of a molecular cascade that induced cellular death and NFT formation.

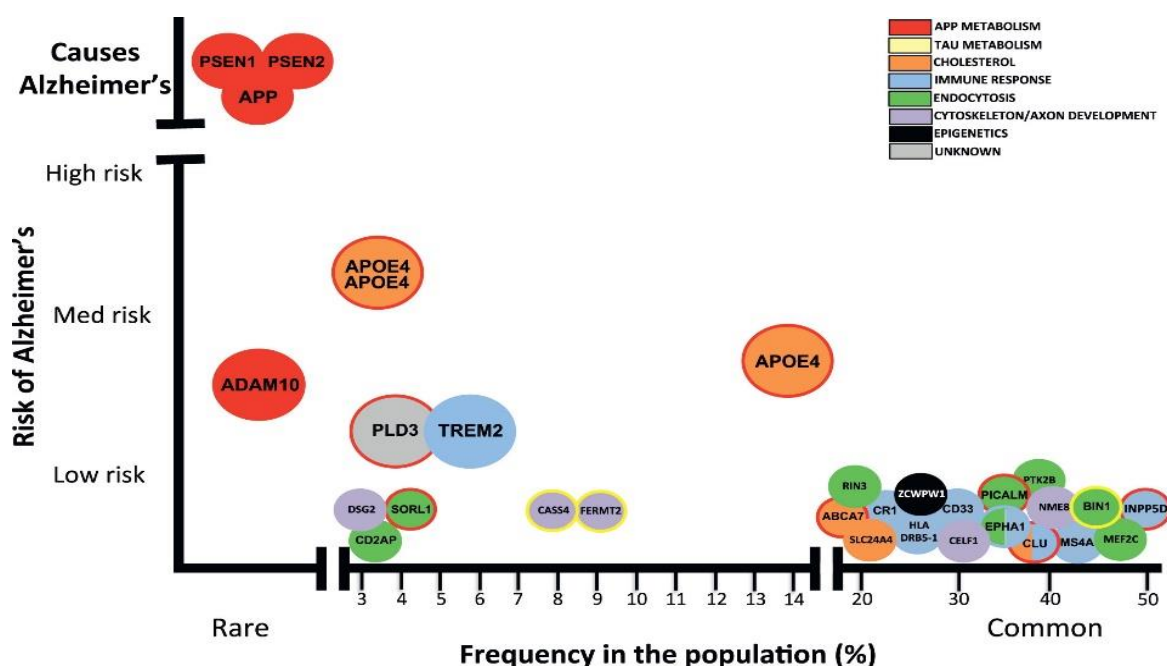


Figure 3 – Genetic risk variants in AD. AD has rare and common genetic variants that are involved in different molecular pathways. Rare genetic variants related to APP metabolism are directly involved in the cause of AD. However, there are other common genetic variants with higher frequency and lower risk in the population, which are related to other relevant pathways (Celeste and Goate et al 2015).

2.1. Function and characterization of the amyloid cascade components

2.1.1. γ -secretase

The first mutations identified in the genes of PS 1 (Fig. 4A) and 2^{38,39} in 1995 in a form of inherited AD, were some of the first breakthroughs to understand the molecular mechanisms involved in AD. In the following years, several publications provided evidence that fibroblasts derived from AD patients⁴³ or the overexpression of PS mutants *in vitro* and *in vivo* affected the ratio of A β 42 versus A β 40^{44–49}. Yet, the function of PS was not fully demonstrated till the first experiments in knock-out (KO) models were performed providing direct evidence that PS was involved in the proteolysis of APP but also other substrates like Notch^{41,50}. Other studies showed that PS were indeed the catalytic subunits of the γ -secretase complex, which were targetable by inhibitors that could turn into possible future AD treatments^{51–53}. The catalytic subunit of the γ -secretase complex was further characterized showing that in mammals PS consisted of two homologous intramembrane proteins that had 9 transmembrane domains^{54,55} and that could cleave itself⁵⁶. In addition, three additional transmembrane protein subunits composed the γ -secretase complex: Nicastrin⁵⁷, Aph-1^{58–60} and Pen-2⁶¹.

Pen-2 is crucial for the endoproteolysis of full length PS into PS-NTF and PS-CTF^{490,491}, while nicastrin and Aph-1 are thought to be important for PS stabilization and trafficking⁴⁹². Moreover, nicastrin has been proposed to participate in the recruitment of Notch1 and APP substrates to γ -secretase^{494,493} but this function has been challenged^{495,496} and recent reports indicate that APH-1 may play that role⁵⁰⁰. APH-1 has also been proposed to participate in the catalytic functions of γ -secretase via its conserved histidine residues in transmembrane sequences 5 and 6^{497,498,499}. Similarly to PS, Aph-1 is also encoded by two different genes in rodents (three in human), thus making possible the formation of 4 different γ -secretase complexes⁶² (Fig. 4B). Furthermore, the formation of the protein complex can be even more complicated due to the different splice variants known to exist for each of the genes⁶³. Interestingly, the divergent effects observed in substrate processing when specific subunits of the complex were knocked out in mice showed that different γ -secretase complexes might target specific substrates⁶².

A



B

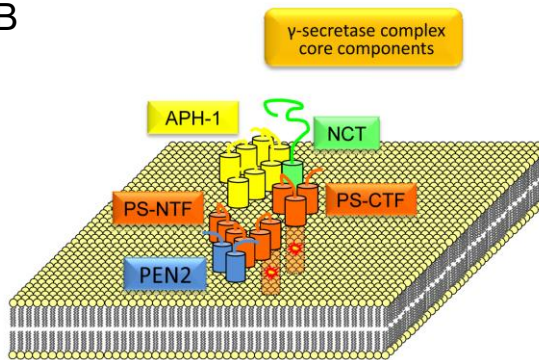


Figure 4 – The γ -secretase complex. A) Peptide sequence of the catalytic subunit of the γ -secretase complex: PS1. PS1 consists of 9 transmembrane domains, which host most of the pathogenic mutations (red). The impact of certain mutations is unknown (blue). More than 300 mutations have been reported in the gene of PS1, being the most common cause of familial AD (Alzforum – PSEN-1 mutations). B) The γ -secretase enzymatic complex consists of 4 different subunits: APH1, PEN2, Presenilin (PS) and Nicastrin (NCT). (Barthet et al 2012).

The function of the γ -secretase is diverse and complex due to the variety of proteolytic and non-proteolytic functions. Indeed, previous studies reported that certain functions related to calcium leakage in ER or in the turnover of telencephalin are independent from the catalytic activity of the complex^{64–66}. Nevertheless, most studies have focused on the proteolytic functions of the complex in a large set of different substrates such as Notch, N-cadherins and APP. It is clear that the Notch signaling pathway induces severe phenotypes for cell development and differentiation in immune system cells^{67–69}, intestinal goblet cells^{69,70} or skin cells^{71,72}, which are affected when the activity of the γ -secretase complex is impaired. One of the most important substrates of γ -secretase besides APP appeared to be Notch. Importantly, Notch cleavage by γ -secretase releases Notch intracellular domain (NICD) in the cytosol, which mediates Notch signaling in the cell. In conditions

where γ -secretase activity is impaired, severe alterations in the development and differentiation of immune system cells^{67–69}, intestinal goblet cells^{69,70} or skin cells^{71,72} are observed, which resemble Notch KO phenotype. Furthermore, other types of substrates processed by PS1 are the cell adhesion molecules E and N-cadherins, which once cleaved release intracellular catenins or disassembly the adherens junctions between cells^{73,74}. Remarkably more than 200 substrates for the γ -secretase have been identified defining other functions related to cancer, cell adhesion, transmembrane clearance or acne inversa⁵⁰¹. Probably one of the best studied substrates of the γ -secretase complex is APP, which will be discussed later.

2.1.2. BACE1

BACE1 is a type I transmembrane protein of 501 amino-acids, part of the aspartyl protease family mostly present in the pancreas and brain. This protein was demonstrated to be the first cleaving enzyme of the amyloidogenic pathway, indispensable in the production of A β peptide⁴⁰. Interestingly, a mutation identified in the gene of APP, which decreases its cleavage by BACE1, indicated BACE1 as a putative therapeutical target in the prevention of AD⁷⁵. In addition, several reports provided evidence of the elevated levels of BACE1 in postmortem brains⁷⁶ as well as its presence in dystrophic presynaptic terminals surrounding A β plaques in AD mouse models and patients^{77–79}.

In recent years, the physiological function of BACE1 has been studied in the nervous system providing evidence of its expression in different cell compartments of the endocytic pathway of neurons such as plasma membrane, lysosomes and endosomes⁸⁰. Therefore, the impaired processing of substrates like APP can induce dysfunctional cellular functions, which are discussed later in this manuscript. BACE1 is also known to be involved in the processing of approximately 40 different substrates^{81–83}, implying a large variety of developmental deficits and phenotypes observed in KO animals. Among them reduced myelination^{84–86}, synaptic plasticity deficits^{87–90}, impaired working memory⁸⁸, altered neuro and astrocytogenesis⁹¹ or impaired axonal^{92–95} and spine development⁹⁶ are some of the best characterized phenotypes. Furthermore, KO models also display other disease-related pathologies such as retinal deficits⁹⁷, schizophrenia^{88,96} and epileptic-like^{88,98,99} phenotypes. BACE1 has a homolog, BACE2, that is mostly expressed in pancreas, kidney and glial cells but not in neurons¹⁰⁰, where it cleaves APP within the A β domain promoting the non-amyloidogenic processing of APP¹⁰¹. Thus, this data together with the fact that A β production is absent in the BACE1 KO suggest that BACE2 is not likely a β -secretase in the nervous system¹⁰².

2.1.3. APP protein family

2.1.3.1. Structure and location

APP was identified as a type I single-pass transmembrane protein that belonged to a conserved gene family consisting of 3 different proteins: APP and the two APP-like proteins (APLPs) APLP1 and APLP2¹⁰³ (Fig. 5A,B). Throughout species, these proteins have been well conserved in *C.elegans*¹⁰⁴ (APL-1), *Drosophila*¹⁰⁵ (APPL), Zebrafish¹⁰⁶ or mammals^{107–109} and are widely expressed in different cell types. While APLP1 is mostly found in the nervous system¹¹⁰, APP and APLP2 are ubiquitously expressed by independent genes in different body organs during and after development^{107,110,111}. Interestingly, APP has three main splice variants: APP770, APP751 and APP695, which is the most prominent variant in the nervous system¹⁰³.

The APP protein family consists of different domains that exert a large variety of functions. APP, together with its 2 paralogs, have a conserved N-terminal (Nter) ectodomain which consists of two different regions: E1 and E2. In the E1 region there are 2 subdomains known as the heparin-binding/growth factor domain and the metal binding domain for copper or zinc, which are linked to the E2 region by an acidic region and a Kunitz-type protease inhibitor region. Interestingly, although the peptide sequence of the E1 and E2 regions is very well conserved in the APP family, one of the most divergent and unique regions for APP is the juxtamembrane region where the A β sequence is located^{103,112}. This region of 42 amino acids has driven a lot of the AD research in the last decades as it is one of the main components of amyloid plaques²⁹. The last region located at the intracellular C-terminal domain (Cter) is the YENPTY domain, which is well conserved among the APP family. The homogeneity found between the different domains explains the overlapping functions between the APP family members (Fig. 5A). This feature together with the generation of different polypeptides driven by the proteolytic processing has made the study of APP very complex^{103,112}.

APP is ubiquitously expressed in different cell types of the brain, including neurons (Fig. 5C). Moreover, at the subcellular level, the 3 family members are present in all neuronal compartments like soma, dendrites and axons^{113,114}. In the intracellular compartments, they are known to mature through the secretory pathway^{115,116} to reach either the cell surface or the axons, where they are mainly transported in vesicles^{117,118} that reach the active zones of synapses^{119,120}. Interestingly, the cellular distribution of APP directly influences its functions as well as its processing by the different secretases. While the surface accumulation of APP favors the non-amyloidogenic processing, the retention of APP in acidic compartments like endosomes^{121,122}, favors the amyloidogenic processing. Much less is known about APLP2 and APLP1, however APLP1 is

particularly well expressed at the cell surface level¹²³. The secretases involved in the cleavage and processing of APP are also known to converge in similar locations such as endosomes^{121,122}, ER and Golgi, making the release and secretion of A β possible from different intracellular compartments at the soma, dendrites or axons¹¹⁶.

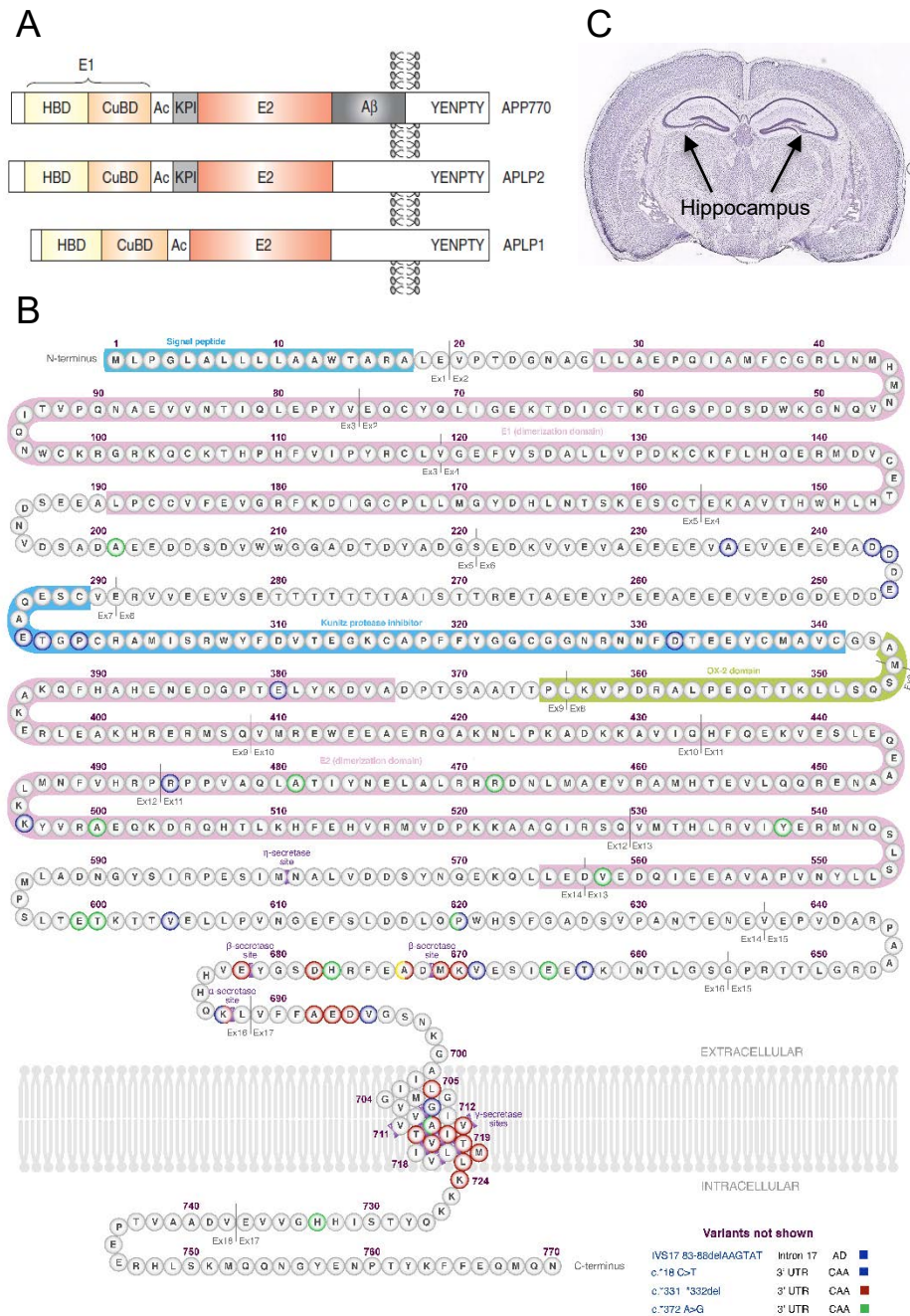


Figure 5 – Structure and location of APP and its paralogs. A) Schematic illustration of APP protein family domain structure. APP family members share conserved E1 and E2 extracellular Nter domains. Each member has an acidic binding domain (Ac) whereas Kunitz- type protease inhibitor (KPI) domain is only present in APP and APLP2. The intracellular Cter domain is also well conserved. The transmembrane A β domain is uniquely present in APP. (Ulrike C.Muller and Hui Zheng 2012); B) APP peptide sequence shows that pathogenic mutations (red) are present in the transmembrane A β domain where APP can be cleaved by the secretases. Some mutations can be non-pathogenic (green) or even protective (yellow). (Alzforum – APP mutations). C) In situ hybridization of APP mRNA in a mouse slice. APP is ubiquitously expressed in neurons in different brain regions including the hippocampus (Allen brain atlas).

2.1.3.2. APP processing pathways

The proteolytic processing of APP is very complex and can happen in two different pathways: the canonical processing and the non-canonical processing¹⁰³. Due to its relevance in AD, in the past years the canonical processing of APP has been well characterized involving three enzymes: BACE1¹¹⁶, α -secretase (ADAM10)^{81,124} and γ -secretase⁴¹. Each of these enzymes is involved in two separated pathways known as the non-amyloidogenic and the amyloidogenic pathways (Fig 6). As stated by its name the amyloidogenic pathway is responsible for the A β production and it is initiated when BACE1 cleaves the amino terminus (596 position) of the A β domain producing the soluble APP β (sAPP β) fragment and the remaining carboxy-terminal fragment attached to the membrane known as CTF β . Subsequent cleavage by the γ -secretase complex will produce two more fragments known as the A β peptide and the APP intracellular domain (AICD). On the other hand, the non-amyloidogenic pathway is initiated by ADAM10 cleaving within the A β region, preventing the formation of A β and releasing the soluble APP α (sAPP α) and the CTF α . This membrane attached fragment will be further cleaved again by γ -secretase producing AICD and the extracellular p3 fragment. Although APLP1 and APLP2 lack the A β domain, the processing and production of different fragments by the secretases is very similar to APP¹⁰³ (Fig. 6). In addition, each of these fragments have diverse functions, which will be further discussed.

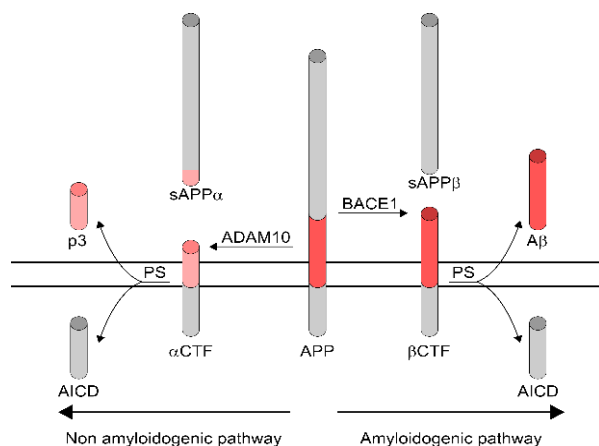


Figure 6 – Canonical APP processing pathways. In the non-amyloidogenic pathway ADAM10 cleaves APP at the amyloid- β region and liberates the sAPP α and α CTF fragment. The α CTF can be further cleaved by PS (γ -secretase) which produces two more fragments: p3 and AICD. In the amyloidogenic pathway APP is first cleaved by BACE1, liberating sAPP β and β CTF. The transmembrane β CTF fragment can be further cleaved by PS producing extracellular amyloid- β peptide and the AICD fragment.

Four non-canonical APP processing pathways have been identified so far and involve other types of enzymes that release other APP fragments with a physiological function, in many cases still to be determined. One of these pathways involves the caspases that can cleave full-length APP intracellularly at Asp664 releasing the intracellular C31 fragment involved in apoptosis¹²⁵. In addition, γ -secretase can also cleave the α/β CTF Δ C31 fragments (α/β CTF deleted of the terminal 31 amino-acids), which releases in the cytosol the Jcasp peptide that corresponds to the juxtamembrane and intracellular part of APP. Interestingly, Jcasp has been recently reported to

inhibit presynaptic transmitter release¹²⁶. Another pathway involves Meprin β , a metalloprotease which cleaves APP in three different positions of the ectodomain and has been proposed to act as another β -secretase¹²⁷. However, the contribution of Meprin β is still controversial and remains to be investigated. Better characterized was the δ -pathway, an asparagine endopeptidase linked to AD due to its capacity to cleave Tau and also APP^{128,129}. The δ -secretase cleaves APP at the ectodomain to release different fragments, some of them cytotoxic within the brain. In addition, compiling evidence in an AD mouse model has shown the involvement of this path in synaptic and behavioral deficits¹²⁹. One of the last secretase discovered was the η -secretase, another metalloprotease that cleaves APP producing CTF η and sAPP η involved in neuronal activity attenuation¹³⁰.

2.1.3.3. APP protein family functions

Over the last years, the work conducted on APP has demonstrated that the APP protein family has multiple biological functions. When APP was discovered, clear evidence showed that the protein resembled a glycosylated cell-surface receptor-like protein¹³¹. Indeed, one of the better-established functions of APP family members is to be adhesion molecules forming *cis* or *trans* dimers through the full-length protein. The E1, E2 (heparin binding domains) and the transmembrane domain contribute to the strength of the *cis* dimerization^{132–136} important for cell signaling and processing of APP by γ -secretase¹³⁷. Moreover, APP and APLPs can also form *trans* homo- or hetero-dimers which work as cell and synaptic adhesion molecules *in vitro*^{135,138,139} and at the neuromuscular junction (NMJ) *in vivo*¹³⁹. Importantly, the *trans*-synaptic function of APP and APLP2 plays a role in synaptogenesis through the copper-binding site domain (E1)¹³⁸.

Another important property of APP and its fragments is to function as ligands that bind to other membrane proteins and adaptors, triggering signaling cascades. It has been reported that APP has more than 200 extracellular and intracellular partner interactions, some of them already studied *in vitro* like Alcadin¹⁴⁰, Reelin¹⁴¹, LRP1¹⁴², sorL1/LR11¹⁴³, Nogo-66 receptor¹⁴⁴, Notch¹⁴⁵ or Netrin¹⁴⁶ and only a few *in vivo*. Some proteins interacting with APP modulate its processing, like BRICHOS domain of integral membrane protein 2 and 3 (Bri2/3). Interestingly, in Danish and British dementias, a mutation leading to loss of BRI2^{147,148} increases APP processing and also the levels of sAPP β , AICD and CTF β ¹⁴⁹. Other proteins that have been reported to interact with APP are extracellular matrix proteins (collagen, laminin and heparin) and heparan sulfate proteoglycans (glypican and syndecan), which interact through the heparin binding domains present in the ectodomain^{150–153}. These proteins have been involved in neuronal migration and differentiation during development as well as soluble proteins like netrin, F-spondin and

pancortins, all direct interactors of APP. Direct or adaptor interactions of APP have also been observed with the lipoprotein receptor family members including the lipoprotein receptor-related protein 4 relevant in the synaptogenesis of the NMJ¹⁵⁴.

In several pathologies, APP has been identified as a marker of axonal damage including multiple sclerosis (MS)^{155,156}, traumatic brain injury¹⁵⁷ or myelopathy¹⁵⁸, suggesting a role in this cell compartment. Consistent with these observations, APP is known to be present in axons where it performs different molecular functions related to axonal growth, transport, pruning and development. As an example, APP is present in axonal growth cones^{117,159}, where it colocalizes with Fe65, which functions as a regulator of actin dynamics through RAC1¹⁶⁰. In addition, *in vivo* experiments have shown that APP can function as a co-receptor for contactin¹⁶¹, promoting axon arborizations in the retinotectal axons. In other cases, the E2 domain of APP makes *cis* interactions with death receptor 6 (DR6) on the axonal membrane, triggering axonal pruning via caspases¹⁶². The fast anterograde and retrograde transport of proteins has also been linked to APP that might work as a membrane cargo receptor for kinesin 1 or dynein^{163,164}. Related to its cell-cell and cell-substrate interactions, APP has been reported to play a role in axonal guidance^{165,166} and also in neurite outgrowth through β 1 integrins¹⁶⁷. Importantly, several APP fragments like AICD, APP-CTFs or sAPP α have also been involved in axonal growth through different mechanisms. For example, *in vitro* overexpression of the APP- β CTF can induce neurite growth through the G-protein-adenylyl cyclase-cAMP cascade, and the accumulation of APP-CTFs upon γ -secretase loss of function leads to axodendritic outgrowth *in vitro* and *in vivo*^{168,169}. Several reports have also provided evidence that the sAPP α is involved in neurotrophic functions including axon growth¹⁷⁰ (Table1).

APP fragment	Physiological functions of APP and its fragments in the canonical processing pathway
APP full length	- Axonal growth, guidance, transport and pruning. - Synaptogenesis and plasticity. - Cell surface receptor working as adhesion molecule or ligand.
sAPP α	- Neurotrophic functions. - Synaptogenesis and plasticity. - Memory and social behavior. - Protective against TBI.
sAPP β	- Neurotrophic functions. - Transcription functions.
CTF α	- Unknown.

CTF β	- Regulation of synaptic proteins such as Syt7. - The accumulation of this fragment can induce serious synaptic impairments like LTP impairments, working memory impairments followed by gliosis and neurodegeneration.
p3	- Unknown.
AICD	- Transcriptional regulation. - AICD overexpression causes hippocampal degeneration, tau phosphorylation and working memory impairments. - Axonal growth.
A β	- Synaptic function: Regulation of neuronal homeostasis that depends on the concentration of the peptide. - Tumor suppressor. - Antimicrobial activity. - Traumatic brain injury recovery. - Blood brain barrier leakage prevention.

Table 1 – Summary of the *in vivo* and *ex vivo* functions of APP fragments.

Studies performed in conditional and constitutive KO and knock-in (KI) mouse models have provided important insights in the physiological roles of APP protein family in synapses. Mutant mice lacking just one of the genes of the protein family are viable and show mild phenotypes mostly related to neurodevelopment. This suggests that the three family members have overlapping functions that can be overtaken by the other two family members in single KO animals¹⁰³. However, with exception of APP/APLP1 KO any other double or triple combination of these proteins is lethal after birth due to the numerous defects such as neuromuscular problems^{171,172} (Table 2). This suggests that APLP2 has unique features that combined either with the absence of APP or APLP1 are lethal. Alternatively, this lethality may be due to higher abundance of APLP2 compared to APP and APLP1 which could not be compensated in APLP2-KO. A common model used to characterize the function of these proteins is the neuromuscular junction (NMJ)¹⁰³. APP/APLP2 KO mice have abnormal terminals, which exhibit impaired neurotransmitter release^{173–176}. In addition, consistent with a role in trans-synaptic adhesion, APP and APLP2 are fundamental for the pre and postsynaptic site assembly, morphology and functioning at the NMJ¹³⁹. Corroborating these results, submandibular ganglia neurons showed smaller active zones with fewer vesicles when APP and APLP2 were absent¹⁷⁶. Triple KO animals for all three APP family members are also lethal after birth and show important anatomical abnormalities including focal dysplasia, cortical loss of Cajal-Retzius cells, cranial problems and impaired position of the cellular migration¹⁷⁷. Similar phenotypes have been reported in a

Fe65/Fe65L1 KO model, which are protein interactors of APP, suggesting that the complexes formed between these proteins mediate similar functions related to normal brain development^{178,179}. The combination of these models with KI alleles expressing extra or intracellular truncations of APP has been a powerful model to delineate the function of the different APP products and domains *in vivo*. The creation of a KI allele expressing the sAPP α in the APP/APLP2 constitutive KO background was able to overcome the lethality and restore all the deficits observed in the APP/APLP2 KO model including grip strength, locomotor activity, body and brain weight, impaired spatial learning and long term potentiation (LTP)^{113,180,181}. Contrary to sAPP α , sAPP β fails in rescuing lethality and any of the phenotypes observed in the APP/APLP2 KO model¹⁸², implying the distinct functional roles of sAPP fragments. Importantly, the soluble fragments constitute 50% of the forms of APP in the brain¹⁸³ and despite sAPP α being 16 amino acids longer than sAPP β ¹³², they have different physiological consequences that might be also explained by their different structure. In agreement with these results, several studies have shown the relevance of these 16 amino-acids in the Cter of sAPP α in restoring LTP and cognition by binding to the α -7 nicotinic receptor¹⁸⁴. The sAPP α/β have recently been also identified to function as a GABABR1a ligand to inhibit synaptic vesicle release and modulate neurotransmission¹⁸⁵. Therefore, the relevance of sAPP α in synaptic plasticity and cognition demonstrates the potential therapeutic properties that this peptide could have in AD patients, where it is reduced in cerebrospinal fluid (CSF) samples together with ADAM10¹⁸⁶.

The fact that APP and APLPs are expressed at presynaptic and postsynaptic sites implies that they might have a function in these compartments. Electrophysiological studies in the APP/APLP2 KO model showed impairments in paired-pulse facilitation, post-tetanic potentiation, spine deficits and LTP maintenance suggesting a role of these proteins in synaptic functioning^{173,176,187,188}. Furthermore, several proteomic studies provided evidence that APP interacts with proteins involved in synaptic vesicle cycle such as synaptophysin, bassoon, synaptotagmin1 or SNARE proteins^{189,190}. Importantly, the absence of APP can cause important changes in the proteomic composition of neurotransmitter release machinery, diminishing synaptic vesicle proteins such as synaptophysin, SV2A and synaptotagmin 1¹⁹¹. Moreover, several reports have described the presence of APP in endocytosed synaptic vesicles and the presynaptic plasma membrane due to synaptic vesicle recycling and vesicle release respectively¹¹⁸. Indeed, the intracellular Cter domain is related to clathrin-mediated endocytosis in different species^{192,193}. Nevertheless, whether APP is more present in synaptic vesicles, synaptic organelles or the plasma membrane is still a matter of debate.

Recently, some of the synaptic molecular mechanisms where APP is involved have started to be revealed. These mechanisms involve direct interaction through the intracellular Cter sequence (YENPTY) or indirect interaction through adaptor proteins like JIP, Shc, Grb2, Numb, X11/mint family and Fe65 family¹⁰³. Interestingly, the conserved intracellular Cter domain of the APP family has been suggested to form a complex with the X11 Munc18 interacting protein (Mint) and the calcium/calmodulin-dependent serine protein kinase (CASK) to mediate synaptic adhesion functions¹¹². The phosphorylation status of this domain at the Thr668 or Tyr682 residues regulates APP interactome by promoting or preventing protein binding^{194,195}. Interestingly, while a substitution of the Tyr682 amino acid has severe developmental and neurotransmitter release implications, modification of the Thr668 residue has no developmental defect^{196,197}. APP fragments containing the Cter domain can also exert synaptic functions. AICD released upon γ -secretase cleavage of APP-CTFs can be transported into the nucleus where they can form a transcription complex with Fe65 and Tip60 which modulates the expression of certain genes, some of which being related to synaptic transmission¹⁹⁸. Furthermore, some of the work performed in this thesis provides evidence that the regulation of certain synaptic markers such as synaptotagmin-7 are mediated by APP-CTFs after γ -secretase absence or inhibition¹⁹⁹.

At the postsynapse level, APP plays a key role in spine density and dynamics. Only aged APP KO mice show deficiencies in dendrite arborization with a reduced dendritic length and fewer branches, suggesting that compensatory mechanisms by APLP1 or APLP2 might fail over time^{200,201}. The spine deficits have not been observed in single KO of APLP1 and APLP2 where spine morphology is normal²⁰², however it is impaired in the APP/APLP2 KO²⁰³. The precise molecular mechanisms of how APP is involved in spine density and development are not fully understood but sAPP α is sufficient to rescue the deficits in different models^{188,203–205}. Another possible explanation is that the trafficking of N-methyl-D-aspartate receptor (NMDAR), which are linked to spine density *in vivo*²⁰⁶, can be regulated by APP^{207–209}. Recent reports proved that the APP protein family can interact with NMDAR, enhancing their presence at the cell surface^{207,210–212}. Importantly, *in vitro* research has assessed the toxic effects of A β oligomers on NMDAR by showing that aberrant activation and entry of excessive Ca²⁺, caused by a glutamate reuptake failure at the synapse, leads to a molecular cascade that induces long-term depression (LTD)²¹³. These oligomeric forms of A β also affect the endocytosis and removal of NMDAR and AMPAR from the synaptic surface leading to synaptic depression and inhibition of LTP.

Constitutive KO	Viability and other phenotypes	Electrophysiology	Behavior
APP KO	- Viable - Lower body and brain weight - Abnormal brain morphology and anatomy - Reduced spine density and branching at CA1 in aged mice	- Normal basal synaptic transmission - Impaired CA3-CA1 LTP in aged mice - Impaired PPF in perforant path –DG synapses - Impairment in GABAergic neurotransmission	- Reduced grip strength - Impaired locomotor activity - Impaired learning and memory in aged mice
APLP1 KO	- Viable - Lower body weight - Normal brain morphology and anatomy	- Normal basal synaptic transmission in CA1 - Normal STP and LTP in aged mice at perforant path to DG and CA3-CA1 LTP in aged mice	- Unknown
APLP2 KO	- Viable - Normal body weight - Normal brain morphology and anatomy - Normal spine density and branching at CA1 in young and aged mice	- Normal basal synaptic transmission in young and aged mice - Normal CA3-CA1 LTP in young and aged mice	- Normal locomotion and cognition
APP/APLP1 KO	- Viable - Lower body weight - Normal brain morphology and anatomy	- Unknown	- Unknown
APP/APLP2 KO	- Lethal - Deficits in NMJ morphology	- Impaired STP and reduced mEPSP frequency at CA3-CA1 synapses in juvenile surviving mice	- Unknown
APLP1/APLP2 KO	- Lethal - Normal brain morphology and anatomy but with NMJ deficits	- Unknown	- Unknown
APP/APLP1	- Lethal	- Unknown	- Unknown

/APLP2 KO	- Deficits in NMJ morphology		
Conditional KO in nervous system	Viability and other phenotypes	Electrophysiology	Behavior
APP KO	- Viable - Normal brain morphology and anatomy - Impaired neurogenesis in hippocampus after	- DG cells present reduced mIPSC frequency	- Normal locomotion and exploration
APP/APLP2 KO – Nervous system and NMJ	- Viable - Normal brain morphology and anatomy - Reduced spine density and branching at CA1 in young mice - Deficits in NMJ morphology and neurotransmission	- Normal basal synaptic transmission - Impaired PPF CA3-CA1 LTP in young mice - Normal AMPA and NMDA receptor frequencies and amplitudes	- Normal locomotion and exploration
APP/APLP1 /APLP2 KO	- Viable - Normal brain morphology and anatomy	- Normal basal synaptic transmission - PPF is enhanced - LTP and NMDA receptor mediated responses are reduced in CA3-CA1 synapses - Hyperexcitability in hippocampal neurons	- Impaired spatial learning and memory in KO forebrain neurons

Table 2 – Characterization of APP protein family in KO models.

2.1.3.4. Physiological functions of A β peptide

APP is mostly known to be the substrate of A β peptide, which has been the focus of many studies performed in the AD field. This peptide consists of 38 to 49 amino-acids and can be produced after the cleavage of APP by BACE 1 and γ -secretase. The peptide sequence of A β is well conserved among species, meaning that it might confer an evolutionary advantage for survival²¹⁴. Besides being present in different cell types of the neuronal tissue, it is also present in non-neuronal tissues such as skin, skeletal muscle or the intestinal epithelium²¹⁵. A β is usually

present in the soluble form after being secreted extracellularly and cleared by the CSF and the vascular system²¹⁶. In physiological and pathological conditions, in human CSF samples the most common form is the A β 40 isoform²¹⁷. Nevertheless, an increase in the fractions of longer A β , like A β 42 or A β 43 is observed in the disease^{218,219}. The formation of amyloid plaques is believed to be formed by different A β species that initially form oligomers and then insoluble fibrils that form β -pleated sheets which compose amyloid plaques. These findings lead to the interpretation that

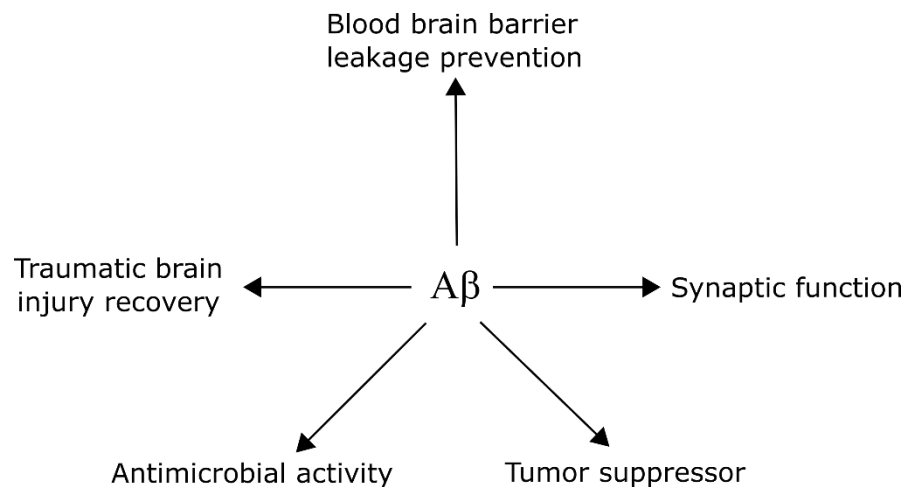


Figure 7 – Physiological roles of A β in vertebrates.

the deposition of A β overtime induces a toxic gain of function that contributes to AD pathology²²⁰. Therefore, most clinical trials have targeted amyloid plaques as the principal cause of the disease as proposed by Hardy and Higgins⁴². However, among the more than 200 clinical trials that have been done between 1984 and 2013, none of them had a positive outcome to reduce cognitive decline in AD patients²²¹.

Despite that physiological functions of A β remain controversial, several research lines have reported solid evidence in the multifunctional nature of this peptide (Fig. 7). One of the first functions proposed was the “Biofloculant hypothesis” proposed by Bishop and Robinson in 2002^{222,223}, suggesting that A β might be a chelator and flocculant of toxic agents like metal ions (Fe²⁺), bacteria, virus and proteins that are released in the extracellular fluid and need to be removed (Fig. 8). Therefore, A β would work as an antimicrobial peptide that traps and kills microbes by mechanisms like phagocytosis of microglia²²⁴. In addition, human A β has been demonstrated to have an antimicrobial activity in humans slowing down the proliferation of seven different bacteria and one fungi species in culture²²⁵. *In vivo* studies in mice and nematodes also provided evidence that overexpressed human A β enhances the resistance against bacteria and yeast²²⁶. The resistance provided by both A β 40 and A β 42 was also shown to be efficient against

viruses like the herpes simplex virus (HSV1) and the influenza virus by neutralizing and preventing the infections of neuron-glia cultures^{227,228}. Interestingly, in AD brains several different pathogens such as HSV1 DNA²²⁹, *Borrelia* DNA and antigens²³⁰, *C.pneumoniae* were found in the core of A β plaques²³¹. Although not being direct evidence for the role of A β against pathogens, clinical trials targeting A β have also provided evidence that depletion of this peptide can induce an increase in infection rates by pathogens^{232–234}.

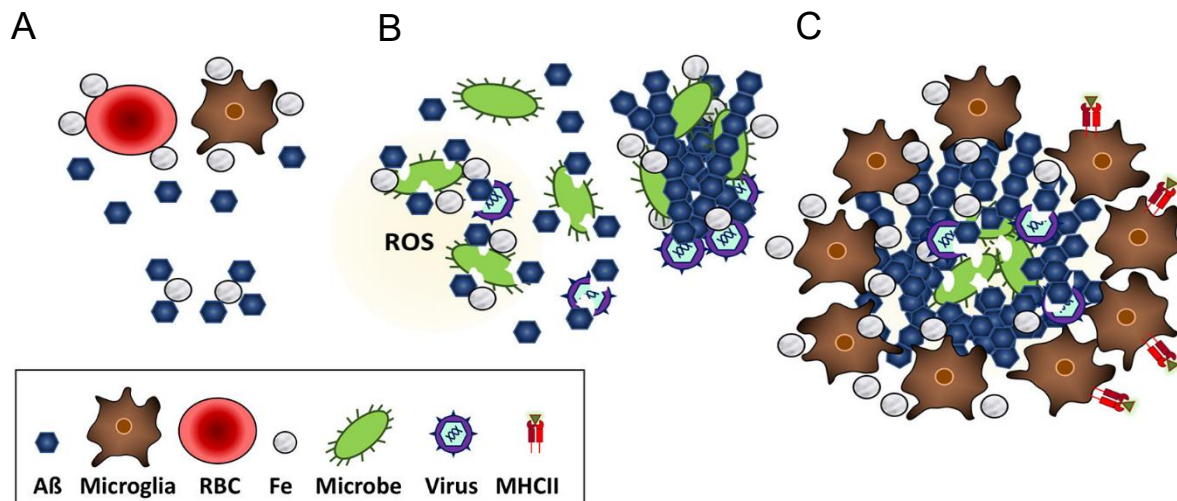


Figure 8 – The antimicrobial and chelator functions of A β against pathogens. A) A β can interact with cells enriched with ferritin and sequester metals acting as a chelator. B) A β can permeabilize, agglutinate and degrade microbes and viruses producing radical oxygen species (ROS). C) Pathogens can be found in the core of A β plaques, which are surrounded by microglia that express the major histocompatibility complex II (MHCII) that will initiate an immune response (Adapted graph from M. Brothers, L. Gosztyla R. Robinson 2018).

A second function attributed to A β has been the protection against certain forms of cancer. There is evidence of the inverse correlation between AD and cancer in humans and rodents^{235,236}. Different studies have shown that elderly patients who are diagnosed with cancer have less chances to develop AD, whereas patients diagnosed with AD are less likely to develop cancer^{237–242}. However, the protective properties of A β against cancer are still debatable given the fact that AD clinical trials do not fully support this hypothesis specially with trials targeting γ -secretase^{234,243–245}. It has also been speculated that clinical trials targeting A β might help in the proliferation of certain oncogenic viruses that can cause up to 18% of cancers²⁴⁶. The research line still remains controversial because *in vitro* studies show that different concentrations of A β can reduce the proliferation rate of certain types of cancer in mice and humans²⁴⁷. Importantly the activation of pro-survival pathways in cancer oppose to pro-death pathways activated in AD questioning the relation between A β and cancer prevention.

Another important property of A β is the relationship with the blood brain barrier (BBB). From early years of research, A β was reported to be present in blood vessels of AD patients suggesting that it might be directly involved in physiological and disease conditions^{248–250}. Indeed, A β plaques contain different proteins and peptides derived from blood and they tend to remain close to blood vessels or capillaries, thereby suggesting a link between BBB deficits and A β aggregation²⁵¹. Other studies have suggested that A β may be a sealing compound of blood vessels allowing endothelial cell adhesion²⁵². In fact, a recent study reported that as we age, the BBB is more permeable in the hippocampus of mild cognitive impaired (MCI) patients compared to age-matched controls or multiple sclerosis patients²⁵³. Again, clinical trials targeting A β with immunotherapies have been critical in testing the consistency of this hypothesis, confirming that when A β is removed brain, edemas and micro-hemorrhages become among the most frequent side effects of these trials^{254,255}. Similar results were observed when AD mouse models or aged lemur primates were treated using A β -immunotherapy²⁵⁶. Importantly, the breakdown of the BBB is known to increase A β deposition in the brain of different mice models in which the number of plaques was severely increased^{257–259}. Viewed from this perspective It is not strange that risk factors related to vascular diseases like hypertension^{260,261}, diabetes^{262,263} are also related to AD.

A β has been also related to traumatic and ischemic brain injuries, as a mechanism to recover. McKenzie *et al* in the 90s already reported that after a traumatic brain injury (TBI), APP levels elevated within 2 hours²⁶⁴ and plaques were distinguishable at 4 hours already^{265–267}. Interestingly, TBI patients with higher titers of A β correlated better with an improved neurological status of the patients^{268,269}. A common characteristic of TBI and MS patients is the accumulation of A β in damaged axonal paths, which can last over years after the injury^{270–272}. However, plaque numbers and APP expression are not necessarily increased in TBI patients compared to age and APOE matched controls^{273,274}, suggesting that for these patients the formation of A β plaques is transient. Similar results have been obtained in mouse models and rats in which a transient buildup of APP, BACE1 and A β has been observed after cortical impact^{275–277}. In addition, interesting experiments in which A β production was inhibited, by genetic deletion of BACE1 or by pharmacological inhibition of γ -secretase, impair the recovery of cortical impacts^{278,279}, stroke models^{280,281} or spinal cord injuries²⁸².

Synaptic plasticity is another key process where A β peptides are known to play a function in the regulation of synaptic activity²⁸³. In periods of neuronal activity, APP can be anterogradely transported to the synapse where it is cleaved and A β peptides released in the synaptic cleft, increasing neurotransmitter release probability of acetylcholine^{284–286}. Importantly, the duration

and concentration of A β peptide has a direct impact on neurotransmitter release, affecting memory. In rodents, A β depletion is known to reduce LTP affecting short and long-term memory that can be rescued after human A β 42 addition^{287–289}. On the other hand picomolar concentrations of human A β 42 show enhanced memory performances^{287,288}, which can be impaired with longer infusions or nanomolar concentrations²⁹⁰. Interestingly, while picomolar concentrations of A β directly activate α 7-nicotinic acetylcholine receptors, nanomolar concentrations can inactivate these receptors^{291–293}. In the absence of α 7-nicotinic acetylcholine receptors there is no effect of A β peptides in memory enhancement, suggesting a direct relation between these proteins^{291–293}. A β is also known to have adverse effects postsynaptically by disrupting the glutamate clearance of astrocytes and inducing aberrant activation of NMDAR²¹³. Finally, clinical trials based on A β reduction have provided further support in the idea that A β depletion can contribute to synaptic dysfunction by inducing adverse effects such as seizures or worsen cognitive impairments^{243–245,294}.

3. New century, new challenges: Synaptic degeneration in AD.

Synapses are the physical junctions between neurons where information is transferred. The transfer of information is a tightly regulated process initiated by the release of synaptic vesicles from the presynapse. Synaptic vesicles carry the neurotransmitters released in the synaptic cleft, which bind and activate receptors at the postsynapse to relay the information. Importantly, the molecular mechanisms triggering the release of neurotransmitters have been broadly studied, implicating a large variety of proteins critical in this precise and rapid physiological event. Early studies in the neuromuscular junction by Bois-Reymond (1818-1896) and Katz (1911-2003) discovered the relevance of action potentials (electrical discharge in the membrane of the cell) and the entry of Ca²⁺ in the presynaptic terminal to trigger the exocytosis of synaptic vesicles²⁹⁵. The molecular mechanisms of vesicle release were further characterized by the discovery of Ca²⁺ sensor proteins like synaptotagmins and the soluble NSF attachment receptor proteins (SNARE proteins: VAMP2, Syntaxin1a and SNAP25) and other synaptic proteins (Munc18-1, Munc13, RIMs, Complexins and others). These proteins undergo a cycle of assembly and disassembly essential for the docking, priming, fusion and recycling of synaptic vesicles during neurotransmission²⁹⁶ (Fig. 9). Importantly, the discovery of these proteins and its mechanisms, constituted a relevant field of research in neurodegenerative diseases such as PD, Huntington's disease or AD.

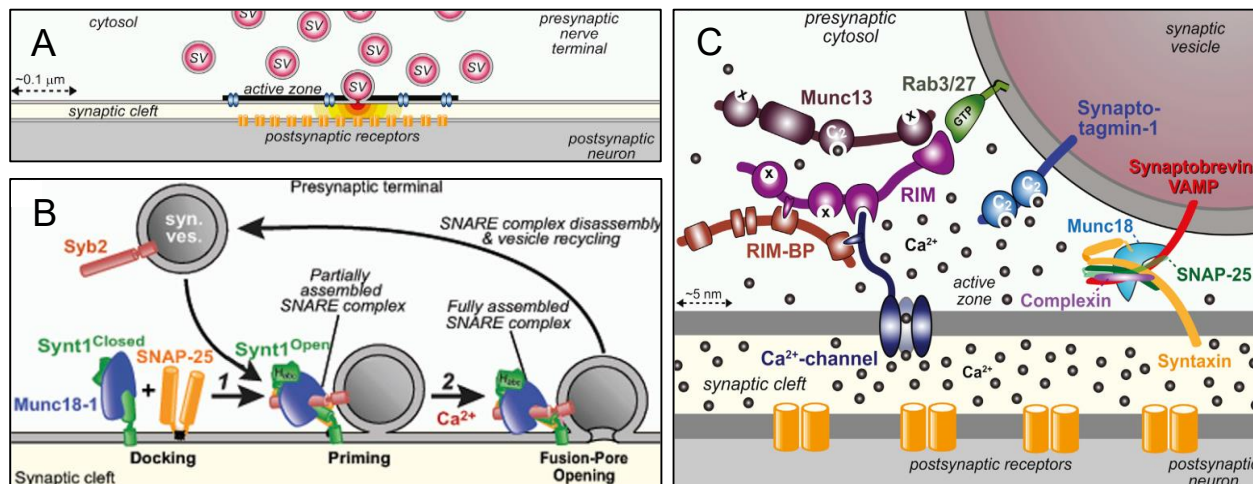


Figure 9 – The presynaptic release machinery. A) Synaptic vesicles (SV) present at the presynaptic compartment ready to fuse in the active zone, where active channels are present (blue). Neurotransmitters are released at the synaptic cleft binding postsynaptic receptors and propagating the action potential to the next neuron. B) Stages of vesicle fusion: Docking, priming and fusion and vesicle recycling. C) Schematic view of the protein components present at the release machinery in a docked vesicle. The SNARE complex is formed by VAMP2 (or synaptobrevin 2) syntaxin-1 and SNAP25. Munc18 and Complexin are fundamental in the assembly of the complex and posterior fusion. The calcium sensor Synaptotagmin 1 is present at the vesicle with its two C2 domains that bind calcium. The plasma membrane contains the protein complex formed by RIM, RIM binding protein (RIMbp), Munc13 and Calcium channels. RIM orchestrates the formation of the complex and binds to Rab proteins (3 or 27) (Sudhof 2013).

Synaptic degeneration and alteration were pointed as one of the earliest events in AD. Indeed, synaptic loss has a strong correlation with cognitive decline in AD and several studies in humans have shown it is one of the earliest events that explain the progressive symptoms observed in AD^{297–300} (Fig. 10). Indeed, the decrease in synapse number observed in MCI and AD patients indicates that synaptic degeneration occurs early^{301,302}. Studies performed in autopsies using immunohistochemistry and biochemical analysis showed that synaptic markers present in membranes and vesicles are decreased in hippocampal and cortical areas of AD patients^{299,303–307}. In addition, the loss of synapses affects glutamatergic but not GABAergic terminals in the hippocampus^{308,309}. Interestingly, comparative correlation studies of the two main hallmarks of AD with synaptic degeneration support the notion that NFT are more associated^{306,310–314} than amyloid plaques with synaptic impairments^{306,310,311,313,315–318}. Studies which investigated changes in gene expression in early AD, revealed an altered expression of proteins related to synaptic vesicle trafficking and release, neurotransmitter receptors, postsynaptic scaffolding proteins and synaptic cell adhesion molecules^{298,319–321}. Whether the decrease of synapses in AD brains is caused by lowered gene expression of proteins related to synaptic physiology will have to be further studied.

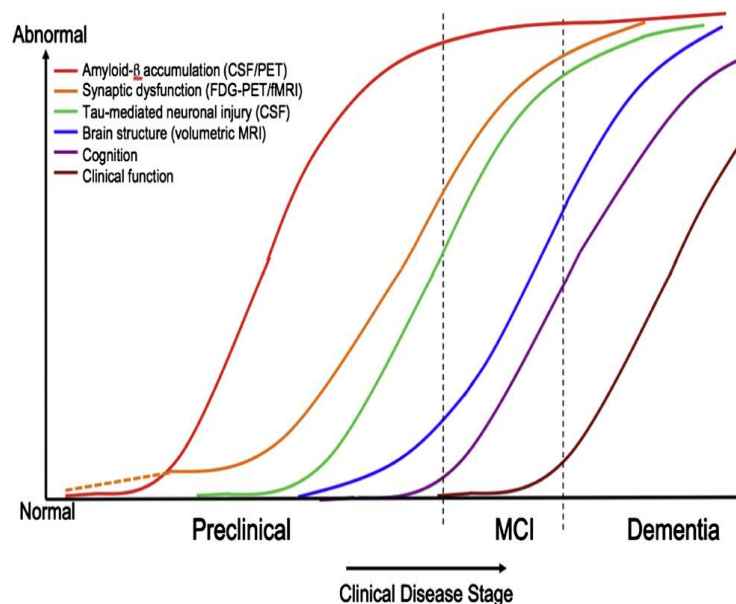


Figure 10 – Hypothetical model of AD progression in different clinical phases. This model is based on the biomarkers found at different phases of the disease. In early preclinical stages of the disease, Amyloid β can be detected together with synaptic dysfunction evidenced by a low metabolic rate of glucose, a proxy of synaptic activity in neurons (Dashed line indicates that in APOE4 carrier's synaptic dysfunction may be detected before A β deposition). As disease progresses the presence of Tau and P-Tau in CSF evidences neuronal damage and progressive changes in the brain structure due to the shrinkage. The first cognitive symptoms show when the patient enters a Mild cognitive impaired stage (MCI) also known as the prodromal stage, which will end with dementia (Sperling, Aisen et al 2011).

Disturbances in the synaptic integrity are also accompanied by changes in the organization of these proteins and the synaptic size^{297,322,323}. Therefore, despite the severe decline in synaptic population, recent studies have pointed out the rise of certain markers depending on the stage of the disease^{324–328}. In addition, the synaptic area per unit of tissue volume can be initially preserved but progressively decreased over time^{297,299,329}. All together, these results suggest that initial compensatory mechanisms might play a critical role in AD in an attempt to preserve cognition. Interestingly, synaptic compensation has also been observed in normal aging indicating that such mechanisms might be relevant in the preservation of cognitive functions^{330–333}. Whether APP or its secretases play a role in these mechanisms remains unknown.

The causes of synaptic degeneration in AD are still debated along several scenarios. One of the possible mechanisms proposed is that the initial production of toxic oligomer species of A β at the synapse induces a progressive damage pre and postsynaptically³⁰⁰. As previously described, toxic oligomer forms of A β can aggregate and induce the dysregulation of AMPA and NMDA receptors at the postsynapse. Importantly, although the concentrations of oligomeric forms of A β can be non-physiological and thus criticizable, synthetic and purified A β oligomers from tissue can damage spines and disrupt synaptic plasticity inhibiting LTP *in vitro*^{334–337} and *in vivo*³³⁸. Recent reports have also proposed that A β could interfere with the function of postsynaptic scaffolding proteins such as post-synaptic density-95 (PSD95)^{339–342}, Shank1³⁴³ or Shank3³⁴⁴. However, while several studies have reported unchanged levels of postsynaptic proteins, others have pointed at presynaptic proteins as the main disturbed proteins in synapses³⁴⁵.

The molecular mechanisms that cause presynaptic protein loss are still the object of study in AD field. Importantly, deficits in neurotransmission induced by A β have been proposed as one of the possible explanations for presynaptic impairment. A series of *in vitro* studies using different A β concentrations (50-500 nM) show that A β 40 enhances vesicle release by increasing the Ca²⁺ miniature currents and a delayed reduction of mEPSCs frequency³⁴⁶. Furthermore, other studies in which A β was incubated³⁴⁷ or overproduced due to APP overexpression, alter synaptic vesicle recycling^{348,349}. However, studies in mouse models did not show presynaptic alterations in paired-pulse facilitation when A β oligomers were incubated in *ex vivo* slices³³⁶ or in APP transgenic mice that lead to the accumulation of A β ³⁵⁰. In contradiction, other transgenic mouse models have shown an impairment in the release probability³⁵¹ but in this case with nanomolar concentrations of A β ³⁵². Some of the mechanisms proposed to explain the presynaptic impairment involve the inhibition of the calcium P/Q channels by A β ³⁴⁷ or the impaired formation of the SNARE complex by Syntaxin1a interaction³⁵³. Overall, the variability of these results indicates that the effect of A β is rather disperse depending on models, synapse type, the concentrations used and the variety of targets. Importantly, while the downstream effects of A β have been one of the main fields of study in the AD field, other explanations should equally be considered.

The complex variation observed among different synaptic proteins in AD suggests that protein abundance could rely on other factors like axonal transport. Importantly, the functional interaction between APP and Tau has been reported in several studies that exemplified how they mutually influence each other roles in axonal transport^{354–356}. Curiously, APP undergoes fast anterograde transport along the axon^{163,357}, which can be altered in AD, affecting the arrival of proteins in different cell compartments including synapses. In addition, alterations in the axonal transport are known to cause neuronal dysfunctions in *Drosophila*, where gene mutations that disrupt axonal transport affect synapse development and the levels of synaptic vesicles and proteins at the active zone^{358,359}. Therefore, the blockade of anterograde and retrograde protein transport can induce a deficit in the quantity of synaptic proteins present at the terminals and cause progressive cell death.

The identification of synaptic protein decrease in AD, significantly contributed to the detection and measure of novel biomarkers in CSF samples. Previous research used CSF samples to measure the levels of A β and P-Tau as a clinical method to diagnose AD³⁶⁰. Nevertheless, in the last decade, the identification of synaptic biomarkers in CSF has constituted a relevant field of research for the early detection and pathological changes of AD. Synaptic and axonal proteins such as neurogranin, chromogranin, neurofilament light polypeptide or the myelin basic protein

have been some of the first candidates to be followed in the progression of different neurodegenerative diseases³⁶¹. Future research will certainly increase a list, which will expand the horizons to diagnose and understand the progressive molecular changes of AD.

4. Mouse models in the AD field

The first mouse models in AD were created in the early 90s to study the physiological and pathological mechanisms of the proteins involved in the disease by mimicking the main hallmarks and symptoms. After the generation of the first KO and transgenic mouse models, the field rapidly expanded, generating a large variety of models that turned out to be valuable tools to study drugs, biomarkers and the mechanisms that trigger AD.

4.1. Models with gene ablations: constitutive vs conditional KO models

After the discovery of the main genes involved in AD, many mice invalidated for those genes were developed. These mice did not present human pathological phenotypes but they were very useful to understand the physiological roles of these proteins in the nervous system³⁶². Specific constitutive KO models for secretases like BACE³⁶³, PS (1 and 2)^{364,365}, ADAM10³⁶⁶ or APP³⁶⁷ were produced, providing important discoveries on their functions as potential therapeutic targets to treat AD. Interestingly, the deletion of some of these genes like PS1 are lethal demonstrating the importance they have in the physiology of organisms³⁶⁴.

As previously discussed (section 2.1.3.3), APP KO animals are viable and fertile with minor anatomical defects but with important behavioral, locomotor and spine density abnormalities over 12 weeks of age^{367,368}. Furthermore, the discovery of the other two members of the APP protein family (APLP1 and APLP2) and the resemblance in the peptide sequence between their domains suggested that these proteins could have functional redundancies^{103,112}. This hypothesis was confirmed when the first animal lines combining the deletion of these genes were produced, revealing that APLP2 has unique properties that are required for normal development and survival^{171,172}. To circumvent the postnatal lethality of the constitutive KOs (APP/APLP2, APLP1/APLP2 and APP/APLP1/APLP2) a new generation of conditional KO mice were produced³⁶⁹ (Table 2). Conditional KO mice are based on the Cre-Lox recombination, used to carry out insertions, deletions, translocations or inversions at specific DNA sites of cells. Therefore, by using specific cell promoters, this technology has been used to delete exon sequences of genes, depleting their expression in specific cell types³⁷⁰ (Fig. 11). This model has been frequently used to study the role of proteins relevant for the understanding of AD. In fact

conditional KO models for BACE1, ADAM10, presenilins and for the APP protein family have already been developed to assess their functions in different cell types of the brain³⁷¹.

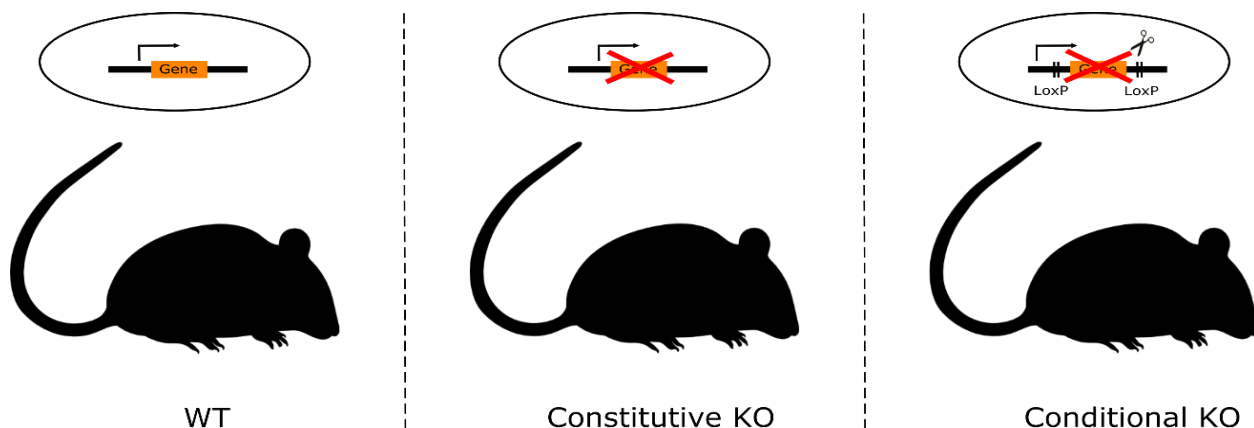


Figure 11 – Mouse models: KO model. KO models are based on the gene deletion. Constitutive KOs are born with the deletion of the gene of interest (yellow), which might be lethal or affect the normal development of the animal. Conditional KOs are normally born and the gene of interest can be deleted after development using a recombinase that can target specific cell types in the tissue of the animal.

4.2. Transgenic mouse models

A transgenic mouse model consists of a mouse whose genome has been altered by inserting one or multiple transgenes through the use of genetic engineering (Fig. 12). The clinical similarities between FAD and SAD pathologies has rationalized the use of mouse models genetically engineered with familial mutations in the APP or presenilin genes to mimic AD. Importantly, these mouse models can reproduce the main hallmarks observed in AD but do not recapitulate all the features present in human patients³⁷².

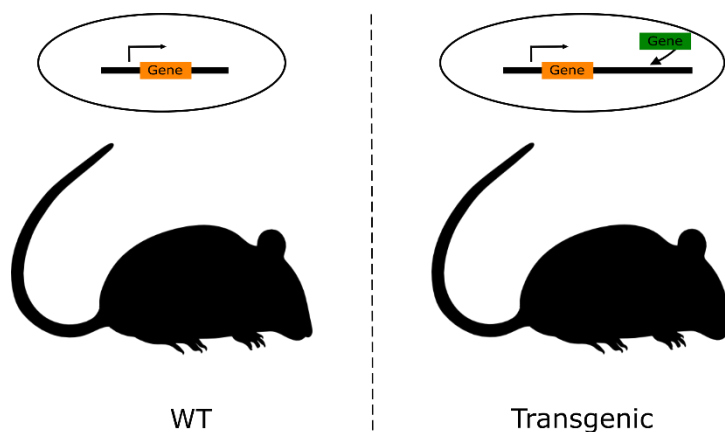


Figure 12 – Mouse models: Transgenic model. Transgenic mouse models are based on the random insertion of a gene (green) in the genome of the animal (yellow). Consequently, the animal will overexpress the protein of interest.

The initial AD transgenic models for APP overexpressed different constructs of APP (APP695 or APP770) with or without FAD mutations using different promoters (Thy1, prion protein or PrP). Some of the earliest models developed were the PDAPP³⁷³, APP23³⁷⁴, J20³⁷⁵, Tg2576³⁷⁶ and

TgCRND8³⁷⁷, which exhibited A β plaques and cognitive dysfunction without developing NFT. Similar to APP, mutations present in the PS genes were also used to develop PS1 and PS2 transgenic mouse lines with one or several mutations to potentiate the pathological effects. Importantly most familial mutations are found in the PS genes but these mutations alone did not induce A β deposition in mouse models. However, models combining APP and PS1 mutations showed their ability to accentuate plaque pathology, causing severe effects such as behavioral deficits, inflammatory response and sometimes loss of synaptic markers in neurons but without NFT. The Swedish mutation³⁷⁸ is the most frequent APP mutation used in combination with other PS mutations. Among the most common models are the Tg2576 and PS1M146L Tg³⁷⁹, APPKM670/671NL Tg and PSA246E Tg^{44,46}, APP751KM670/671NL-V717I Tg and PSM146L Tg³⁸⁰, APP KM670/671NL-V717I and PSEN1M233T/L235P KI³⁸¹ or the APP KM670/671NL and PS1DExon9³⁸².

The APP KM670/671NL and PS1DExon9 has been a frequent double transgenic model used in AD research. The model was created by breeding mice that expressed the APP 695 isoform with the Swedish mutation and the S-9 mice line expressing human PS1 lacking exon 9 (Fig. 13). The double transgenic are viable and fertile developing plaques at 3 months, having a much higher amyloid burden compared to the single APP KM670/671NL mutant³⁸³ at similar age. A progressive cognitive impairment occurs after amyloid plaque deposition turning more severe over time³⁸².

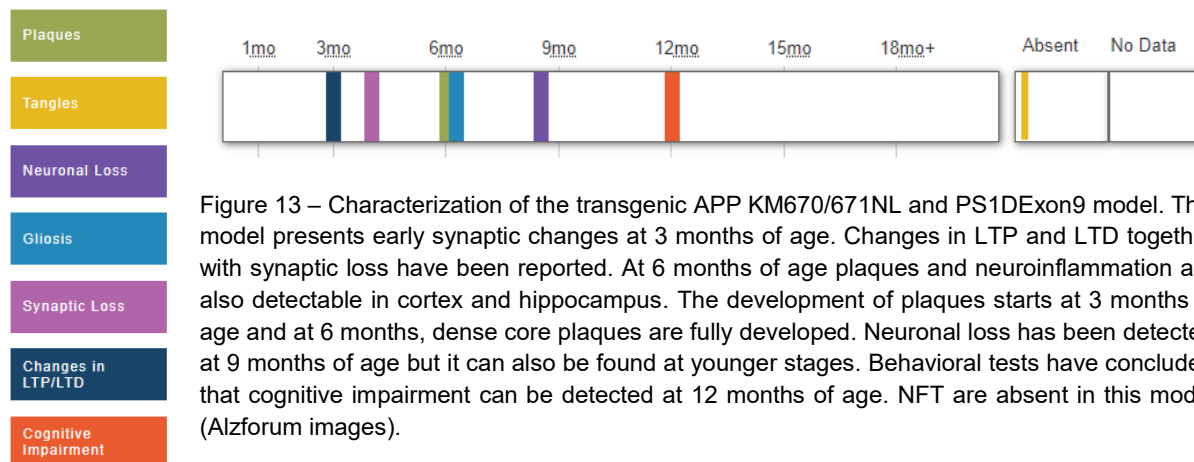


Figure 13 – Characterization of the transgenic APP KM670/671NL and PS1DExon9 model. This model presents early synaptic changes at 3 months of age. Changes in LTP and LTD together with synaptic loss have been reported. At 6 months of age plaques and neuroinflammation are also detectable in cortex and hippocampus. The development of plaques starts at 3 months of age and at 6 months, dense core plaques are fully developed. Neuronal loss has been detected at 9 months of age but it can also be found at younger stages. Behavioral tests have concluded that cognitive impairment can be detected at 12 months of age. NFT are absent in this model (Alzforum images).

APP and PS1 transgenic mouse models were also backcrossed with mutants of Tau models in an effort to better replicate AD pathology with plaques and tangles^{384–386}. In general, these triple transgenic mouse models exhibit a similar pathology to AD patients but the overexpression of multiple genes can make the interpretation of the data rather difficult by causing artificial phenomena and by preventing single genotype-phenotype correlations. More interesting, are the

findings in which transgenic APP mouse models were backcrossed with Tau KO mouse, shedding some light in the relationship of these two proteins. Interestingly, these model demonstrated that cognitive deficits associated with APP mutations are prevented in a Tau KO, which had no effect on the deposition of A β or other APP fragments³⁸⁷. Furthermore, the lack of NFT observed in mouse models overexpressing A β can be linked to endogenous differences between mouse and human Tau. One study showed that the crossing of APP E693 Δ -Tg model with a normal human Tau resulted in the formation of NFT³⁸⁸. Thus, endogenous Tau is necessary for APP and A β pathological effects in cognition, LTP or the hyperexcitability of neurons^{387,389,390}.

Many of the models described above were used as preclinical models to evaluate therapies targeting A β . Subsequently, these models were used to screen the effect of BACE1 and γ -secretase complex inhibition to reduce A β levels in the brain^{391–393}. Other approaches were the immunotherapies directly targeted against A β plaques³⁹⁴. Even though these therapies were successful to reduce A β levels and reverse cognitive deficits in transgenic models, they abruptly failed in phase III clinical trials with humans³⁹⁵.

Many of the criticisms received by the first generation of transgenic mouse models came from the failure of A β therapies in clinical trials. Nevertheless, these models faithfully reproduced some of the main features of the disease and also the side effects observed in clinical trials. However, one of the main concerns in these models were the overexpression of APP together with its fragments and A β ³⁷². Consequently, any of the phenotypes observed were technically hard to assess due to difficulties in distinguishing the effects of the different APP fragments. Apart from the APP overexpression, each of the model's phenotypes are difficult to compare due to the differences in the genetic constructions, promoters and mouse strains^{396,397}. In addition, the transgene is randomly inserted in the genome, making genetic disruption possible. Taken together, these limitations as well as the intrinsic differences between species could explain some of the differences observed between experimental results and clinical trials.

4.3. KI mouse models

The drawbacks driven by APP overexpression made necessary the creation of new models overcoming this issue. Therefore, a new generation of mouse models based on the knock-in technology were produced. This technology consists of a one-for-one substitution or insertion of a DNA sequence into a specific genetic locus (Fig. 14).

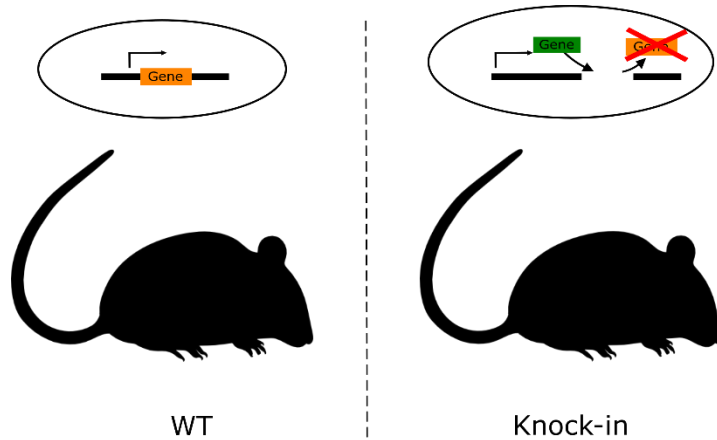


Figure 14 – Mouse models: KI model. KI mouse models are based on the substitution of one gene for another. Therefore, the gene of interest (yellow) is deleted and substituted by another modified gene (green). The animals are born without overexpressing the protein of interest.

Consequently, the study of APP and its secretases without overexpression made possible a better physiological characterization of each of these proteins. Furthermore, the progressive discovery of new gene mutations present in the APP gene such as Swedish, Iberian or arctic led to the development of new KI models with one or several mutations that induced an increase of the A β 42/A β 40 ratio without APP overexpression^{398,399}. Importantly, mutations like the Swedish are capable of increasing the levels of APP fragments like the β -CTF without having major cognitive effects^{398,399}. Similarly to transgenic mouse models, APP-KI like the NL-F (APP KM670/671NL Swedish and I716F Iberian mutations) or the NL-G-F (APP KM670/671NL Swedish, APP E693G arctic and APP I716F Iberian mutations) mouse models develop pathological traits like A β deposition in cortex and hippocampus, neuroinflammation or synaptic alterations^{398,399}. In addition, memory and learning dysfunctions are also developed but at later stages (18 months for the NL-F and 6 months NL-G-F) (Fig. 15). Double KI mouse models combining APP and PS1 have also been produced showing a more aggressive A β pathology compared to the single KI but less aggressive than the transgenic³⁷².

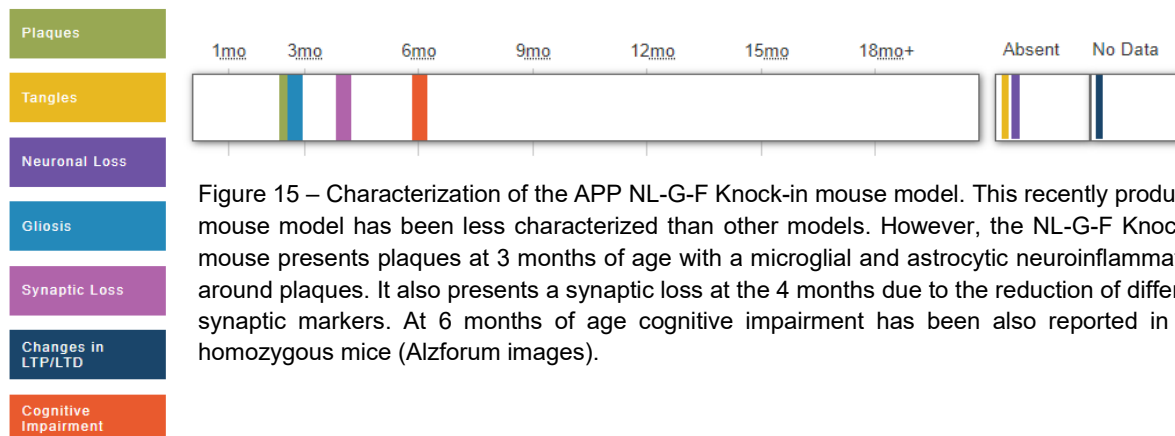


Figure 15 – Characterization of the APP NL-G-F Knock-in mouse model. This recently produced mouse model has been less characterized than other models. However, the NL-G-F Knock-in mouse presents plaques at 3 months of age with a microglial and astrocytic neuroinflammation around plaques. It also presents a synaptic loss at the 4 months due to the reduction of different synaptic markers. At 6 months of age cognitive impairment has been also reported in the homozygous mice (Alzforum images).

Like other transgenic models KI mouse models do not exhibit tau pathology, suggesting that impairments in the processing of APP and A β deposition can induce cognitive dysfunction in AD. However, cognitive deficits can also be triggered by Tau dysfunction as demonstrated by the irreversible neurodegeneration and cognitive dysfunction observed in Taupathies⁴⁰⁰. Therefore KI mouse models represent a mild preclinical AD phase with other limitations such as less severe behavioral phenotypes or non-neuronal loss³⁷². Nevertheless, the combination of first and second generation mouse models has been critical in differentiating some of the clinically relevant mechanisms present in AD from overexpression artifacts. Extensive research with a new generation of models will be necessary to validate new and old results. Consequently, the inclusion and combination of new mouse models with humanized genes (Tau, APOE and APP) together with pluripotent stem cells^{401,402,402} and non-human primate models for AD⁴⁰³ will be crucial to have models that replicate the disease features.

5. The hippocampus

5.1. Anatomy

Due to its relation with memory and learning, a brain structure with particular interest in AD has been the hippocampus. In the 60s and 70s, several psychology studies identified the hippocampus as a severely affected area in AD, which would turn this anatomical region into the target of many studies. In mammals, the hippocampus is a highly organized brain structure located at the limbic system, which is involved in diverse functions such as memory, learning, motivation and emotions. In the hippocampus, two different regions folded into one another can be distinguished: the dentate gyrus (DG) and the *Cornu Ammonis* (CA)⁴⁰⁴.

The DG is a narrow concave region lamina that envelopes the CA4 region in humans, better known as the hilus in mice. This structure consists of three well defined regions known as the molecular layer (ML), the granule cell layer (GCL) and the polymorphic layer. The ML can be divided in the outer ML that is the external two thirds and receives axons from the perforant path and the inner ML, which is in contact with the GCL and receives fibers from the commissural and septal nuclei. The polymorphic layer fuses the GCL with the CA4 and it is crossed by mossy fiber (MF) axonal projections that travel from GCL to the CA3⁴⁰⁴ (Fig. 16 B).

The CA region has a heterogeneous distribution of areas divided in four different fields named from CA4 to CA1. The CA4 is a large region formed by scattered large cells present within the concavity of the hippocampus⁴⁰⁴. Forming the concavity of the DG there are the CA3 cells, whose dendrites project to the *stratum lucidum*, a region where the synaptic input coming from the giant

MF synapses connect the GCL to the CA3 cells. Then, the CA2 region is composed of a densely packed group of pyramidal cells, whose existence has been debated in several models but they have been clearly observed in mice and primates. Finally, the CA1 is one of the largest regions formed by small pyramidal scattered cells, which belong to the *stratum pyramidale* and can be divided between the *stratum profundum* and the *stratum superficiale*. The end of CA1 marks the beginning of the *subiculum*, a region which is one of the most important outputs of the hippocampus.

From the hippocampal sulcus, the CA regions can be divided in six different layers: the molecular zone that contains *lacunosum moleculare*, the *stratum lacunosum*, the *stratum radiatum*, the *stratum pyramidale*, the *stratum oriens* and finally, the *alveus* (Fig. 16 B).

- stratum lacunosum moleculare: this layer is adjacent to the hippocampal sulcus for half of the hippocampus and it contains few interneurons and the extremity of the apical dendrites from the pyramidal neurons that are connected with perforant fibers.
- stratum lacunosum: this layer contains axons that are formed by perforant fibers and Schaffer collaterals.
- stratum radiatum: this region consists of apical dendrites from the pyramidal neurons that connect with Schaffer collaterals, fibers from septal nuclei and commissural fibers.
- stratum pyramidale: it is the region containing the pyramidal neurons of the CA, which have its base with axons facing the alveus and the apex with dendrites facing the hippocampal sulcus. Some of the pyramidal neurons also project dendrites to the stratum oriens. Pyramidal neurons can either project to the septal nucleus or to other pyramidal neurons forming association fibers called Schaffer collaterals that curve back reaching the stratum radiatum (CA3-CA1).
- stratum oriens: this poorly defined layer is on top of the pyramidal cell layer, and therefore it is crossed by axons of the pyramidal neurons that reach the alveus. Other cell types like basket cells can also be found here.
- alveus: the alveus is the most external layer, covering the intraventricular surface that contains efferent axons of the hippocampal and subicular neurons but also afferent axons from the septum. The efferent axonal projections enter the fimbriae that will form the body of the fornix that is one of the major outputs of the hippocampus.

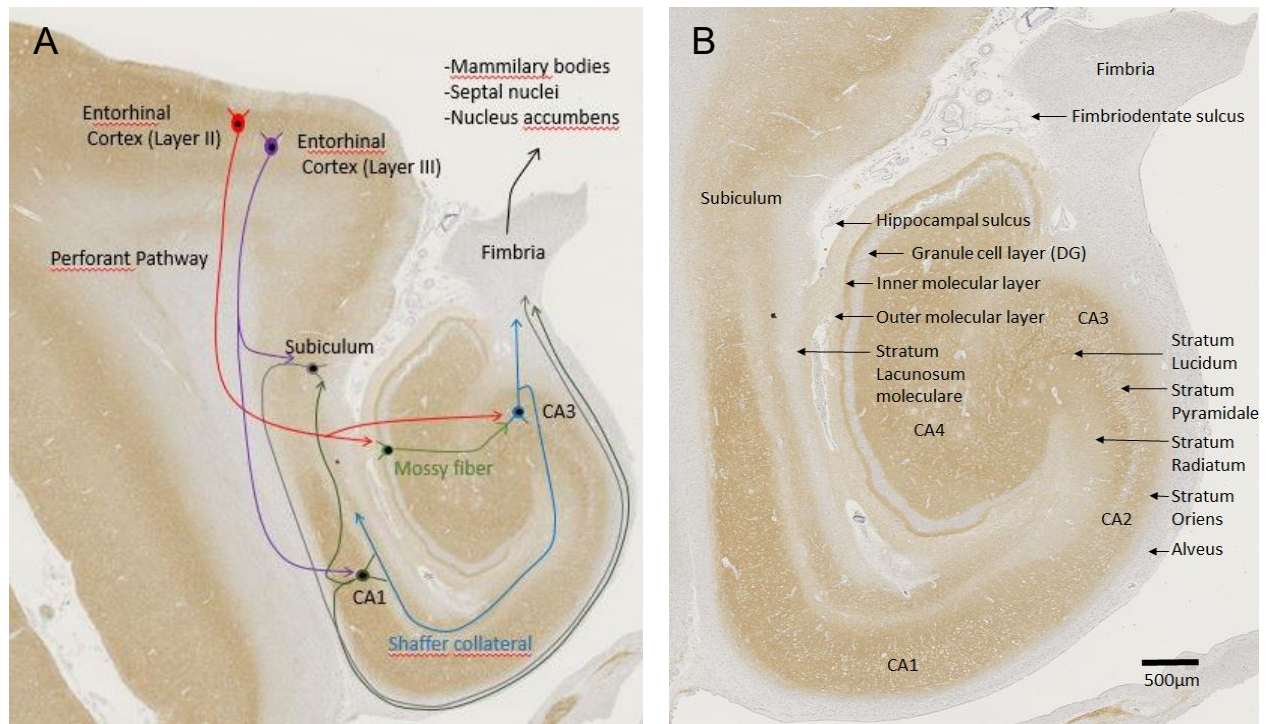


Figure 16 – Anatomy of the human hippocampus. A) Schematic overview of the synaptic circuit connections in the hippocampus. B) Anatomical regions and layers present in the hippocampus.

5.2. Functional plasticity of the MF-CA3 synapses in the hippocampus

The MF input to the CA3 cells is part of the trisynaptic circuit formed by the EC projections to the GCL, from which MF axonal projections are sent to CA3 cells, which send Schaffer collaterals to the CA1 region. The flow of information is critically encoded by the GCL, which receives strong input from layer II of the EC and gives output to the CA3 cell region (Fig. 16 A, 17A). Importantly the unmyelinated MFs cross the hilus entering the CA3 region and forming synapses at the stratum lucidum in parallel to the pyramidal cell layer^{404,405}. These glutamatergic synapses are known as the giant MF boutons (3-10µm in diameter), which act as high-pass filters that allow the encoding of information into inhibitory neurons like basket cells and excitatory neurons like CA3 pyramidal cells⁴⁰⁵. Each pyramidal CA3 neuron gets around 50 different MF inputs into large dendritic spines called Thorny excrescences⁴⁰⁷, located in the proximal apical dendrites⁴⁰⁶ (Fig. 17 A). In addition, MF axonal bundles contain *en-passant* boutons that can innervate local inhibitory interneurons present in the *hilus* and in the *stratum radiatum* respectively, which provide feedback inhibition at the granule or pyramidal cell layers^{408,409}. Additionally, *en-passant* boutons can also excite hilar mossy cells, which provide feedback excitation to the GCL⁴⁰⁸. All together, these physiological mechanisms constitute an efficient system to tune and modulate the

recruitment of small neuronal memory ensembles at the CA3 region during learning and memory⁴¹⁰.

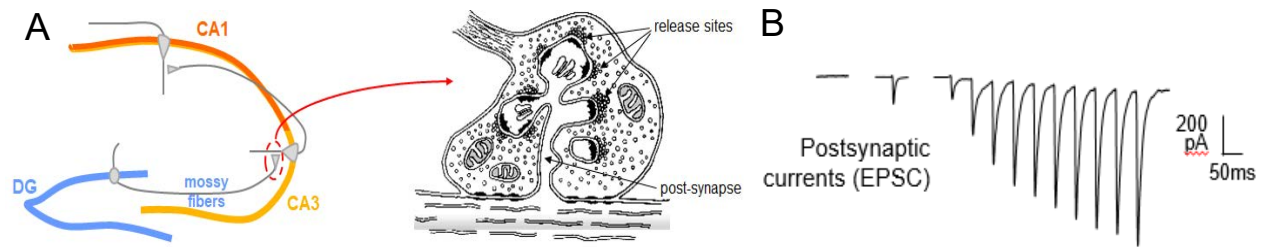


Figure 17 – The MF-CA3 synapse. A) Schematic illustration of the MF-CA3 giant synapse bouton in mouse hippocampus B) Postsynaptic currents illustrating the short-term plasticity changes in the MF-CA3 synapses.

The unique morphological features of MF boutons partly explain the synaptic properties of these synapses. MF boutons have around 18 to 45 distinct active zones⁴¹¹, where synaptic vesicles, neuropeptides⁴¹², adenosine⁴¹³ or Zn^{2+} ions⁴¹⁴ are released. In addition, from a total of 25000 vesicles present in the bouton, between 1400 and 5700 are present in the releasable pool⁴¹⁵. Thus, even though these synapses are characterized by a low release probability (0,01-0,05)⁴¹⁶, the postsynaptic potential may show a high amplitude (30mV) because of the activation of numerous active zones in a single MF bouton^{417,418}. Indeed, crosstalk of calcium signals between different active zones, which stimulate synaptic vesicle release, occurs under conditions of short-term plasticity at MF-CA3 synapses. Such conditions consist in the increase of the frequency of action potentials in a short time period (a burst), which increases the residual calcium and elevates the release probability, driving frequency facilitation^{417,419} (Fig. 17 B). Interestingly, a single MF axon makes ten times more synapses in interneurons than pyramidal cells, which causes that under low-frequency transmission, disynaptic feed-forward inhibition blocks monosynaptic excitation onto CA3 pyramidal cells. However, under high-frequency facilitation, excitation by MF boutons dominate feedforward inhibition and activate CA3 pyramidal cells^{420,421}. These synaptic properties permit to change and control the input-output information, always dependent on the spiking frequency coming from the GCL. Although the firing rate of granule cells is low, under learning conditions, these cells can discharge high frequency spike bursts that can induce LTP *in vivo* and *in vitro*^{422–424}. These synaptic changes are frequently accompanied by substantial structural changes in the number of synapses but also in the remodeling of the circuitry and ultrastructure of synaptic boutons⁴⁰⁵.

6. Past, present and future research of the human brain to understand AD

When looking at the research history in human brain, anatomical studies by ancient Greeks and Egyptians constituted one of the initial approaches to understand the complexity of the nervous

system. Over the centuries until our days many anatomists provided useful and precise illustrations of the nervous system, which would constitute the first milestone in the history of neuroscience. In the XVIII and XIX centuries, the development of optics, electricity and chemical methods were a breakthrough in the characterization of cellular and functional properties of the nervous system. In addition, the advent of psychology at the end of the XIX century was also relevant for the development of clinical tests that would help in evaluating the cognitive functions of patients with different diseases. However, posterior to the patient death autopsies and histopathological studies have been frequently used to complete the diagnosis of the disease.

In the XIX and XX centuries, the contributions made by Nissl, Otto Deiters, Golgi or Ramon y Cajal among others were extremely important in the characterization of different neuronal cell types and marked the beginning of modern neuroscience. These methods inspired the development of other techniques such as immunolabeling conjugated to benzidin tetraedro, which was pioneered in 1934 by J.R. Marrack who discovered that antibodies could be specifically used to localize antigens⁴²⁶. However, the low sensitivity of this method under optical microscopy pushed the development of techniques with the same principle but with a different approach. Thus, in 1942 *in vitro* and *in vivo* studies using fluorescent antibodies would turn into a reality, transforming the field of bioimaging. After 1960, the development of immunohistochemical methods with enzyme-conjugated antibodies turned out to be very useful because it was possible to see the reactions through optical microscopy, facilitating the use and spread of the technique to researchers and pathologists⁴²⁷. In AD the use of dyes like Congo Red (1922) and Thioflavin (1959) were commonly used to stain the main hallmarks of the disease⁴²⁸. In addition, the use of antigen retrieval methods facilitated the labelling of epitopes in fresh and fixed human samples that further increased the use of immunohistochemistry for diagnostic and publishing purposes on AD⁴²⁷. Hence, the development of immunohistochemistry helped in the qualitative characterization of AD related proteins and hallmarks in different regions of the human brain.

Advances in DNA sequencing provided valuable insights in the causative and risk genetic variants of AD. Initially, some of the causative genes were discovered by performing genetic linkage studies in families, which were very useful for single gene disorders like FAD. Nevertheless, for the more complex sporadic form of the disease, genome wide association studies (GWAS) constituted an alternative that used large numbers of data based on human individuals. GWAS studies associated specific traits or diseases to single nucleotide polymorphisms (SNPs)⁴²⁹. Interestingly, besides the APP or PS genes, other genes related to cholesterol and lipid metabolism, immune response, endocytosis or Tau metabolism have also been linked to the risk

of developing AD⁴³⁰. A clear example was the detection and confirmation of the type $\epsilon 4$ allele of the apolipoprotein E (APOE4), as the most prominent risk variant in AD⁴³¹. This gene identified in chromosome 19 was coding for a cholesterol carrier that could confer a three to eight-fold increased risk factor depending if one or two copies of the gene are present respectively. Therefore, GWAS studies not just confirmed the results found in FAD cases but also expanded the knowledge of new genes and potential pathways involved in the disease.

Over the history, ethical concerns became stricter and non-invasive techniques turned into a reality that changed the treatment and diagnosis of neurological diseases like AD. Importantly, the XX century was marked by the development of non-invasive techniques such as electroencephalogram (EEG), transcranial magnetic stimulation (TMS), magnetic resonance imaging (MRI) or PET (positron emission tomography), which constituted a revolution in the understanding of the entire human brain. In addition, other more invasive methods based on the detection of AD biomarkers in blood and CSF samples are also clinically relevant to understand the progressive changes of the disease in human patients⁴³². Induced pluripotent stem cells (IPSCs) have been another alternative human model to reproduce human genetics and cellular mechanisms in AD. Derived cells from AD patients can constitute an easy strategy to test the effects of drugs and mutations in 2D or 3D cell culture systems of neurons, microglia or astrocytes⁴³³. Furthermore, although IPSCs constitute an alternative interesting model, it has several disadvantages such as the genetic and epigenetic variability between different patient lines or the use of pluripotent cellular properties in aging diseases.

Research on the human brain also benefited from the advances made in imaging and analytical techniques. After the invention of light microscopy, the development of electron (1931) and confocal (1967) microscopy was a notarial step forward to visualize at a detailed level cellular structures in human tissue. Interestingly the use of 2D imaging in human specimens started to provide interesting qualitative and quantitative results in the AD field. However, the limited resolution of optic microscopy imposed by the diffraction limit of light (200nm) determined by Ernst Abbe⁴³⁴, pushed the development of super-resolution techniques. These techniques known as STED, STORM and PALM allowed the acquisition of images with higher resolutions (10nm) than those imposed by the diffraction limit of light⁴³⁵. Therefore, higher resolution facilitated the study and visualization of small structures like synapses in cell cultures and slices. In addition, the development of more recent techniques such as array tomography allows the combination of optical fluorescence and electron microscopy in ultrathin cryosections in human brain slices⁴³⁶. Importantly, this method provides quantitative data in high-resolution images that are imaged

volumetrically, improving spatial resolution specially in the z-stack. In fact different antigens can be labeled allowing the localization of proteins at the ultrastructural level and therefore a better precision to define the molecular architecture in 3D samples of AD patients⁴³⁷.

The progress in imaging techniques has been accompanied by new generation methods that provide chemical and physical properties to tissues, allowing the 3D imaging of entire specimens. Among these methods CLARITY, a technique based on tissue clearing to visualize intact biological samples is one of the most prominent examples⁴³⁸. This technique transforms biological tissues into a transparent hydrogel-tissue hybrid by removing lipid layers and preserving molecular and structural information. Importantly, the use of this technique to study the brain in its entire 3D complexity will certainly be a breakthrough to study neuronal circuits and the distribution of gene and protein expression. In addition, in the last years other tissue clearing techniques such as CUBIC or DISCO have been developed suggesting an increased interest for tissue clearing techniques⁴³⁹.

The complexity of the brain will also require the use of techniques that combine 3D imaging with good resolution, which will help in the mapping of the entire brain connectome. Interestingly, the development of methods such as expansion microscopy based on the physical expansion of the tissue are also raising interest⁴⁴⁰. One of the main reasons is because expanded samples are clarified and can be imaged at the nanoscale resolution using conventional microscopes for any type of tissue including human samples. A recently developed technique known as magnified analysis of the proteome (MAP) can combine the expansion of entire specimens with the proteome preservation and the 3D imaging⁴⁴¹. It is relevant to underline that the imaging of large scale volumes in 3D requires the use of microscopes such as the light sheet microscopes, which are ideal to image large 3D samples in high resolution and in a short time period⁴⁴². The storage and analysis of the data produced by these samples is another big challenge that automatized analytical programs based on AI will face. Therefore, the fast advances in tissue engineering together with new imaging methods and analytical tools will provide a new approach to map gene and protein expression as well as the brain connectome at the synapse level in the entire human brain. The discoveries in this research field will certainly be a breakthrough not just to study neurodegenerative diseases but also to understand the human brain as an entire entity, which will be used in other fields of science to change the society we live in.

MATERIALS AND METHODS

The materials and methods used to obtain the results of this thesis can be grouped in three different sections. One section concerning the data obtained in mouse models, a second section about the data obtained in human brain tissue and a third section about the image acquisitions and statistical analysis used. For each of the models used specific methodology and protocols were used.

Section 1 – Mouse

Mouse models

For the studies performed in this thesis, several different mouse lines were used:

-PS1 and PS2 KO mice

This mouse model consisted of a single or combined KO for PS1 and PS2 genes. The exon number 7 of PS1 gene was flanked by loxP sites allowing its deletion by the Cre recombinase. PS2 mouse model was a constitutive KO disrupting exon 5 by the introduction of a frame shift between exon 4 and 6. By crossing these two mouse lines a new model of PS1fl/PS2KO was created and used in our studies.

-APP and APLP2 KO mice

This model consisted of a single or combined KO for APP and APLP2 genes. Both genes were flanked independently with 3 lox p sites present between exon1 and the neoR cassette present 1.1kb upstream of the start codon (ATG)³⁶⁹.

-APP and PS1 KO mice

This model was obtained by crossing the two single KO mouse lines for APP and PS1, obtaining a double conditional KO for these genes.

-APP/PS1 transgenic mice

This model consisted of a heterozygous APP/PS1 mice coexpressing human APP Swedish mutation (KM670/671NL) and PS1ΔExon9³⁸². Mice were obtained from crossing heterozygous female transgenic mice with B6C3F1/J male mice. Only female mice were used in our studies.

-APP NL-G-F KI mice

This model consisted of a KI mice model expressing the human Swedish (KM670/671NL), Iberian (I716F) and Artic (E693G) mutations³⁹⁸. Slices of 5 μm came from 12 month old females, which

were fixed in PFA 4 % and mounted with paraffin.

Mouse immunohistochemistry

In order to perform immunohistochemistry mice were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg body weight). Animals were then perfused intracardially with 0.9 % NaCl for 1 min and then with 4 % paraformaldehyde in 0.1 mM PBS (PFA 4 %) for 4 min. Brains were then dissected and post-fixed for 8 h in PFA 4 %. Coronal slices were cut by using a vibratome (Leica VT1200S) and permeabilized in PBS 0.3 % Triton X-100 (Sigma Aldrich - #9002-93-1) at RT for 2 hours. Brain slices were then incubated overnight at 4°C with the primary antibody diluted in PBS-T. In addition, for some experiments slices were also incubated at RT for 5 minutes with an autofluorescence quencher (TrueBlack® Lipofuscin Autofluorescence Quencher - Biotium #23007). After 3 washes in PBS 1X, slices were incubated at RT with secondary antibodies (Thermo fisher scientific - Invitrogen) for 2 hours. Finally, as a penultimate step, slices were incubated in Methoxy-X04 or in DAPI for 10 minutes before getting mounted in Fluoromount™.

Electron microscopy

Electron microscopy was performed on samples from the cortex of APP/PS1 animals to visualize the structural content of certain structures. To this aim, mice were anesthetized injecting intraperitoneal urethane. After 5 minutes, mice were perfused intracardially with 35°C oxygenated (saturating carbogen 95% O₂ and 5% CO₂) ACSF 1X (in mM: 120 NaCl, 26 NaHCO₃, 2.5 KCl, 16.5 glucose, 2.8 pyruvic acid, 0.5 ascorbic acid, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, pH 7.4, osmolality of 305 mOsm) for 4 minutes. Afterwards 2.5 % paraformaldehyde in 0.1 % glutaraldehyde was perfused intracardially for 10 minutes until the animal fixation. Brains were dissected, post-fixed in PFA for 8 hours and sliced coronally at 250 µm. Sections were permeabilized using sodium borohydride 1% (Sigma Aldrich #452882) at RT for 30 minutes. Slices were then stained with DAB for APP Cter and Nter or Aβ 1-16.

Brain sections were post-fixed at RT in 1 % osmium tetroxide in the dark. Slices were then dehydrated in an ascending series of ethanol dilutions from 50 to 100 % for 10 minutes each. Furthermore, slices were lifted into a 50 % mix (vol.) of Epon 812 resin (Electron Microscopy Sciences) and pure ethanol for 2h at RT and finally in pure resin overnight. After another bath in pure resin for 5 hours, slices were finally embedded between 2 sheets of aclar, and polymerized at 60°C for 48 hours.

Regions of interest colored with toluidine blue were cut at 500 nm thick sections by using an

ultramicrotome and a Diatome diamond knife (Leica EM UC7). Sections of 70 nm thick were then collected on copper grids (Electron Microscopy Sciences) and observed with a transmission electron microscope Hitachi H7650 equipped with a Gatan Orius CCD camera.

Electrophysiology

For the slice preparation, animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg). After mice were anesthetized, they were perfused transcardially with ice cold artificial cerebrospinal fluid (aCSF (in mM): 120 NaCl, 50 Sucrose, 1.25 NaH₂PO₄, 27 NaHCO₃, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 2.8 pyruvic acid, 16.5 glucose, 0.5 ascorbic acid, pH 7.3 adjusted by saturating with carbogen (95% O₂ and 5% CO₂), and an osmolarity of 305 mOsm) for 1 min. After perfusion the brain was quickly dissected and removed from the skull in the cold aCSF solution. Immediately after, fresh parasagittal slices of 250 µm from both hemispheres were cut with vibratome (VT 1200S, Leica Microsystems, Nussloch, Germany) and gently deposited for 20 min in a chamber filled with aCSF at 33 °C (without sucrose). The aCSF together with slices were constantly oxygenated with carbogen (95% O₂, 5% CO₂) even during the recording of cells. Whole-cell patch clamp recordings were used to record CA3 neurons in the hippocampus at RT. The set-up was equipped with a contrast microscope (Eclipse FN-1, Nikon, Champigny sur Marne, France), and a camera (CoolSNAP EZ, Roper, Evry, France). For these experiments voltage-clamp recordings were performed using a patch electrode (4 ± 0.5 MΩ) filled with an internal solution ((in mM): 125 CsCH₃SO₃, 2 MgCl₂, 5 phosphocreatine, 4 NaCl, 10 EGTA, 4 Na₂ATP, 10 HEPES pH 7.3 adjusted with CsOH, and an osmolarity of 300 mOsm). Furthermore, Bicuculline (10 µM) together with CGP was added in the aCSF going through the recording chamber to inhibit GABA-A and GABA-B receptors. By using optogenetics, MF-CA3 excitatory postsynaptic currents (EPSCs) were evoked by light pulses of 1 ms at the ML of the DG. Membrane potential was kept constant at -70 mV and the baseline stimulation frequency for all experiments was 0.1 Hz. Signals were amplified using a HEKA EPC10 amplifier (Lambrecht, Germany), filtered at 3.3 kHz and digitized at 10 kHz via PatchMaster software (Lambrecht, Germany). Data were analyzed using IGOR PRO 6.3 (Wavemetrics, Lake Oswego, OR).

Lentiviral vectors and cloning

All the lentiviruses used in the projects were produced by the viral vector facility of Bordeaux "TransBioMed". The developed lentiviruses were based on the pRRLsin-PGK-MCS-WPRE plasmid, in which the PGK promoter was exchanged with the C1q12 minimal promoter. After the promoter was inserted in the lentiviral backbone cDNA sequences of interest were inserted in the

5'–3' order: ChIEFtom, 2A peptide from porcine teschovirus-147 and the Cre-recombinase or the eGFP. Two different Cre-dependent constructs were also produced to allow conditional expression of ChIEFtom and other proteins. Behind C1ql2 promoter, a Lox-Stop-Lox cassette was inserted followed by the cDNA of ChIEFtom and another protein of interest. The second strategy was based on a double-inverted open-reading frame (DIO - two doublets of lox sequences (loxP and lox 2722)), which flanked an inverted sequence cassette of ChIEFtom, 2A peptide and Syt7. Therefore, the expression of the desired proteins would be dependent on Cre-recombinase, which would cut, flip and reinsert the sequence, allowing the expression of the viral sequence.

Mice stereotaxic injection and surgery

3 to 4 week old mice were used to perform stereotaxic injections. Animals were anesthetized using isoflurane inhalation and injected with intraperitoneal buprenorphine to prevent post-surgery pain. One μL viral injections (5×10^6 particles) were performed in head bar fixed animals using a micropump and a syringe (Nanofil WPI) at a rate of 100 nl/min in the DG (Y: 2.2mm from lambda; X: ± 2.0 mm from sagittal suture; Z: 2.0 mm from the skull). After adding stitches, animals were injected with rydamil, a second pain killer with a prolonged effect over time. Animals were fully recovered and ready to be used 3 weeks after surgery.

Image analysis

Images of focal planes in the region of the MF were acquired in red (ChIEFtom) and far-red (synaptotagmin1, synaptobrevin2, synaptotagmin7, syntaxin1) channels. For every acquisition all settings were always fixed (laser power, gain, offset). Images were analyzed using ImageJ program. A first region of interest (ROI1) was defined and selected by using the default mode threshold in the red channel. Consequently, this approach defined and delineated the MF tract allowing the quantification of the pixel intensity in the far-red channel where the synaptic protein of interest was present. Furthermore, a second ROI of an adjacent region not genetically manipulated such as the stratum radiatum was also measured. Thus a ROI/ROI2 ratio was performed slice per slice to normalize the data. Between 40 and 50 measurements from 6 to 8 different mice were acquired for each genotype and each presynaptic protein studied. For the imaging and co-localization analysis in neuronal cultures, focal plane images were acquired for different cells in green (APP), red (Syt7), and far-red (MAP2) channels. The settings (laser power, gain, offset) were fixed between cells as well as the pixel size (120 nm). Images were analyzed by using Image J plugin JACoP49. Fluorescence was analyzed by excluding the cell body and

applying a mean filter (radius = 1 px). For each image acquired the JACoP plugin was used to apply the Costes auto thresholding pixel intensity in each of the channels. Then, Manders coefficient was applied by the plugin to determine the amount of co-localized pixels in each channel, represented as 0 (no co-localization) or 1 (perfect co-localization).

Section 2 - Human

The usage of human brain tissue for the understanding of neurodegenerative diseases such as AD is crucial. Importantly, large cohort studies of patients are also critical to have solid and conclusive results, which can be compared with animal models. An important part of the work performed in this thesis has focused on the replication of results in human and mouse models to overcome the limitations of each of them. In addition, certain steps in the protocols of human brain slices differ from the mouse models.

Immunohistochemistry on human postmortem brain slices

-DAB immunostaining

A Dako EnVision + Dual Link System-HRP (#K4065) was used to perform stainings with DAB. Paraffin embedded hippocampus sections were sliced at 5 µm and mounted on microscope glasses. Slices were incubated 2 times in xylene (≥98.5 %, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent) for 10 minutes and hydrated for 3 minutes in different baths ranging from 99% to 70% ethanol concentrations. Then, slices were incubated in a pressure cooker (Biocare medical) at 110 °C for 30 min either in citrate buffer (10 mM Citric acid (Sigma Aldrich 251275), 0,05 % Tween-20 (Sigma Aldrich P7949), pH 6) or formic acid 70 % ((Sigma Aldrich F0507) pH 2) depending on the protein to be stained. Brain sections were washed with PBS-Tween 0,05 % (PBS-T) and blocked with a peroxidase enzyme blocker for 5 minutes (Dako Dual Endogenous Enzyme Block S2003) and goat serum (NGS; Thermo Fisher Scientific PCN5000) for 20 minutes at RT. Slices were then incubated at room temperature (RT) for two hours with one primary antibody diluted at 1/500: APP C-terminal Y188 rabbit antibody (Abcam #32136; used on slices treated with citrate buffer) or APP C-terminal C1/6.1 mouse antibody (BioLegend; citrate buffer). After 3 washes in PBS-Tween slices were incubated with peroxidase-conjugated secondary antibody. Slices were then washed and incubated for 2 minutes with a 3,3'-diaminobenzidine chromogen solution. Brain slices were again washed and incubated with Hematoxylin (Sigma Aldrich #51275) for 5 minutes at RT before getting dehydrated in baths of 3 minutes ranging from 70% to 99% ethanol and finally in xylene for 10 min. Slices were finally mounted with Vecta mount (VectaMount® Permanent Mounting Medium #H-5000) and dried

overnight. Preabsorption controls in human slices were performed by using the APP C-terminal peptide sequence KMQQNGYENPTYKFFEQMQN (Proteogenix) at 1 µg/ml and Y188 at 1 µg/ml (equal mass concentration, i.e. excess molarity of the adsorption peptide).

-Immunofluorescence

Brain sections were sliced, hydrated and treated for epitope retrieval as for the DAB immunostaining. In addition, slices were washed and blocked with NGS, before incubation for 2 hours at RT with one or a combination of primary antibodies diluted at 1/500: APP C-terminal Y188 rabbit antibody (Abcam #32136; used on slices treated with citrate buffer) or APP C-terminal C1/6.1 mouse antibody (BioLegend; citrate buffer), APP Nter JRD32 mouse antibody against the APP-E1 domain (gift from Johnson and Johnson; Formic acid), APP Nter 22C11 mouse antibody (eBioscience #14-9749-82; citrate buffer), BACE1 rabbit antibody D10E5 (Cell Signaling #5606; citrate buffer), PS1-CTF 33B10 mouse antibody (gift from N. Robakis; citrate buffer), Aβ42 mouse antibody 12F4 (BioLegend # 805501; formic acid), Aβ 1-16 DE2 mouse antibody (Sigma Aldrich MAB5206; citrate buffer). Then, slices were incubated with an autofluorescence quencher (TrueBlack® Lipofuscin Autofluorescence Quencher - Biotium #23007). After 3 washes in PBS 1X, slices were incubated at RT for 2 hours with secondary antibodies diluted at 1/500 (coupled with Alexa 488 or 568 - Thermo Fisher Scientific) and with Methoxy X04 (5 µM) (Tocris Bioscience #4920) or DAPI (300 nM) for 20 minutes. Finally, slices were mounted in Fluoromount™ (Southern Biotech #0100-01) and dried overnight.

Image analysis

Image acquisitions were exported to tiff files in independent channels by using NDP.view2 software (Hamamatsu). Using Image J, images were then calibrated and processed as 8-bits grayscale images (0-255). A high manual threshold was first applied to the APP accumulations and plaques to remove any signal coming from other non-interesting structures. The structures of interest were then saved separately as regions of interest (ROIs) and enlarged by delineating new boundaries 15 µm away from their detection perimeter for posterior analysis. The two ROIs were then merged and used as an overlay to extract the objects from the initial image. Finally, from the extracted images colocalization studies were performed between different channels by using the Mander's overlap coefficient (MOC). Images were analyzed by using the JACop plugin (S. Bolte & F. P. Cordelieres 2006), which provides the proportion of co-localized pixels ranging from 0 (no co-localization) to 1 (perfect co-localization). For some markers, the threshold setting was done automatically by using the plugin 'Auto threshold', which provides a set of different automatized algorithms. APP C-terminal accumulations and plaques (methoxy X04) were selected by using

the 'Max entropy' algorithm that implements the Kapur-Sahoo-Wong thresholding method (Kapur *et al* 1985). For A β antibody stainings the algorithm 'Yen' was applied (Yen *et al* 1995 and Sezgin *et al* 2004) and for other markers such as APP Nter, BACE1 and PS1 a manual individual threshold was applied. Manual thresholding was only applied when none of the automatized algorithms was able to detect reliably markers without a clear signal to noise ratio. Coste's method was used as a statistical test to evaluate the correlation between different channels (Kenneth W. Dunn *et al* 2011). The average pixel intensity for each of the proteins was also measured within the surface of the APP accumulation and 15 μ m outside by using the ROI manager function and the gray value distribution histogram.

DAB stainings were used to count APP accumulations with an automated macro that was segmenting, counting and measuring the size of each accumulation in different regions of the brain slice. Any misdetections were avoided when the user was invited to check for any false-positive counts or by adding any miss-counted accumulations. (https://github.com/fabricecordelieres/IJ-Macro_APP-Plaques-NanoZoomer).

Sholl analysis (Sholl 1953) was used to measure the area and number of APP accumulations around amyloid plaques labelled for A β 42. For this aim an automated macro was used to isolate and count the number of APP accumulations in intervals of 5 μ m, up to 50 μ m distance away from plaques. The area and number of A β 42-positive plaques per hippocampal region was also provided by the macro. (https://github.com/fabricecordelieres/IJ-Macro_APP-Aggregates-Per-Plaques-NanoZoomer).

Section 3 - Image acquisitions and statistical analysis

Brain slice imaging

Human and mouse brain slices were all imaged using the bright field microscope Nanoscope 2.0HT (Hamamatsu). Slices were imaged with a 20X objective and fixed additional lens 1.75X. For every experiment, imaging settings and acquisitions were maintained constant between different patient groups.

Confocal acquisitions with a Leica SP8 White Light Laser 2 were also performed. An inverted stand DMI6000 (Leica Microsystems, Mannheim, Germany) together with a 20x objective (NA 0.75) was used to acquire images. Image settings (laser power, gain, offset) were fixed between different animals.

Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to perform all the

statistical analysis. Experimental data were first tested for normal distribution using d'Agostino and Pearson tests. Data were represented using scatter plots in which the mean or median was included. Normally distributed data were compared by using a two-tailed t-test and data that was non-normally distributed were compared using a two-tailed Mann-Whitney rank test. Two-way ANOVA was used for analysis with 2 different factors (number of APP accumulations and patient groups) and represented as histograms with error bars ($\pm$ SEM). Differences between groups were considered statistically significant when $p < 0.05$.

RESULTS

Chapter 1 - Physiological role of Presenilin in the regulation of Syt7

1.1. Context and aims of the study

Genetic studies on familial forms of AD revealed the genes of APP, PS1 and PS2^{46,39} in the pathological mechanisms of AD. Interestingly, the proteins encoded by these genes are part of an enzymatic pathway that cleaves APP into different fragments⁴¹. As a transmembrane protein, APP has diverse functions in different cell compartments such as synapses in neurons. Importantly, APP and PS1 are both present at the presynaptic active zone and interact with proteins involved in vesicle release and recycling, which are essential processes in synaptic transmission^{118,119,120}. Further support for the role of AD-related genes in synaptic transmission has been suggested from the strong correlation between synapse loss and cognitive impairment in AD^{297–300}. Therefore, the research carried in this project aimed to understand the molecular mechanisms by which PS and APP modulate synaptic transmission and plasticity. As a model, we used the MF-CA3 synapse from single or combined conditional KO mouse lines for PS1, PS2 and APP. Consequently, by targeting the hippocampal DG with different viruses, we could express different fragments of APP together with Cre recombinase and channelrhodopsin. With the combination of these molecular tools we performed a set of experiments with different techniques like electrophysiology combined with optogenetics, imaging and biochemistry.

Our results show that the presynaptic depletion of PS decreases the level of the Ca^{2+} sensor Syt7, inducing an impairment in the presynaptic plasticity. In addition, we discovered that the accumulation of APP-CTF fragments driven by the suppression of the catalytic subunit of the γ -secretase complex correlates with Syt7 depletion. The creation and analyses of PS/APP double KO model demonstrated that APP-CTFs cause Syt7 reduction. The importance of these results made us conceive the idea that Syt7 could be a potential new biomarker for synaptic degeneration in AD. Given the fact that there is a need for early diagnosis and risk evaluation in preclinical stages of AD, finding new biomarkers besides A β and Tau is of relevant interest. Therefore, we started a collaboration with Kaj Blennow's laboratory in Sweden to detect and quantify the levels of Syt 7 in CSF samples from AD patients. In parallel to this collaboration and together with Susanne's Frykman laboratory at the Karolinska Institutet (Sweden), we initiated a new project to measure the changes of Syt7 and APP in human brain hippocampal sections. To this aim, we combined biochemistry experiments together with cutting edge immunofluorescence imaging in a cohort of 3 different groups of patients. The 3 groups consisted in a mixed gender cohort of 11

controls, 4 FAD cases and 12 SAD cases (Table S1 – Manuscript from Chapter 3). Importantly, one of the most interesting groups to test Syt7 levels was the FAD group, in which patients were presenting different missense point mutations in the gene of PS1. These heterozygous mutations were PS1 G206A, PS1 I143T and PS1 H163Y.

PS1 G206A

The G206A point mutation variant affects a coding sequence of exon 7 (GGT to GCT) in the fourth transmembrane domain of PS1. The mutation was first discovered in families from Puerto Rico and Dominican Republic and it is known to be linked to more than 70 families with a Caribbean-Hispanic ancestry^{460,461,462}. The onset of the first cognitive symptoms start appearing between 40 and 73 years of age and the classic AD hallmarks including amyloid plaques and NFT can be found. Furthermore, for some of these patients MRI studies proved cortical atrophy and tempoparietal hypometabolism revealed by FDG-PET⁴⁶³. Other studies in *in vitro* cell cultures showed an increase in the A β 42/A β 40 ratio⁴⁶⁴. Interestingly the presence of the PS1 G206A mutation in HEK-293 cells together with the Swedish mutation produced a 2.2-fold increase in A β 42 compared to cells without the PS1 mutations⁴⁶⁰.

PS1 I143T

The I143T point mutation variant is present in a coding sequence of exon 5 (AAT to ACT) in the fourth transmembrane domain of PS1. The mutation was discovered in a Belgian family⁴⁶⁵ but it has also been found in other families worldwide such as Colombian, French, Japanese, Chinese or Swedish^{466,467,468,469,470}. The patients with this mutation have a mean age onset of 35 years old with severe cognitive and locomotor symptoms such as disorientation, memory loss, dystonia, alexia, apraxia, agnosia, dysphasia, epilepsy⁴⁶⁸. In addition, one of the neuropathological studies showed that these patients presented plaques and NFT in many brain regions such as cerebellum, hippocampus, amygdala and cortex, with special affectation in the frontal lobes⁴⁶³. In some cases, A β 42 and A β 43 were increased at the core of plaques compared to A β 40⁴⁶³. *In vitro* studies containing the PS1 mutation in COS-1 cells have shown that there is an increase in the A β 42/A β 40 ratio⁴⁷². Similar results were also obtained in HEK293 cells expressing the Swedish mutation or in *in vitro* assays using purified proteins⁴⁷².

PS1 H163Y

The H163Y point mutation variant is present in a coding sequence of exon 5 (CAT to TAT) in the fourth transmembrane domain of PS1. The mutation was discovered in several generations of a

Swedish family with a variable onset of the disease between 44 and 65 years old. The most common symptoms were memory loss, epileptic seizures and psychiatric problems⁶³. Furthermore, presymptomatic patients had hypometabolism of glucose and posterior studies showed the presence of widespread amyloid plaques⁴⁷⁵. CSF samples from some patients revealed decreased levels of A β 42 and A β 38⁴⁷⁴. Exceptionally, some carriers of the mutation died young but without dementia or had an incomplete penetrance of the mutation⁴⁷³. As for many other PS1 mutations, studies in COS-1 cells cotransfected with the PS1 H163Y mutation and the Swedish mutation showed an increase in the A β 42/A β 40 ratio⁴⁷². Consistent results were also obtained using *in vitro* assays of purified proteins⁴⁶⁴.

1.2. Manuscript

See PDF of the manuscript in the next page.

ARTICLE

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OPEN

Presenilin-mediated cleavage of APP regulates synaptotagmin-7 and presynaptic plasticity

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Mutations of the intramembrane protease presenilin (PS) or of its main substrate, the amyloid precursor protein (APP), cause early-onset form of Alzheimer disease. PS and APP interact with proteins of the neurotransmitter release machinery without identified functional consequences. Here we report that genetic deletion of PS markedly decreases the pre-synaptic levels of the Ca^{2+} sensor synaptotagmin-7 (Syt7) leading to impaired synaptic facilitation and replenishment of synaptic vesicles. The regulation of Syt7 expression by PS occurs post-transcriptionally and depends on γ -secretase proteolytic activity. It requires the substrate APP as revealed by the combined genetic invalidation of APP and PS1, and in particular the APP-Cterminal fragments which interact with Syt7 and accumulate in synaptic terminals under pharmacological or genetic inhibition of γ -secretase. Thus, we uncover a role of PS in presynaptic mechanisms, through APP cleavage and regulation of Syt7, that highlights aberrant synaptic vesicle processing as a possible new pathway in AD.

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The etiology of Alzheimer disease (AD) is complex. The familial forms of Alzheimer disease (FAD) are caused by mutations of two presenilin (PS) paralogs PS1 or PS2¹ or of the amyloid precursor protein (APP)² indicating a central role of these genes in the disease. PS is the catalytic subunit of the intramembrane protease γ -secretase which cleaves several type 1 transmembrane proteins including APP³. The involvement of both PS and APP in the same enzymatic reaction indicates a possible role for APP fragments processed by PS in AD etiology. Within the amyloidogenic pathway, APP is first cleaved by β -secretase (BACE1) producing the APP β C-terminal fragment (APP- β CTF), a transmembrane stub further processed through the intramembrane proteolytic activity of γ -secretase which releases A β peptides and the APP-intracellular domain (AICD). While molecular studies have mostly focused on A β , possible roles for all three APP fragments in AD have been reported⁴. At the clinical level, synaptic dysfunction and loss are the leading pathological correlates of cognitive impairment in AD^{5–7} suggesting a role for AD-related genes in synaptic transmission.

Accordingly, the roles of both PS and APP in synaptic transmission and plasticity have been studied in several cellular and mouse models (reviewed in ref. ⁸ for PS and in ref. ⁹ for APP). Both proteins are abundantly expressed in the presynaptic active zone^{10–12}, where they interact with proteins of the synaptic release machinery^{13–15}. Therefore, the physiological role of both PS and APP in presynaptic mechanisms through interactions with proteins of the presynaptic release machinery needs to be investigated. To address this, we investigated the presynaptic function of PS and APP at hippocampal mossy fiber (Mf) synapses formed between the dentate gyrus (DG) and CA3 pyramidal cells (Mf-CA3 synapses). Mf-CA3 synapses are large synapses with multiple release sites and are characterized by a large dynamic range of presynaptic plasticity¹⁶. Moreover, Mfs are endowed with a prominent expression of γ -secretase both in rodents¹⁷ and in humans¹⁸. We identified Syt7 as a key presynaptic actor linking the absence of PS or inhibition of γ -secretase activity to presynaptic accumulation of APP-CTFs and impaired presynaptic mechanisms.

Results

Combined DG-specific gene deletion with light activation. We have developed optogenetic tools that combine cell-specific activation with cell-specific gene deletion of DG granule cells (GCs) to study the role of PS at Mf-CA3 synapses. The expression of the C1ql2 gene is largely restricted to the DG in the hippocampus¹⁹. We cloned a sequence of the promoter rich in CpG islands which includes a 1 kb fragment upstream of the transcriptional start site and part of the 5' untranslated region of the mRNA sequence to create a C1ql2 minimal promoter (Supplementary Fig. 1a). The minimal promoter was used in a lentiviral bicistronic construct to control the expression of both ChIEF (a variant of Chr2, see ref. ²⁰) fused to the red fluorescent protein tomato and the Cre recombinase, both cDNAs being separated by a 2A sequence (Fig. 1a). A similar strategy combining expression of ChIEF and Cre was recently applied using an internal ribosomal entry site (IRES) sequence²¹. In brain sections from mice injected with this construct, ChIEFtom is selectively expressed in the DG (Fig. 1b). Moreover, an extensive study of C1ql2-mediated expression of reporter genes validated the bicistronic 2A strategy, the efficiency of the method, the specific targeting of mature Mfs (Supplementary Fig. 1b–e). We used this tool in PS1floxed/PS2KO mice to characterize the properties of Mf-CA3 synapses lacking PS presynaptically, but nonetheless expressing PS1 postsynaptically (Fig. 1c). We confirmed by WB experiments the absence of PS1 protein upon PS1 gene deletion following Cre expression in

neuronal cultures from PS1floxed/PS2KO mice (Supplementary Fig. 1f).

We first recorded from ChIEF-expressing GCs in the current-clamp mode, and observed that a 1 ms light-pulse was sufficient to trigger an action potential (AP) (Supplementary Fig. 1g). Trains of light pulses at 20 Hz reliably triggered trains of APs up to several seconds without failures (Supplementary Fig. 1g). We did not observe any impact of PS deletion on the excitability of DG GCs and responsiveness to light pulses. At the light intensity used in this study (100% light intensity, i.e. 2 mW/mm² using a $\times 63$ objective and 470 nm light), GCs fired systematically in both genotypes (Supplementary Fig. 1h). Moreover, the threshold for spike discharge, the amplitude, and the half-width of the AP were similar in both genotypes (Supplementary Fig. 1i).

By illuminating the dendritic layer of the DG, we could activate a restricted population of GCs, and consequently a restricted number of Mfs (Fig. 1b, c and Supplementary Fig. 1j). Mf-EPSCs evoked in CA3 pyramidal cells using this stimulation protocol displayed properties comparable to those of Mf-EPSCs evoked by minimal electrical stimulation²². Mean EPSC amplitude, failure rate and short-term plasticity, including paired-pulse facilitation and low-frequency facilitation (at 1 Hz) were comparable (Supplementary Fig. 1k) between the two modes of stimulation.

Loss of PS impairs presynaptic facilitation and SV recycling.

We virally transduced the C1ql2-ChIEFtom-2A-Cre construct into PS1floxed/PS2-KO mice (PS1 conditional KO mice backcrossed to constitutive PS2^{-/-} mice). This allowed the recording of Mf-EPSCs from presynaptic PSKO Mf-CA3 synapses when the dendrites of Cre-expressing GCs are stimulated by light (Fig. 1c). We compared the properties of EPSCs at WT (ChIEFtom2A-Cre expression in WT mice) and PSKO Mf-CA3 synapses (Fig. 1d). The mean basal amplitude of Mf-EPSCs, their variance and the failure rate were comparable between the two genotypes when GCs were stimulated at low frequency (0.1 Hz), suggesting similar initial release probability (Fig. 1d, e and Supplementary Fig. 1l). To further study basal synaptic transmission, we recorded Mf-EPSCs in the presence of 32 μ M of Cd²⁺ (a bivalent cation that blocks Ca²⁺ channels), leading to partial inhibition of transmitter release at this concentration (Supplementary Fig. 1m). Under these conditions, 95% of Mf-EPSCs at 1 Hz stimulation were estimated to be due to the quantal release of glutamate (see Methods). No difference in the quantal amplitude of Mf-EPSCs between the two genotypes was observed (Fig. 1f, mean: WT = 42.4 pA; PSKO = 43.8 pA/median: WT = PSKO = 38.0 pA). Altogether, these results indicate that the absence of PS does not impair basal synaptic properties at low-frequency of stimulation.

We then compared short-term plasticity between the two genotypes. Paired-pulse facilitation (40 ms interval) and frequency facilitation, observed when switching the frequency of stimulation from 0.1 to 1 Hz, did not differ between WT and PSKO Mf-CA3 synapses (Fig. 1g, h). In contrast, the large facilitation triggered by short trains of light stimulation of GC dendrites at 20 Hz (train facilitation) was markedly smaller at PSKO Mf-CA3 synapses (Fig. 2a). Interestingly, the deletion of presynaptic PS1 alone was sufficient to cause a deficit in train facilitation (Supplementary Fig. 2a). The deficit in train facilitation is unlikely to result from changes in initial release probability as indicated above. Alternatively, PS inactivation could alter the excitability of DG GCs during trains of stimulation. However, we did not observe any difference in spiking activity of GCs in response to trains of light stimulation in GC dendrites. The threshold for spike discharge, the amplitude and the half-width of APs recorded in DG GCs along the 20 Hz train did not differ between WT and PSKO GCs (Supplementary

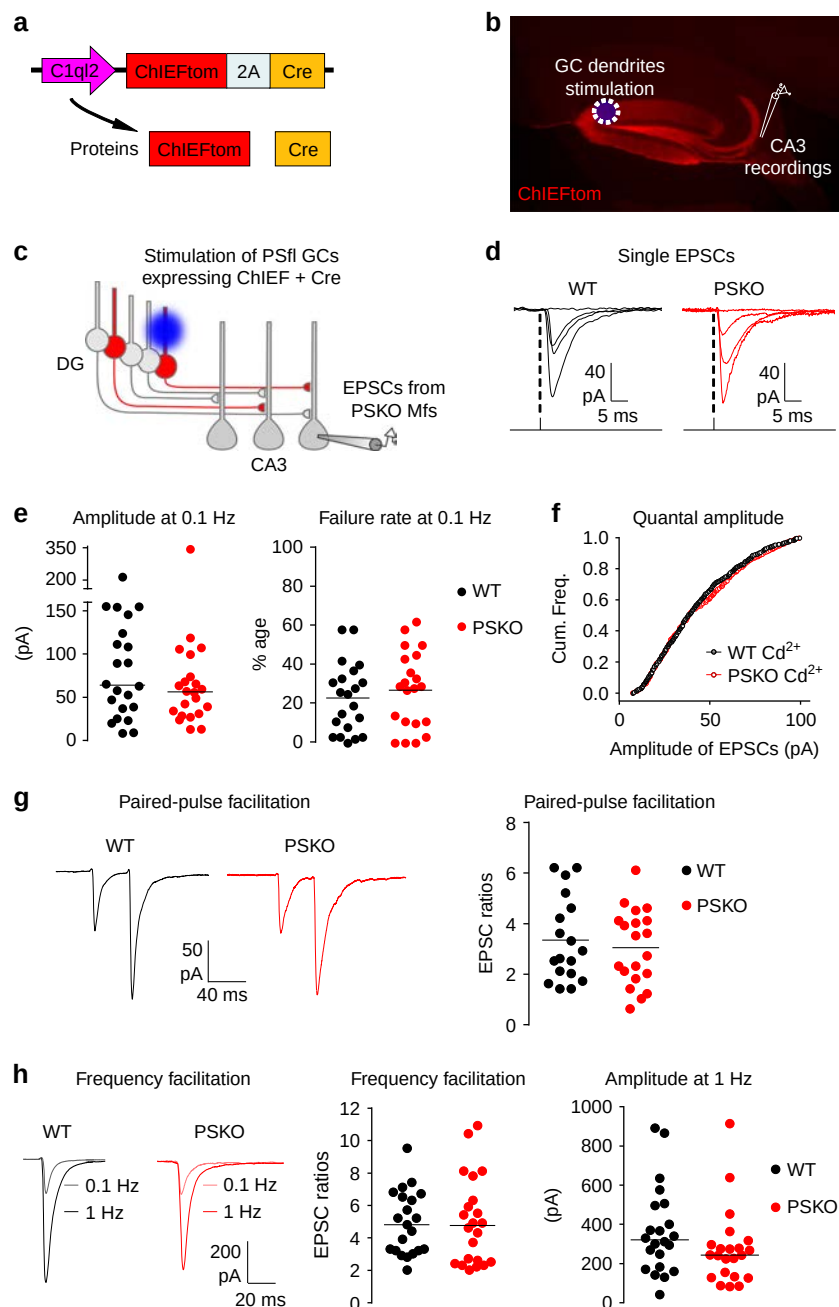


Fig. 1 Study of PS presynaptic function at Mf/CA3 synapses using an optogenetic strategy. **a** Scheme of lentiviral construct which co-expresses ChIEF fused to the fluorescent protein tomato and the Cre recombinase. The promoter C1ql2 restricts expression to DG granule cells (GCs). **b** Picture of a parasagittal section of a mouse brain injected with a virus coding for ChIEFtom. **c** Strategy to selectively study Mf/CA3 synapses that are PSKO presynaptically and intact postsynaptically. The blue light triggers the firing of granule cells that express ChIEF and by obligation (bicistronic construct) the Cre recombinase. In PS “flox” conditional knock-out mice, the activated GCs do not express PS; EPSCs evoked in CA3 neurons by light pulses in the DG arise from presynaptic PSKO Mf terminals. **d** Representative traces of CA3 EPSCs evoked by single light-pulses focused as in (**b**). **e** Basal transmission at Mf/CA3 synapses is not altered by PS genotype. Scatter plots with medians of the amplitude, and means of the failure rate of EPSCs recorded in CA3 neurons at 0.1 Hz in WT or PSKO condition ($n = 22/\text{genotype}$). **f** Cumulative frequencies of EPSCs amplitude in presence of Cd^{2+} . **g** Representative recordings of paired-pulses EPSCs evoked by two light-pulses separated by 40 ms, and analyses of paired-pulse ratio. The facilitation is represented by the amplitude of the second EPSC normalized to the amplitude of the first EPSC. **h** Representative traces of EPSCs evoked by light-pulses at 0.1 or 1 Hz, and analyses of facilitation. The frequency facilitation is represented by the amplitude of EPSCs obtained at 1 Hz normalized to the amplitude of EPSCs obtained at 0.1 Hz. The raw amplitude of EPSCs at 1 Hz does not differ between WT and PSKO

Fig. 2b). Overall, inactivation of PS at Mf-CA3 synapses neither affected release probability nor quantal amplitude under basal conditions, but strongly decreased facilitation induced by trains of Mf stimulation by affecting short-term plasticity mechanisms.

The broad dynamic range of EPSC amplitudes from failures (0.1 Hz) to large currents in response to high-frequency bursts of stimulation represents a high-pass filter for the transfer of information at Mf-CA3 synapses. We explored the impact of the short-term plasticity deficit on spike transfer at DG-CA3

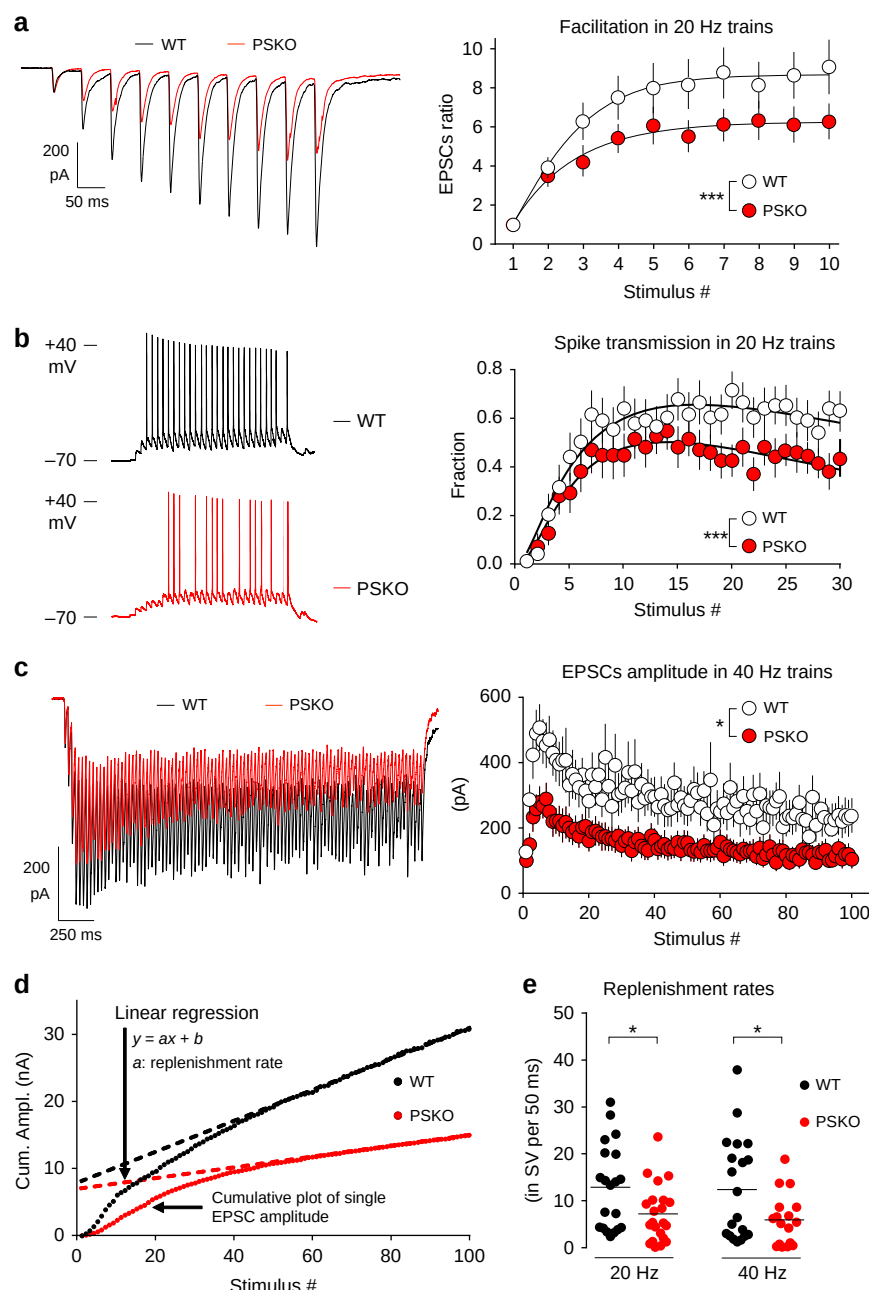


Fig. 2 PS promotes presynaptic facilitation, spike transmission, and SV replenishment. **a** Representative traces of EPSCs evoked by 10 repetitive light-pulses at 20 Hz, and analysis of the facilitation within the train. The facilitation is represented by the amplitude of EPSCs normalized to the amplitude of the first EPSC. The facilitation is strongly impaired by absence of presynaptic PS. Two-way ANOVA, $n = 19$ in WT, 16 in PSKO, $p = 0.0004$. **b** Representative recordings of CA3 spikes in response to 30 stimulations at 20 Hz, and plot of the fractions of spike transmission (for 16 recorded neurons per genotype; five sweeps per neuron) in WT or PSKO genotype. The highest rate of transmission (over 50%) is reached around 15 stimuli and the plot in PSKO is lower than that in WT condition (two-way ANOVA, $n = 16$ in WT, 18 in PSKO $p < 0.0001$). **c** Representative EPSCs evoked by 100 stimuli at 40 Hz, and the analysis of their amplitude. After the initial rising phase (facilitation), the amplitude drops and reaches a steady state that is reduced by absence of PS (two-way ANOVA, $n = 20$ in WT, 17 in PSKO, $p = 0.03$). **d** Graph of the cumulative amplitude of Mf-EPSCs in WT or PSKO condition from trains of stimulation plotted against the stimulus number. A linear regression fits the second part of the cumulative plot where a steady state is reached. The slope is proportional of the replenishment rate. **e** Scatter plots with means of replenishment rates calculated neuron per neuron are represented by genotype and frequency. Absence of PS decreases the rates of replenishment (unpaired *t*-test $p < 0.05$; $n = 20$ in WT, 22 in PSKO). Errors bars represent s.e.m.

connections by performing current-clamp recordings of CA3 pyramidal cells while stimulating GC dendrites with light. In minimal stimulation conditions, the amplitude of Mf-EPSPs at low stimulation frequencies (0.1–1 Hz) is small and only occasionally triggered spike discharge in CA3 PCs (Supplementary Fig. 2c). Spike transmission increased dramatically in

response to trains of stimulation at 20–40 Hz (Fig. 2b and Supplementary 2d) confirming the role of Mf-CA3 synapses as high-pass filters for information transfer within the hippocampal circuit²³. Within a train of 30 stimuli, the fraction of spike transmission at WT Mf-CA3 synapses increased from 0 to 0.63 after 15 stimuli and then slowly decreased (Fig. 2d). In the

absence of PS, spike transfer at DG-CA3 connections was markedly less efficient reaching on average a fraction of 0.45 spikes per stimulus after 15 stimuli (Fig. 2b). Altogether, these results indicate that PS is essential for presynaptic facilitation that supports high-pass filtering of spike transfer at DG-CA3 connections.

Synaptic transmission depends on the priming of release-competent synaptic vesicles (SVs), a mechanism dynamically regulated by the replenishment of the readily releasable pool of SVs²⁴. Because inactivation of PS impacts Mf-CA3 synapses under conditions of trains of presynaptic stimulation, we postulated that the replenishment of release-competent SVs could be impaired. To test this hypothesis, we used long trains of stimulation at 20–40 Hz that involve the recycling pool of SVs (Fig. 2c and Supplementary 2e). We estimated the replenishment rate of SVs at Mf-CA3 synapses by plotting the cumulative amplitude of Mf-EPSCs against stimulation number²⁵ (Fig. 2d). The rate of replenishment was estimated for each recorded CA3 cell by fitting the linear part of the curve for 100 stimuli trains at 20 Hz by linear regression (Fig. 2d), and taking into account the average quantal size measured above (Fig. 1f). The replenishment rate at Mf-CA3 synapses was reduced approximately by half in PSKO conditions as compared to WT conditions (WT and PSKO values, from 13 to 7 SVs per 50 ms, Fig. 2e).

Unaltered presynaptic terminals. We investigated whether the reduced facilitation and impaired replenishment rate of SVs resulted from the degeneration of presynaptic Mf terminals lacking PS, possibly leading to smaller Mf boutons with fewer release sites. To investigate this possibility, we studied the morphology of Mf boutons expressing membrane-targeted YFP as a morphological fluorescent marker²⁶, by confocal imaging followed by 3D reconstruction. In both genotypes, Mf boutons had an average volume of 16 μm^3 (on 200 Mf boutons, Supplementary Fig. 2f). We then stained Mf boutons for Bassoon, a presynaptic marker for release sites and we performed stimulated-emission-depletion (STED) microscopy to evaluate differences in the number of Bassoon puncta between WT and PSKO Mf boutons. We did not observe any difference in the number (16.7 in WT; 17.0 in PSKO) and in the area (0.073 μm^2 in WT; 0.078 μm^2 in PSKO, $n = 82$ Mf boutons from three mice per genotype) of Bassoon-positive puncta (Supplementary Fig. 2g) suggesting that impaired facilitation at Mf-CA3 synapses in PSKO does not result from fewer active zones. These morphological data indicate that the impairment of presynaptic function in PSKO is not caused by a broad alteration of the structure or composition of Mf terminals but more likely results from a distinct alteration at the molecular level.

Marked drop of Syt7 axonal expression in the absence of PS. Recent evidence indicates that the Ca^{2+} sensor synaptotagmin 7 (Syt7) is necessary for both presynaptic facilitation²⁷ and replenishment of release-competent SV²⁸, two presynaptic mechanisms being impaired in the absence of PS. We tested whether the functional impairment of PSKO Mf-CA3 synapses results from decreased levels of Syt7 by immunolabelling Syt7 in mouse brain slices. We observed that Syt7 is an abundant protein in the mouse brain (Supplementary Fig. 3a–c), and is enriched in the *stratum lucidum* (SL) where Mfs make synaptic contacts with CA3 pyramidal cells, in agreement with its role in synaptic facilitation²⁷. Notably, expression of Syt7 in the SL contrasts with the adjacent *stratum radiatum* (SR) where recurrent CA3/CA3 inputs make synapses devoid of marked presynaptic facilitation (Fig. 3a). In brain slices in which PS is absent only in DG-GCs, the labelling of Syt7 in the SL was dramatically decreased,

indicating a loss of Syt7 in PSKO Mf axons (Fig. 3a and Supplementary 3a, d). We measured the mean pixel intensity of staining in the SL and normalized it to the mean pixel intensity in the SR, a region not genetically manipulated (Fig. 3b). We observed an average decrease of 40% (Fig. 3c), a result likely minimized by the infection rate of GCs by the Cre-expressing lentivirus (transduction in around 50% of GCs). We then prepared protein lysates from laser capture microdissections of the SL and hilus to quantify the expression level of the different Syt7 splice variants. The Syt7 antibody labeled two bands at 46 and 80 kDa (Fig. 3d), corresponding to different splice variants²⁹. The shorter variant carries an N-terminal transmembrane region followed by two C-terminal Ca^{2+} -binding domains (C2A and C2B)³⁰ and has been shown to rescue presynaptic facilitation in Syt7KO mouse²⁷. The longer isoform includes extra amino acids of unknown function in the spacer domain localized between the transmembrane region and the C2A domain. We observed a dramatic decrease in the expression of the 46 kDa splice variant of Syt7 (Fig. 3d, e) in agreement with decreased staining in brain slices (Fig. 3a and Supplementary Fig. 3a, d).

We then tested whether the functional impairment of SV release at PSKO Mf-CA3 synapses could result from fewer SVs available or from a limited amount of molecules necessary for SV release. To test these possibilities, we labeled brain slices against VAMP2, the most abundant SV protein; against synaptotagmin 1 (Syt1), the Ca^{2+} sensor necessary for synchronous SV release; against syntaxin1, a SNARE complex protein integrated at the plasma membrane necessary for the release of SV (Fig. 3f); and against Bassoon (Supplementary Fig. 2g). We measured the mean pixel intensity of staining in the SL and did not observe any difference between genotypes for these presynaptic markers (Fig. 3g). Thus, the deficit in protein level observed for Syt7 is not shared by other major proteins of the presynaptic machinery.

The PSKO phenotype is caused by decreased expression of Syt7. We thus postulated that impaired facilitation and SV replenishment at PSKO Mf-CA3 synapses are the functional consequences of decreased Syt7 expression. To address this hypothesis, we tested whether the molecular re-expression of Syt7 in PSKO Mfs was sufficient to recover the functional impairment in synaptic plasticity and SV replenishment rate. We used a strategy involving an injection of PSfl mice with two viral vectors: a first virus allows expression of Cre in GCs leading to deletion of PS and to expression of Cre-dependent ChIEF_{tom} and of Syt7 which is brought by a second virus (Fig. 4a). While GCs infected by only one virus do not respond to light, double-infected GCs lack PS, yet express Syt7 and can be controlled by light. Under this condition, in PSKO, Syt7 was correctly re-expressed in Mf axons (Fig. 4b). The EPSCs evoked by light stimulation in GC dendrites displayed basal synaptic properties similar to WT or to PSKO Mf-CA3 synapses (Supplementary Fig. 4a). However, presynaptic facilitation in response to high frequency trains of stimulation (Fig. 4c, d) and the SV replenishment rate (Fig. 4e, f) recovered to levels not significantly different from WT levels. Besides, the over-expression of Syt7 in WT does not significantly alter presynaptic facilitation or the replenishment rates of SVs (Supplementary Fig. 4b,c). Hence, normal synaptic properties can be rescued in PSKO Mf-CA3 synapses by re-expression of Syt7, demonstrating that the decreased expression of Syt7 is responsible for the PSKO functional phenotype.

γ -secretase activity regulates Syt7 protein levels. We then asked whether the loss of γ -secretase proteolytic activity, normally mediated by PS, was involved in the decreased expression of Syt7. We investigated this possibility by treating rat neuronal cultures

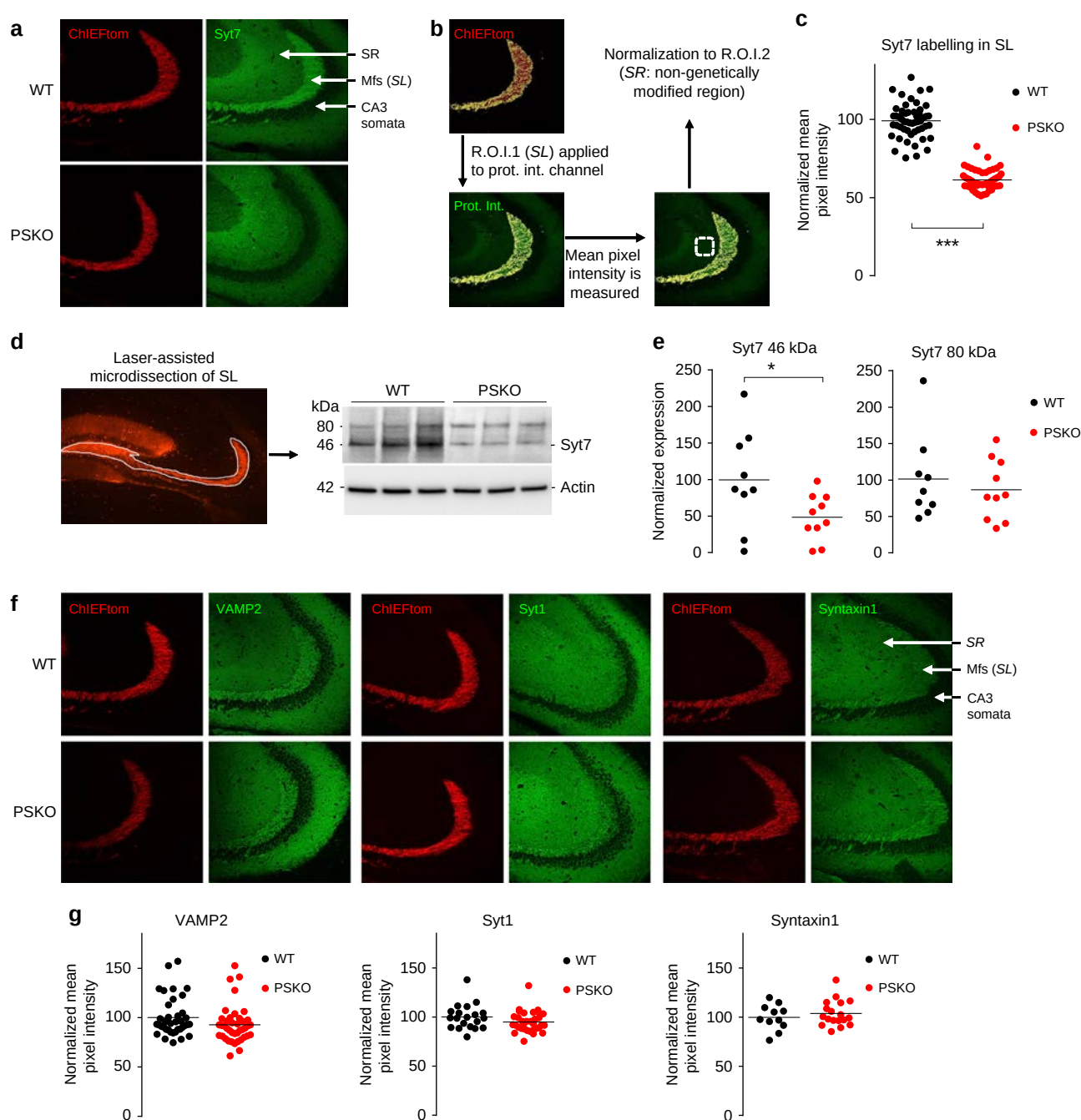


Fig. 3 PS supports the presynaptic expression of Syt7. **a** Confocal images of the *stratum lucidum* (SL) where the Mfs can be distinguished in the red channel (ChIEFtom) and stainings of Syt7 depicted in green. Syt7 staining in PSKO Mfs was negligible in comparison to WT. The phenotype presented here using antibody 105173 from Synaptic System is reproduced in Supplementary Fig. S3d with the antibody N275/14 from Millipore. **b** To quantify the intensity of the stainings a mask corresponding to the SL was created in the red channel. This first region of interest (ROI) was applied to the green channel where the mean pixel intensity of the protein of interest was measured only in the SL. To normalize the results, the mean pixel intensity was also measured in a second ROI: the *stratum radiatum* (SR). The staining intensities in the SL were normalized to the intensities in the SR. **c** Scatter plots of the mean pixel intensity in the SL normalized to the mean pixel intensity in the SR. A reduction of 40% of intensity is observed while approximately half of the Mfs are projected from PSKO GCs. $n = 53$ slices from nine mice in WT, $n = 50$ slices from eight mice in PSKO, $p = 0.0004$. **d** Confocal image of the hippocampus depicting the area dissected with a laser to prepare protein lysates, and western blot from these lysates showing a decreased expression of Syt7. **e** Quantitative analysis of western blot in **c**. A 50% decrease in the expression of the 46 kDa band of Syt7 was detected. **f** Confocal images of the *stratum lucidum* (SL) where the Mfs can be distinguished in the red channel (ChIEFtom) and stainings of VAMP2, Syt1 or Syntaxin1A are seen in green. **g** Scatter plots of the mean pixel intensity in the SL normalized to the mean pixel intensity in the SR measured in images as in **e**. Staining patterns and intensities do not differ between genotypes. For VAMP2, $n = 38$ slices from eight mice in WT, $n = 41$ slices from nine mice in PSKO. For Syt1, $n = 20$ slices from five mice in WT, $n = 33$ slices from seven mice in PSKO. For Syntaxin1, $n = 11$ slices from four mice in WT, $n = 18$ slices from seven mice in PSKO.

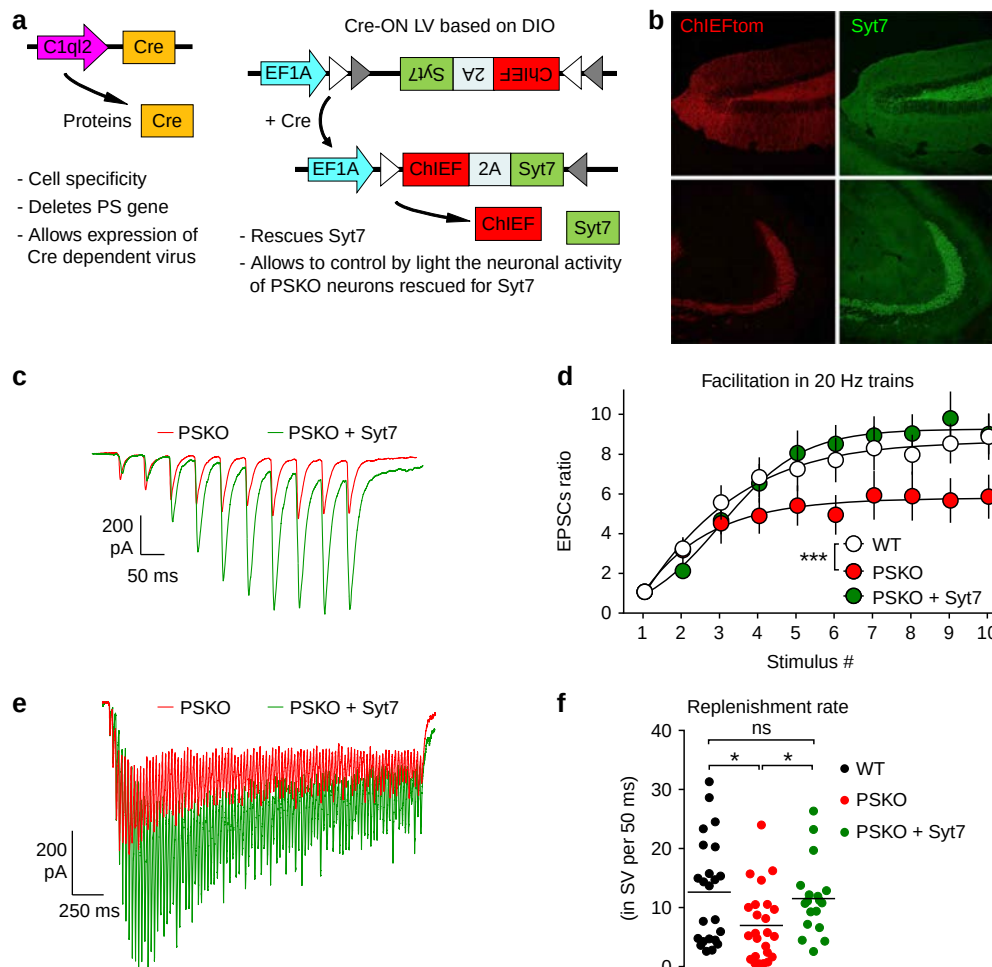


Fig. 4 Impaired facilitation and SV replenishment in PSKO Mfs is caused by decreased Syt7 expression. **a** Schemes of the two lentiviruses (LV) used to rescue Syt7 expression in PSKO GCs controlled by light. C1ql2 promoter restricts the expression of Cre to GCs where the floxed PS gene is deleted. The Cre also allows the Cre-ON LV to express Syt7 and ChIEF. **b** Confocal images showing the rescued expression of Syt7 in the hilus (up) and in the stratum lucidum (down) using the strategy described in **a**. **c** Representative traces of EPSCs evoked by 10 repetitive light pulses at 20 Hz. **d** Analysis of presynaptic facilitation in 20 Hz trains. Facilitation is represented by the amplitude of the EPSCs normalized to the amplitude of the first EPSC. Facilitation, impaired in absence of presynaptic PS, recovers to WT level by re-expression of Syt7. (Two-way ANOVA, $n = 24$ in WT, 23 in PSKO, 24 in PSKO + Syt7, $p = 0.001$). Errors bars represent s.e.m. **e** Representative EPSCs evoked by 100 stimuli at 40 Hz. The amplitude of EPSCs is rescued by Syt7 re-expression. **f** Scatter plots with means of replenishment rates calculated per neuron are represented by genotype. The replenishment rate impaired by the absence of PS is rescued by re-expression of Syt7.

with a γ -secretase inhibitor (GSI: L685,458 at 10 μ M) for 4 days (from 10 to 14 DIV) and preparing protein lysates for western-blot (WB) analysis. The Syt7 antibody labelled two bands at 46 and 80 kDa (Fig. 5a), as in the microdissected brain samples. Interestingly, the intensity of the lowest band was decreased by half in conditions of GSI treatment (Fig. 5a, c), indicating that blocking γ -secretase proteolytic activity downregulates Syt7 expression. In contrast, the expression levels of VAMP2, Syt1, and Syntaxin1 (Fig. 5b, d) were not modified by GSI treatment. Hence, the regulation of Syt7 by γ -secretase activity is specific among the presynaptic markers tested.

This involvement of γ -secretase activity indicates a possible role for γ -secretase substrates expressed at the presynaptic site. APP, the main proteolytic substrate of γ -secretase is first processed by β -secretase (BACE1) producing the β C-terminal fragment of APP (APP- β CTF), a transmembrane stub further cleaved by the intramembrane proteolytic activity of γ -secretase releasing A β peptides and the AICD (Fig. 5e). We hypothesized that the PSKO phenotype, including the decreased expression of Syt7 results from the absence of AICD because this γ -secretase

product has been reported to regulate transcription³¹. We performed laser capture microdissection of the GC layer to prepare RNA samples used for qRT-PCR of Syt7 mRNA, and did not observe any change between the two genotypes (Fig. 5f). We further addressed the possible involvement of AICD in presynaptic mechanisms by testing whether the molecular rescue of AICD in PSKO GCs could recover the functional phenotype. Once again we used a strategy based on the combination of a Cre-expressing lentivirus with a Cre-ON lentivirus (Fig. 5g and Supplementary 5a). This allowed the optical control of activity of GCs which were PSKO but still expressed AICD (Fig. 5h). In this condition, we did not observe any recovery of presynaptic facilitation (Fig. 5i) while basal transmission was not affected (Supplementary Fig. 5b,c). The lack of evidence for an impairment at the transcriptional level indicates that Syt7 protein is dysregulated post-transcriptionally.

Syt7 downregulation results from accumulation of APP-CTFs. In the absence of PS and its γ -secretase proteolytic activity, unprocessed substrates APP-CTFa/ β (APP-CTFs) may

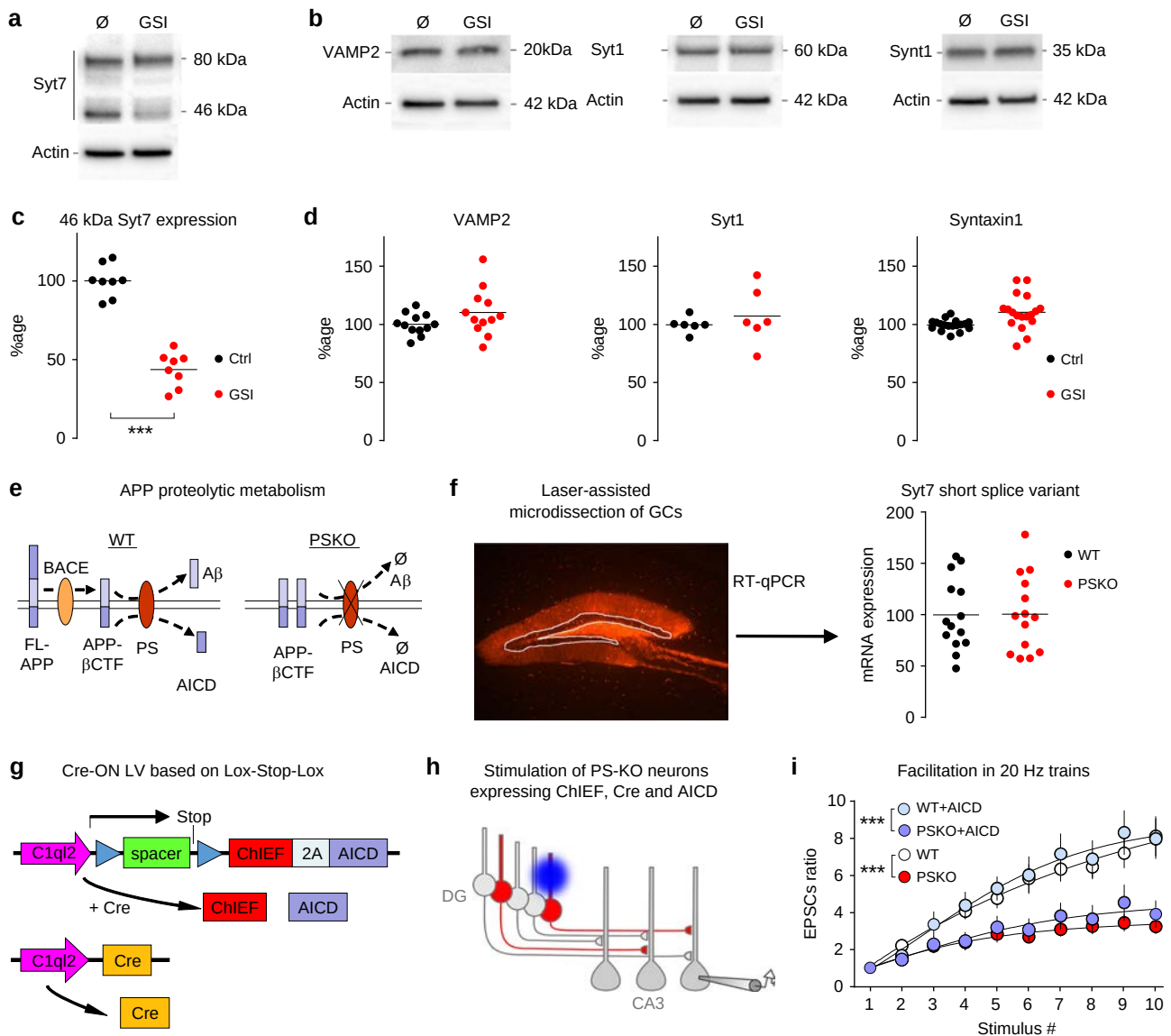


Fig. 5 γ -secretase activity regulates Syt7 protein levels. **a, b** Western blots of protein samples from neuronal cultures treated or not with 10 μ M GSI from 10 to 14DIV. The 46 kDa Syt7 band decreases dramatically when γ -secretase is inhibited. γ -secretase inhibition does not impair expression of VAMP2, Syt1, and Syntaxin1A. **c, d** Scatter plots of the western-blot quantification of samples from neuronal culture treated or not with GSI. Syt7 expression is decreased by half in treated condition as seen in **a**, while the expression of VAMP2, Syt1, and Syntaxin1A is stable under GSI condition as seen in **b**. **e** Schemes of APP proteolytic metabolism in WT and PSKO conditions. Full-length (FL) APP is first cleaved by BACE producing β CTF, a transmembrane stub, further degraded by PS (γ -secretase) into A β and APP IntraCellular Domain (AICD). In absence of PS, β CTF cannot be catabolized and thus accumulates. **f** Confocal image of the hippocampus depicting the GCs layer dissected with a laser to prepare RNA samples, and RT-qPCR analysis of the samples indicates that mRNA level of Syt7 is not impaired in absence of PS. **g** Schemes of the two lentiviruses (LV) used to rescue AICD expression in PSKO GCs controlled by light. C1q12 promoter restricts the expression of Cre to GCs where the PS floxed gene is deleted. The Cre also removes the Lox-Stop-Lox sequence allowing the Cre-ON LV to express AICD and ChIEF. **h** Strategy to selectively study Mf/CA3 synapses that are presynaptically PSKO, yet express AICD. In PS “floxed” conditional knock-out mice, double-infected GCs do not express PS (Cre deletes PSfl gene), yet express AICD (Cre dependently), and ChIEF (bicistronic construct) allowing the control of their activity by light. GCs infected by a single virus do not respond to light. **i** Facilitation of EPSCs amplitude measured along trains of 10 stimuli at 20 Hz in WT or PSKO-expressing AICD. The impaired facilitation of PSKO is not rescued by expression of AICD. Two-way ANOVA, $n = 8$ in WT, 11 in PSKO, $p = 0.017$. Errors bars represent s.e.m

accumulate. We studied the expression level of different fragments of APP by staining brain slices with antibodies directed against either the Nterminal or Cterminal epitopes of APP. In control conditions, we observed that the SL was moderately stained with the Cterminal antibody relatively to the CA3 somata and barely stained with the Nterminal antibody (Fig. 6a). Interestingly, in conditions where PS is selectively suppressed in Mf axons, we observed a striking increase of APP staining with the

Cterminal but not with the Nterminal antibody indicating that an APP fragment lacking the Nterminal epitope accumulates in PSKO axons (Fig. 6a, b). Using protein samples obtained by laser microdissection of PSKO Mfs, we distinctly identified APP-CTFs as the APP fragment accumulating in Mf axons (Fig. 6c, d). In Mfs, the initial ectodomain shedding of APP is likely performed by BACE1 which is abundantly expressed in these axons (ref. ³² and Supplementary 6a). In PSKO Mfs, the accumulation

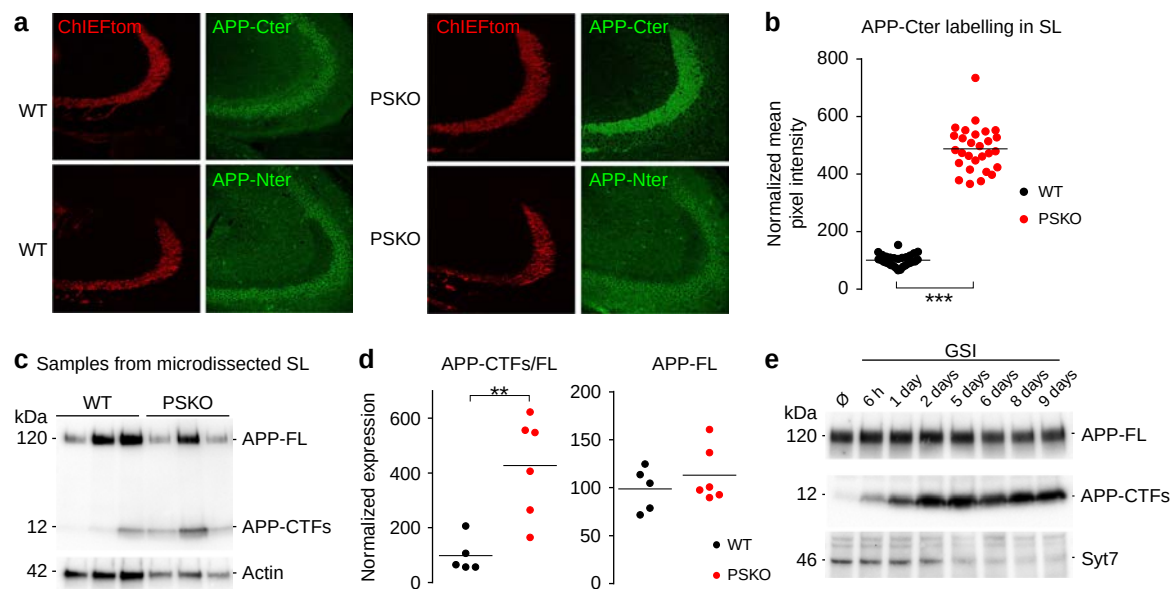


Fig. 6 γ -secretase proteolytic activity regulates the axonal expression of APP- β CTF. **a** Confocal images of the Mf tracts distinguished in the red channel (ChIEFtom) and of stainings of APP Cterminal with Y188 antibody (upper panel) and Nterminal with JRD32 antibody (lower panel). In WT, Mfs are labelled with the Cterminal but barely with the Nterminal antibody. In PSKO, Mfs are strongly labelled with the Cterminal but not with the Nterminal antibody. **b** Scatter plots of the mean pixel intensity of APP-Cter in the SL normalized to the mean pixel intensity in the SR. A five-fold increase of the intensity is observed in PSKO condition compared to WT. **c** Samples prepared as in (Fig. 3c) show a striking increase in APP-CTFs levels in dissected Mfs detected with Y188 antibody against APP-Cter. **d** Quantitative analysis of western blot in c. A 400% increase in the expression of APP-CTFs was detected. **e** Western-blot of samples prepared from neuronal cultures treated with GSI from 6 h to 9 days in vitro. APP-CTFs accumulate while Syt7 drops

of APP- β CTF in terminals suggests that it can neither be retrogradely transported nor be catabolized by enzymes other than PS. We induced an accumulation of APP-CTFs in neuronal cultures similar to the one observed in Mfs by blocking PS function with a γ -secretase inhibitor (GSI). Interestingly, we observed an inverse correlation between Syt7 expression level and APP-CTFs, which progressively accumulates in protein lysates from cultures treated from 6 h to 9 days with GSI (Fig. 6e), suggesting that accumulation of APP-CTFs prevents the normal expression of Syt7.

Amongst other γ -secretase substrates identified so far only a few of them are known to be expressed at the presynaptic site and could potentially play a role in the presynaptic phenotype observed^{33,34}. We identified N-cadherin and Neurexin1 as additional candidate substrates that may accumulate in pre-synaptic Mf terminals upon deletion of PS. We studied the accumulation of the transmembrane stubs of these proteins produced after ectodomain shedding in neuronal cultures treated with GSI (Supplementary Fig. 6b–e). We also stained hippocampal slices using antibodies against the Cterminal fragments of these two proteins. We observed, as already reported, that Ncad-CTF1 accumulates in neuronal cultures³⁵ but not in PSKO Mf terminals. The Cterminal stub of Neurexin does not accumulate in cultured neurons treated with GSI nor in PSKO Mfs (Supplementary Fig. 6b–e).

To address whether there was a causal relation between APP-CTFs accumulation and Syt7 downregulation, we crossed the PS1fl mouse line with an APPfl mouse line³⁶. In the resulting APPfl/PS1fl mouse line, the expression of Cre removes both PS1 and APP in the same cells; thus even though the enzyme is removed, no substrate (APP-CTFs) can accumulate (Fig. 7a). We compared the expression level of Syt7 in APPfl/PS1fl to PS1fl and WT (Fig. 7b, c). In PS1KO Mfs from PS1fl mice, the level of Syt7 expression was significantly decreased compared to WT. However, the extent of this reduction was less pronounced than the reduction observed in PSKO Mfs from PS1fl/PS2KO mouse. This

is in agreement with the milder impairment of presynaptic facilitation observed in PS1KO compared to PSKO (removal of both PS1 and PS2) conditions (Supplementary Fig. 2a). In Mfs expressing neither PS1 nor APP, the expression level of Syt7 was comparable to WT. Thus, the absence of APP prevents the decreased expression of Syt7 in Mfs, demonstrating a causal relationship between APP processing and downregulation of Syt7. While the level of Syt7 differs between PSKO and APPKO/PS1KO conditions, γ -secretase products of APP processing ($A\beta$, AICD) cannot be produced in both conditions, indicating that the Syt7 deficit does not result from the absence of $A\beta$ or AICD. In contrast, accumulation of APP Cterminal stubs can occur in PSKO but not in APPKO/PS1KO cells establishing APP-CTFs as the causative APP fragments involved in Syt7 regulation.

We then performed experiments to better understand the molecular mechanism underlying the functional interaction between APP and Syt7. We tested whether the two proteins were expressed in synaptic compartments and whether they could physically interact. We detected both APP and Syt7 in synaptosomes prepared from adult mouse brain (Supplementary Fig. 7a) in agreement with previous studies^{10,12,29}. Co-immunoprecipitation of the two proteins (in both directions) demonstrated that they are present in the same protein complexes (Fig. 7d). Additionally, we observed that the two proteins colocalize in neuronal cultures with a Manders coefficient of 0.77 (Supplementary Fig. 7b). Interestingly, colocalization increases (Manders coefficient of 0.94) following treatment of the neurons for 4 days with GSI suggesting that accumulated APP-CTFs also colocalize with Syt7 (Supplementary Fig. 7b).

We further investigated the relationship between APP-CTFs and Syt7 by performing cell fractionation on an iodixanol gradient. We observed that Syt7 was enriched in the lighter fractions from neuronal culture extracts characterized by high expression of VAMP2 (Fig. 7e). Interestingly, APP-CTFs was detected in the same fractions while full-length APP was

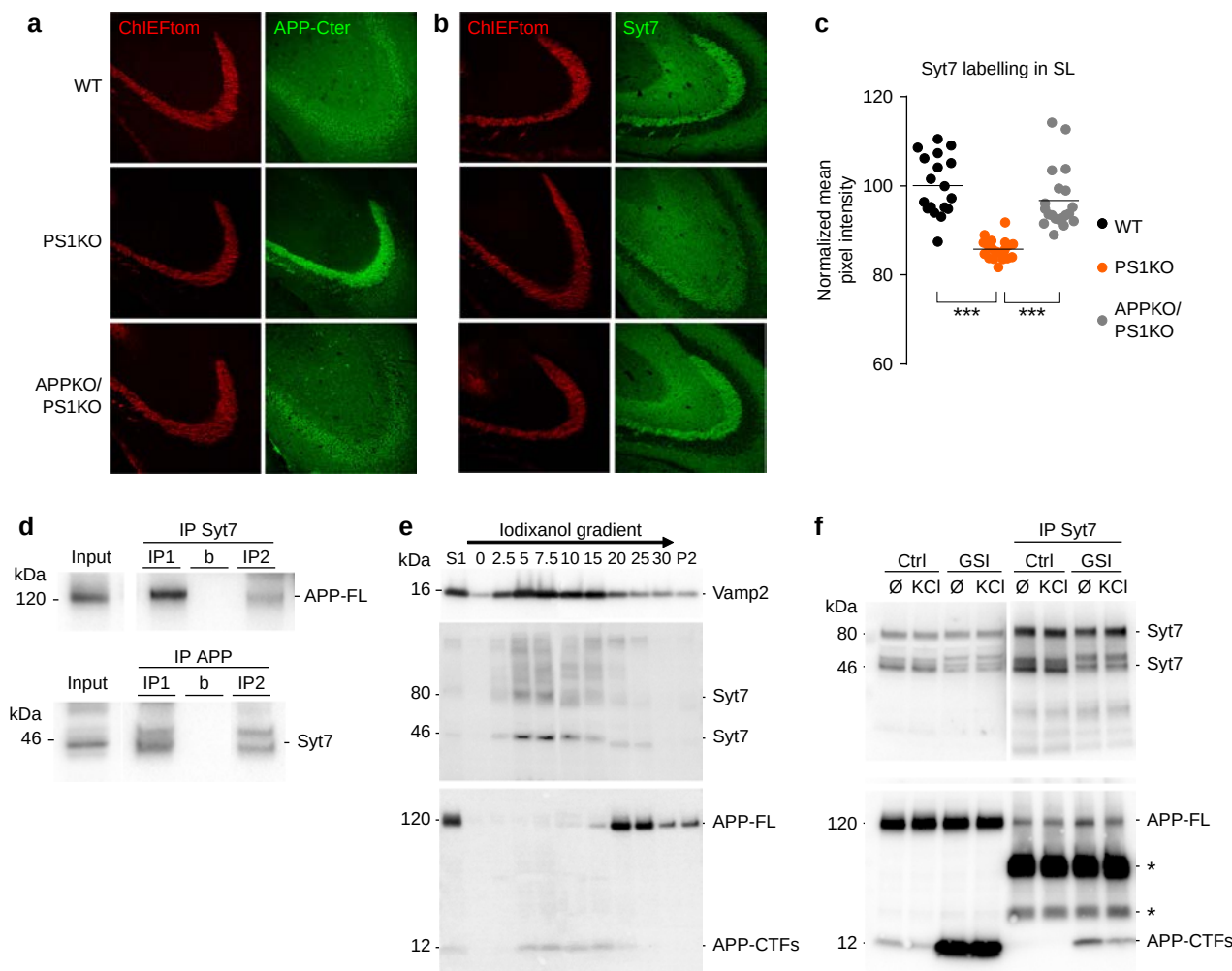


Fig. 7 PS regulation of Syt7 expression depends on APP-CTFs, new interacting partners of Syt7. **a** Confocal images of ChIEFtom (red channel) and APP-Cterminal (detected with C1/6.1 antibody, green channel) in the Mf tracts in WT, PS1KO, and PS1KO/APPKO conditions. Mfs are faintly labelled with the APP antibody in WT and barely in APPKO/PS1KO. In contrast, PS1KO Mfs display strong labelling of APP. **b** Confocal images as in **a** displaying Syt7 labelling. Mfs are strongly labelled in WT and in APPKO/PS1KO but faintly in PS1KO. **c** Scatter plots of the mean pixel intensity in the SL normalized to the mean pixel intensity in the *stratum radiatum* report a reduction of 18%. In this condition, 50% of the GCs are infected by a Cre-expressing LV thus ~50% of the Mfs are projected from non-infected (PS expressing) GCs. **d** WB from synaptosomal samples prepared from whole brains of adult mice. Top: IP of Syt7 pulled-down APP-FL. Bottom: IP of APP pulled-down Syt7. The characterization of the synaptosomal fraction is depicted in Supplementary Fig. 7. b: beads + immuno-precipitating antibody without sample. **e** Western-blot of cellular fractions from cultured neurons separated on an iodixanol gradient. VAMP2, Syt7, and APP-CTFs are enriched in light fractions while APP-FL is present in heavier fractions. **f** Samples prepared from cultured neurons treated or not with GSI from 10 to 14 DIV and with or without KCl (30 mM) for 15 min before lysis. In the inputs, detection of APP Cterminal with the Y188 antibody indicates that GSI induces CTFs accumulation. IP of Syt7 pulled-down APP-FL and CTFs. Asterisks indicate light and heavy chains of the immuno-precipitating antibody. Syt7/CTFs interaction is decreased by KCl treatment suggesting that it is regulated by neuronal activity

expressed in the more heavy fractions (Fig. 7e). This suggests a possible interaction between APP-CTFs and Syt7 in the lighter cellular compartments of the neurons. We thus studied a possible interaction between Syt7 and the APP fragments. Immunoprecipitation of Syt7 from protein lysates of neuronal cultures allowed co-precipitating APP indicating that the two proteins interact (Fig. 7f). When neurons were treated with GSI, APP-CTFs dramatically accumulated and was massively co-precipitated with Syt7 (Fig. 7f). Moreover, when the neuronal cultures were treated with KCl to depolarize the neurons and induce AP firing, the interaction between Syt7 and APP-CTFs decreased, indicating that the interaction was dynamically regulated by neuronal activity and possibly by SV release (Fig. 7f).

Discussion

Understanding the physiological function of AD-related genes is fundamental because dysregulation of their normal function likely contributes to AD pathogenesis. Because APP and PS are abundantly expressed in presynaptic compartments^{10–12}, where they interact with proteins of the release machinery, it is essential to understand their physiological role in presynaptic mechanisms. To address this, we have developed versatile optogenetic tools that help circumvent difficulties inherent to studies of presynaptic mechanisms with classical electrophysiological methods. Indeed, following genetic manipulations in a subpopulation of neurons, stimulation with an extracellular electrode activates both the axons from genetically manipulated cells and those from non-modified cells. We cloned a new minimal promoter of the C1ql2

gene and used it in a lentiviral vector to target expression of ChIEF in DG together with a second transgene, e.g. Cre recombinase. C1ql2-driven expression of ChIEF and Cre allowed the combination of region-specific activation with gene deletion in the DG of PS-conditional KO mice. Using this approach, we examined the effects of the genetic deletion of PS in presynaptic GCs on the morpho-functional properties of Mf-CA3 synapses.

We discovered that PS controls the levels of the Ca^{2+} sensor Syt7 presynaptically. Indeed, in the absence of presynaptic PS, Syt7 dramatically drops in axons and terminals. Interestingly, four other presynaptic markers tested were not impaired by the absence of PS, two being SV proteins (VAMP2 and Syt1) and two being active zone proteins (Bassoon and Syntaxin1A). Moreover, the absence of PS does not impact on the morphology of presynaptic terminals indicating, all together, that the functional phenotype is not caused by degenerative processes within presynaptic terminals.

The reduced expression of Syt7 in PSKO Mfs impairs two essential presynaptic functions: presynaptic facilitation and replenishment of release-competent vesicles. Indeed, Syt7 has recently been shown to be essential for presynaptic facilitation²⁷ at several central synapses. Its role is especially noteworthy at Mf-CA3 synapses where the large presynaptic train facilitation is nearly abolished in Syt7KO²⁷. Thus, the impaired short-term plasticity observed at PSKO Mf-CA3 synapses is consistent with the decreased expression of Syt7 in Mf terminals. Additionally, Syt7 is necessary for the replenishment of release competent SVs²⁸. We demonstrated that re-expression of Syt7 in PSKO Mf-CA3 synapses rescued presynaptic function, providing a causal link between the drop of Syt7 expression and the functional phenotype. Of note, quantal and basal transmission were unchanged in agreement with the observation that Syt7 does not determine the initial probability of release^{27,28}.

Next, we investigated whether this novel synaptic function of PS depends on its γ -secretase proteolytic activity. In neuronal cultures, we observed that inhibiting γ -secretase activity pharmacologically decreased Syt7 expression within a few days indicating a role for γ -secretase. The investigation of molecular mechanisms involving γ -secretase is complex due to its diverse functions and molecular targets⁸. APP is a major substrate of PS and is highly enriched in synapses, and particularly at a presynaptic level¹². In the absence of PS or when γ -secretase activity is inhibited, the substrates of PS, APP-CTFs, cannot be processed into A β (or p3) and AICD. Thus, in the PSKO condition, only the absence and not the excess of A β (or p3) and AICD could potentially explain the PSKO phenotype. Because AICD has been proposed to act as a transcription factor³¹, the decline in Syt7 level could result from a deficit in AICD. However, the analysis of Syt7 mRNA and rescue experiments of AICD expression in PSKO GCs show that the absence of AICD does not play a role in the regulation of Syt7 expression and function.

We observed that the substrates of PS, APP-CTFs, accumulate in Mf axons in the absence of PS, or in cultured neurons when γ -secretase is pharmacologically inhibited. These fragments of APP are involved in the deficit in Syt7 expression observed in PSKO axons, since deletion of APP in APPKO/PS1KO prevents the drop of Syt7 expression. Moreover, there was an inverse relationship between APP-CTFs and Syt7 expression in neuronal cultures. Finally, we show that Syt7 is a new interacting partner of APP-CTFs. The mechanism linking the accumulation of APP-CTFs to downregulation of Syt7 will need further investigation. A possible hypothesis is that CTFs sequester Syt7 in compartments preventing its functional roles and causing its degradation. This interpretation is supported by the well-documented function of PS and APP in axonal transport^{37–40} and by the observation that the absence of PS impairs the cell surface expression and the

shuttling of several proteins between the cell interior and the cell surface^{41,42}. Syt7, as a protein expressed both in SVs and at the plasma membrane^{43,44} could be sequestered in the wrong compartment by accumulation of APP-CTFs. Our results are based on the pharmacological inhibition of γ -secretase activity or genetic invalidation of PS. They suggest that in physiological conditions, presynaptic γ -secretase activity regulates the abundance of Syt7, a key presynaptic protein involved in presynaptic plasticity which interacts with APP-CTFs. It will be interesting to investigate the physiological conditions under which γ -secretase activity is regulated, hence modulating the amount of APP-CTFs and consequently of Syt7.

Although our results mainly deal with the investigation of the physiological role of PS, they may also be of importance for the physiopathology of AD. FAD mutations of PS1 also cause APP-CTFs accumulation due to decreased enzymatic activity of γ -secretase, limiting the catabolism of APP-CTFs⁴⁵. Interestingly, genetic evidence indicates a relationship between the level of APP- β CTF catabolized by γ -secretase and AD. Indeed, APP- β CTF is highly produced when the APP gene contains mutations causing the early onset form of AD (the Swedish mutations K595N/M596L²) and is produced to a much lower extent when APP contains a mutation (A598T) protecting from AD⁴⁶. Overall our data highlight the notion that presynaptic dysfunction may play a role in AD in relation to APP processing by PS.

Methods

Ethical approval. All experiments were performed in accordance with the European Union (EU) guidelines (directive 2010/63/UE) and under the authorization of the 'Pole in Vivo' of the IINS (#A33 12 061). The experimental protocol (#50120174-A) has been authorized by the local ethical committee (registered at the National Committee about Animal Experimentation).

Animals. The animals used in this study were PS1floxed #007605, PS2-KO #005617 mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) and C57BL6/J wild type (WT). In the PS1floxed mice, loxP sites are present on either side of exon 7 of the targeted gene and allow deletion of this exon by Cre-mediated recombination. In the PS2 mouse line, a targeting vector was used to disrupt exon 5 and introduce a frame shift between exons 4 and 6. We developed a PS1fl/PS2KO mouse line by crossing the PS1floxed and the PS2KO mice together. Mice were genotyped using the following primers: PS1fl-F: CAGGCACACTCACCCACAGA, PS1fl-R: GAAAATCATATCCCTACACTA; PS2WT-F: GAGAGAAGGCACCA GGATAGGTT, PS2WT-R: AGTGTCTGGAGCAGCAGATAC and PS2Mut-F: CGAAGGAGCAAAGCTGCTATTGG, PS2Mut-R: CTGGGTCTCCAGCAG TC TTC. We developed an APPfl/PS1KO mouse line by crossing the APPfloxed³⁶ and the PS1floxed mice together. APP gene was genotyped using the following primers: 1: AACGCAGGGAGGAGTCAGGG, 2: TGCATGTCTAGTCTAATGGAGGC, and 3: ATCTGCC CTTATCCAGTGAAATGAACC.

Cloning C1ql2 promoter and generation of lentiviral vectors. The lentiviruses were produced by the viral vectors facility of Bordeaux "TransBioMed". The lentiviruses developed here are based on the pRRLsin-PGK-MCS-WPRE plasmid where PGK promoter has been exchanged with the C1ql2 minimal promoter. This sequence was cloned from mouse genomic DNA using the following primers EcoRV/C1ql2-F: ATATATGATATCagcaccacatagcagc; BamHI/C1ql2-R: ATATATGGATCCgctctggaactgat ctg. The 1.3 kb sequence was then inserted between EcoRV and BamHI restriction sites of the lentiviral backbone. After the promoter, the following cDNA sequences were inserted in the 5'–3' order: ChIEFtomato, 2A peptide from porcine teschovirus-1⁴⁷, finally the Cre recombinase or the eGFP. A Cre-dependent construct was created to allow conditional expression of ChIEFtomato and AICD. Behind C1ql2 promoter, a Lox-Stop-Lox cassette was inserted followed by the cDNA of ChIEFtomato, 2A peptide and AICD. A second Cre-ON construct was created based on a double-inverted open-reading frame (DIO) strategy. Briefly, a cassette including ChIEFtomato, 2A peptide and Syt7 was placed in an inverted manner between two doublets of lox sequences (loxP and lox 2722). For 3D reconstruction of Mf boutons, a lentiviral vector expressing myristoylated YFP (mYFP) was used (generously provided by C. Lois, California Institute of Technology, Pasadena, CA) described in ref. ²⁶.

Viral gene transfer and stereotaxic delivery. Mice (P22–P30) were anesthetized by isoflurane inhalation and injected with buprenorphine to prevent post-surgery pain. 1 μL of viral solution (5×10^6 particles) was injected using a micropump and

syringe (Nanofil WPI) at the rate of 100 nl/min in the DG (Y: 2.2 mm from lambda; X: ± 2.0 mm from sagittal suture; Z: 2.0 mm from the skull) to infect GCs. This site targets a rather dorsal part of the hippocampus but the spreading of viruses allowed the infection of a large part of the hippocampus (radius of roughly 1 mm) to produce numerous slices for IHC or electrophysiology. Experiments were performed at least 3 weeks post-injection.

Slice preparation. Mice were anesthetized using ketamine/xylazine mix (100 mg/kg/10 mg/kg; i.p.) and perfused transcardially with ice cold artificial cerebrospinal fluid (aCSF) for 40–60 s. The brain was quickly removed from the skull and chilled in ice-cold aCSF containing the following (in mM): 120 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 16.5 glucose, 2.8 pyruvic acid, 0.5 ascorbic acid, pH 7.4 adjusted by saturating with carbogen (95% O₂ and 5% CO₂), and an osmolality of 305 mOsm. Parasagittal slices (300 μ m) were cut from brain hemispheres using a vibratome (VT 1200S, Leica Microsystems, Nussloch, Germany). Slices were then transferred to aCSF for 20 min at 33 °C and thereafter maintained at room temperature until required.

Electrophysiological recordings. A slice was transferred to a recording chamber where it was submerged and continuously perfused with oxygenated (95% O₂, 5% CO₂) aCSF at RT. Whole-cell patch clamp recordings were performed on neurons identified with a differential interference contrast microscope (Eclipse FN-1, Nikon, Champigny sur Marne, France) equipped with a camera (CoolSNAP EZ, Roper, Evry, France). Voltage-clamp recordings were performed on CA3 pyramidal neurons using patch electrode (4 ± 0.5 M Ω) filled with an internal solution containing the following (in mM): 125 CsCH₃SO₃, 2 MgCl₂, 4 NaCl, 5 phospho-creatine, 4 Na₂ATP, 10 EGTA, 10 HEPES pH 7.3 adjusted with CsOH, and an osmolality of 300 mOsm. Bicuculline (10 μ M) was added to the bath to inhibit GABA-A receptors. Mf-CA3 excitatory post synaptic currents (EPSCs) were evoked by light pulses of 1 ms over GCs dendrites in the *stratum moleculare*. The baseline stimulation frequency for all experiments was 0.1 Hz. Current-clamp recordings were performed on GCs or CA3 cells patched with an internal solution containing the following (in mM): 115 KCl, 10 KCl, 0.02 CaCl₂, 4 MgATP, 0.3NaGTP, 15 phospho-creatine, 0.2 EGTA, 10 HEPES pH 7.3 adjusted with KOH and an osmolality of 300 mOsm. Spike transmission experiments were performed in current clamp mode while using the “low-frequency voltage clamp” function of EPC10 amplifier to keep the membrane potential of the recorded CA3 neurons constant at -70 mV between recording sweeps. Signals were amplified using a HEKA EPC10 amplifier (Lambrecht, Germany), filtered at 3.3 kHz and digitized at 10 kHz via PatchMaster software (Lambrecht, Germany). Data were analyzed using IGOR PRO 6.3 (Wavemetrics, Lake Oswego, OR).

Analysis of quantal transmission. The characteristics of quantal neurotransmission at Mf/CA3 were determined by recording EPSCs in CA3 neurons while stimulating with light-pulses at 1 Hz in presence of 32 μ M of cadmium. We used the following model to estimate the probability of release at individual releasing site (active zone) and we estimated the contamination of mono-quantal release by bi-quantal release in the experiments in presence of 32 μ M cadmium. In this condition, the failure rate is of 88%, hence the probability of release is 12%. This total probability is the sum of the probability of release at individual release sites (we considered 20 sites/Mf bouton here) and possibly of concomitant release from two sites (concomitant release from more than two sites are negligible and not considered here). For the simplicity of the demonstration, we postulate the release sites to be equivalent.

Thus, Eq. (1): $P(\text{tot}) = 0.12 = P(s_1) + P(s_2) + \dots + P(s_{20}) + P(s_1 + s_2) + P(s_1 + s_3) + \dots + P(s_{19} + s_{20}) = 20 P(s) + 190 P(s + s)$

Eq. (2) of second order is: $190 P(s + s) + 20 P(s) - 0.12 = 0$ and its solutions are:

$$P(s)_1 = -0.11095 \text{ and } P(s)_2 = +0.00569$$

Thus, Eq. (3): $P(s + s) = (0.12 - 20 \times 0.00569)/190 = 0.0000316$. In our recordings, the ratio of release from two sites compared to one site is: $(190 \times 0.0000316)/(20 \times 0.0057) = 0.0526$, i.e. 5.26%. In our condition (32 μ M of cadmium) for the analysis of quantal release, 5% of the EPSCs were possibly coming for the concomitant release from two sites.

Immunostaining. WT and PSKO mice were anesthetized with intraperitoneal administration of pentobarbital (50 mg/kg body weight) and were perfused intracardially with 0.9% NaCl for 1 min followed by 4% paraformaldehyde in 0.1 mM PBS for 3 min for imaging experiments. Brain were removed and postfixed in 4% PFA for 8 h. Coronal sections of the hippocampus (50 μ m) were cut on a vibratome (Leica VT1000S). Sections were permeabilized in 0.3% TritonX100 for 2 h at room temperature. Then sections were incubated with primary antibody diluted at 1/500 in PBS-tween for 48 h at 4 °C (APP N-terminal JRD32 mouse antibody is a non-commercial antibody directed against the N-terminal APP-E1 domain⁴⁸, APP C-terminal Y188 rabbit antibody Abcam #32136 or APP C-terminal C1/6.1 mouse antibody BioLegend, BACE1 rabbit antibody D10E5 Cell Signaling #5606; syntaxin1 rabbit antibody Alomone # ANR-002; synaptotagmin7 mouse antibody MABN665, clone N275/14 Millipore; from Synaptic Systems: bassoon rabbit antibody #141013, synaptoporin rabbit antibody #102002, synaptotagmin1 mouse

antibody #105011, synaptotagmin7 rabbit antibody #105173, synaptobrevin2 mouse antibody #104211). After washes in PBS-T, sections were incubated with secondary antibodies for 3 h (coupled to either Alexa 488 or 555 or anti-rabbit Abberior STAR 635P). Slices were incubated during the penultimate wash in DAPI (300 nM) for 10 min before a last PBS wash and mounted in Mowiol-based solution. For low-magnification acquisition, an up-right widefield microscope (Zeiss AxioPhot2) was used with a $\times 2.5$ objective. The camera was a CoolSnap HQ2 (Photometrics, Tucson, USA) controlled by MetaMorph.

Whole brain slice imaging. WT or PS1fl/PS2KO mice were injected with lentivirus encoding ChIEFtom2A-Cre on the right hemisphere and ChIEFtom2A-GFP on the left hemisphere using stereotaxic surgery. After fixation and brain slicing, whole brain sections, fluorescently labelled against synaptotagmin1, synaptotagmin7, or synaptobrevin2 were acquired using the digital slide scanner NanoZoomer 2.0HT (Hamamatsu).

Confocal fluorescence microscopy and image analysis. Confocal acquisition were performed using a Leica SP8 White Light Laser 2 on an inverted stand DMI6000 (Leica Microsystems, Mannheim, Germany) and with a $\times 20$ (NA 0.75) objective. Images of focal planes in the region of the SL were acquired in the red (ChIEFtomato) and far-red (synaptotagmin1, synaptotagmin7, synaptobrevin2, syntaxin1) channels. The settings (laser power, gain, offset) were fixed between genotypes. Images were analyzed using ImageJ. A region of interest (ROI) restricted to the Mf tract was defined in the red channel using the default mode threshold of ImageJ followed by the creation of a selection corresponding to this ROI. Then average pixel intensity was measured in the same ROI but in the far-red channel. The mean pixel intensity of a second area (ROI2) in the *stratum radiatum* (SR), an adjacent region not genetically manipulated was measured. A ROI/ROI2 ratio was performed slice per slice to normalize the data (Fig. 3b). Between 40 and 50 measurements from 6 to 8 mice were acquired per genotype and per presynaptic proteins studied.

Imaging of neuronal cultures and co-localization analysis. Confocal acquisition were performed using a Leica SP8 White Light Laser 2 on an inverted stand DMI6000 (Leica Microsystems, Mannheim, Germany) and with a $63\times$ (NA 1.4) objective. Focal plane images were acquired for different cells in green (APP), red (Synt7), and far-red (MAP2) channels. The settings (laser power, gain, offset) were fixed between cells as well as the pixel size (120 nm). Images were analyzed by using Image J plugin JACoP⁴⁹. Fluorescence was analyzed by excluding the cell body and applying a mean filter (radius = 1 px). For each image acquired the JACoP plugin was used to apply the Costes auto thresholding pixel intensity in each of the channels. Then, Manders coefficient was applied by the plugin to determine the amount of co-localized pixels in each channel, represented as 0 (no co-localization) or 1 (perfect co-localization).

Mf boutons analysis after 3D-stack acquisition. Confocal acquisition was performed using a Leica SP8 described above and with a $\times 63$ (NA 1.40) objectives. Images of the Mf boutons expressing myristoylated YFP in the CA3b-c region of hippocampus were captured with an interval of 0.2 μ m in z-axis and a pixel size of 70 μ m. 200 Mf boutons from five animals per genotype were analyzed. High-resolution 3D stacks were generated using Imaris 7.1.1 software (Bitplane) to characterize and analyze presynaptic terminals. Illustrations correspond to 3D reconstructions smoothened by a median filter.

STED microscopy and image analysis. STED acquisitions were performed on the same microscope using a 775 nm depletion laser and a $\times 100$ (NA 1.40) objective. Images of the Mf bouton were first acquired on the yellow channel (myristoylated YFP) to obtain a mask of the Mf bouton. An intensity threshold was applied to define the region contour of the Mf bouton. Then, a STED image of bassoon puncta was acquired in far-red with a pixel size of 20 nm. To count the number of bassoon clusters per Mf bouton and to measure their surface, images were treated by a segmentation program allowing clusters to be detected as single objects, which were then automatically counted within the contour of Mf boutons. Analyses were performed with Image J software (National Institutes of Health, New York, USA).

Laser-assisted microdissection and RTqPCR. Laser capture microdissection (LCM) was performed using a MicroBeam microdissection system Version 4.8 equipped with a P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). Microdissection was performed at $\times 5$ magnification. Samples were collected in adhesives caps (P.A.L.M. Microlaser Technologies AG, Bernried, Germany).

cDNA were synthesized from total RNA using RevertAid Premium Reverse Transcriptase (Fermentas) and primed with oligo-dT primers (Fermentas) and random primers (Fermentas). qPCR was performed using a LightCycler[®] 480 Real-Time PCR System (Roche, Meylan, France). The PCR data were exported and analyzed with a software (Gene Expression Analysis Software Environment) developed at the NeuroCentre Magendie. For the determination of the reference gene, the Genorm method was used. Relative expression analysis was corrected for

PCR efficiency and normalized against two reference genes: the peptidylprolyl isomerase A (Ppia) and non-POU domain containing, octamer-binding protein (Nono). The relative level of expression was calculated using the comparative ($2^{-\Delta\Delta CT}$) method. Primers sequences are reported in supplementary Table 1.

Cell fractionation in iodixanol gradient. Neuronal cultures were homogenized in buffer A (130 mM KCl, 25 mM NaCl, 25 mM Tris-HCl pH 7.4, 1 mM EGTA, protease inhibitors) and centrifuged at 1000×g, 10 min. The supernatant S1 was then transferred to gradient of iodixanol in buffer A and centrifuged at 122,000 ×g, 40 min. Each fractions was then diluted in buffer A and centrifuged at 126,000 ×g, 40 min. Pellets were suspended in lysis buffer and analyzed by western-blotting.

Cell fractionation to prepare synaptosomes. Synaptosomes were prepared from mouse brain following a published protocol⁵⁰. Briefly, tissue samples were homogenized by 12 strokes at 900 rpm with a glass/Teflon potter in 2 mL of ice cold isosmolar buffer (320 mM sucrose, 4 mM HEPES, pH 7.4). Then, the homogenate was centrifuged at 1000×g for 8 min at 4 °C. The supernatant S1 was then centrifuged at 12,500×g for 13 min at 4 °C. The pellet P2 was resuspended in isosmolar buffer and laid on top of a discontinuous sucrose gradient (composed of one phase at 0.8 M sucrose, 4 mM HEPES pH 7.4 and one phase of 1.2 M sucrose, 4 mM HEPES pH 7.4). The sample was centrifuged at 50,000×g for 1 h 10 min at 4 °C. The synaptosomal fraction at the interface of the two layers of sucrose was collected with a 1 mL syringe and a 25 G needle by piercing the tube wall. The sample was resuspended in lysis buffer, compared to homogenate and subjected to immunoprecipitation.

Co-immunoprecipitation and western-blotting. Analysis of the expression of the different polypeptides expressed by lentiviral constructs was tested by transfecting HEK293 cells (from ATCC) with the non-liposomal reagent X-tremeGENE (Roche). Cells were scraped and lysed 2 days after transfection. Analysis of the expression level and the interaction of endogenous neuronal proteins were made from lysates of cortical neurons cultures for 9 DIV in Neurobasal (Gibco) and then up to 14DIV in BrainPhys. The γ -secretase inhibitor (L685,458) was applied from 9 DIV up to lysis. Cells were lysed in a dodecylmaltoside-based lysis-buffer (Hepes 50 mM, pH 7.4; NaCl 100 mM, Glycerol 10%, DDM 0.5%). Immunoprecipitation was performed using an antibody against Syt7 on 600–1000 μ g of cell lysates. Immunoglobulins–proteins complexes were pull down by addition of magnetic beads (surebeads) and magnets. Samples were completed with Laemmli buffer. Proteins in samples were separated by western-blotting using 4–20% gradient Tris–tricine gels. Membranes were probed for the proteins of interest and chemoluminescent bands detected with a CCD camera (ChemiDoc—BioRad). Full-length blots can be found in Supplementary Fig. 8.

Statistical analysis and graphical representations. Statistical analyses were performed with Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Values were first tested for normality (D'Agostino and Pearson omnibus test). Data were presented as scatter plots with mean or median according to the result of the normality test. Normally distributed data were compared using a *t*-test (two-sided) while Mann–Whitney rank test (two-sided) was used for non-normal data set. Analyses involving two factors (genotype, stimulus number) were presented as points with error bars ($\pm$ SEM) and non-linear fits. Statistical differences were tested using two-way ANOVAs. Cumulative frequency distributions were presented as fractions and tested using a Mann–Whitney rank test. Statistical differences were considered as significant at $p < 0.05$.

Drugs. All drugs were obtained from Tocris biosciences or Sigma-Aldrich.

Data availability

All data are available from the authors upon request.

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Author contributions

G.B., T.J.S., J.R.-M. and F.B. conducted the experiments, G.B., U.M., and C.M. designed the experiments and wrote the manuscript.

Additional information

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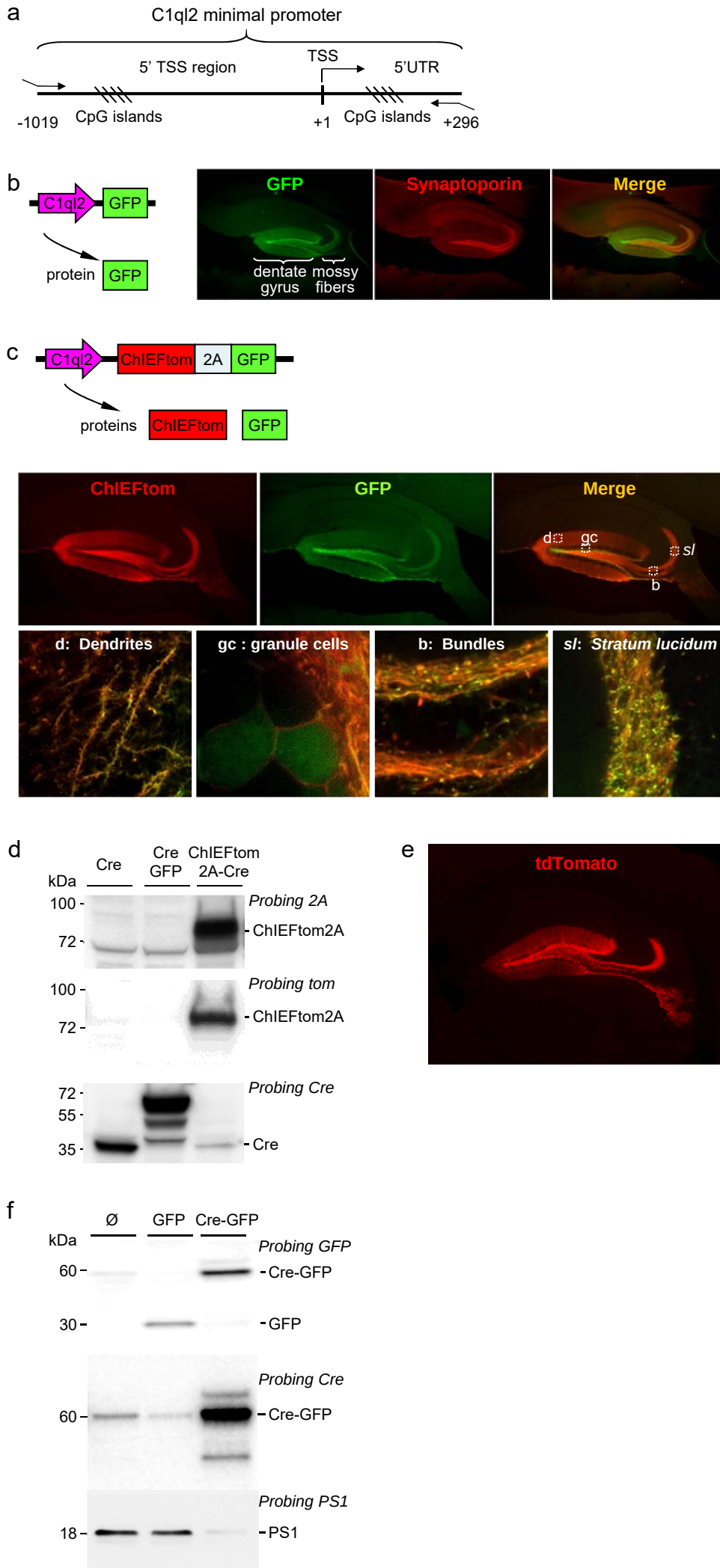
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Supplementary information

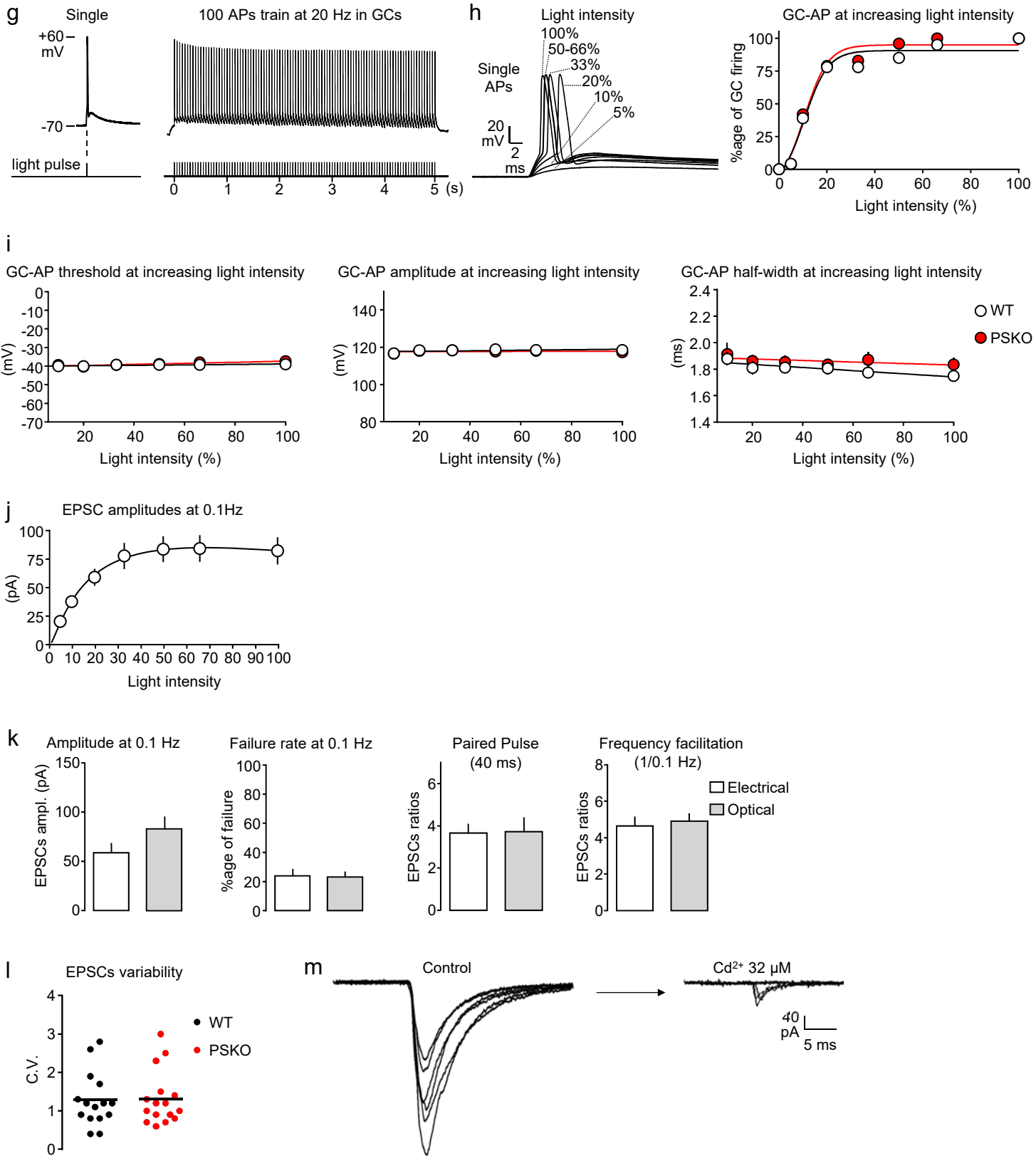
Presenilin-mediated cleavage of APP regulates
synaptotagmin-7 and presynaptic plasticity

Barthet *et al.*

Supplementary figure 1

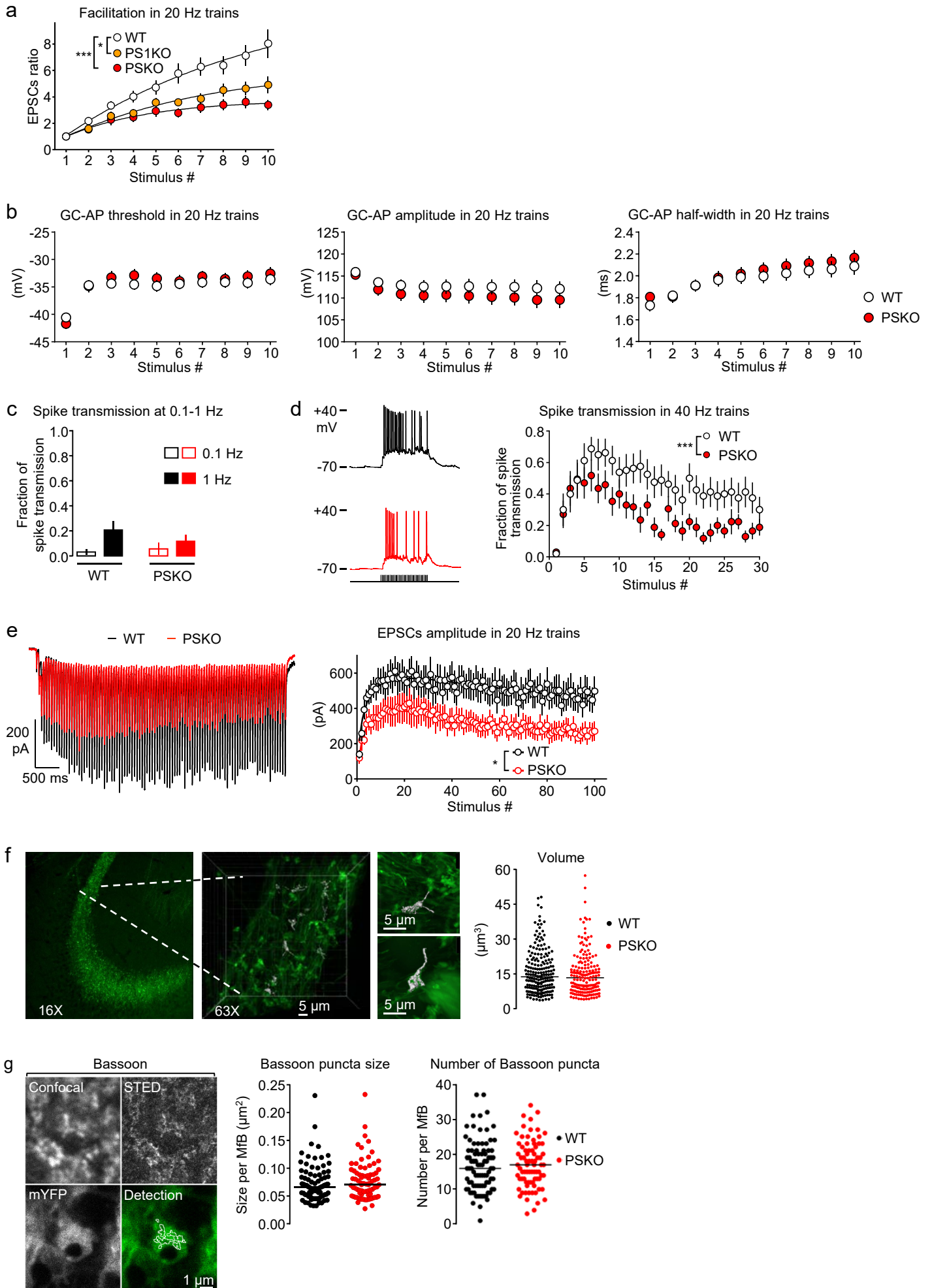


Supplementary figure 1



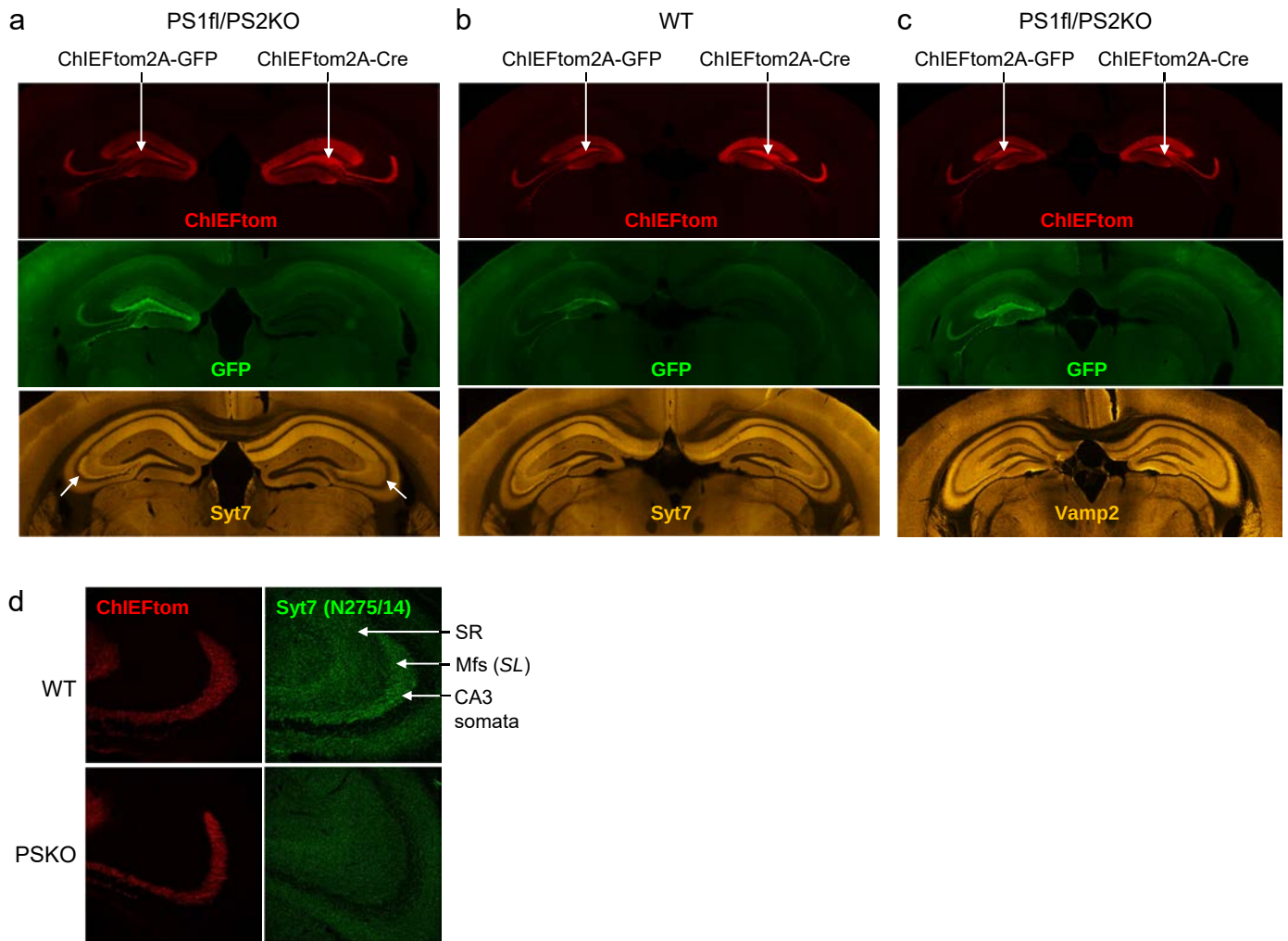
Supplementary figure 1 Combined DG-specific gene deletion with light activation. **a** Schematic representation of the mouse C1ql2 minimal promoter cloned from genomic DNA. The 1315 bp sequence includes a region proximal to the transcription start site (5' TSS) and part of the 5' untranslated region of the C1ql2 mRNA (5' UTR). The sequence is rich in putative transcription factor binding sites (CpG islands). **b** Expression of GFP controlled by the C1ql2 promoter targets mature Mfs labelled by an antibody against synaptoporin. **c** *Top panels*, scheme of a bicistronic DNA construct that allows the co-expression of ChIEFtom and GFP under the control of the C1ql2 promoter. *Middle panels*, pictures showing the co-expression of ChIEFtom and GFP in the DG. Small squares indicated in the merge panel are presented enlarged in the lower row of panels. *Lower panels*, images acquired using a confocal microscope with a 20x or 60x objective showing that the two proteins ChIEFtom and GFP are well expressed in all cell compartments (dendrites, GCs soma, Mf bundles, Mf boutons in the stratum lucidum (SL). The fluorescence of mTomato fused to membrane bound Chr2 is prominent in dendrites and fibers whereas the fluorescence of cytosolic GFP is prominent in soma. The two polypeptides are well separated as shown by the expression of the membrane-bound ChIEFtom at the GC cell surface and of the free GFP in the cytosol (panel "gc: granule cells). However, infected neurons do not display any sign of degeneration, toxicity or aggregates of Chr2. **d** Western blots of ChIEFtom2A-Cre constructs (right) showing that ChIEFtom is fused to the remaining 2A peptide while Cre recombinase is free. **e** Expression of Cre recombinase under the control of the C1ql2 promoter allows the recombination of floxed-tdTomato gene in Ai9 mouse line leading to the expression of free cytosolic tdTomato in GC soma. **f** Western blots of samples from neuronal cultures prepared from PS1floxed embryos. Neurons were non-infected ($\emptyset$), infected with GFP- or with CreGFP-expressing viruses. Cre prevents most of PS1 protein expression. **g** Electrophysiological properties of ChIEF-expressing GCs studied in the whole-cell current clamp mode. A single action potential (AP) is evoked by a light pulse at 470 nm of 1 ms. A long burst of 100 stimuli at 20 Hz for 5 s triggers a long train of APs without failure. **h** Representative traces of single AP evoked in GC at increasing light intensity, and plot of the percentage of GC firing in function of light intensity. 1 ms pulses of 470 nm light were shined on top of GC using a 63x objective. Light intensity was increased up to 2 mW/cm² (100%). **i** Properties of the GCs spikes in function of the intensity of light pulses. Plots of the threshold, amplitude and half-width of the GC action potential (GC-AP) are depicted. **j** Plot of EPSC amplitudes in function of light intensity recorded from 20 CA3 pyramidal cells. 1 ms pulses of 470 nm light were shinned on top of GC dendrites using a 63x objective. Light intensity was increased up to 2 mW/cm². **k** Comparison of averaged values of Mf-EPSC amplitude, failure rate, paired-pulse and frequency facilitation recorded in CA3 pyramidal cells when stimulating Mfs with an extracellular electrode placed in the hilus or by stimulating GC dendrites with light. **l** Coefficient of variability (C.V.) of the amplitude of Mf-CA3 EPSCs recorded at 0.1 Hz in WT and PSKO conditions. **m** Representative Mf-EPSCs recorded in CA3 pyramidal cells in control conditions or in presence of 32 μ M Cadmium²⁺ (Cd²⁺). The presence of Cd²⁺ dramatically increases the failure rate and decreases the amplitude of the EPSCs. Errors bars represent s.e.m.

Supplementary figure 2



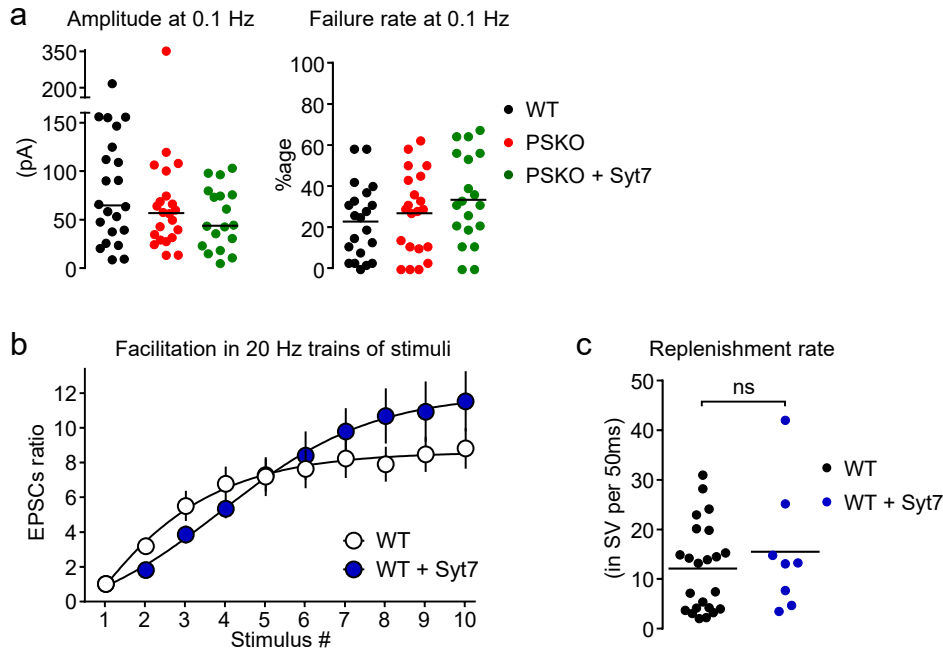
Supplementary figure 2 Electrophysiological properties of GCs, Mf-CA3 neurotransmission and Mf boutons morphology. **a** Analysis of Mf-EPSCs recorded in CA3 pyramidal cells while stimulating with ten repetitive light pulses at 20 Hz in WT, PS1KO and PS1/PS2KO conditions. Facilitation is represented by the ratio of EPSC amplitudes relative to the amplitude of the EPSC evoked by the first stimulus. Note that facilitation is impaired in the absence of PS1 alone. Two-way ANOVA, $p=0.0304$. **b** Properties of GCs spikes evoked by 10 light pulses of 1 ms at 20 Hz on GCs in WT and PSKO conditions. Plots of the threshold, amplitude and half-width of the GC action potential (GC-AP) are depicted. **c** Histograms of the mean fraction of spike transmitted in CA3 pyramidal cells recorded in the current-clamp mode at 0.1 or 1 Hz in WT and PSKO conditions. Note that in average the fraction of spike transmitted is low in all conditions even if a few neurons do fire quite reliably at 1 Hz. **d** Representative traces of APs in CA3 pyramidal cells in response to the stimulation of GC dendrites at 40 Hz in WT (black) and PSKO (red) conditions. Plot of the fractions of spike transmission (for 16 recorded neurons per genotype; 5 sweeps per neuron) in WT or PSKO genotype is shown. Note that at 40 Hz the highest rate of transmission is reached around 5 stimuli and that the plot in PSKO is lower than in WT condition (Two-way ANOVA, $p<0.0001$). **e** Representative Mf EPSCs obtained by illuminating the dendrites of GCs with 100 stimuli at 20 Hz. After the peak reached within ten stimuli, the amplitudes of Mf-EPSCs decrease while the charge of the train increases. The raw amplitude of Mf-EPSCs is plotted against the stimulus number. Note that Mf-EPSC amplitudes in PSKO condition are lower than in WT. Two-way ANOVA, $n=20/\text{genotype}$, $p<0.05$. **f** Confocal images of the *stratum lucidum* region of the hippocampus in brain slices where the Mfs projects onto CA3 pyramidal cells. A zoom in using a 63x objective allows to observe Mf boutons. 3D reconstruction of some Mf boutons after acquisition of Z-stack are depicted in grey. A zoom on two large Mf boutons allows to appreciate their large volume and complex structure. The scatter plots with medians of the volume of Mf boutons in WT and PSKO conditions are represented. **g** Evaluation of Bassoon puncta number and surface using STED microscopy. A representative image of a Mf bouton labelled with membrane-bound YFP (mYFP, bottom left panel) and stained against Bassoon (upper left panel in confocal; upper right panel in STED microscopy) is shown. Detected Bassoon positive puncta are delimited with white lines. Scatter plots of average size and the number of Bassoon puncta per Mf bouton are depicted. 76 Mf boutons have been analyzed per genotype. The absence of PS does not impact on the number of putative releasing sites. Errors bars represent s.e.m.

Supplementary figure 3



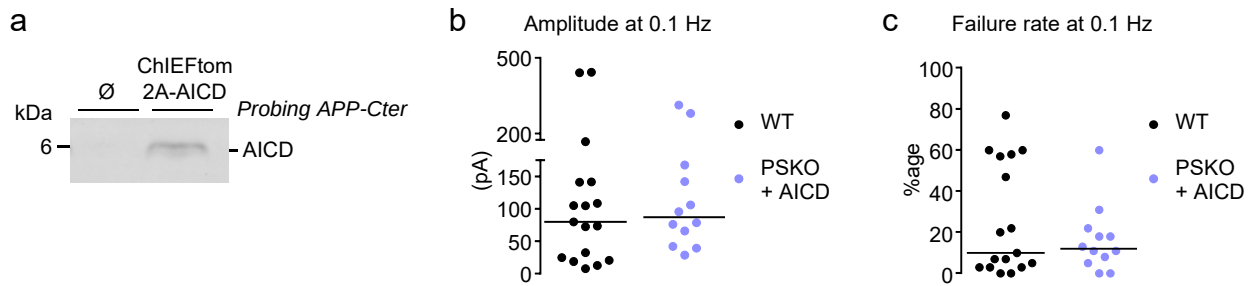
Supplementary figure 3 Effect of targeted deletion of PS in GCs on Syt7 and Vamp2 expression. **a-c** Pictures of coronal sections from mice injected in the left hemisphere with a LV expressing ChIEFtom and GFP and on the right hemisphere with a LV expressing ChIEFtom and Cre. **a**, in PS1fl/PS2KO mice, a decrease of Syt7 staining is observed only in the SL where PS1 has been removed (compare the two oblique arrows). In WT condition stained for Syt7 (**b**) or in PS1fl/PS2KO mice stained for Vamp2 (**c**), no difference is observed between the two hemispheres. Note that the absence of PS2 alone does not noticeably affect the staining of Syt7 compared to WT (compare the left hemispheres of WT and PS1fl/PS2KO conditions). **d** Confocal images of the *stratum lucidum* (SL) where the Mfs can be distinguished in the red channel (ChIEFtom) and stainings of Syt7 depicted in green. The Syt7 staining in PSKO Mfs was negligible in comparison to WT and indicates that PS supports the expression of Syt7.

Supplementary figure 4



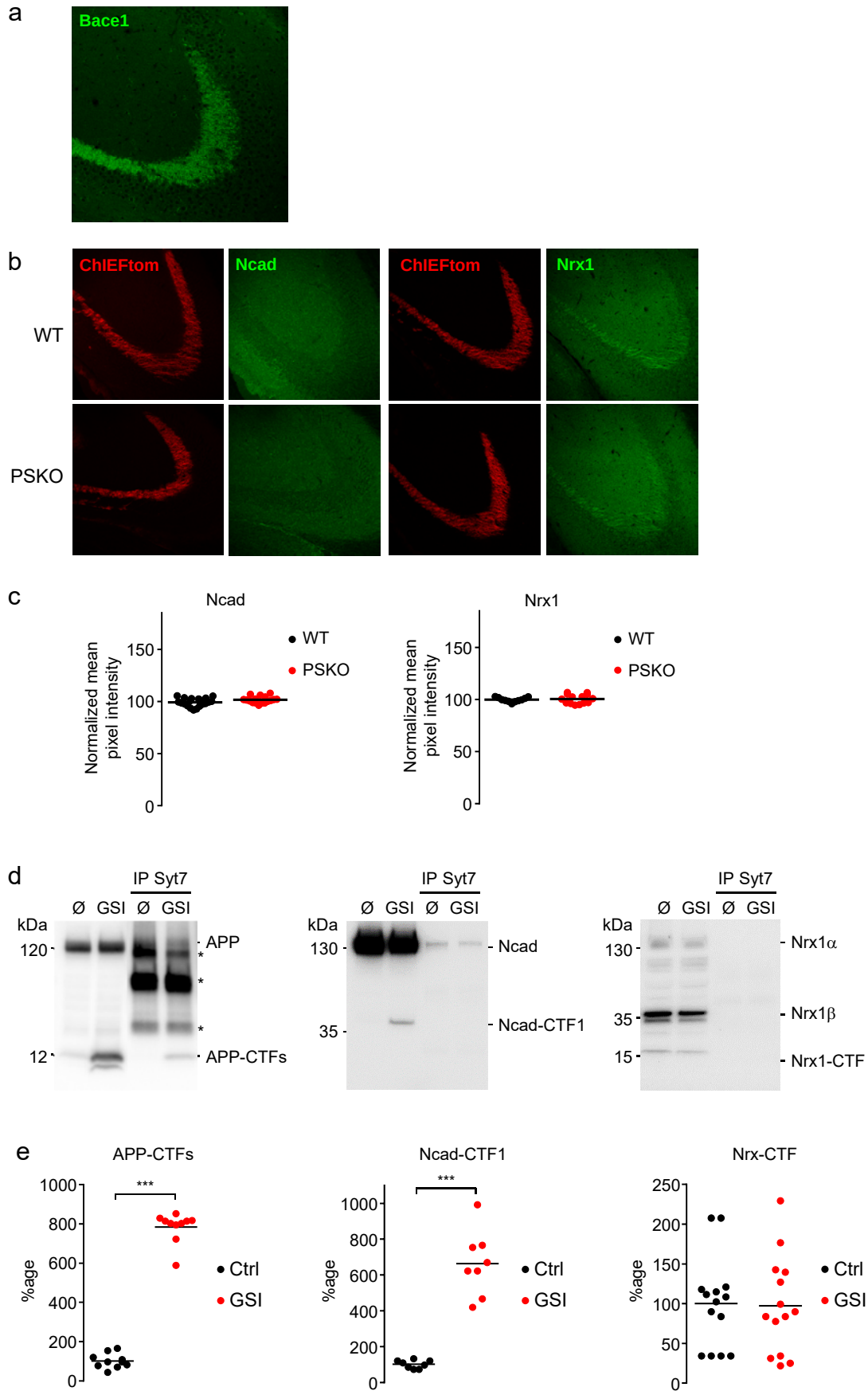
Supplementary figure 4 Basal transmission at PSKO Mf-CA3 synapses rescued for Syt7. **a** Basal transmission at Mf-CA3 synapses in WT, PSKO and PSKO+Syt7 conditions. Scatter plots with medians of the amplitude, and means of the failure rate of EPSCs recorded in CA3 neurons at 0.1 Hz. **b** Analysis of presynaptic facilitation in 20 Hz trains. Facilitation is represented by the amplitude of the EPSCs normalized to the amplitude of the first EPSC. The over-expression of Syt7 in WT does not significantly alter presynaptic facilitation. n=24 in WT, 14 in WT+Syt7. **c** Scatter plots with means of replenishment rates at 20 Hz calculated neuron per neuron are represented by genotype. The replenishment rate of WT+Syt7 condition does not differ from the WT condition. Errors bars represent s.e.m.

Supplementary figure 5



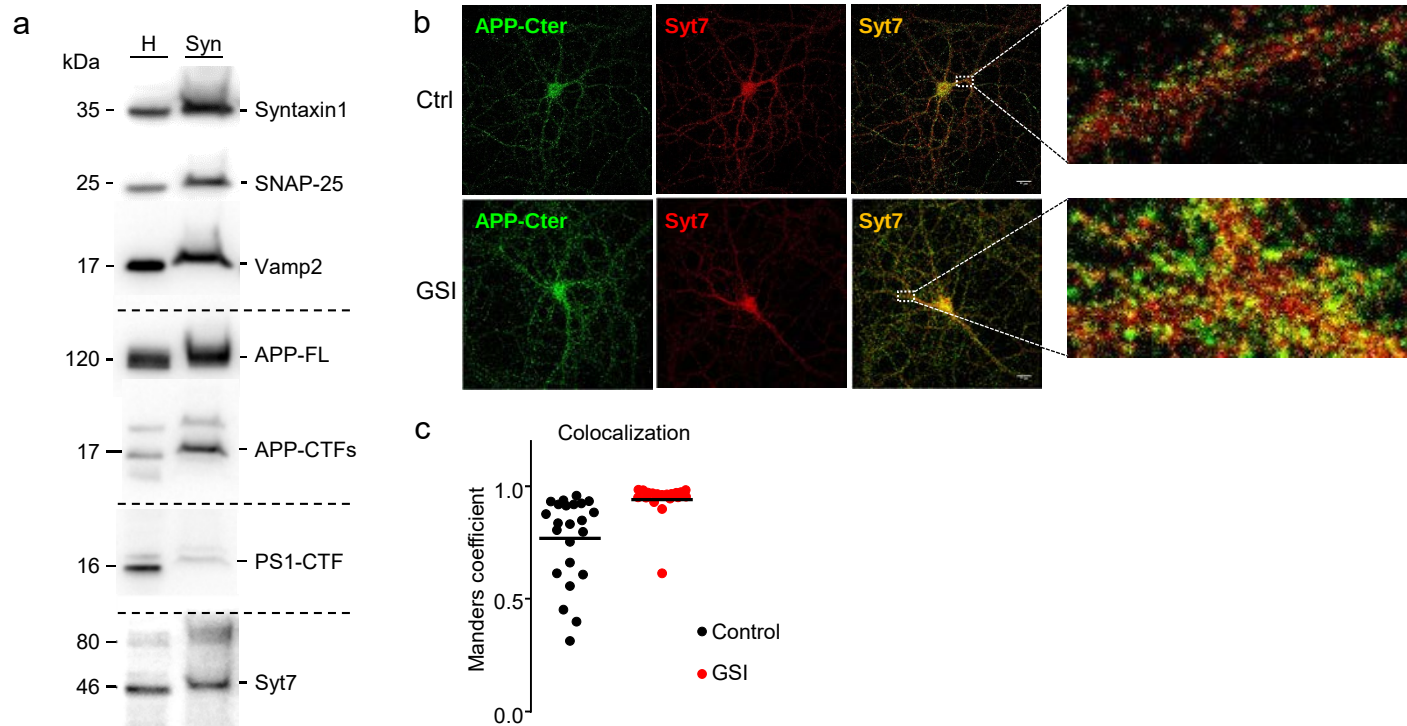
Supplementary figure 5 Basal transmission at PSKO Mf-CA3 synapses with rescue of AICD. **a** Western blots of (LoxStopLox)-ChIEFtom2A-AICD construct (right) showing the expression of AICD at the protein level. **b** Basal transmission at Mf-CA3 synapses in WT and PSKO with re-expression of AICD. Scatter plots with medians of EPSCs amplitude recorded in CA3 pyramidal cells in the whole cell voltage-clamp mode while stimulating GCs with light at 0.1 Hz. **c** Scatter plots with mean failure rates in conditions as in (**a**).

Supplementary figure 6



Supplementary figure 6 Expression of γ -secretase substrate candidates in Mfs and accumulation of C-terminal stubs upon γ -secretase inhibition. **a** Confocal images of the Mfs tracts in WT and PSKO conditions observable in the red channel (ChIEFtom) and green channel (staining for Ncadherin Cterminal (Ncad) or Neurexin Cterminal (Nrx1)). Mfs are faintly labelled by the Ncad antibody and more prominently by the Nrx1 antibody. The absence of PS does not alter labelling with these two antibodies. **b** Scatter plots of the mean pixel intensity in the SL normalized to the mean pixel intensity in the *stratum radiatum* for Ncad and Nrx1 labelling. **c** The absence of PS does not increase the intensity of the labelling indicating that Cterminal stubs of Ncad or Nrx1 do not accumulate in Mfs. **d** Western-blot of protein extracts from neuronal cultures treated or not with a γ -secretase inhibitor (GSI). GSI induces the accumulation of APP-CTFs and Ncad Cterminal stub (Ncad-CTF1) but not of the band expected to be Neurexin1-CTF (16 kDa). IP of Syt7 pull down APP-FL, APP-CTFs and Ncad but not Ncad-CTF1 or Neurexin1 of any form. **e** Quantitative analysis of the CTF bands detected in western blot of starting materials from neuronal cultures treated or not with GSI as in (d). An increase of 800% in the expression of APP-CTFs was detected. An increase of 650% in the expression of Ncad-CTF was detected. The expression level of Neurexin-CTF was unchanged by treatment with GSI.

Supplementary figure 7



Supplementary figure 7 Characterization of synaptosomal protein pattern and colocalization between Syt7 and APP in neurons. **a** Characterization by WB probing of synaptosomal protein lysate (Syn) versus protein lysate from the crude homogenate (H) of adult mouse brain. 'Syn' is characterized by an enrichment in Syntaxin1a and SNAP-25. While PS1 is detectable, APP (both FL and CTF) and Syt7 are abundant in 'Syn'. **b** Confocal images of APP-Cter (green channel) and Syt7 (red channel) in neuronal cultures treated (GSI) or not (Ctrl) with GSI. Zoom-in representations are depicted in the right. APP colocalizes with Syt7 in both conditions. **c** The extent of colocalization of images as in (**b**) is assessed using the Manders coefficient (see Methods). The colocalization is higher in neurons treated with GSI.

Supplementary figure 8

Supplementary figure 8 Original blots shown in full molecular weight range.

Figure 3d (Syt7)

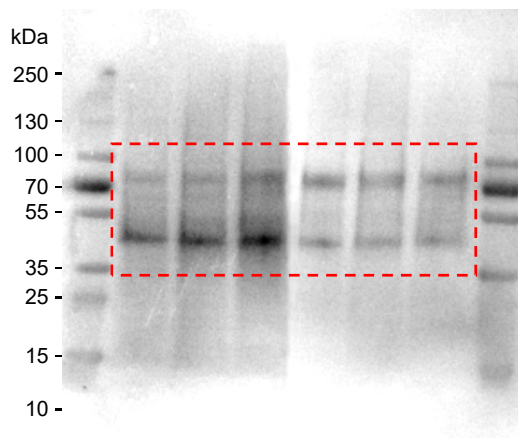


Figure 5a (Syt7)

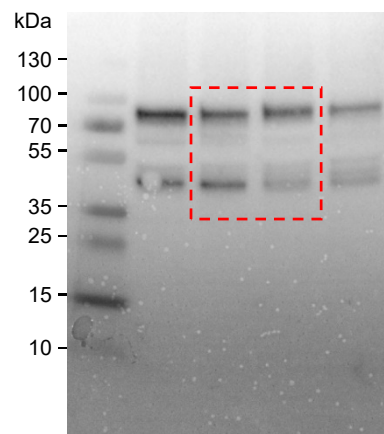


Figure 5b (Vamp2)

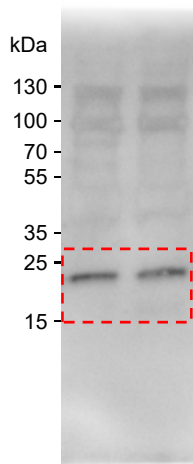


Figure 5b (Syt1)

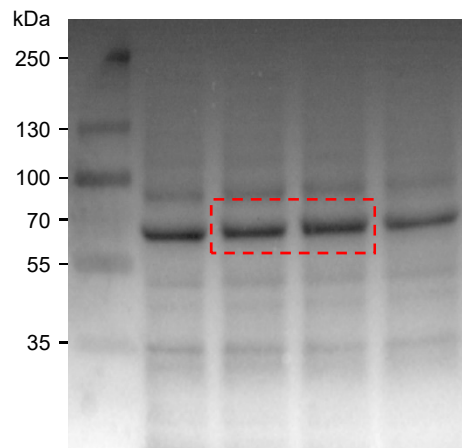


Figure 5b (Syntaxin1)

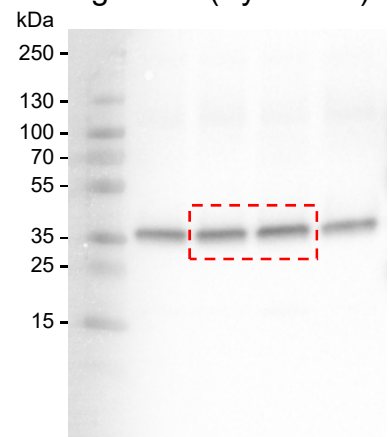


Figure 6e (APP-FL and CTFs)

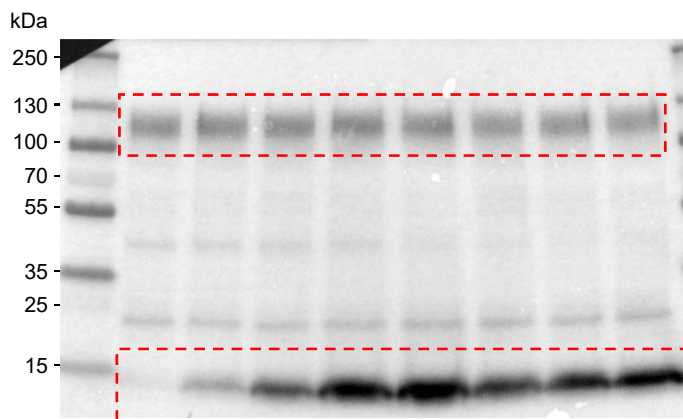
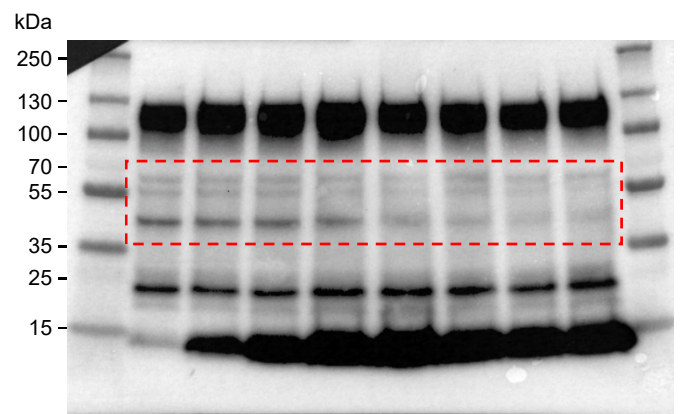


Figure 6e (Syt7)



Supplementary figure 8

Figure 7d (APP-FL)

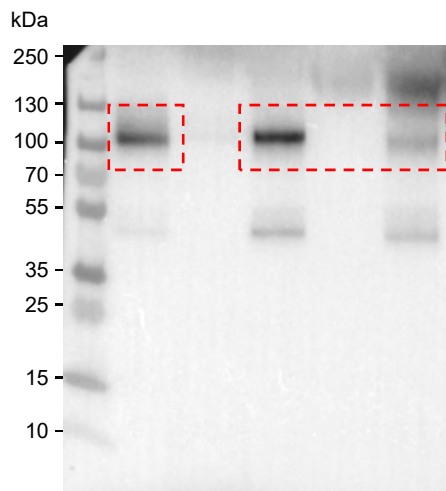
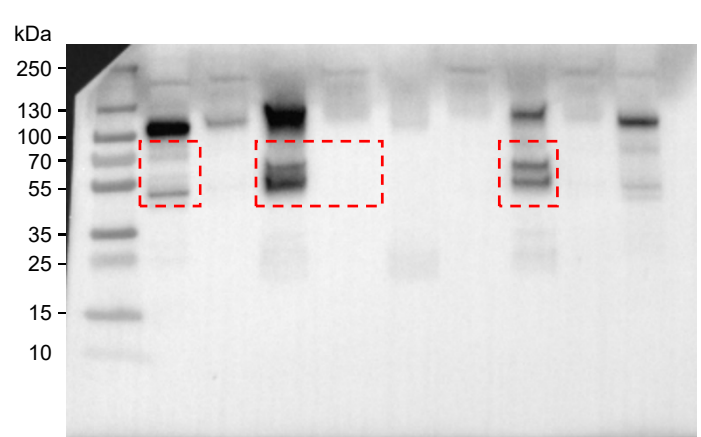
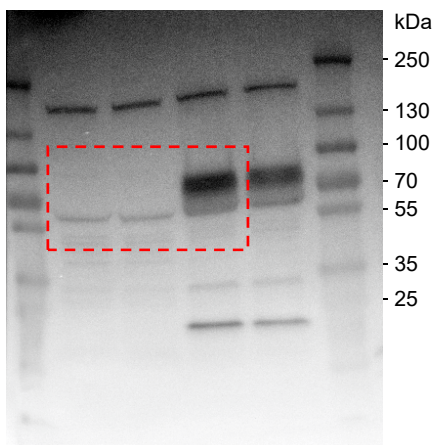


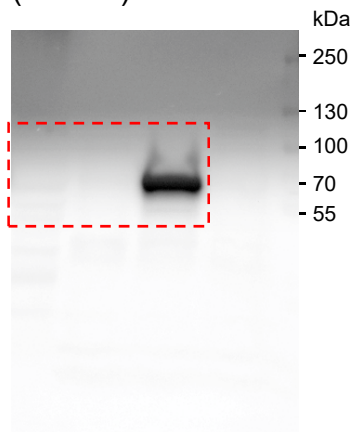
Figure 7d (Syt7)



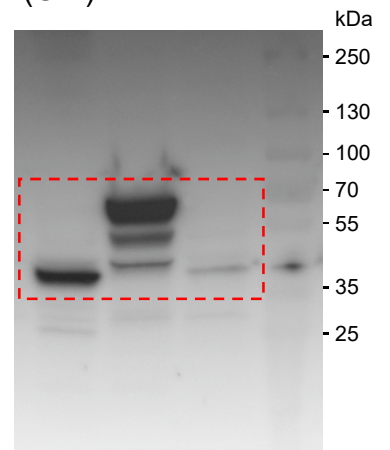
Supplementary figure 1d
(2A)



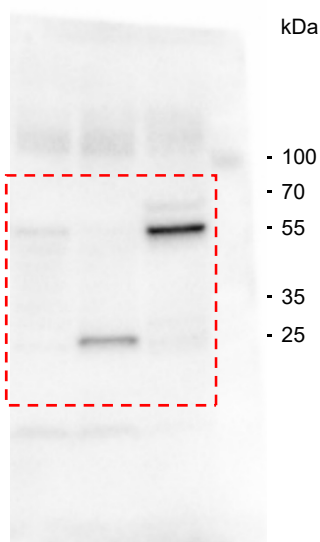
Supplementary figure 1d
(tomato)



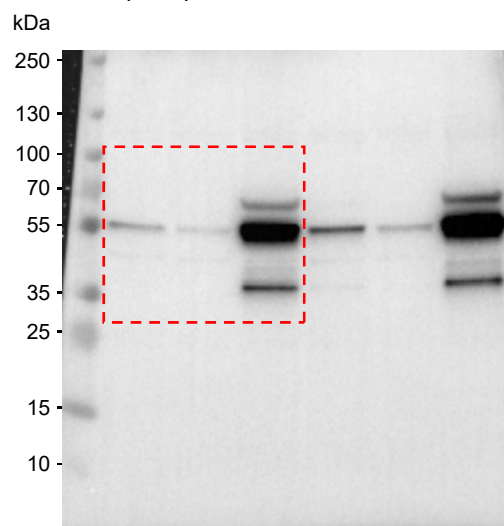
Supplementary figure 1d
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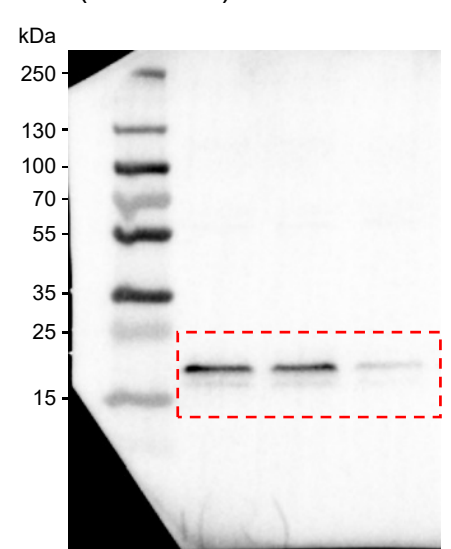
Supplementary figure 1f
(GFP)



Supplementary figure 1f
(Cre)

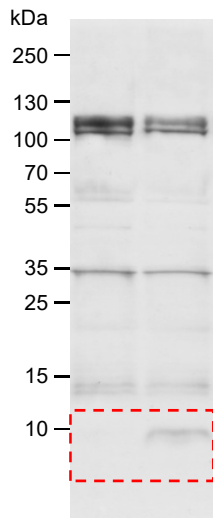


Supplementary figure 1f
(PS1-CTF)

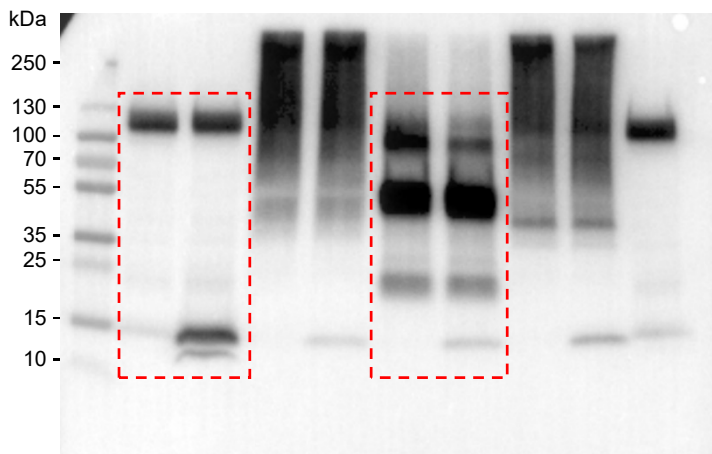


Supplementary figure 8

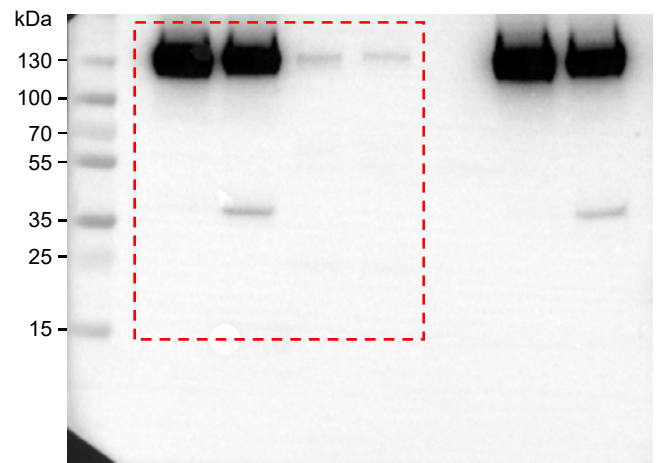
Supplementary figure 5a (APP, AICD)



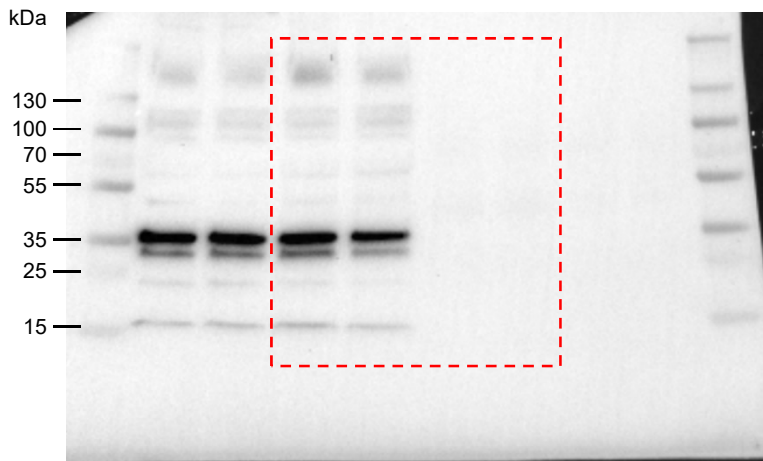
Supplementary figure 6d (APP, AICD)



Supplementary figure 6d (Ncad)

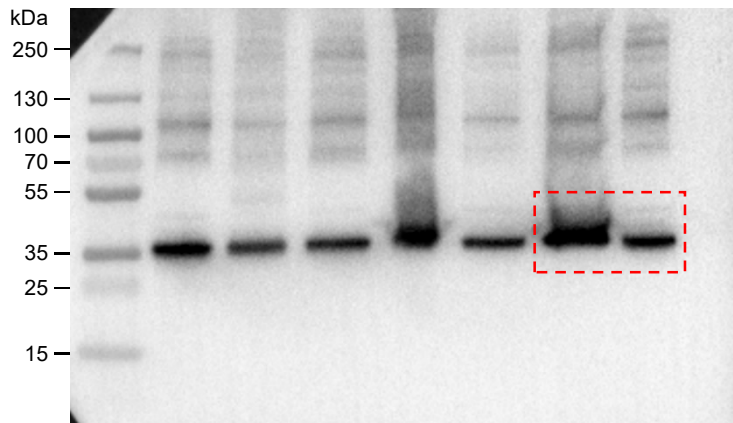


Supplementary figure 6d (Nrx)

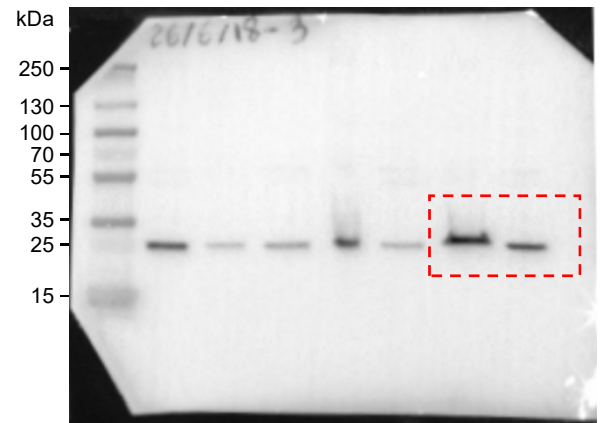


Supplementary figure 8

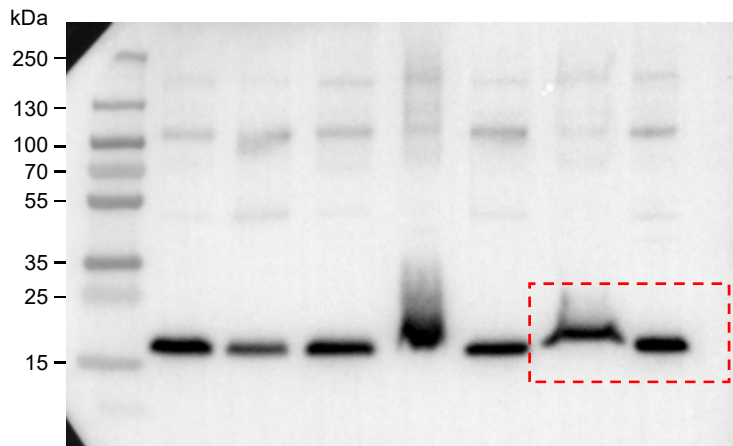
Supplementary figure 7a (Syntaxin)



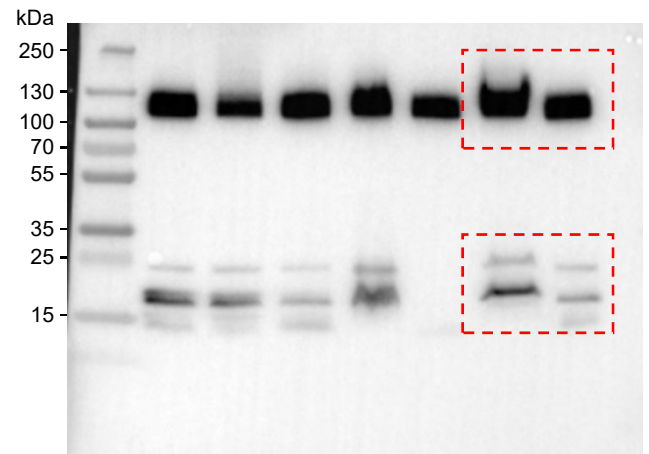
Supplementary figure 7a (SNAP25)



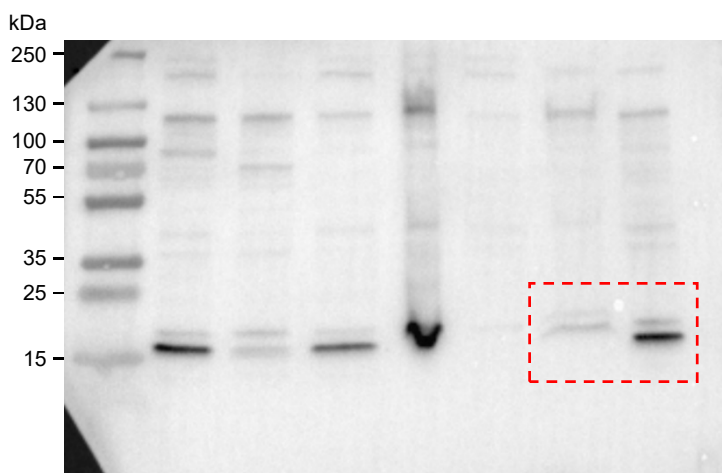
Supplementary figure 7a (Vamp2)



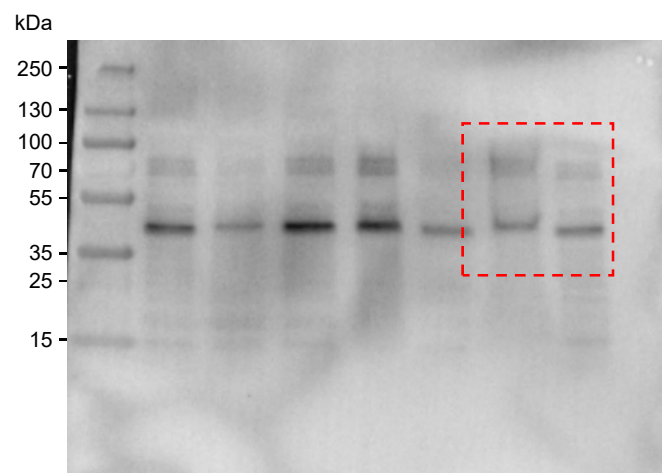
Supplementary figure 7a (APP)



Supplementary figure 7a (PS1-CTF)



Supplementary figure 7a (Syt7)



1.3. Results in human tissue

The results obtained in AD mouse models encouraged us to continue our research in the human AD brain. Therefore, by using FAD (PS1 mutations) and SAD patients we assessed the impact of synaptic proteins such as Syt7. For that purpose, we performed immunofluorescence stainings to analyze the pixel intensity in 6 different regions of the hippocampus including ML, CA4, CA3/CA2, CA1, subiculum and EC (Fig. 1A, B). We also complemented these results by using western blotting (WB) and evaluated the differences in protein concentration in frontal cortex homogenates. Interestingly, the quantifications show important insights in the abundance of Syt7.

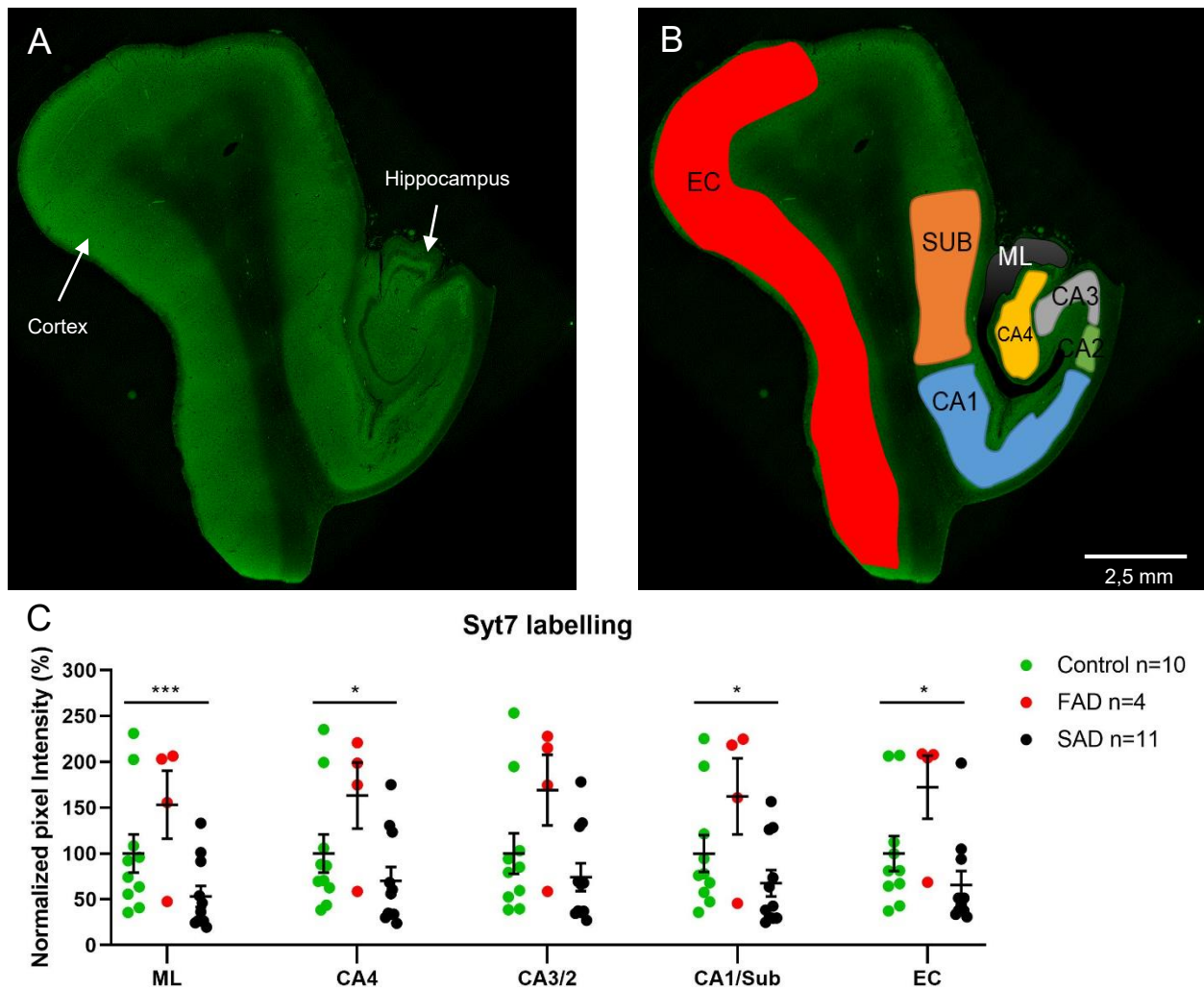


Figure 1 – Representation of a human hippocampus and para-hippocampal cortex. A) Human brain section labelled for Syt7. Cortex and hippocampus are indicated. B) Hippocampal subregions and entorhinal cortex (EC) are depicted on top of a human brain section as in 'A'. C) Quantifications of Syt7 in regions depicted in 'B'. ML, CA4, CA1 and EC were less stained for Syt7 in SAD condition compared to control (2-way ANOVA).

Syt7 was notably present in human slices showing a clear enrichment in regions where synapses are predominantly present such as the ML or the pyramidal cell layers (Fig. 1A). Similar to the

PSKO mouse model, Syt7 had a general decrease in the entire slice. The analysis performed in different regions (Fig. 1B) proved a significant decrease of Syt7 ($30\% \pm 50\%$) in the SAD patients for all the regions except for CA3 (Fig. 1C). One of the regions with the highest differences was the ML, a region of special interest that focused our research in chapter 4 of this thesis. In addition, although the FAD patients did not show any significant differences, the number of patients was insufficient to have any strong conclusions.

We also performed experiments in brain homogenates from the frontal cortex of 4 FAD patients and 4 control patients (Table 1). The aim of this experiment was to investigate if any of the Syt7 splice variants were affected by mutations in the PS1 gene. Interestingly, besides the six major isoforms observed between 100 and 40 kD, the Syt7 antibody detected 3 more bands between 31 and 12 kD (Fig. 2A). These bands were only found in the human brain homogenates since we did not detect them in mouse. Despite the fact that we still ignore the specificity and nature of these new peptides, in FAD patients we observed a higher Syt7 intensity of the 24 and 12 kD bands when compared to the controls (Fig. 2B).

FAD case number	Gender	Braak stage	Age of onset	Age of death	APOE	PMD (h)
Control 1	M	-	-	80	3:4	16
Control 2	M	-	-	69	3:3	22
Control 3	M	-	-	71	3:3	12
Control 4	M	-	-	77	2:4	22
PS1-I143T	M	VI	37	45	3:3	-
PS1-I143T	F	VI	36	43	3:3	-
PS1-H163Y	M	V	55	63	2:4	5
PS1-H163Y	M	V	47	68	3:4	-

Table 1 – Clinical information from patients used in WB. PMD (Postmortem delay).

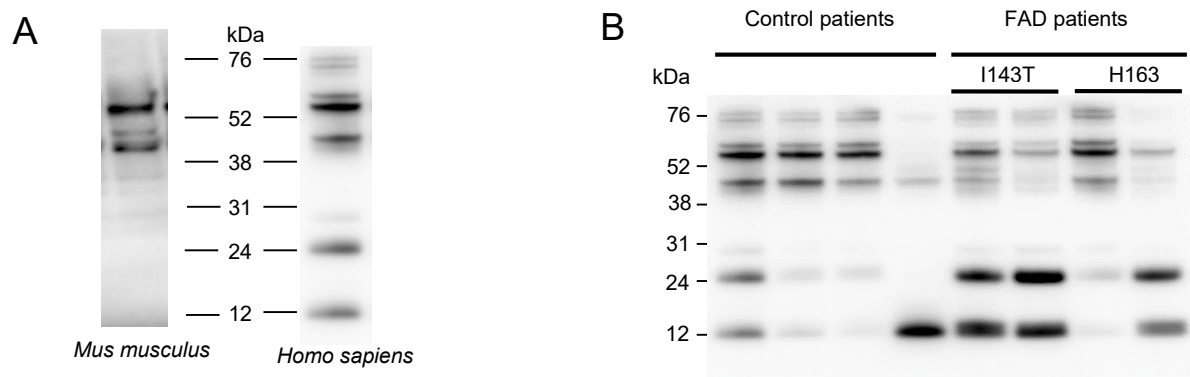


Figure 2 – WB probing Syt7 in mouse and human. A) Comparison of the different splice variants in mouse and human. Humans have 3 extra bands between the 31kD and 12kD. B) WB comparing Syt7 splice variants in control and FAD patients with different PS1 mutations.

1.4. Discussion and perspectives

The regulation of Syt7 and synaptic transmission by PS and APP

The investigation of AD related genes and their protein mechanisms are fundamental to understand the disease etiology. In this chapter we combined the use of different methods to study the presynaptic molecular functions of APP and PS, two ubiquitously expressed proteins present in the synapses of neurons^{118,119,120}. The absence of the γ -secretase activity prevents the production of certain APP fragments like A β , AICD or the p3 fragment, leading to the increase of CTFs and sAPP substrates (Fig 6-Introduction). These peptides play specific roles in synapses, implying that the regulation and quantity of these peptides might have direct consequences on the synaptic pathology. As an example, the AICD fragment has been shown to function as a transcription factor, which regulates gene expression^{198,476}. However, this was not the case for the Syt7 gene, in which mRNA levels were not changed under PSKO conditions and thus not caused by deficits of the AICD fragment. This result together with Syt7 rescue demonstrated that synaptic impairments were post-translationally induced by a molecular cascade involving APP.

The lack of the catalytic subunit of the γ -secretase complex in the GCL increased the levels of APP-CTFs in MF. Thus, APP-CTFs and Syt7 showed an inverse correlation, which was further studied by proving evidence of their interaction with immunoprecipitation and WB. Accordingly, the APP/PS1 KO model prevented Syt7 decreased levels, demonstrating the direct role of APP-CTFs in the molecular mechanism. Since, high levels of APP- β CTF fragment have already been involved in the cytotoxic pathology of AD^{477,478}, it is tempting to speculate how this fragment might trigger the axonal and synaptic degradation of Syt7. Curiously, the absence of PS affects the cellular location of certain proteins like APP, which can switch between the intracellular space

and the cell surface⁴⁸⁹. These changes might also affect Syt7 location from vesicles and plasma membrane to other subcellular compartments where it gets degraded. However, the excess of APP-CTFs might function as a molecular trigger to arrest and deplete Syt7 in organelles like late endosomes or lysosomes, where it can also be found. Another relevant aspect is the cellular location where Syt7 degradation occurs. Although somatic degradation is a possibility, the axonal transport of lysosomes to synapses has been reported, suggesting that Syt7 might be degraded *in situ* in axons or synapses. Importantly, anterograde kinesin mediated transport requires APP and its secretases^{163,164}, which might control the arrival of lysosomes or proteins responsible for Syt7 degradation. Further research will be needed to assess if APP and its fragments are involved in the transport of such cargoes and its effect on synaptic protein regulation.

PS deletion at the GCL of the hippocampus did not affect the levels of other synaptic proteins fundamental for vesicle release in synapses (SV proteins (Syt1 and VAMP2) or plasma membrane proteins (Syntaxin1a and Bassoon)), demonstrating that Syt7 diminishment was not due to a general presynaptic degeneration. Consistent with this data, under basal conditions no changes were found in the release probability and quantal amplitude between the WT and the PSKO. However, the short-term plasticity impairment observed in the 20 and 40Hz long and short train facilitation indicated an impairment in the replenishment rate of SV. Previous research has shown the presence of Syt7 close to the plasma membrane⁴⁷⁹ or in an internal tubular membrane network of different endosomal compartments at the active zone⁴⁸⁰.

Although it is still unknown how each of the Syt7 splice variants function, it has been demonstrated that a truncation mutant speeds-up vesicle recycling⁴⁸¹. Our research supports this data, demonstrating that the diminishment of a short (46kDa) variant is sufficient to slow down vesicle recycling and replenishment of the pool. Interestingly, other publications have shown the implication of Syt7 in the asynchronous release of vesicles⁴⁸³ by the C2 domains but also in the maintenance of the readily releasable pool together with Syt1⁴⁸².

Our results on PS, APP and Syt7 reveal some of the physiological functions of these proteins at the presynapse. However, the results obtained here suggest that these proteins might be relevant in the synaptic pathophysiology of AD. Certain mutations in the genes of PS and APP can cause the early onset form of AD, inducing an increase in the levels of APP CTFs. Therefore, this project highlights the relevance of AD related proteins in the regulation of synaptic proteins that could be important in synaptic degeneration. For this reason, we pursued other studies on synaptic protein degeneration in the human AD hippocampus.

Impairments of Syt7 in human patients

In this chapter we also studied Syt7 as a potential new biomarker for synaptic degeneration in human AD patients. Interestingly, in recent years, different studies have identified Syt7 as a relevant synaptic marker involved in the pathophysiology of other types of diseases. As an example, Syt7 KO animals have shown to suffer from bipolar-like behavioral abnormalities such as mood cycling symptoms⁴⁸⁴. In addition, bipolar patients have lower levels of Syt7 mRNA in plasma samples, suggesting that it could be an important factor for bipolar symptoms⁴⁸⁴. Another study identified an upregulation of Syt7 microRNA (miRNA) in gray matter regions of multiple sclerosis patients⁴⁸⁵. These patients exhibited a misdistribution of Syt7, characterized by an enrichment in the cell body compared to axon, suggesting an impairment in axonal transport. In our immunofluorescence studies we revealed that Syt7 levels are generally decreased in all gray matter regions of SAD patients but with a tendency towards the increase in FAD patients. Although the number of FAD patients (4) did not allow us to get conclusive results, the tendency towards the Syt7 increase could be explained by the increased levels of the two lower bands found in the WB of human cortical tissue. Alternatively, the differences observed between FAD and SAD groups may simply be due to the differences in age. While the SAD patients were as old as the controls, the FAD group consisted of a younger group of patients that had a faster and more aggressive progression of the disease. It would be very interesting to compare the FAD group with an aged-match control group (and not the control group we used here which was aged-matched with the SAD group).

Further research will have to assess the specificity of these bands, which might be degrading products of the six splice variants of Syt7 also present in the human brain. Surprisingly, the PS1 heterozygous mutations from FAD patients studied here did not affect the levels of Syt7. The different results between PSKO mouse model and human FAD cases likely results from the difference in γ -secretase proteolytic activity between the two conditions. Importantly, whereas γ -secretase proteolysis is abolished in the PSKO mouse, PS1 mutations in FAD patients impair the function. As a consequence, the decrease of Syt7 might be only noticeable when there is a full genetic or pharmacological inhibition of the γ -secretase complex. In addition, in humans we cannot exclude compensatory mechanisms exerted by the second PS1 allele or by PS2, which are fully functional.

Future research to understand the molecular mechanisms of PS, APP and Syt7 in AD

Syt7 belongs to a large protein family constituted of 17 other synaptotagmins that function as Ca^{2+} sensors relevant in the regulation of neurotransmitter release and hormone secretion. In addition,

synaptotagmins have a conserved structure and control over different functions at various cellular locations and organs, which show the versatility of these proteins. The regulation of Syt7 in synapses by the γ -secretase complex could imply that other synaptotagmins present at the plasma membrane might follow a similar pathway. Consequently, future research could investigate if the inactivation of the γ -secretase complex affects other members of the synaptotagmin protein family.

In addition to electrophysiological approaches, regulation of Syt7 function by PS and APP could be investigated in neuronal cell cultures with genetically encoded optical indicators like pHluorin⁴⁸⁶. These indicators are commonly used to study exocytosis and recycling of synaptic vesicles under the pharmacological or genetic inactivation. As a perspective to our study, I propose to monitor SV cycling in PS or APP-KO cultures or under the pharmacological inhibition of γ -secretase. Moreover, co-transfections of tagged APP and Syt7 could also shed light on the cell compartments where these proteins interact and if the observed changes happen locally at synapses, soma or if they are due to deficits in the axonal transport. To test this last hypothesis, microfluidic chambers in combination with fluorescent recovery after photobleaching (FRAP) could be used to study impairments in the trafficking of vesicles and organelles through axons.

The research performed in human tissue gives relevance to Syt7 as a potential new biomarker in AD patients. However, besides biochemistry and imaging, other methods could be used to confirm these results and implement synaptic proteins as a diagnostic tool to evaluate the stage and advancement of the disease. The use of CSF samples has been clinically validated in MCI and AD patients as a potential diagnostic tool^{360,432} that could predict the cognitive decline progression in a population of patients. The levels of certain synaptic markers like the postsynaptic protein neurogranin have a good correlation with cognitive decline in the prodromal phase of Alzheimer's, suggesting that other synaptic proteins might also be affected³⁶¹. Given our results, Syt7 might be a good candidate to study in CSF samples from patients with MCI and AD. Furthermore, the use of PET imaging to measure synaptic density in proteins such as SV2A⁴⁸⁷ is turning into a reality that could help to evaluate synaptic protein changes over time in human patients.

Chapter 2 - Physiological role of APP in excitatory neurotransmission in MF-CA3 synapses

2.1. Context and aims of the study

Many functions have been attributed to the APP protein family^{103,112} but its role in presynaptic plasticity remains unclear. Importantly, APP interacts with proteins of the neurotransmitter release machinery^{189,190} suggesting that it might play a role at the presynaptic compartment. Several studies have revealed the importance of these proteins in the binding and regulation of channels crucial for synaptic plasticity and the integrity of synaptic function^{184,185,443}. The aim of this study was to investigate the presynaptic function of APP and APLP2 in a conditional KO model (APP/APLP2 cKO) at the hippocampal MF synapses to CA3 cells. The viral expression was under the control of the C1ql2 promoter, which transduced a variant of channelrhodopsin (ChIEF) fused to tomato and the Cre-recombinase enzyme (C1ql2-ChIEFtom-2A-Cre). This approach allowed us to combine cell-specific activation with light and gene deletion at the same time. Therefore, while cell stimulation was performed at the dendritic ML of the DG, the electrophysiological properties were recorded at the CA3 cells under the presynaptic suppression of APP and APLP2 (Fig. 2A).

2.2. Results

Loss of APP and APLP2 impairs presynaptic facilitation in MF-CA3 synapses

By using WB and immunohistochemistry, we first confirmed the absence of APP and APLP2 protein expression in cell cultures (Fig. 1A) and brain slices (Fig. 1B, C) transfected with Cre-recombinase.

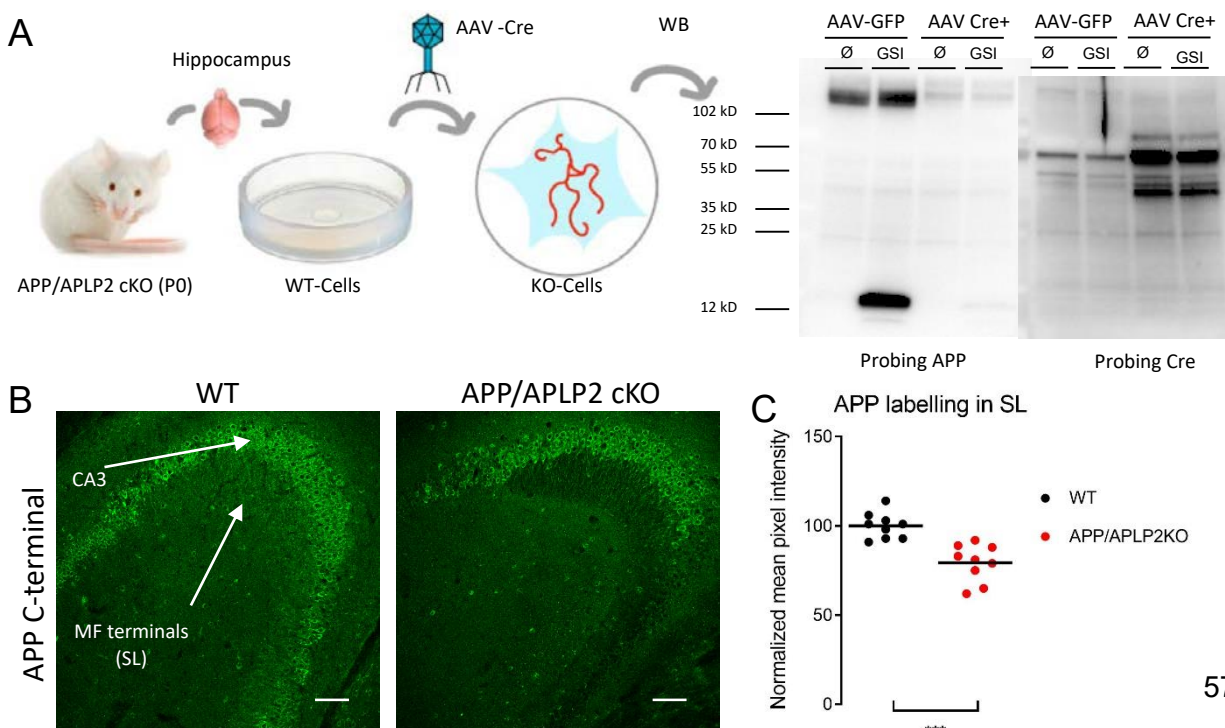


Figure 1 – Expression of APP in the APP/APLP2 cKO model. A) Hippocampal neuron cultures were prepared and transfected with a virus expressing either GFP or Cre-recombinase. Probing for APP or Cre-recombinase in WB showed that when Cre-recombinase was expressed APP was depleted. A condition with gamma secretase inhibitor (GSI) was also added to visualize the APP- CTFs. B) Stainings of APP Cter at the hippocampal CA3 region of a WT hemisphere and an APP/APLP2 cKO, where MF terminals project. APP levels are reduced in the SL of the KO condition in which the DG was injected with the Cre-recombinase. C) Normalized quantifications of the APP Cter in the stratum Lucidum (SL) show significant decrease levels of APP after Cre-recombinase expression (scale bar 100µm).

In addition, the expression of the C1ql2-ChIEFtom-2A-Cre construct in the DG of APP/APLP2 cKO allowed to measure the presynaptic release properties of these proteins by recording the activity of CA3 cells (Fig. 2A). The electrophysiological properties of the KO model were compared to control mice injected with the same construction but without the Cre-recombinase expression. The failure rate (Fig. 2B) and the mean basal amplitude of the EPSCs were compared at 0,1Hz, showing no differences in the initial release probability (Fig. 2C, D). Thus, the basal synaptic properties at low-frequency stimulation were not affected in the APP/APLP2 KO model.

Short-term plasticity between the 2 groups was also compared by increasing frequencies from 0,1Hz to 1Hz and using paired-pulse stimulation (40ms interval). By using the 0,1Hz and 1Hz recordings, we could calculate the frequency facilitation, which is a ratio of the EPSC amplitude averages for each frequency (Fig. 2F). While the frequency facilitation and the amplitude at 1Hz did not differ between the groups; the paired-pulse facilitation in the APP/APLP2 cKO had a tendency towards the decrease but the difference is not statistically significant (Fig. 2G).

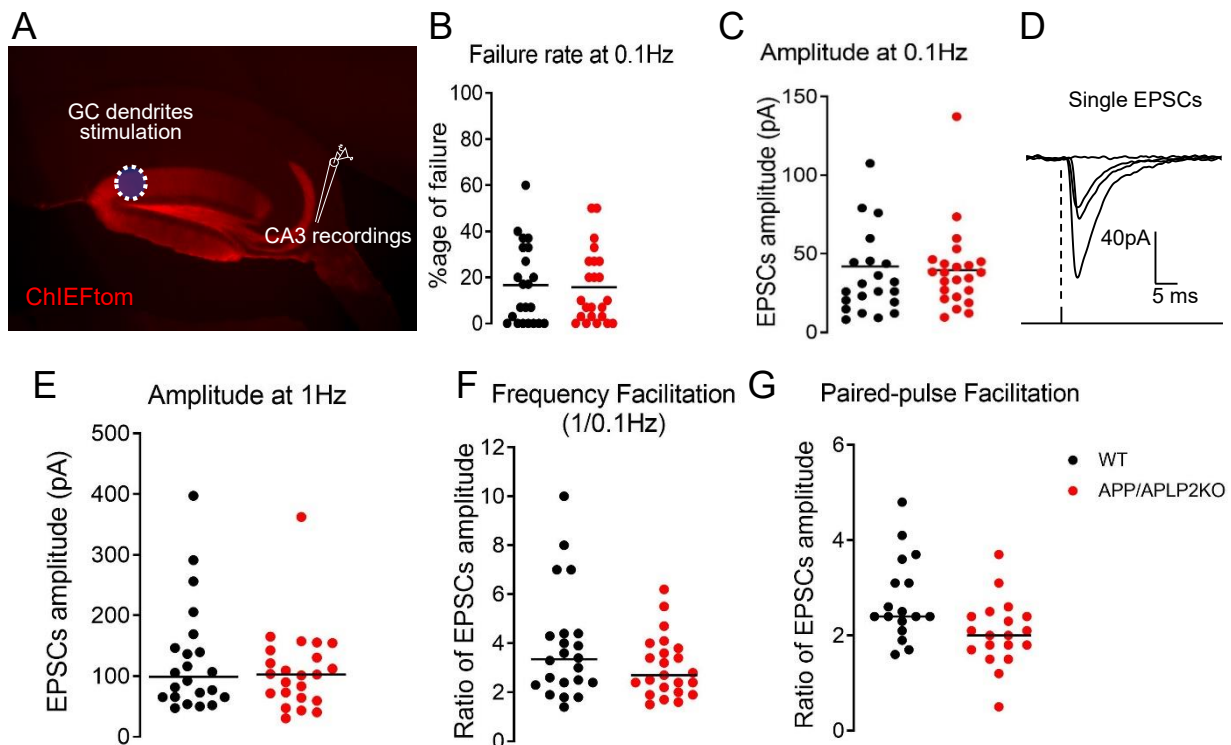


Figure 2 – Basal transmission properties and short-term plasticity properties of the APP/APLP2 cKO. A) DG infection using the C1qI2-ChIEFtom-2A-Cre viral construction B) Failure rate at 0,1Hz shows no differences between groups. C) Mean basal amplitude of EPSC at 0,1Hz shows no differences between groups. D) Representation of recorded EPSC. E) Mean basal amplitude of EPSC at 1Hz shows no differences between groups. F) Frequency facilitation (1/0,1Hz) shows no differences between the groups. G) Paired-pulse facilitation at 1Hz shows a tendency to reduction but no significant differences between groups.

Interestingly, the facilitation triggered by trains of 10 light pulses at 20Hz was significantly smaller in the APP/APLP2 KO MF-CA3 synapses (Fig. 3A, B). Overall, these results show that the deletion of APP and APLP2 do not affect the initial release probability but decrease the facilitation induced by high-frequency facilitation. In addition, these results indicate that these proteins are involved in short-term plasticity mechanisms at the presynaptic compartment of MF-CA3 synapses.

Synaptic transmission depends on the release and recycling of vesicles at the presynaptic compartment. Therefore, since APP/APLP2 cKOs have impaired trains of presynaptic stimulations, we hypothesized that the synaptic vesicle replenishment could be impaired. To test this hypothesis, we induced long trains stimulation of 100 light pulses at 20Hz, which involve the recycling pool of synaptic vesicles. These recordings showed that for longer trains of stimulation the facilitation of the EPSCs amplitude was also decreased in our model, suggesting and impairment in the recycling pool of vesicles (Fig. 3C).

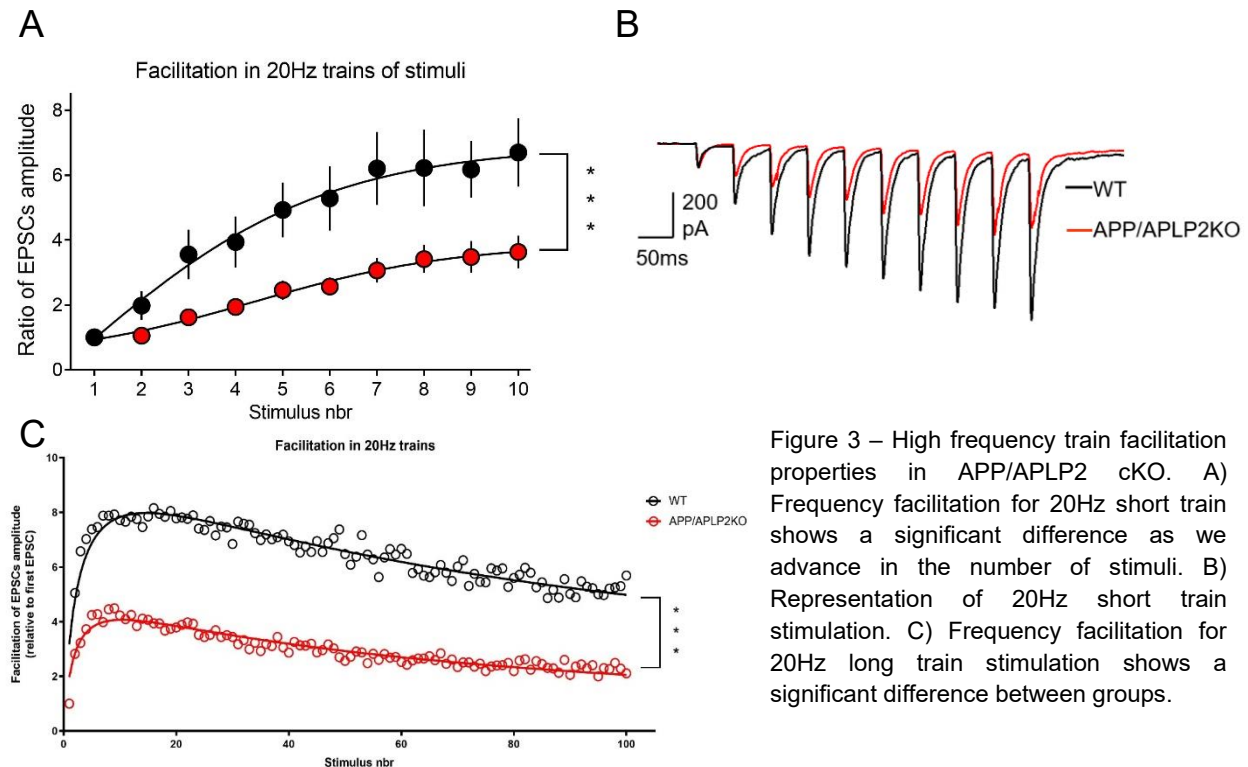


Figure 3 – High frequency train facilitation properties in APP/APLP2 cKO. A) Frequency facilitation for 20Hz short train shows a significant difference as we advance in the number of stimuli. B) Representation of 20Hz short train stimulation. C) Frequency facilitation for 20Hz long train stimulation shows a significant difference between groups.

Loss of APP and APLP2 does not alter presynaptic protein expression

We then tested if the impairment observed in synaptic plasticity could be due to changes in the levels of proteins that are present at the presynaptic compartment and have a relationship with APP. More precisely, we hypothesized that the impaired presynaptic facilitation, a phenotype resembling the one observed in PSKO condition, would rely, like in PSKO, on the reduction of Syt7 abundance. Consequently, we labeled brain slices for different synaptic proteins related to neurotransmitter release such as Munc13, Tau, Syt1, Syt7 and VAMP2 (Fig. 4). Surprisingly, none of these proteins showed major differences in the pixel intensity at the SL where MF projections innervate CA3 cells, demonstrating that none of these proteins deficits could explain the impairment observed in short-term plasticity.

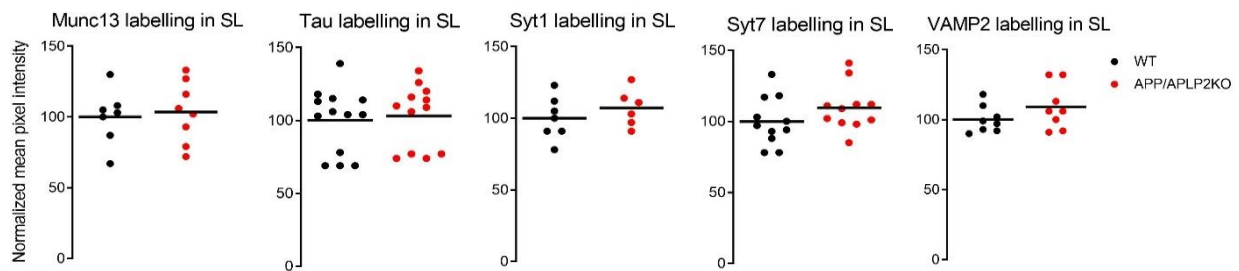


Figure 4 – Pixel intensity quantifications in APP/APLP2 conditional KO for Munc13, Tau, Syt1, Syt7 and VAMP2

2.3. Discussion and perspectives

Studying the physiological function of AD related genes is crucial to understand the pathogenesis of the disease. The presence of APP together with the secretases of the canonical pathway have been reported in the presynaptic compartments of synapses^{118,119,120}. In addition, APP interacts with different synaptic proteins relevant for the release and recycling of synaptic vesicles, suggesting a relevant function in synaptic transmission^{189,190}. Here, we used a set of different optogenetic, electrophysiology and imaging tools at the MF-CA3 synapses to study the presynaptic mechanisms of the APP protein family.

The role of APP protein family in synapse maintenance and formation have been characterized in different synapses of the peripheral and central nervous system^{173,174,176,187,188}. Here, we provide evidence that the loss of APP and its homolog APLP2 results in an impairment of the synaptic plasticity for the train facilitation, characteristic of the MF excitatory synapses. These results clearly put forward impaired presynaptic function in these animals. Interestingly, other studies performed in other excitatory synapses such as the neuromuscular junctions^{173,174} or the Shaffer collateral-CA1 synapses showed pronounced deficits in other types of short and long term plasticity such as PPF and in the induction and maintenance of LTP^{187,188}. In contrast, basal

transmission was found to be normal in these studies, including ours, suggesting that APP and APLP2 are not directly involved in the releasing properties of synaptic vesicles but more in their replenishment rate at the presynapse. In line with these results, in the absence of APP and APLP2, we did not detect a deficit in proteins crucial for the anchoring or release of synaptic vesicles like, Syt 1 Munc13 or VAMP2²⁹⁶. However, the YENPTY domain of APP has been related to clathrin mediated endocytosis in *in vitro* models^{192,193}. In addition, APP is also present in early and late endosomes^{121,122}, which play a key role in the recycling and reuse of synaptic vesicles during neurotransmission. Although the precise molecular mechanism has not been investigated here, the interaction of APP with proteins directly involved in the vesicle recycling like clathrin, dynamin1 or endophilin^{189,190} suggests that APP and APLP2 could form a multiprotein complex involved in the clathrin mediated endocytosis. Further research will need to study the individual and interdependent functions that these two proteins exert in different synaptic processes such as vesicle recycling.

In the immunohistochemistry experiments, we hypothesized that the impaired presynaptic facilitation observed in the APP/APLP2 cKO would resemble the Syt7 phenotype observed in the PSKO model. Surprisingly, this was not the case meaning that the PSKO phenotype does not result from dominant negative effect of accumulated APP-CTFs on full-length APP but depends on a gain of toxic function of APP-CTFs. Importantly, the loss or gain of function of the γ -secretase complex has been in debate for many years in AD research. The fact that FAD mutations in the genes of PS leads to γ -secretase complexes still able to cleave APP and other substrates argues against loss of function mechanisms in FAD. Instead, the aberrant function of the enzyme and thus the wrong processing of substrates like APP might be the source of toxicity as suggested by longer A β species or accumulation of CTF fragments.

The relationship between APP and Tau remains poorly understood and therefore it was an interesting candidate to test in the APP/APLP2 cKO. A recent study showed the importance of Tau in presynaptic dysfunction and its association with synaptic vesicles⁴⁴⁶. Furthermore, another study in human stem-cells showed that neurons from different AD forms differ in APP processing affecting the levels of Tau⁴⁴⁵. Consequently, we hypothesized that the presynaptic deficits observed in the APP/APLP2 cKO could be explained by changes in the Tau protein. Nevertheless, we failed in replicating these results suggesting important differences between the models used.

A possible strategy to define the molecular pathways where APP is involved could be to test the effects of its processed fragments or different drugs to rescue the phenotypes observed in the APP/APLP2 KO. These experiments would enable us to investigate the interaction and assess

the effect of APP fragments in one specific channel or protein. The viral expression of proteins using specific cell promoters is a valid strategy that we successfully implemented for some proteins like Syt7 in the MF-CA3 circuit¹⁹⁹. Therefore, the use of APP/APLP2 KO animals to express different fragments (CTFs, sAPP α and sAPP β) or mutants of APP (Swedish mutation or Icelandic mutation) would allow us to study the impact of these mutations in synaptic transmission.

Certain studies in single constitutive KO have investigated the role of these individual proteins in synapses. For example, single APP constitutive KO mice have normal basal neurotransmission but age-dependent LTP and PPF impairments, which are normal in APLP2 KO animals^{103,180}. Deficits in memory and learning have also been described in APP KO¹⁸⁰, meaning that APP plays an important function in synaptic plasticity and cognitive functions. Thus, these results suggest that APP and APLP2 share initial synaptic properties that can diverge during ageing. Although we did not study the individual synaptic properties of APP or APLP2 in our model, we overcame the developmental limitations by the conditional deletion of APP/APLP2 in adulthood (Cre/Lox).

An important question to be answered is if the synaptic impairments observed are due to the lack of full length APP and APLP2 or their proteolytic fragments. Several studies have demonstrated that sAPP α re-expression is sufficient to rescue cognitive and synaptic plasticity properties^{180,184,447}. Interestingly, the release of sAPP α is dependent on the neuronal activity⁴⁴⁸ and therefore it can be increased by protocols of stimulation used in electrophysiology to trigger plasticity. A recent study showed that the expression of sAPP α in the APP/APLP2 KO mouse is sufficient to rescue LTP and PPF in a mechanism that depends on the binding and allosteric potentiation of the $\alpha 7$ -nAChRs¹⁸⁴. Importantly, sAPP β fails in showing significant effects, indicating that the additional 16 amino acids present at the Cter domain of the sAPP α trigger this effect. However, the physiological expression of $\alpha 7$ -nAChRs in the pre- and the post-synaptic compartments gives a complex interpretation and distinction in the functional extent of this peptide in each synaptic compartment. Therefore, the use of APP/APLP2 KO in combination with tools that allow the specific deletion or inhibition of these channels either pre or post will be important to delineate the function of these peptides in each compartment. Furthermore, APP protein family has recently been demonstrated to regulate the excitability of synapses, interfering with the function of other channels like GABA β R¹⁸⁵ or voltage gated potassium channels⁴⁴³.

This project could be also continued by studying the role of APP as an atypical G protein-coupled receptor in the nervous system. G protein-coupled receptors regulate cellular responses through heterotrimeric G proteins, which constitute a common cellular signaling pathway. Previous research has demonstrated that the intracellular Cter domain of the APP protein family can

interact with and activate G proteins. Experiments performed in purified brain membrane showed that APP specifically co-immunoprecipitated with Gαo proteins providing physical and functional evidence of its interaction. Importantly, the stimulation of G protein can trigger the activation of the adenylate cyclase/ cyclic adenosine monophosphate/ protein kinase A signaling pathway (AC/cAMP/PKA), which plays a major role in the induction of LTP in MF-CA3 synapses. The induction of LTP at this synapse depends on the local increase of Ca^{2+} concentration, which activates Ca^{2+} dependent adenylate cyclases (AC1-AC8)^{454,455}. After its activation, the enzyme catalyzes the conversion of ATP into cAMP that can bind to the regulatory subunits of PKA, activating the catalytic subunits that will phosphorylate proteins involved in neurotransmitter release machinery like Rab3A⁴⁵⁷, synaptotagmin-12⁴⁵⁸, RIM1α⁴⁵⁹ or Munc13-1⁴⁵⁶. The phosphorylation and posterior interaction of these proteins turns out to be important in the induction of LTP at the MF-CA3 synapse⁴¹¹.

The role of APP and APLP2 in the AC/cAMP/PKA cascade has never been addressed in the MF-CA3 synapses or other central synapses. The presynaptic impairments observed in APP/APLP2 KO animals could be explained by the inactivation of the AC/cAMP/PKA pathway, which might be hypothetically activated by APP acting as a single pass receptor⁴¹¹. Indeed, the activation of APP as a receptor has been observed with different molecules that interact with the extracellular Nter fragment. For example, the transmembrane APPfl and the sAPPα interaction in neurons can modulate stress signaling by activating the PI3K/Akt pathway⁴⁴⁹. The impact of this ligand/receptor interaction as a possible mechanism regulating the potentiation of MF-CA3 synapses remains to be understood. However, as sAPPα can rescue the synaptic deficits in APP/APLP2 KO, it is tempting to speculate that sAPPα might regulate different molecular pathways related to synapse potentiation, including the adenylate cyclase/cAMP/PKA pathway. Future research will be needed to study the effect of APP dimerization and the binding of other potential ligands to APP as a G-protein-interacting receptor during synaptic transmission and development.

Chapter 3 - Deciphering APP misdistribution in Alzheimer's disease reveals its presynaptic accumulation around amyloid plaques enriched in APP-Nter

3.1. Context and aims of the study

Aggregation of full-length proteins is a frequent feature found in different neurodegenerative diseases^{452,453}. As an example, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and frontotemporal dementia are respectively characterized by the aggregation of α -synuclein, Huntingtin and TDP-43 proteins within intraneuronal inclusion bodies. However, research in AD has given a central role to A β peptide, a by-product of APP, which is the main component of extracellular amyloid plaques. Amyloid plaques have been proposed to be the cause of AD by initiating a cascade of pathological processes that lead to neuronal cell death⁴². Therefore, in contrast to other neurodegenerative diseases where full-length protein aggregation is a common pathological characteristic, in AD this feature has been well characterized for extracellular and intracellular A β but not for its substrate APP. Thus, the organization and distribution of APP and its processed fragments in the human brain has been poorly characterized and requires better analytical tools.

The research carried out in this project focused on characterizing the distribution of APP in the human hippocampus of AD patients. In general, most molecular AD studies have been performed in AD mouse models that present relevant hallmarks for the disease. However, these models are based on FAD mutations and fail in showing the complete set of factors that trigger AD pathology. Furthermore, in general the data provided in human tissue is qualitative and descriptive limiting the interpretation of the results. In order to overtake these limitations, in this chapter I present a study in which we used a set of semi-quantitative methods that were applied in entire human hippocampal sections of FAD and SAD patients combined with two different AD mouse models (APP/PS1 and APP-NL/G/F mouse). By using immunofluorescence and electron microscopy, we show that APP together with its secretases accumulate around dense-core amyloid plaques that contain A β and APP Nter. In addition, these accumulations contain presynaptic proteins and phosphorylated Tau in multivesicular bodies that are potential degradation sites for the formation of amyloid plaques.

3.2. Manuscript

See PDF of the manuscript in the next page.

Deciphering APP misdistribution in Alzheimer's disease reveals its presynaptic accumulation around amyloid plaques enriched in APP-Nter

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Key words: APP, Alzheimer's disease, amyloid, protein accumulation, presynaptic proteins.

Summary

In Alzheimer's disease (AD), a central role is given to the extracellular deposition of A β peptides, remotely produced by the proteolysis of the amyloid precursor protein (APP). Contrarily, the distribution in AD brains, of APP itself and its fragments other than A β , is not clearly characterized. Here, we combined an extensive set of methodological and analytical tools to investigate neuropathological features of APP in the human AD hippocampus and in two mouse models of AD. We report that in the hippocampus of AD cases, APP remarkably concentrates in extra-somatic microscopic entities whereas its abundance in neuronal soma markedly vanishes, revealing that APP is misdistributed in AD. The extra-somatic accumulations of APP are indicative of faster progression of the disease, their density being greater in the early-onset form of the disease than in the late onset form. Interestingly, their density is also increased in the vicinity of dense-core amyloid plaques, but not of diffuse plaques. We also report that dense-core amyloid plaques are remarkably enriched in APP-Nter, but not in APP-Cter domain, consistent with the large amount of BACE1 observed in the APP accumulations surrounding them. This indicates the presence of a potentially pathologic, non-soluble APP-Nter fragment secreted in dense-core plaques. To investigate the subcellular compartment in which APP concentrates, we labelled neuritic markers (SMI312, myelin-binding protein, Tau, MAP2) and pre- or post-synaptic markers. We report a selective enrichment in presynaptic proteins (Syt1, VAMP2) and phosphorylated-Tau which contrasts with the depletion in post-synaptic proteins within APP accumulations. Ultrastructural analyses unveil that APP concentrates in autophagosomes and in abundant multivesicular bodies together with presynaptic vesicle proteins. Altogether, our data reveals that APP is misdistributed from the soma to impaired presynaptic sites in AD and highlights presynaptic APP accumulations as a potential source of A β and Nter peptides to supply dense-core amyloid plaques.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease (ND) characterized by a severe cognitive impairment and by a prominent injury of the hippocampus. Despite important breakthroughs in the genetic and in the cellular biology of AD pathology, the etiology of the disease is still unknown and today no curative therapy is available. The brain of AD patients presents numerous amyloid plaques, hallmarks that are at the basis of the amyloid cascade hypothesis (Hardy and Higgins, 1992) which is supported by compelling genetic evidence. Indeed, the early-onset, hereditary form of the disease (FAD: familial AD) is caused by missense mutations either in the amyloid precursor protein (APP) (Goate *et al.*, 1991) or presenilin (Sherrington *et al.*, 1995), respectively the substrate and the enzyme necessary for the production of A β peptides which compose the amyloid plaques (De Strooper *et al.*, 1998).

Remarkably, AD stands apart from most other NDs by the extracellular location of amyloid plaques which are proposed to be formed by the deposition of remotely produced A β peptides circulating in the extracellular fluids (Meyer-Luehmann *et al.*, 2006; Eisele *et al.*, 2010). In contrast, many NDs have in common to be proteinopathies characterized by the aggregation and accumulation of a main culprit protein inside neurons (Ross and Poirier, 2004; Soto and Pritzkow, 2018). For example, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and frontotemporal dementia are characterized by the aggregation of α -synuclein, Huntingtin and TDP-43 proteins within intraneuronal inclusion bodies. Moreover, the pathological mechanisms involved in AD are also proposed to involve extracellular processes. Indeed, the amyloid cascade hypothesis states that before precipitating in plaques, A β peptides of different forms circulate in the extracellular space and initiate neuronal injuries by binding cell surface receptors located at synapses (Selkoe, 2002). Besides, this central role attributed to a by-product of APP degradation, A β peptides, also contrasts with the mechanisms involved in most other NDs in which full-length proteins are misfolded.

In comparison to the extensive knowledge about A β peptides roles in AD, relatively limited information is available about the distribution and the abundance of APP, and its fragments other than A β , in the human brain. Here, we sought to quantitatively investigate the distribution of the different APP domains in human AD hippocampus compared to healthy controls in an unbiased approach. Additionally, we compared within AD condition, brains with the early-onset, familial form of the disease, to brains afflicted with the late-onset form. Finally, we contrasted results obtained in human brains to the AD mouse models APP/PS1 and APP-NL/G/F.

We thoroughly labelled the Cter, Nter and A β domains of APP with a set of six different antibodies, and we combined triple-fluorescence labeling and electron microscopy. We report

a dramatic reduction in APP labelling in neuronal somata of AD patients, which inversely correlates with the abundance of distinct microscopic entities in which APP remarkably concentrates. We performed an in depth investigation of the molecular characteristics and the relevance to AD pathology of these accumulations of APP, qualitatively reported in early histopathological descriptions (Arai *et al.*, 1990; Cummings *et al.*, 1992). We report that APP accumulations concentrate β - and γ -secretases, and preferentially accumulate around dense-core amyloid plaques containing A β and APP-Nter in the hippocampus of AD patients, but not around diffuse plaques. In addition, we observed a striking concentration of presynaptic, but not postsynaptic, markers in APP accumulations. Importantly, in human AD cases, APP accumulations are more abundant in the early-onset familial form, and in AD mouse models, their abundance correlates with neuronal loss. These features suggest a role for APP misdistribution from the soma to impaired presynaptic sites in AD pathology, and highlights presynaptic APP accumulations as potential sources of substrates to supply dense-core plaques with amyloid peptides.

Results

Misdistribution of APP in the hippocampus of AD patients

We compared the distribution of APP in the human hippocampus of AD cases versus controls, by collecting paraffin-embedded hippocampal sections from two brain banks: the Netherlands brain bank (NBB) and the Karolinska Institutet brain bank (KIBB) (see Methods). Three groups were constituted: 11 controls patients (mean age 79.6 years old, 73%F and 27%M), 12 AD patients with the late-onset, sporadic form of the disease (SAD; mean age 81 years old, 92%F and 8%M) and 4 patients with the early-onset, familial form (FAD; mean age: 48,5 years old, 50%F and 50%M) (summarized in Table 1; further clinical data tables in TableS1-3).

We first labeled APP using two specific antibodies against the APP C-terminal domain (APP-Cter): the C1/6.1 antibody and the Y188 antibody (Figure 1A). The sections were imaged with a Nanozoomer 2.0HT slide scanner (Hamamatsu) allowing the acquisition of full sections without mosaicism and a resolution of 454 nm/pixel (55947 DPI). Preadsorption with a recombinant APP-Cter peptide abolished the staining (Figure S1A-B). In sections from the control group, APP appeared to be mostly abundant in neuronal soma using a detection with 3,3'-diaminobenzidine (DAB) as a chromogen (Figure 1B-C, S1C). In the hippocampus, the dentate gyrus granule cell layer (GCL) and the pyramidal cell layer in CA4 and CA3 are characterized by a dense DAB staining of APP (Figure 1B-C, S1C). Surprisingly, in both FAD and SAD conditions, the somatic DAB staining of APP appeared faint in the different neuronal layers revealed by the hematoxylin staining (Figure 1B, S1C). In the FAD and SAD groups,

APP was prominent in remarkably intense stainings, which appeared as extra somatic protein accumulations spread in the different hippocampal regions (Figure 1B, S1C). In close-up acquisitions, in the molecular layer (ML) of the dentate gyrus or in the CA4 region, the intense clusters of APP appear like distinct microscopic entities (Figure 1C and S1D) which we further refer to as APP accumulations.

The density of APP accumulations was considerably higher in the hippocampus of AD patients compared to hippocampus from controls, in which they were almost never detected (Figure 1D, S1E). Importantly, more APP accumulations were detected in the hippocampus of FAD patients as compared to SAD (Figure 1D, S1E), albeit the Braak and the amyloid stages (Table S1-3) were comparable between the two AD groups. Besides, the age of onset of the disease was as expected, lower in the FAD cases than in the SAD cases. Thus, the number of APP accumulations inversely correlates with the age of onset of the disease which suggests that APP accumulations could play a role in the progression of the disease and represent a marker of the severity of AD. Besides, the APP accumulations were larger in the cornu ammonis (CA) than in the ML (Figure 1E, S1F).

While giving an excellent sensitivity due to the enzymatic amplification of the detection, the DAB staining approach considerably limits the molecular characterization of the detected objects. Consequently, we developed an immuno-fluorescence approach combining an antigen retrieval method and autofluorescence quenching to perform triple labeling in human brain tissue (see Method and Controls in Figure S1G). We first verified the specificity of the two antibodies against APP-Cter by comparing fluorescence intensity in WT versus APP-KO mice. We observed that the C1/6.1 antibody was fully specific showing no labelling in APP-KO mouse brains (Figure S1H). The Y188 antibody was also specific in the grey matter without any neuronal labeling in APP-KO mouse brains (Figure S1I). However, some white matter tracts remained fluorescent in the APP-KO condition revealing that the white matter staining was not specific for APP (Figure S1I, S1C). We first co-stained human hippocampal sections with both antibodies against the APP-Cter (C1/6.1 and Y188) and observed a high colocalization between the two antibodies within APP accumulations (Figure S1J, K). We developed a method, based on the subcellular colocalization analysis JACoP (Bolte and Cordelières, 2006), to calculate the Mander's overlap coefficient (MOC), an indicator of relative colocalization between two channels (Figure S1L, M). The proportion of pixels positive for C1/6.1 labelling which overlapped the pixels positive for Y188 was defined as MOC (C1/6.1 / Y188) and was 0.95. The proportion of Y188 pixels overlapping the C1/6.1 pixels was defined as MOC (Y188 / C1/6.1) and was 0.74 (Figure S1N). This nomenclature of MOCs was used in the entire manuscript. Then, we measured the intensity of the fluorescent labeling of APP-Cter inside versus outside accumulations. APP labeling was nine times brighter inside

accumulations indicating a considerable enrichment in APP within these hallmarks (Figure S1O).

The observation of accumulations with the APP-Cter antibodies raised the question of whether the APP accumulations also contain the Nter counterpart of APP, which may suggest the presence of full-length APP. To address this question, we labeled the human hippocampal sections with two different antibodies against the Nter domain of APP (Figure 1A), the standard 22C11 antibody and the highly specific JRD32 antibody (Figure S1P). From here, our study was performed on the cohort of samples from the NBB which contains eight control cases, eight sporadic and one familial cases (hereafter combined as AD). In human hippocampal sections from controls, both antibodies against the Nter domain clearly labeled neuronal soma as did the Cter antibodies (Figure 1F and S1Q). Besides, in FAD and SAD conditions, Nter antibodies strongly stained APP accumulations detected with Cter labeling (Figure 1F, G and S1Q, R). Nter and Cter antibody labelings displayed a high MOC (Figure 1H and S1S). Whereas the joint presence of APP-Cter and Nter fragments is possible, the high MOC suggests that the full-length APP protein is also present in the APP accumulations. We next investigated whether there was a correlation between the burden of APP accumulations, mostly present within neuropil areas, and a seeming reduction in APP expression in neuronal soma observable in the DAB staining (Figure 1B). Taking advantage of the linearity of the fluorescent signal, we measured the mean pixel intensities of APP in neurons of the GC or CA3 layers. There was significant decrease in the mean pixel intensities of APP labelling in GC soma of AD cases compared to controls (Figure 1I) which contrasts with the dramatic enrichment in APP inside accumulations (Figure S1O). Moreover, an inverse correlation was observed between APP accumulations burden in neuropil areas (i.e. the surface covered by APP accumulations in each human case) and APP somatic expression suggesting that APP accumulations might result from a cellular misdistribution (Figure 1J).

APP accumulations surround dense-core amyloid plaques

We further sought to characterize the topographical organization of APP accumulations in relation to amyloid plaques. For this, we revealed amyloid plaques with three parallel labelings: an antibody against the Nter of A β peptides (clone 6E10 against amino-acids 1-16 of A β), another antibody against the free Cter of A β 42 peptides (clone 12F4) (Figure 1A) and methoxy-X04, a fluorescent derivative of Congo red. Of important note, epitopes of the C1/6.1 and Y188 Cter antibodies are distinct from the A β peptide sequence (Figure 1A) and thus the detection of APP accumulations cannot be due to the detection of A β peptides. We tested the specificity of A β antibodies in WT and in APP/PS1 mice in which a human APP transgene is overexpressed. The A β 42 and the A β 1-16 antibodies labeled amyloid plaques in APP/PS1

mice but no staining was observed in WT mice, indicating high specificity for the human A β peptide (Figure S2A-B). However, the A β 1-16 antibody being able to also recognize APP, we discriminate between APP and amyloid plaques by combining APP Cter, A β 1-16 and methoxy-X04 labelings. Besides, the secondary antibody alone gave no staining (Figure S2C).

We characterized APP accumulations in human brain slices in relation to the two main types of plaques, the diffuse plaques and the dense-core plaques (Mesulam and Geula, 1994), which can be distinguished by the presence of A β 42 and by congophilic labeling (e.g. using methoxy-X04). Most amyloid plaques in the hippocampus were characterized by an intense methoxy-X04 labeling in which A β 1-16 and notably A β 42 staining were present (Figure S2D-F and 2A-C). These plaques present APP accumulations in their periphery (Figure 2A, B and S2H) indicating that in the hippocampus, most amyloid plaques are of the dense-core type and that they are characterized by APP accumulations in their vicinity. By contrast, numerous amyloid plaques were stained for A β 1-16 but not for A β 42 or methoxy-X04 in the human cortex of controls (Figure S2I-K). These diffuse plaques did not display APP accumulations in their surrounding (Figure S2J) indicating that diffuse plaques, described as benign because they are numerous in some non-demented aged humans (Katzman *et al.*, 1988; Delaère *et al.*, 1993), are not associated with APP accumulations. Inversely, APP accumulations are specific features of the harmful, dense-core amyloid plaques.

In an effort to better assess the distribution of APP accumulations relative to amyloid plaques, we performed a Sholl analysis (Sholl, 1953) to count the number of APP accumulations in intervals of 5 μ m, up to 50 μ m away from amyloid plaques used as references (Figure S2L). This analysis revealed that APP accumulations surround dense-core amyloid plaques, with a progressive decrease away from the plaque's boundaries (Figure 2D). Interestingly, the amyloid load correlates with the extent of APP accumulations, the total area covered by amyloid plaques in the hippocampus being larger in patients exhibiting a larger total area of APP accumulations (Figure 2E). Moreover, Bland-Altman analyses of the difference of areas covered by A β 42 and APP accumulations plotted against their average areas revealed that the data sets were of good agreement (Figure S2M). This indicates that APP accumulations and amyloid plaques probably are pathologic features which are not independent of each other even though they only poorly colocalize (Figure 2F).

APP accumulations contain the secretases to produce APP-Nter and A β peptides

The detection of both APP-Cter and -Nter around dense-core plaques raises the possibility that proteolysis of full-length APP within accumulations plays a role in the generation and or maintenance of amyloid plaques. We thus characterized the distribution of the enzymatic machinery necessary to cleave APP in the accumulations. In the control hippocampus, BACE1

(β -secretase) was prominently observed in neuronal soma reminiscent of the expression of APP, in addition to a fainter labeling of the neuropil (Figure 3A). In AD condition, BACE1 was found around amyloid plaques as previously reported (Zhang *et al.*, 2009; Kandalepas *et al.*, 2013) (Figure 3A, B). More precisely, it was located in APP accumulations but not inside methoxy-X04 positive fibrillary plaques, as revealed by the MOC of APP-Cter over BACE1 of 0.68 (Figure 3C) whereas the MOC of methoxy-X04 to BACE1 overlap was of only 0.05 (Figure S3A). Moreover, the average intensity of BACE1 labeling within APP accumulations was 84% higher compared to the average intensity 15 microns outside the edge of the accumulations, demonstrating the enrichment of BACE1 within the APP accumulations (Figure 3D).

The high abundancy of APP and BACE1 in the same locations suggests the possibility that APP-Nter domains (e.g. sAPP β) are released from the accumulations. Co-stainings for APP-Nter together with methoxy-X04, unexpectedly revealed a remarkable abundance of APP-Nter in the core of amyloid plaques (Figure 3E and S3B). The colocalization between APP-Nter and the core of the plaques was specific of the Nter domain since the MOC between methoxy-X04 and APP-Nter staining was 0.6 whereas the MOC between methoxy-X04 staining and APP-Cter was only 0.14 (Figure 3F and S3C). Moreover, the intensity of the fluorescent labeling of APP-Nter inside amyloid plaques was 2.3 times brighter than in the neuropil, indicating a marked enrichment of APP-Nter inside dense-core plaques (Figure 3G). These results suggest that in addition to A β , APP-Nter can accumulate at the core of the plaques possibly representing another aggregated APP derivative in addition to A β .

We then investigated whether γ -secretase, the second APP protease necessary to produce A β was also present within APP accumulations. We labeled PS1, the catalytic subunit of γ -secretase, using 33B10 antibody (Georgakopoulos *et al.*, 1999). In all conditions, the most prominent labeling was found at the level of neuronal soma (Figure S3D). In AD conditions, PS1 staining was also found overlapping with APP accumulations (Figure S3D, E) with a moderate MOC of APP-Cter to PS1 overlap of 0.4 (Figure S3F); the MOC of methoxy-X04 to PS1 overlap was of 0.16 (Figure S3H). Moreover, comparative analysis of staining intensity revealed a modest enrichment of PS1 within APP accumulations (Figure 4H; 20% increase over the intensity outside APP accumulations). Altogether, we report that the complete machinery necessary to produce APP-Nter and A β , i.e. the substrate APP and the proteolytic enzymes BACE1 and PS1, is present around plaques and could serve as local production sites.

APP accumulations have a distinctive neuritic markers composition enriched with presynaptic proteins

In order to explore the subcellular compartments in which APP accumulates, we investigated the co-localization of these accumulations with a set of neuritic markers. Indeed, dystrophic neurites of different molecular contents (e.g. expressing neurofilaments or phosphorylated forms of Tau (pTau)), have long been reported around amyloid plaques (Masliah *et al.*, 1993; Su *et al.*, 1994; Sharoar *et al.*, 2019). We labelled: the dendritic marker MAP2, the phospho-neurofilament (SMI312), the myelin basic protein (MBP) of the myelinated axons, the non-phosphorylated (AT8) and phosphorylated forms of Tau. The distribution of these neuritic markers in controls are shown in Figure S4. The colocalization with APP accumulations was low for all these markers, except for phospho-Tau which displayed a MOC of 0.53 (Figure 4A-F) and which was enriched almost two-fold within APP accumulations (intensity ratio of 1.86) (Figure 4G).

Since neurites could correspond to either postsynaptic or presynaptic compartments, we searched for the presence of pre- or post-synaptic proteins in APP accumulations. We immunostained the post-synaptic protein Shank 2 and APP, and in parallel we detected amyloid plaques with methoxy-X04. Whereas a prominent staining of dendrites and spines could be observed in both control and AD conditions (Figure 5A), we did not detect any Shank 2 within APP accumulations or plaques (Figure 5A, B and MOCs S5G and S5C). As indicated above (Figure 4A), the dendritic protein MAP2 was absent from dense-core plaques, as previously reported (D'Andrea and Nagele, 2002), and from APP accumulations, further indicating that APP does not accumulate together with dendritic or post-synaptic proteins. However, the distinctive lower intensity in MAP2 staining where dense-core plaques and its APP surroundings seat (Figure 4A), indicates that post-synaptic compartment is not preserved within these hallmarks.

We then stained Syt1, VAMP2 and Syntaxin1A as a set of presynaptic proteins in relation to APP accumulations and amyloid plaques. The three proteins were abundantly found in the neuropil over the entire hippocampal slice (Figure 5C, S5A, B). Interestingly, the two synaptic vesicle proteins Syt1 and VAMP2 were highly enriched within APP accumulations (Figure 5D, E, G, H) whereas they were absent from the core of the plaques (Figure S5C). Syntaxin1A, a cell-surface protein present in presynaptic active zones, was also present within APP accumulations (Figure 5F, G and S5B) although not enriched inside them (Figure 5H).

The observation that APP accumulations contain presynaptic proteins indicates that they may originate from axons or axon terminals. We questioned whether the APP accumulations were preferentially segregated in the white matter. Using MBP staining (Figure 4C and S5D) to delineate white matter tracts, we observed that APP principally accumulated in the grey matter (Figure S5E) and when found in the white matter, the accumulations were significantly smaller (Figure S5F). Collectively, these results indicate that APP accumulates together with

presynaptic proteins related to vesicle release and P-Tau, presumably at presynaptic sites from unmyelinated axons.

AD mouse models recapitulate the features of APP accumulations observed in human

In order to investigate the formation and the dynamics of APP accumulations, we aimed to study them in AD mouse models. Therefore, we first investigated whether AD mouse models faithfully recapitulated the presence of APP accumulations observed in human brains. We studied two mouse models: the APP/PS1 model which overexpresses two FAD human genes (APP with Swedish mutations and PS1 Δ Exon9 (Jankowsky *et al.*, 2004)), and the APP knock-in model NL/G/F (with four APP mutations but no overexpression (Saito *et al.*, 2014), (Figure S6D-G)). In both models, we observed intense APP accumulations surrounding amyloid plaques, as in human (APP/PS1: Figure 6A, B and S6A-C; APP-NL/G/F: S6D, E). Besides, the APP accumulations contained the APP-Nter domain (Figure 6C, D and S6F, G; MOCs S6A) and β - and γ -secretases (Figure 6E, F) like in human AD brains. Finally, as observed in human AD brain, APP accumulations display a much lower labelling intensity of the post-synaptic protein Shank2 (Figure 6G) whereas Syt1 (Figure 6H) and Vamp2 stainings were prominent (Figure S6H).

Thus, the two mouse models tested faithfully recapitulate the features of APP accumulations. From a histopathological point of view, both APP/PS1 and APP-KI NL/G/F properly exhibit APP-related AD hallmarks. However, it is noteworthy that AD-mouse models present numerous APP accumulations and dense-core plaques in the cortex, contrary to human for which the APP accumulations were more frequent of the hippocampus. Besides, the transgenic model, sometimes criticized for overexpressing the FAD genes, displayed hallmarks similar to the KI model, simply in a more abundant manner.

Ultrastructural analyses reveal presynaptic proteins and APP accumulations within multivesicular bodies

We further characterized APP accumulations at the ultrastructural level using electron microscopy (EM) in 9-months old female APP/PS1 mice, which exhibit abundant APP accumulations and amyloid plaques. In order to localize the structures of interest, hippocampal samples were immunostained with DAB and were then processed in osmium tetroxide and epoxy resin. We first analyzed the samples stained for APP-Nter taking advantage of its presence in both APP accumulations and plaques. We identified amyloid plaques given their distinctive shapes with radial projections at the micrometric scale (Narang, 1980; Wisniewski *et al.*, 1989) (Figure 7A, left), and their fibrillary structures at the sub-micrometric scale (Figure 7A, right). Outside the boundaries of the amyloid plaques (Figure 7A, left), we frequently

observed lysosomes connected to autophagosomes which were highly stained by the DAB reaction associated with either APP-Cter or -Nter antibody (Figure 7B). We also observed numerous multi-vesicular bodies and autophagic vacuoles all over the tissue surrounding the plaques (Figure 7A, C), as previously reported (Nixon *et al.*, 2005; Sanchez-Varo *et al.*, 2012), consistent with an impairment of the autophagy-lysosomal pathway currently debated in the AD field (Lee *et al.*, 2010; Neely *et al.*, 2011; Zhang *et al.*, 2012; Kwart *et al.*, 2019).-

In an effort to better characterize the membrane accumulations observed above, we performed stainings on cryosections based on the Tokuyasu method. We stained APP-Nter with DAB and labeled APP-Cter using immuno-gold particles. We observed abundant DAB staining of APP-Nter at the level of membranous accumulations (contrasted in white on dark background in cryosections) indicating that APP accumulates where membranes accumulate (Figure 7D). This observation was consistent with the presence of a transmembrane isoform of APP bearing Nter antigenicity, i.e. the full-length APP. Importantly, we observed in the same area some immuno-gold puncta revealing the presence of APP-Cter in the same location as APP-Nter (Figure 7D). This suggests again that full-length APP accumulates in membranes of the autophagy lysosomal pathway.

We then investigated the ultrastructural location of the synaptic proteins enriched within APP accumulations. Cryosections stained for APP-Cter with DAB and labeled for VAMP2 using immuno-gold particles revealed the presence of the presynaptic protein in multi-vesicular bodies positive for APP-Cter (Figure 7E). This result suggests that the multi-vesicular bodies derive from misprocessed synaptic vesicles containing APP.

APP accumulations inversely correlate with neuronal density

The current report of APP accumulations surrounding amyloid plaques raises the question of their participation in the pathological processes in AD and first of all in neuronal loss. We first evaluated whether APP accumulations correlated with neuronal loss in the somatosensory cortex of APP/PS1. Using NeuN labeling as a neuronal reporter, we counted neurons in 100 μm -side squares, used as regions of interest (ROIs) along the sagittal to temporal axis in each slide (Figure 8A). ROIs have been placed one next to the other and did contain one, several or none APP accumulations. The analysis revealed an inverse relationship between APP accumulations and neuronal density, the larger the area covered by the APP accumulations was, the fewer neurons were present (Figure 8B). This result may be explained by neuronal dispersion due to the surface occupied by the APP accumulations within a finite area. To address this possibility, we counted the number of neurons in the full layer V from the somatosensory cortex or of the full ML of the dentate gyrus in slices from 2, 5 and 9 months-old APP/PS1 mice (Figure 8C). In both brain regions, we observed a significant and

progressive decrease in the number of neurons starting at 5 months in APP/PS1 mice compared to WT (Figure 8D). This result indicated that the decrease in neuronal density observed close to APP accumulations was not caused by neuronal dispersion but by neuronal loss, in which APP accumulations may participate. Together with the observation that FAD cases exhibit more APP accumulations than SAD cases (Figure 1D), these results suggest a relationship between the number of APP accumulations and the severity of the disease.

Discussion

Here, we performed a detailed characterization of the distribution of APP fragments in the human AD brain. We report that the somatic distribution of APP, prominent in the control condition, is markedly decreased in AD condition. On contrary, we observed extra-somatic microscopic entities in AD cases of remarkably intense APP stainings. These entities likely correspond to the clusters of APP reported around plaques soon after the cloning of APP (Arai *et al.*, 1990; Cummings *et al.*, 1992). Since then, the molecular characteristics and the relevance to AD pathology of these accumulations of APP largely remained to be investigated. We describe APP accumulations as distinct microscopic entities which consist of densely packed APP (enrichment factor by 9), found in the vicinity of dense-core amyloid plaques but not of diffuse plaques. In contrast, the dense-core of the amyloid plaques is almost devoid of APP-Cter whereas APP-Nter is concentrated by a factor of two. The APP accumulations are detected with four different antibodies against the Cter and the Nter, which colocalize at both the micrometric (Figure 1) and nanometric scales within membranes (Figure 7), suggesting that they might contain FL-APP.

APP misdistribution with presynaptic proteins: AD as a presynaptic failure

A striking feature of APP accumulations is their selective enrichment in proteins of the presynaptic vesicle machinery (Syt1 and Vamp2) (Figures 5-7), whereas uniquely postsynaptic or dendritic proteins (such as Shank2 and MAP2) appear to be absent. Under physiological conditions, APP shows remarkable abundance in presynaptic compartments (Laßek *et al.*, 2013; Wilhelm *et al.*, 2014; Lundgren *et al.*, 2015), in which it interacts with several proteins of the synaptic release machinery (Kohli *et al.*, 2012). This raises the possibility that the enrichment of APP accumulations in presynaptic proteins results from the local sequestering of presynaptic proteins through interaction with APP. Interestingly, the ultrastructural analysis of APP accumulations did not provide evidence for synaptic vesicles but revealed synaptic vesicle proteins residing within the numerous multivesicular bodies observed in APP accumulations. The accumulations of presynaptic proteins in multivesicular bodies indicates

that the proteinopathy within these hallmarks is not specific of APP and that presynaptic impairment is a characteristic of AD.

The accumulation of presynaptic proteins contrasts with the absence of post-synaptic proteins in APP accumulations. Recent reports indicated that amyloid plaques are also mostly devoid of post-synaptic proteins (Hadley *et al.*, 2015; Sadleir *et al.*, 2016). Whereas APP accumulations are selectively enriched with presynaptic proteins, this does not imply that they display preserved post-synaptic compartments. On the contrary, we found that both MAP2 and Shank2 stainings are faint within dense-core amyloid plaques and APP accumulations, indicating that dendrites passing through these hallmarks are either absent or injured (dendritic spines alteration in AD are reviewed here (Herms and Dorostkar, 2016)).

Our observation of the selective accumulation of presynaptic proteins in discrete microscopic regions such as APP accumulations also contrasts with recent discoveries of broad downregulation of presynaptic proteins in AD brains (de Wilde *et al.*, 2016; Barthet *et al.*, 2018; Haytural *et al.*, 2020). Interestingly, we report that APP itself follows a complex pattern of misdistribution. Indeed, in addition of the accumulations found mostly within neuropil areas, we observed a marked reduction in the staining of APP in neuronal somata of AD hippocampus (Figure 1B-C, I) where APP is normally prominent. Importantly, this decrease correlates with the extent of APP accumulations burden (Figure 1J) suggesting that the two phenomena depend on the same cellular mechanisms, e.g. a slow-down of axonal retrograde transport. Indeed, an impairment of axonal transport represents a simple and straight-forward explanation for these complex variations in proteins abundance. A traffic jam would lead, on one hand, to the pathological accumulation of proteins at the site of blockage, and on the other hand, to their deficit where they exert their physiological function. Clinical and neuropathological studies have long indicated that synaptic impairment correlates strongly with cognitive deficits in AD (DeKosky and Scheff, 1990; Terry *et al.*, 1991; Selkoe, 2002; Scheff *et al.*, 2007). Moreover, recent evidence points to a specific impairment of presynaptic proteostasis in AD (Barthet and Mülle, 2020). How the misdistribution of proteins crucial for neurotransmitter release contributes to the impairment of memory and cognition in AD is a key question that remains to be addressed.

The presence of MVB harboring presynaptic vesicle proteins in APP accumulations, may represent the endpoint of an axonal degeneration process. Indeed, APP accumulation is a marker of axonal damage in several pathologies including multiple sclerosis (Ferguson *et al.*, 1997; Kuhlmann *et al.*, 2002), myelopathy (Umehara *et al.*, 2000), herpes simplex encephalitis (Mori *et al.*, 2005), or even traumatic brain injury (Gentleman *et al.*, 1993; Rorke-Adams, 2011). Given the well-established fast axonal transport of APP (Koo *et al.*, 1990; Sisodia *et al.*, 1993;

Lazarov *et al.*, 2007; Gunawardena *et al.*, 2013), APP may accumulate in AD as a result of axonal injury. The cause of such axonal injury could be diverse and possibly related to the destabilization of axonal microtubules by alterations (e.g. over-phosphorylation) of the microtubule-stabilizing protein Tau (Weingarten *et al.*, 1975). Indeed, phosphorylated-Tau (P-Tau) is enriched within APP accumulations but not its non-phosphorylated form (Figure 4). This observation offers the possibility of a functional interaction in the same compartment between the two main pathological actors of AD, APP and Tau. This hypothesis is supported by recent evidence that deletion of Tau reduces BACE1 around plaques and slows plaque formation in an AD mouse model (Peters *et al.*, 2019). Together, our results support a role of presynaptic APP in AD pathology, within P-Tau positive dystrophic neurites, where synaptic vesicle proteins are misdistributed, in keeping with a presynaptic failure hypothesis (Barthet and Mülle, 2020).

APP accumulations as local sources for A β production

The classical paradigm of amyloid plaque formation proposes that A β peptides, ubiquitously produced in the brain and in the periphery, would circulate in extracellular fluids before seeding and aggregating within amyloid plaques (Meyer-Luehmann *et al.*, 2006; Eisele *et al.*, 2010). However, this rationale seems inconsistent with the long observed decrease in A β concentration in cerebrospinal fluid and plasma of AD patients compared to non-demented controls (Motter *et al.*, 1995) and with the decreased proteolytic efficacy and processivity of γ -secretase containing PS1 with FAD mutations (Bentahir *et al.*, 2006; Cacquevel *et al.*, 2012; Woodruff *et al.*, 2013; Sun *et al.*, 2017).

One of the alternative paradigms of amyloid plaque formation, formulated by Fisher in 1907 in his early description of AD pathology, proposes that amyloid plaques derive from dystrophic, degenerating neuronal processes (reviewed in (Gouras *et al.*, 2005)). The collection of data we are presenting here, namely the striking accumulations of APP within distinct microscopic entities (Figure 1), the localization of APP accumulations in the close vicinity of dense-core plaques (Figure 2, 6), their content in secretases (Figure 3, 6), advocates that APP accumulations represent a local source for A β peptides composing the plaques. Indeed, the localization in the same compartments of APP and its secretases (BACE1 and PS1; Figure 3) could favor enzyme-substrate collisions and therefore A β production. This alternative formation of A β peptides is also supported by the remarkable correlation between APP accumulations and amyloid plaques areas (Figure 2). This model may also help understanding how abundant amyloid plaques can be formed in AD brains, despite the lower concentration of A β in extracellular fluids compared to controls.

Role of APP accumulations in AD pathology

The number of APP accumulations correlates with faster progression of the disease, as indicated by the higher density observed in the early-onset form of the disease (FAD cases) than in the late-onset form (SAD cases) (Figure 1D and S1E). This suggests that APP accumulations could play a role in the progression of the disease. Besides, the Braak Tau stage and the amyloid stage were not higher in our FAD cohort compared to the SAD cohort (Table S2, 3). Thus, APP accumulations may represent an additional reliable indicator of the severity of the disease. Finally, we observed an inverse correlation between the number of APP accumulations and neuronal loss (Figure 8).

The molecular mechanisms by which APP accumulations may be pathogenic have not been investigated here, but it is tempting to speculate on their role as local sources to fuel amyloid plaques as described above. Alternatively, APP accumulations may trigger degeneration through impairing presynaptic function, e.g. by co-aggregating or misrouting synaptic vesicles proteins (Barthet *et al.*, 2018). Indeed, intriguing observations that the genetic deletion, or the loss of function (using neurotoxins), of several presynaptic proteins leads to neurodegeneration have been reported. For example, loss of Munc18, which prevents neurotransmitter release, leads to a rapid neurodegeneration (Verhage *et al.*, 2000; Heeroma *et al.*, 2004; Dudok *et al.*, 2011); genetic deletion of CSP α revealed that it cooperates with α -synuclein and SNAP25 to prevent neurodegeneration (Fernández-Chacón *et al.*, 2004; Chandra *et al.*, 2005; Sharma *et al.*, 2012); and use of botulinum neurotoxins revealed a direct role of syntaxin 1 and SNAP-25 in neuron survival (Berliocchi *et al.*, 2005; Peng *et al.*, 2013). Whatever the mechanisms involved in APP pathological function, targeting APP in AD therapeutical trials could be promising. Indeed, despite numerous clinical trials targeting A β peptide (β -secretase or γ -secretase inhibitors, active or passive immunotherapy against A β peptides), no treatment to cure or even to slow down AD progression is available (Mangialasche *et al.*, 2010; Huang *et al.*, 2020). There is thus, a huge need for alternative approaches in AD clinical research. Based on the present results, we propose the Nter domain of APP, very accessible to the binding of therapeutic molecules due to its extracellular topology, as a therapeutic target in an attempt to prevent APP accumulation and misprocessing.

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Author contributions

T.J-S., M.P., V.K. conducted the experiments, F.C. developed ImageJ macros for images analyses, S.F. supervised the experiments performed at the Karolinska Institute, U.M. provided research materials and advices, T.J-S., G.B. designed the experiments, T.J-S., C.M., G.B. wrote the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Methods

Human brain tissue

The use of human brain material was approved by the ethical and research committees after the consent of patients or relatives for research use. Paraffin-embedded samples were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam (www.brainbank.nl) or from and the Karolinska Institutet brain bank (KIBB). All Material has been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB.

We used sections from the hippocampus of 11 control patients (mean age 79.6 ± 6.93 , 73%F and 27%M, Table 1), 12 sporadic AD patients (mean age 81 ± 9.6 , 92%F and 8%M, Table 2) and 4 familial AD cases (all cases with different PSEN1 mutations, mean age 48.5 ± 9.7 , 50%F and 50%M Table 3).

Mice

In agreement with the Bordeaux/CNRS Animal Care committee, heterozygous APP/PS1 female mice co-expressing human APP Swedish mutation (KM670/671NL) and PS1 Δ Exon9 were used (Jankowsky *et al.*, 2004). Mice were obtained from crossing heterozygous female transgenic mice with B6C3F1/J male mice. Animals were caged in groups of 4 to 10 with access to water and food. Rooms were kept at a stable temperature with 12 h light/dark cycle under pathogen-free conditions until surgery. After surgery mice were kept in single cages. Brain sections (5 μ m) were also obtained from from 12-month- old female APP-KI NL-G-F mice expressing the Swedish (KM670/671NL), Iberian (I716F) and Artic (E693G) mutations (Saito *et al.*, 2014). Sections were fixed in PFA 4 % and mounted with paraffin.

List of antibodies

Antibody		Company	Reference	Antigen retrieval treatment	Species	Dilution
APP-Cter Y188	-	Abcam	#32136	Citrate buffer or formic acid 70%	Rabbit	1:500
APP-Cter C1/6.1	-	BioLegend	# SIG-39152	Citrate buffer	Mouse	1:500
APP-Nter JRD32	-	Johnson and Johnson	-	Formic acid 70%	Mouse	1:500

APP-Nter 22C11	-	eBioscience	#14-9749-82	Citrate buffer	Mouse	1:500
BACE1 - D10E5		Cell Signaling	#5606	Citrate buffer	Rabbit	1:500
PS1-CTF 33B10	-	N. Robakis laboratory	-	Citrate buffer	Mouse	1:500
A β 42 -12F4		BioLegend	# 805501	Formic acid 70%	Mouse	1:500
A β 1-16 - DE2		Sigma Aldrich	MAB5206	Citrate buffer	Mouse	1:500
MAP2		Sigma Aldrich	M4403	Citrate buffer	Mouse	1:500
MBP		Abcam	ab216668	Citrate buffer	Rabbit	1:500
NeuN		Merck	#ABN78	Citrate buffer	Rabbit	1:500
Syntaxin1a		Alomone	#ANR-002	Citrate buffer	Rabbit	1:1000
Synaptotagmin-1		Synaptic Systems	#105011	Citrate buffer	Mouse	1:500
VAMP2		Synaptic Systems	#104211	Citrate buffer	Mouse	1:500
Shank2		Synaptic Systems	#162 204	Citrate buffer	Guinea pig	1:500

Immunohistochemistry on human postmortem brain sections

DAB immunostaining

A Dako EnVision + Dual Link System-HRP (#K4065) was used to perform stainings with DAB. Paraffin-embedded hippocampus sections (5 μ m) were sliced and mounted on microscope glasses. Sections were incubated 2 times in xylene ($\geq$ 98.5 %, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent) for 10 minutes and hydrated for 3 minutes in different baths ranging from 99% to 70% ethanol concentrations. Then, sections were incubated in a pressure cooker (Biocare medical) at 110 °C for 30 min either in citrate buffer (10 mM Citric acid (Sigma Aldrich 251275), 0,05 % Tween-20 (Sigma Aldrich P7949), pH 6) or formic acid 70 % ((Sigma Aldrich F0507) pH 2) depending on the protein staining. Brain sections were washed with PBS-Tween 0,05 % (PBS-T) and blocked with a peroxidase enzyme blocker for 5 minutes (Dako Dual Endogenous Enzyme Block S2003) and goat serum (NGS; Thermo Fisher Scientific PCN5000) for 20 minutes at RT. Sections were then incubated at room temperature (RT) for two hours with one primary antibody. After 3 washes in PBS-Tween, sections were incubated with peroxidase-conjugated secondary antibody. Sections were then washed and incubated 2 minutes with 3,3'-diaminobenzidine chromogen solution. Sections were washed again and

incubated with Hematoxylin (Sigma Aldrich #51275) for 5 min at RT before being dehydrated in baths of 3 min ranging from 70% to 99% ethanol and finally in xylene for 10 min. Sections were finally mounted with Vecta mount (VectaMount® Permanent Mounting Medium #H-5000) and dried overnight. Preabsorption controls in human sections were performed by using the APP C-terminal peptide sequence KMQQNGYENPTYKFFEQMQN (Proteogenix) at 1 µg/ml and Y188 or C1/6.1 at 1 µg/ml (equal mass concentration, i.e. excess molarity of the adsorption peptide).

Immunofluorescence

Brain sections were sliced, hydrated and treated for epitope retrieval as for the DAB immunostaining. In addition, slices were washed and blocked with NGS, before incubation for 2 hours at RT with one or a with a combination of primary antibodies. Then, sections were washed in PBS-Tween and incubated with an autofluorescence quencher (TrueBlack® Lipofuscin Autofluorescence Quencher - Biotium #23007) for 5 minutes at RT. After 3 washes in PBS 1X, sections were incubated at RT for 2 hours with secondary antibodies diluted at 1/500 (coupled with Alexa 488 or 568 - Thermo Fisher Scientific) and with Methoxy X04 (5 µM) (Tocris Bioscience #4920) or DAPI (300 nM) for 20 minutes. Finally, sections were mounted in Fluoromount™ (Southern Biotech #0100-01) and dried overnight.

Mouse immunohistochemistry

Mice were first anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg body weight). Mice were then perfused intracardially with 0.9 % NaCl for 1 min and then with 4 % paraformaldehyde in 0.1 mM PBS (PFA 4 %) for 4 min. Brains were then dissected and post-fixed for 8 h in PFA 4 %. Coronal sections of 50 µm were cut on a vibratome (Leica VT1200S) and permeabilized in PBS 0.3 % Triton X-100 (Sigma Aldrich - #9002-93-1) at RT for 2 hours. Brain sections were then incubated overnight at 4°C with the primary antibody diluted in PBS-T. In addition, sections were also incubated at RT for 5 min with an autofluorescence quencher (True black). After 3 washes in PBS 1X, sections were incubated at RT with secondary antibodies for 2 hours. Finally, slices were incubated in Methoxy-X04 or in DAPI for 10 min before getting mounted in Fluoromount™.

Brain section imaging

Brain sections were all imaged using the bright field microscope Nanozoomer 2.0HT (Hamamatsu) with a 20X objective and fixed additional lens 1.75X. For every experiment, imaging settings and acquisitions were maintained constant between different patient groups.

Image analysis

Image acquisitions were exported as .tiff files in independent channels by using the NDP.view2 software (Hamamatsu). Using Image J, images were then calibrated and processed as 8-bits grayscale images (0-255). A high manual threshold was first applied to the APP accumulations and plaques to remove any signal coming from other non-interesting structures. The structures of interest were then saved separately as regions of interest (ROIs) and enlarged by delineating new boundaries 15 μm away from their detection perimeter for posterior analysis. The two ROIs were then merged and used as an overlay to extract the objects from the initial image. Finally, from the extracted images, colocalization studies were performed between different channels by using the Mander's overlap coefficient (MOC) (Figure S1J, K). Images were analyzed by using the JACop plugin (<http://www3.interscience.wiley.com/cgi-bin/fulltext/118727584/PDFSTART>; 2006), which provides the proportion of co-localized pixels ranging from 0 (no co-localization) to 1 (perfect co-localization). For some markers, the threshold setting was done automatically by using the plugin 'Auto threshold', which provides a set of different automatized algorithms. APP C-terminal accumulations and plaques (methoxy X04) were selected by using the 'Max entropy' algorithm that implements the Kapur-Sahoo-Wong thresholding method (Kapur *et al.*, 1985). For A β antibody stainings, the algorithm 'Yen' was applied (Yen *et al.*, 1995). For Syntaxin1a and Shank2 the 'Moments' algorithm and for myelin basic protein the 'Default' algorithm was respectively applied. Manual thresholding was only applied when none of the automatized algorithms was able to detect reliably markers without a clear signal to noise ratio, i.e for APP N-terminal, VAMP2, syt1, BACE1 and PS1. Costes method was used as a statistical test to evaluate the correlation between different channels (Dunn *et al.*, 2011). The p value for all the images tested in this study was equal to 1. The average pixel intensity for each of the proteins was also measured within the surface of the APP accumulation and 15 μm outside by using the ROI manager function and the gray value distribution histogram.

DAB stainings were used to count APP accumulations with an automated macro that was segmenting, counting and measuring the size of each accumulation in different regions of the brain section. Any misdetections were avoided when the user was invited to check for any false-positive counts or by adding any miss-counted accumulations (https://github.com/fabricecordelieres/IJ-Macro_APP-Plaques-NanoZoomer)

Sholl analysis (Sholl, 1953) was used to measure the area and number of APP accumulations around amyloid plaques labeled for A β 42. For this aim, an automated macro was used to isolate and count the number of APP accumulations in intervals of 5 μm , up to 50 μm distance away from plaques in human and mouse sections. The area and number of A β 42-positive

plaques per hippocampal region were also provided by the macro (https://github.com/fabricecordelieres/IJ-Macro_APP-Aggregates-Per-Plaques-NanoZoomer).

In the mouse brain, similar analyses were performed by using the MOC and Sholl analyses. JACop plugin was used to calculate the MOC in individual plaques and APP accumulations. Thresholds were set automatically for each of the markers stained: APP-Cter and APP-Nter ('Percentil' algorithm), A β 1-16 ('Yen' algorithm), Methoxy ('Max entropy' algorithm). Neuron cell counting was performed at the somatosensory cortex and the molecular layer by using the cell counter plug in of Image J.

Electron Microscopy - ultrastructure studies

Electron microscopy was performed on samples from the cortex of 2 WT and 3 APP/PS1 mice to visualize the structural content of APP accumulations. To this aim, mice were anesthetized injecting intraperitoneal urethane. After 5 min, mice were perfused intracardially with 35°C oxygenated (saturating carbogen 95% O₂ and 5% CO₂) ACSF 1X (in mM: 26 NaHCO₃, 2.5 KCl, 16.5 glucose, 2.8 pyruvic acid, 0.5 ascorbic acid, 120 NaCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, pH 7.4, osmolarity of 305 mOsm) for 4 minutes. Afterwards, 2.5 % paraformaldehyde in 0.1 % glutaraldehyde was perfused intracardially for 10 min until animal fixation. Brains were dissected, post-fixed in PFA for 8 hours and sliced coronally at 250 μ m. Sections were permeabilized using sodium borohydride 1% (Sigma Aldrich #452882) at RT for 30 min. Sections were then stained with DAB for APP-C and N-terminal or A β 1-16.

Brain sections were post-fixed at RT in 1 % osmium tetroxide in the dark. Slices were then dehydrated in an ascending series of ethanol dilutions from 50 to 100 % for 10 min each. Furthermore, slices were incubated into a 50 % mix (vol.) of Epon 812 resin (Electron Microscopy Sciences) and pure ethanol for 2 h at RT and finally in pure resin overnight. After another bath in pure resin for 5 hours, slices were finally embedded between 2 sheets of aclar, and polymerized at 60°C for 48 hours.

The regions of interest were cut at 500 nm thick sections by using an ultramicrotome and a Diatome diamond knife (Leica EM UC7). Sections of 70 nm thick were then collected on copper grids (Electron Microscopy Sciences) and observed with a transmission electron microscope Hitachi H7650 equipped with a Gatan Orius CCD camera.

Electron Microscopy - Tokuyasu Immunolabeling with gold particles

2 APP/PS1 and 2 WT mice at 9 months were sacrificed and chemically fixed by using the protocol previously described. Sections were then stained with DAB for APP-C or N-terminal. After chemical fixation, small cortical sections were incubated in 2.3M sucrose letting them rinse overnight at RT. Sections were then mounted with pins and immersed in liquid nitrogen

to slice them at 70 nm with a 35° angle Diatome diamond knife at -120°C. Sections were collected in copper grids to be washed with water and incubated with PBS 1x – glycine (0.05mM) for 15 min at RT to start the immunolabelling. Sections were first incubated with a blocking solution of goat conjugate for 30 min and then in a PBS1x-0,1% BSA solution in different baths. The primary antibodies used were then added after diluting them in a PBS1x-0,1% BSA solution. The sections were then washed with PBS 1x-0.1% BSA and incubated with the secondary antibody coupled with gold particles (goat anti-rabbit 10 nm with a dilution of 1:40 for APP C-ter and goat anti-mouse 15 nm with a dilution of 1:50 for VAMP2). After six washes of 5 min each with PBS 1x-0,1% BSA, sections were postfixed with 2% glutaraldehyde. The next wash was done in distilled water and a heavy metal contrast incubation was performed in the dark with aqueous uranyl acetate containing 2% methylcellulose for 10 min in ice. Finally, the grids were gently dried using filter paper.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was used to perform all the statistical analyses. Experimental data were first tested for normal distribution using d'Agostino and Pearson tests. Data were represented using scatter plots in which the mean or median was included or as histograms of the means. Normally distributed data were compared by using a two-tailed t-test and data that were non-normally distributed were compared using a two-tailed Mann-Whitney rank test. Two-way ANOVA was used for analysis with 2 different factors (number of APP accumulations and patient groups) and represented as histograms with error bars ($\pm$ SEM). Differences between groups were considered statistically significant when $p < 0.05$.

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Table 1

Table 1 - Clinical resume table

Case	Gender	Braak stage	Age of onset	Age of death	PMD (h)	Brain weight (g)	CSF PH	Brain bank	Number of patients
Control	F/M (73/27%)	I-III	-	79,6	8,54	1204	6,75	NBB/KI	11
SAD	F/M (92/8%)	V-VI	72,1	81	11,08	1061	6,43	NBB/KI	12
FAD	F/M (50/50%)	V-VI	40,7	40,7	3,5	1101	6,22	NBB/KI	4

M – male, F – female, KI – Karolinska Institutet, NBB – Netherlands Brain Bank

Main figures

Figure 1

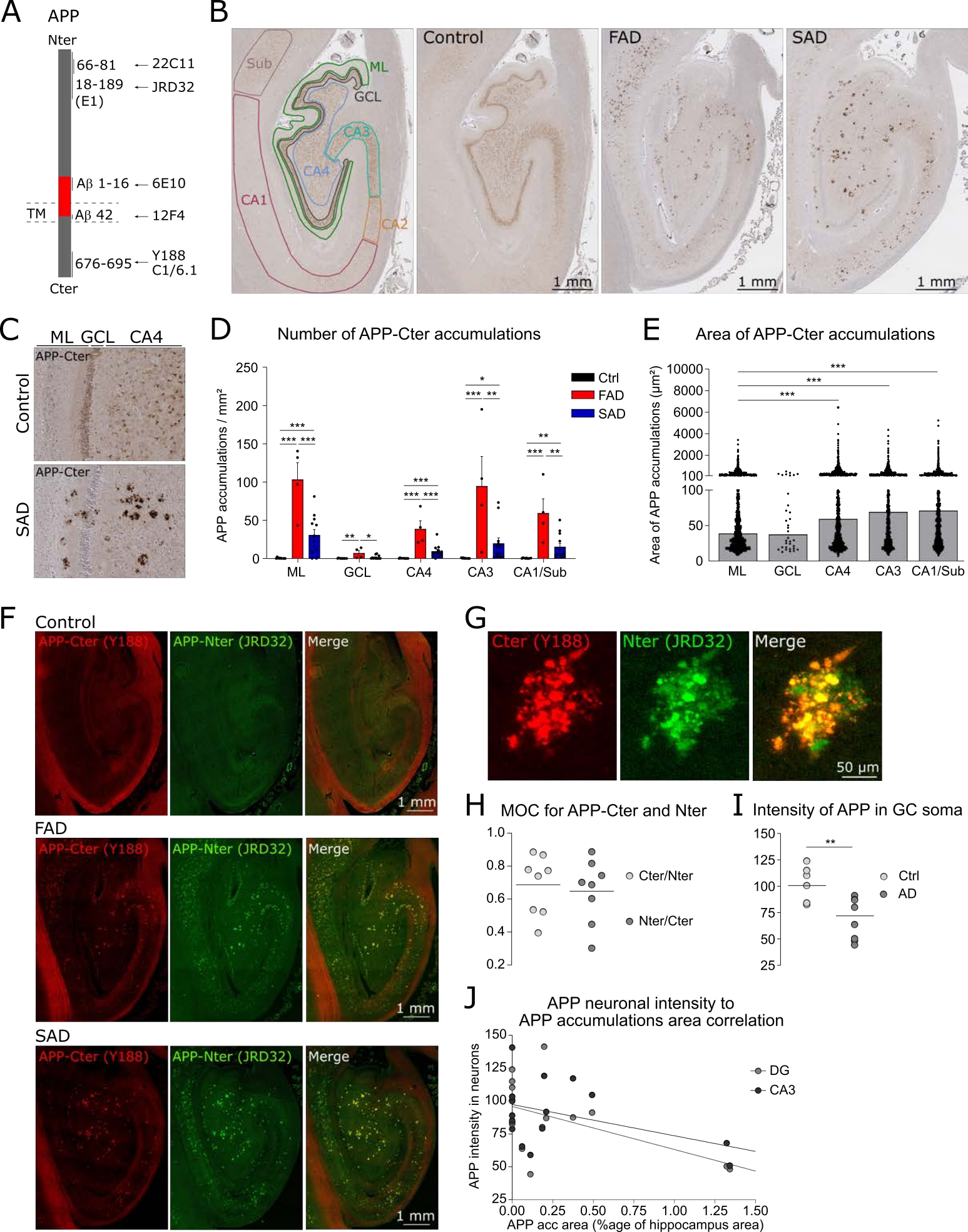


Figure 1. Misdistribution of APP in the hippocampus of AD patients (A) Scheme of full-length APP showing the epitopes in the Nter, Cter and A β domains of six antibodies against APP. The red segment represents the A β sequence spanning through the transmembrane (TM) domain. (B) Pictures of human hippocampal sections stained by DAB against APP-Cter with C1/6.1 antibody. Cases from control, FAD and SAD groups are illustrated. The first panel on the left depicts different anatomical regions where the subsequent quantifications have been performed. In controls, APP is stained mostly in the soma of neuronal layers of the dentate gyrus granule cells (GCL), CA4 and CA3. In both FAD and SAD cases, intense extra-somatic APP stainings are observable. (C) Image magnification of the dentate gyrus as in 'B'. In the SAD case, intense extra-somatic APP accumulations are observable in the dentate gyrus molecular layer (ML), GC and CA4 regions. (D) Histogram of the number of APP-Cter accumulations in different hippocampal regions in the control (ctrl), FAD or SAD conditions. APP accumulations are barely detected in Ctrl whereas they are abundant in AD. The number of accumulations is higher in FAD compared to SAD condition. (E) Histogram of the area of APP accumulations in different hippocampal regions. The CA regions contain larger APP accumulations than the ML. (F) Human hippocampal sections fluorescently immuno-labeled against APP-Cter with Y188 antibody and APP-Nter with JRD32. In controls, both Cter and Nter APP antibodies mostly label the soma of neurons in the GCL, CA4 and CA3. In addition, Y188 nonspecifically stained the white matter tracts (see FigS1C). In both FAD and SAD cases, intense extra-somatic APP stainings, co-labeled with the two antibodies are observable. (G) Close-up picture of an APP accumulations co-labeled with APP-Cter and Nter antibodies. (H) Scatter plot of Manders overlapping coefficients (MOC) (Method described in FigS1L-M) between APP-Cter and Nter labelings. The analysis revealed a high colocalization between the two channels suggesting the presence of full-length APP in the accumulations. (I) Scatter plot of the intensity of APP labelling in the GC soma normalized to the mean intensity in controls. The intensity is significantly reduced in the AD group (J) Graph of the mean pixel intensity of APP staining in neuronal somas in function of the area covered by APP accumulations (normalized to the hippocampus area). The linear regression of the data presents a negative slope. The Pearson correlation coefficient was -0.53 for DG ($p=0.035$), -0.43 for CA3 ($p=0.046$), indicative of an inverse correlation between the extent of APP accumulations and APP intensity in the neuronal soma.

Figure 2

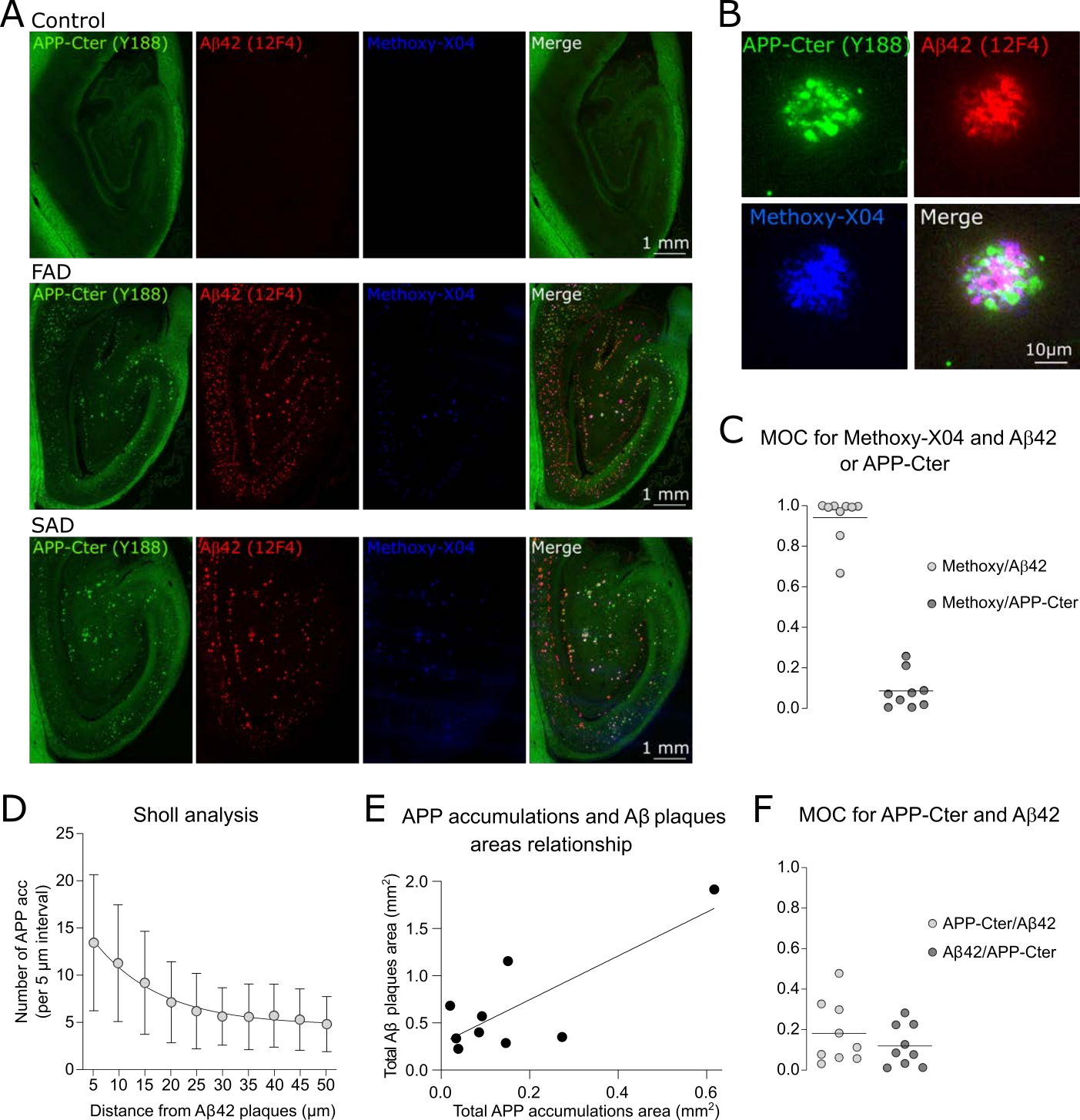
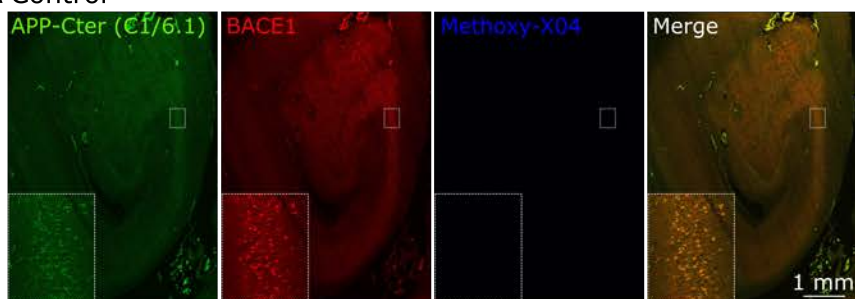
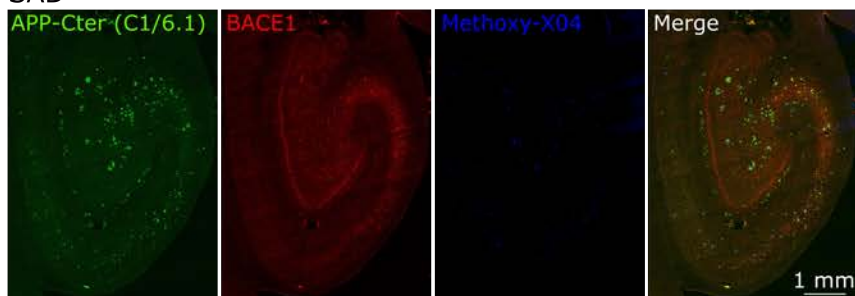
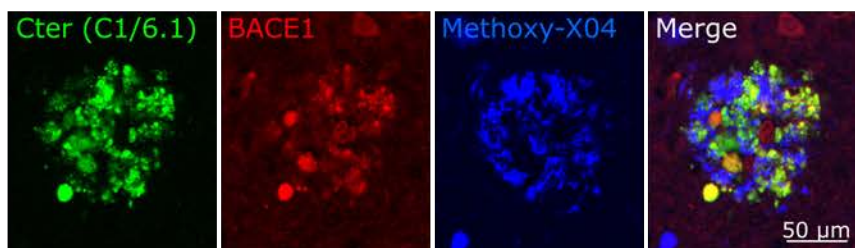
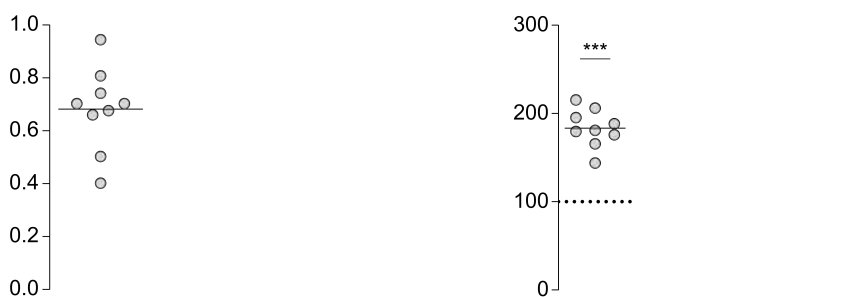
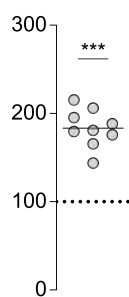
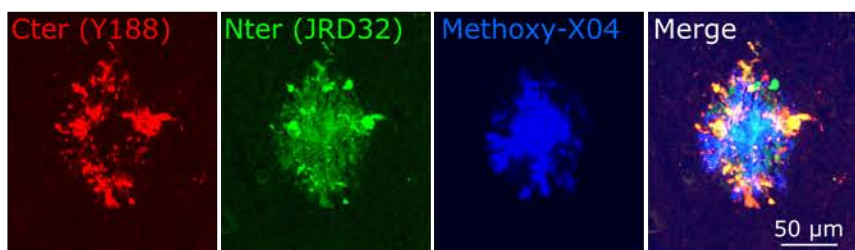
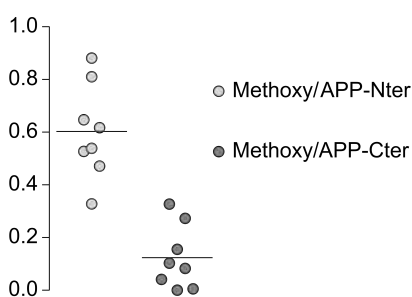


Figure 2. APP accumulations surround dense-core amyloid plaques (A) Human hippocampal sections fluorescently immuno-labeled against APP-Cter and A β 42 with respectively Y188 and 12F4 antibodies and with methoxy-X04. In both FAD and SAD cases, intense A β 42 and methoxy-X04 labelings are observable in close proximity to APP accumulations whereas no staining is observable in control. (B) Close-up images of APP accumulations surrounding an amyloid plaque co-labeled with A β 42 and methoxy-X04. A β 42 and methoxy-X04 colocalize well together but not with APP-Cter. (C) Scatter plot of the MOC between methoxy-X04 and A β 42 or APP-Cter. The analysis revealed a high colocalization between methoxy-X04 and A β 42 but not with APP-Cter indicating that amyloid plaques and APP accumulations are located nearby but non-overlapping. (D) Graph of a Sholl analysis of the number of APP accumulations in function of the distance from the edge of A β 42 plaques. More APP accumulations are present close to the amyloid plaques. (E) Graph of the total area covered by amyloid plaques in the hippocampus of AD cases in function of the total area of APP accumulations. The linear regression of the data presents a positive slope. The Pearson correlation coefficient was 0.79 ($p=0.011$), indicative of a positive correlation between the amyloid load and the extent of APP accumulations. (F) Scatter plot of MOC between APP-Cter and A β 42. The analysis revealed a low colocalization between the two channels indicating that amyloid plaques and APP accumulations are non-overlapping.

Figure 3

A Control**SAD****B****C** MOC for APP-Cter and BACE1**D** BACE1 intensity inside APP acc**E****F**

MOC for Methoxy and APP

**G**

APP intensity in the core of the plaque

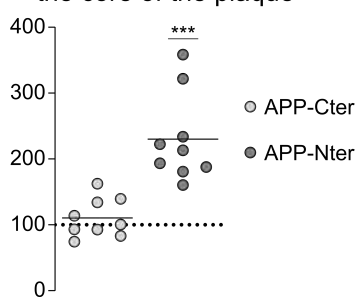
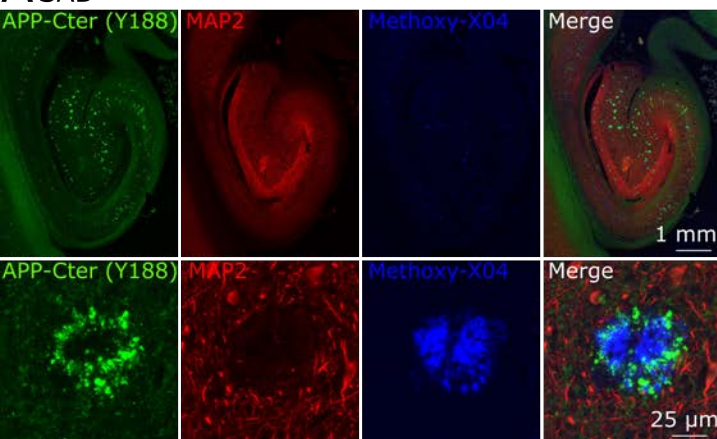
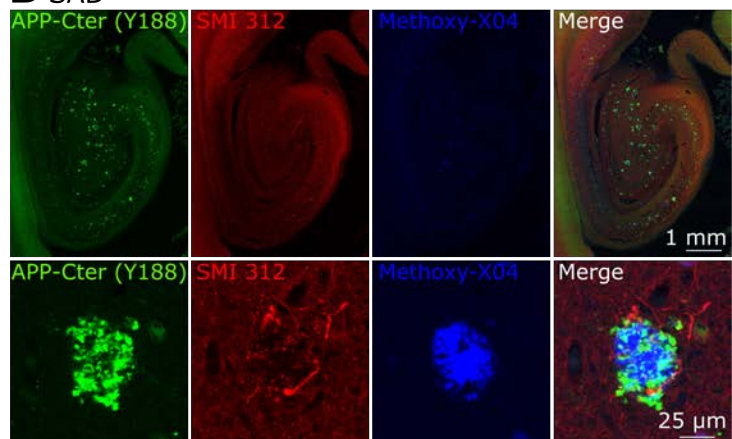
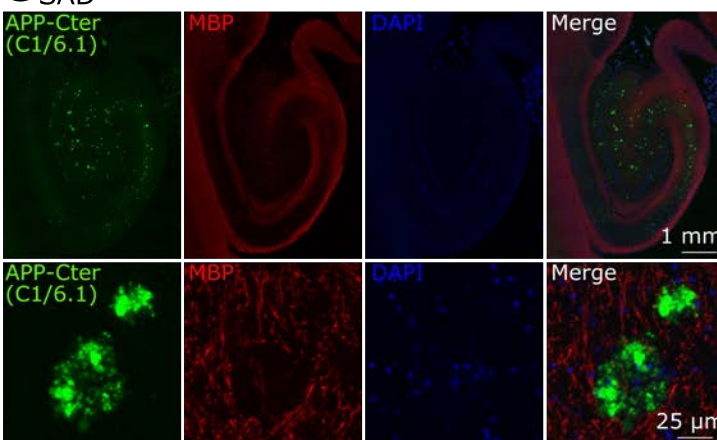
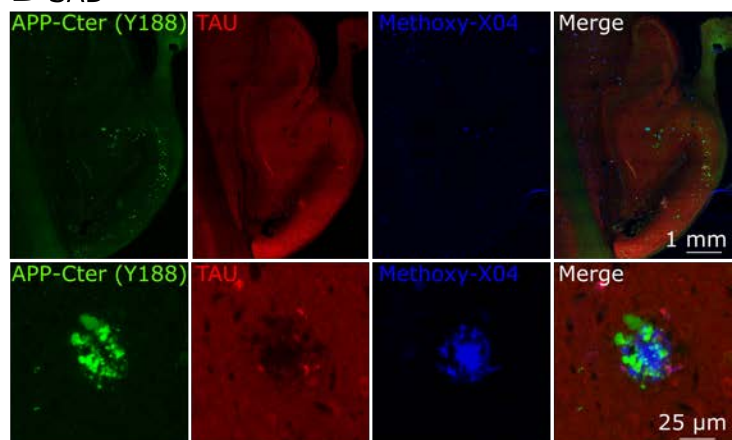
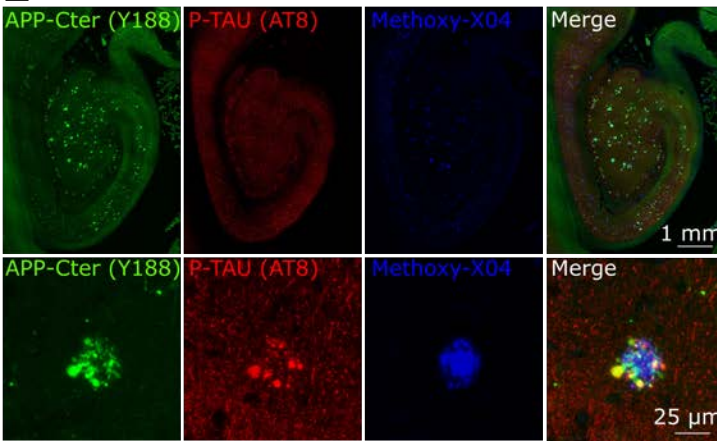
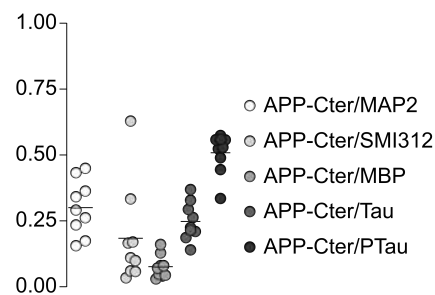


Figure 3. APP accumulations contain the secretases to produce A β peptides and APP-Nter enriched in the core of amyloid plaques (A) Human hippocampal sections immunolabelled against APP-Cter, BACE1 and methoxy-X04. BACE1 is prominently observed in neuronal soma reminiscent of the expression of APP, in addition to a fainter labeling of the neuropil. In both FAD and SAD cases, BACE1 staining is observable in APP accumulations. (B) Close-up pictures of stainings as in 'A' showing BACE1 and APP accumulations colocalizing in the surrounding of an amyloid plaque labeled with methoxy-X04. (C) Scatter plot of the MOC between APP-Cter and BACE1. (D) Scatter plot of the intensity of BACE1 labeling within APP accumulations. The data are normalized to the intensity outside the APP accumulations represented here by a dashed line. The analysis revealed that BACE1 is enriched inside the APP accumulations. (E) Close-up pictures of hippocampal amyloid plaques labelled with APP-Cter, Nter and methoxy-X04 revealed a remarkable abundance of APP-Nter in the core of dense-core amyloid plaques surrounded by APP. (F) Scatter plot of the MOC between methoxy-X04 and APP-Nter or Cter. The analysis revealed a high colocalization between methoxy-X04 and APP-Nter but not with APP-Cter. (G) Scatter plot of the intensity of APP-Cter or Nter labelings within the core of the plaques labeled with methoxy-X04. The data are normalized to the intensity outside the plaques and accumulations represented here by a dashed line. The analysis revealed that APP-Nter is enriched in the core of the plaques whereas APP-Cter is not.

Figure 4

A SAD**B** SAD**C** SAD**D** SAD**E** SAD**F**

MOC for APP-Cter to neuritic markers

**G**

P-Tau intensity in APP accumulations

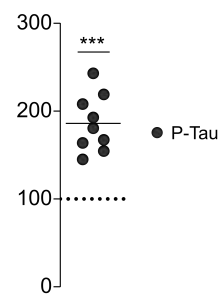


Figure 4. Phospho-Tau concentrate in APP accumulations (A) Pictures of a human hippocampal section labeled against APP-Cter and MAP2 and with methoxy-X04. The lower panels show a hippocampal dense-core plaque (revealed by methoxy-X04) and surrounded by APP-Cter. It corresponds to a dark area in the MAP2 channel. (B) Pictures as in (A) but with SMI312. The lower panels show a hippocampal dense-core plaque (revealed by methoxy-X04) and surrounded by APP-Cter. Some SMI312 positive neurites surrounds the plaques but do not colocalize with APP-Cter. (C) Hippocampal sections stained for APP, MBP and DAPI. APP accumulations are mostly found out of regions labelled for MBP. The close-up pictures show the absence of colocalization between MBP and APP. (D) Hippocampal sections stained for APP, Tau and methoxy-X04. Close-up pictures show few enlarged neurites positive for Tau and negative for APP. (E) Hippocampal sections stained for APP, phosphorylated-tau (P-Tau) and methoxy-X04. Close-up pictures show several enlarged neurites positive for P-Tau which colocalize with APP. (F) Scatter plot of the MOC between APP-Cter and the neuritic markers labeled in Fig4. The colocalization is low for MAP2, SMI312, MBP, and Tau but high for P-Tau (0.53). (G) Scatter plot of the intensity of P-Tau labeling within APP accumulations. The data are normalized to the intensity outside the APP accumulations represented here by a dashed line. The analysis revealed that P-Tau is enriched inside the APP accumulations.

Figure 5

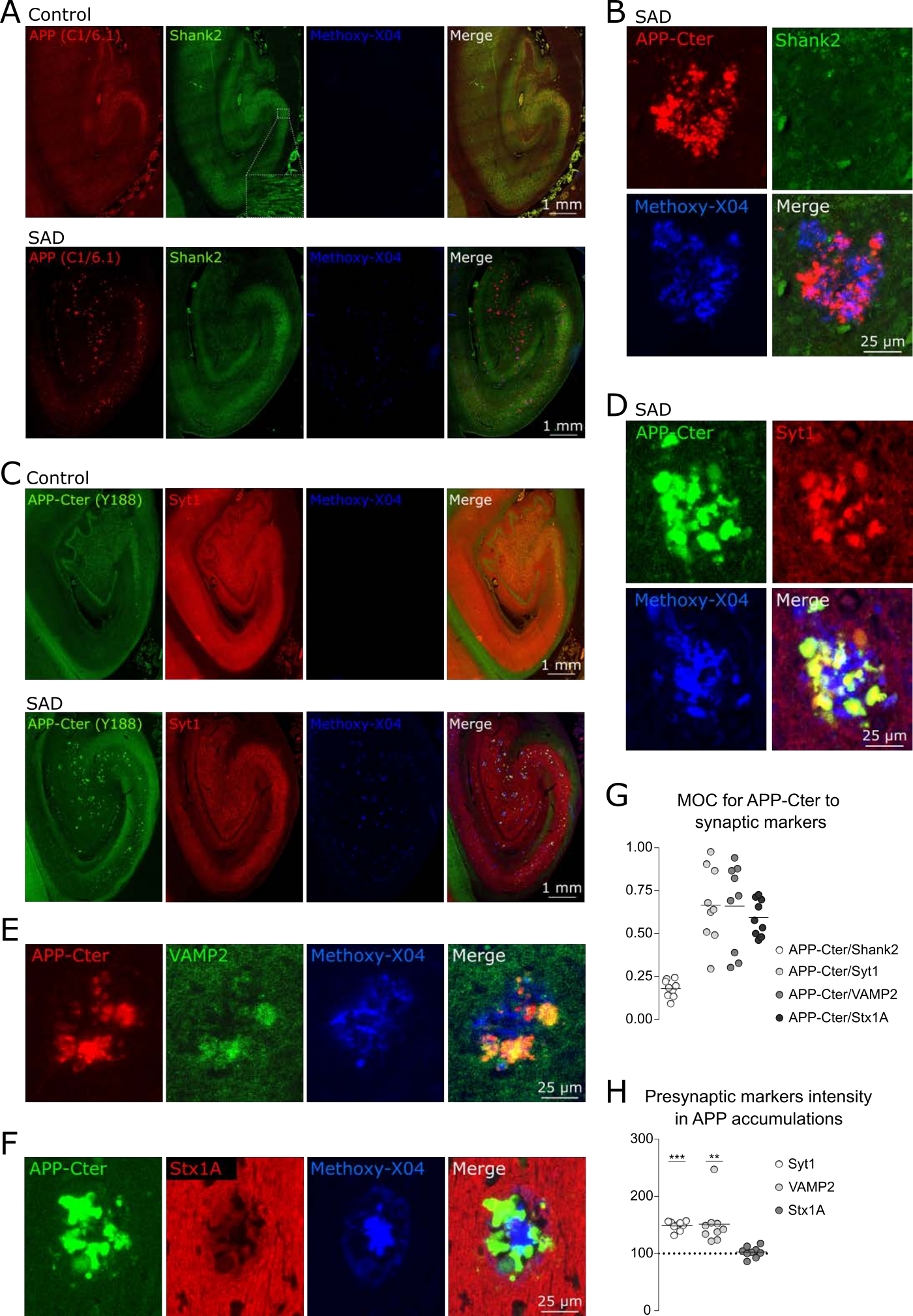


Figure 5. Synaptic vesicle proteins but not postsynaptic proteins concentrate within APP accumulations (A) Human hippocampal sections immuno-labeled against APP-Cter and Shank2 and with methoxy-X04. Shank2 is prominently observed in neuronal soma and in the neuropil in both control and AD condition. (B) Close-up pictures of stainings as in 'A' showing the absence of Shank2 in APP accumulations surrounding an amyloid plaque labeled with methoxy-X04. (C) Human hippocampal sections immuno-labeled against APP-Cter and synaptotagmin-1 (Syt1) and with methoxy-X04. In controls, Syt1 is prominently observed in the neuropil, especially in presynaptic terminals in the CA4 region. In AD, Syt1 strikingly accumulates at the level of APP accumulations. (D) Close-up pictures of stainings as in 'C' showing the presence of Syt1 in APP accumulations close to an amyloid plaque labeled with methoxy-X04. (E) Close-up pictures of stainings showing the presence of VAMP2 in APP accumulations close to an amyloid plaque labeled with methoxy-X04 (full-hippocampus acquisition related to this condition are shown in FigS5A). (F) Close-up pictures of stainings showing the presence of syntaxin-1a in APP accumulations nearby an amyloid plaque labeled with methoxy-X04 (full-hippocampus acquisition related to this condition are shown in FigS5B). (G) Scatter plot of the MOC between APP-Cter and the synaptic markers labeled in Fig5. The colocalization is high for the presynaptic markers Syt1, VAMP2 and Stx1A but low for Shank2. (H) Scatter plot of the intensity of presynaptic markers labelings within APP accumulations. The data are normalized to the intensity outside the APP accumulations represented here by a dashed line. The analysis revealed that synaptic vesicle proteins Syt1 and VAMP2 are enriched inside the APP accumulations whereas the cell surface protein Stx1A is not.

Figure 6

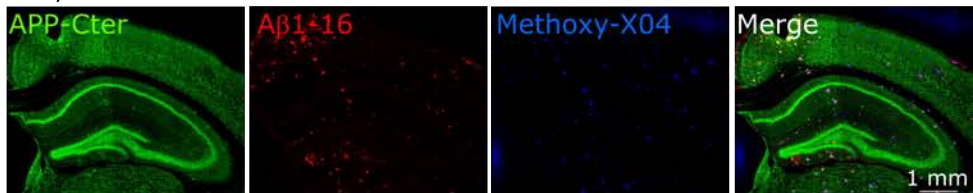
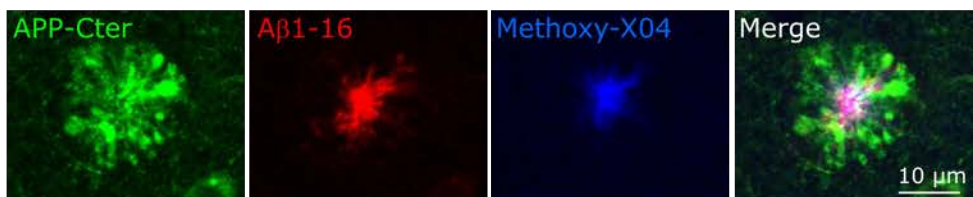
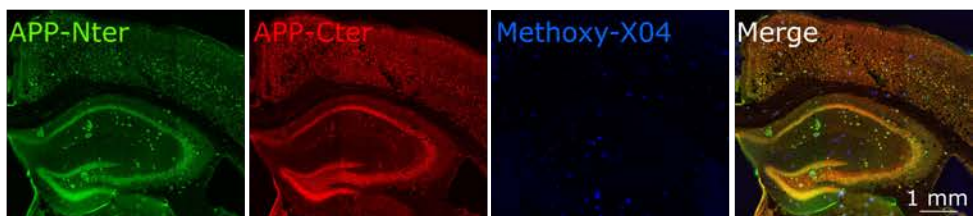
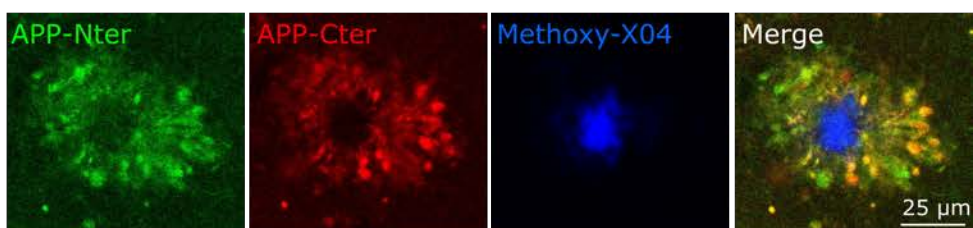
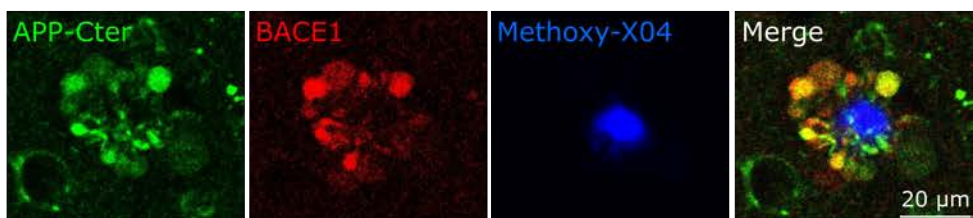
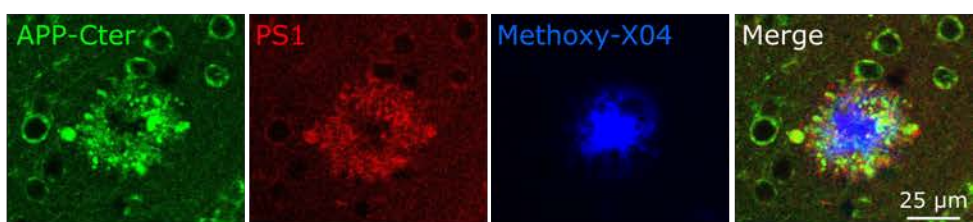
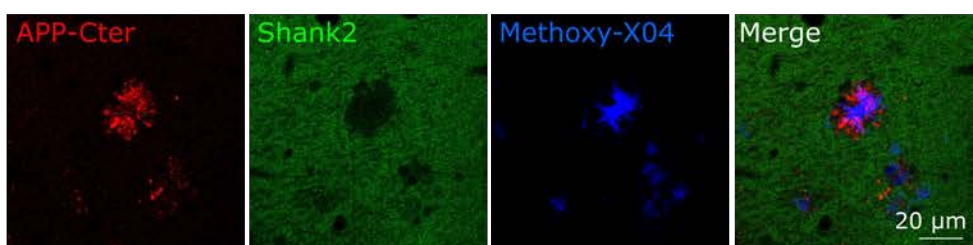
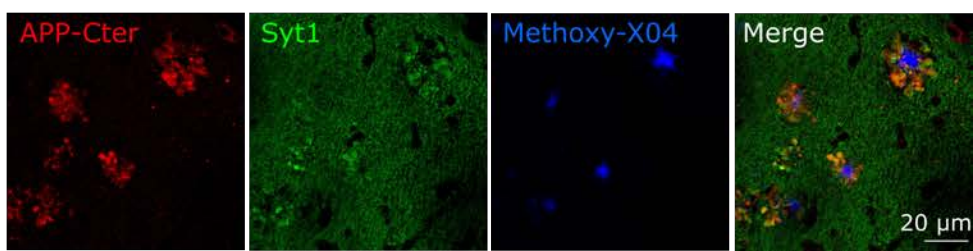
A APP/PS1**B****C****D****E****F****G****H**

Figure 6. AD mouse models recapitulate the features of APP accumulations observed in human (A, C) APP/PS1 mouse hippocampal sections immuno-labelled against APP-Cter and either A β 1-16 in 'A' or against APP-Nter in 'C'. (B, D) Close-up pictures of stainings as in 'A, C' showing that amyloid plaques are surrounded by APP accumulations as in human AD conditions. (E, F) Close-up pictures immuno-labelled against APP-Cter and either BACE1 in 'E' or PS1 in 'F' showing that APP accumulations contain APP-secretases necessary for A β production as in human AD conditions. (G, H) Close-up pictures of stainings showing the absence of Shank2 and the presence of Syt1 in APP accumulations surrounding amyloid plaques labelled with methoxy-X04.

Figure 7

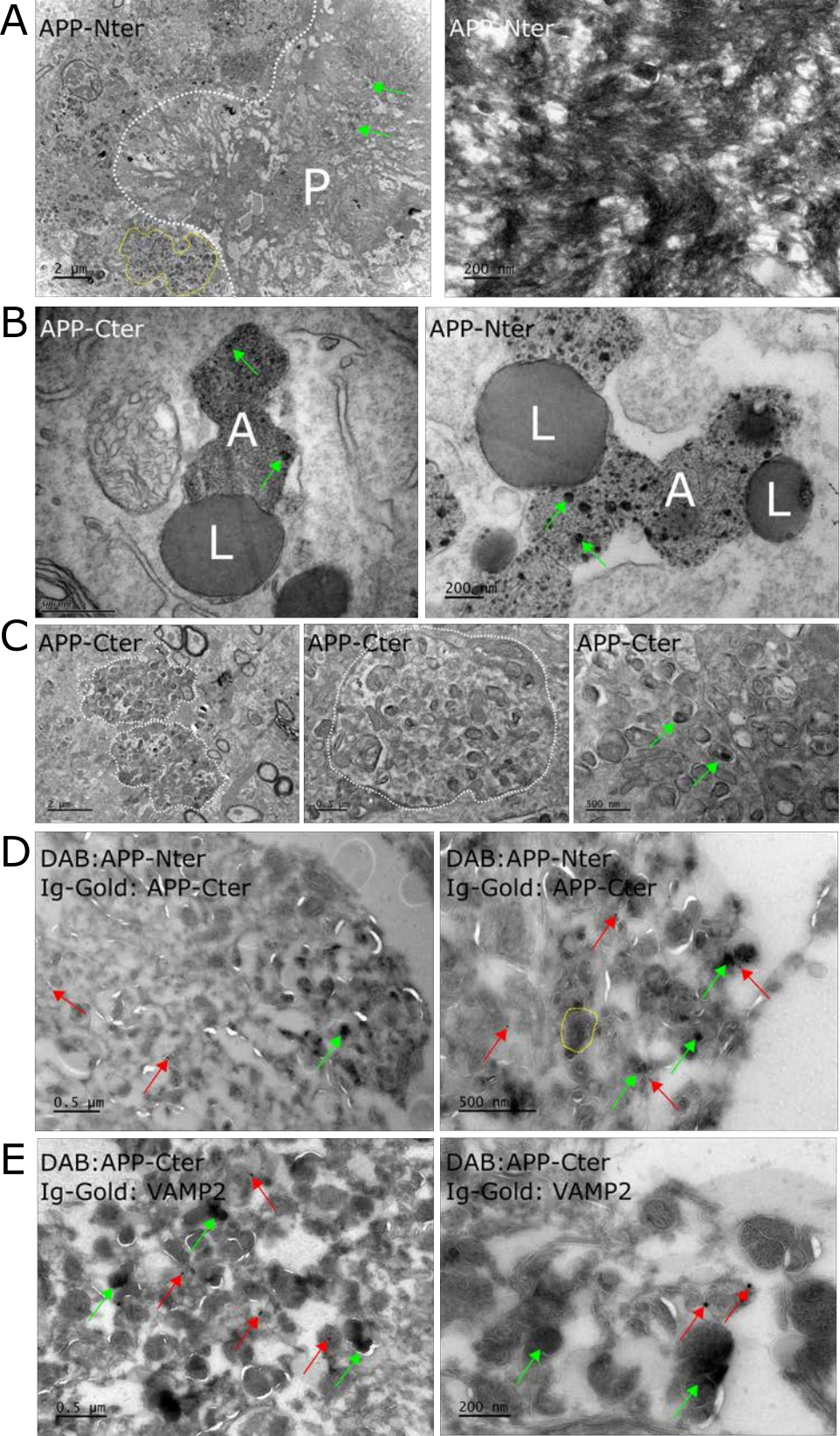


Figure 7. Electron microscopy analyses reveal presynaptic proteins and APP accumulations within multivesicular bodies (A) Electron microscopy images of a hippocampal section from an APP/PS1 mouse labeled with osmium (membrane marker) and with an antibody against APP-Nter (DAB labeling). *Left*, an amyloid plaque (P) is marked with DAB (positive for APP-Nter, green arrows) and delimited by a white broken line. Outside the plaque, round membranous organelles, positive for APP-Nter, are abundant. One cluster is highlighted inside a yellow border. *Right*, the fibrillary structure of the amyloid plaque is observable at the sub-micrometric scale. (B) Outside the boundaries of amyloid plaques, lysosomes (L) connected to autophagosomes (A) highly stained by the DAB reaction associated with either APP-Cter or -Nter antibody (green arrows) are frequently observed. (C) Clusters of multi-vesicular bodies and autophagic vacuoles are observed all over the tissue surrounding the plaques. (D) Hippocampal cryosections (Tokuyasu method) from an APP/PS1 mouse labeled with the antibody against the Nter domain JRD32 (electron dense DAB labeling, green arrows) and with the antibody against the Cter domain Y188 (antibody labeled with gold; black dots visible at high magnification, red arrows). The membranes appear finely white on the cryosections. The tissue contains numerous multivesicular bodies; one of them is highlighted inside a yellow border. They are positive for APP-Nter (DAB) and APP-Cter (Ig-Gold). (E) Hippocampal cryosections labeled with an antibody against the Cter domain of APP (DAB labeling, green arrows) and with an antibody against VAMP2 (antibody labeled with gold; black dots visible at high magnification, red arrows). The membranes appear finely white on the cryosections. The tissue contains numerous multivesicular bodies, positive for both APP-Cter (DAB) and VAMP2 (Ig-Gold).

Figure 8

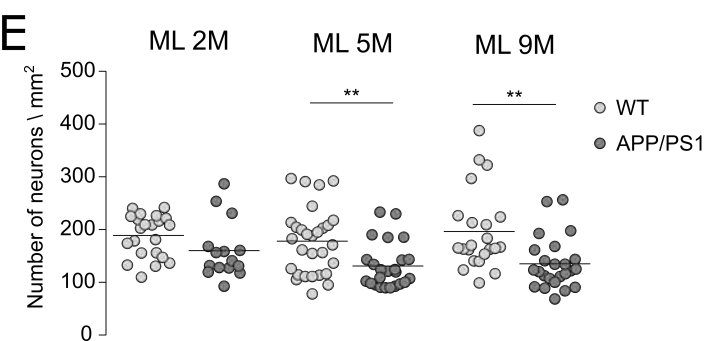
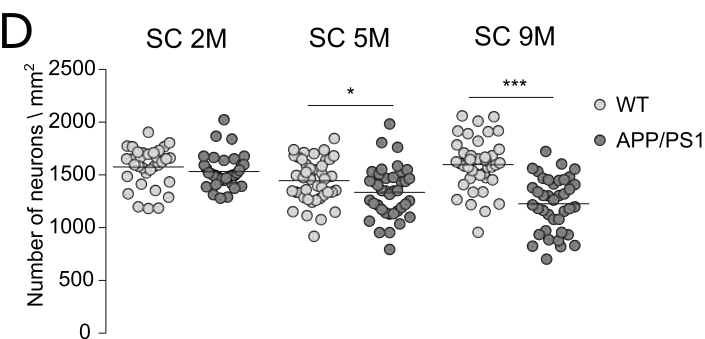
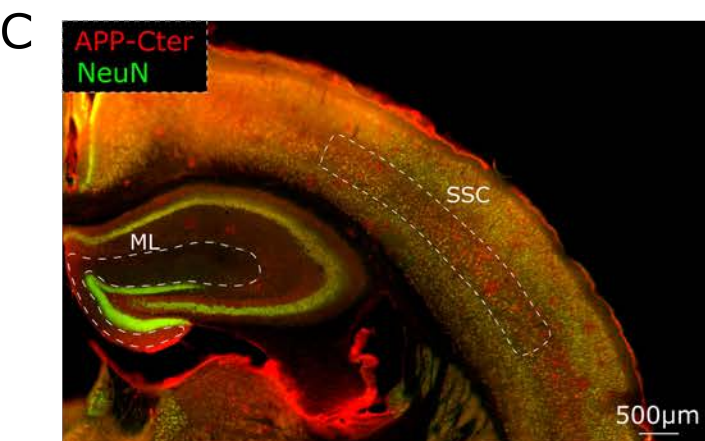
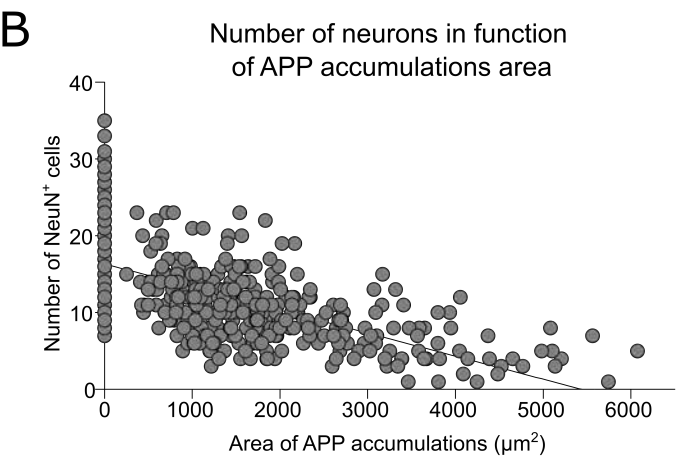
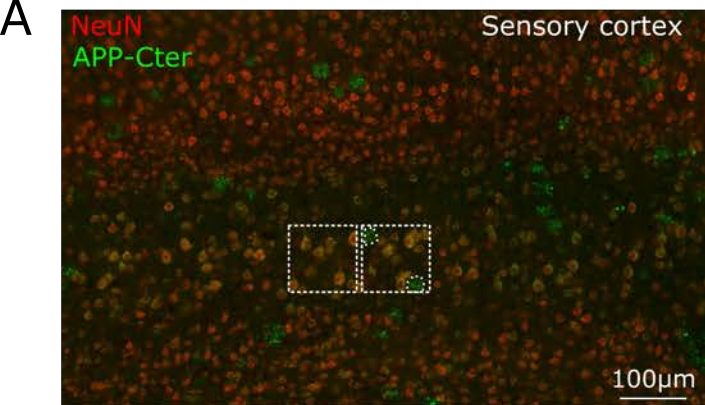


Figure 8. The number of APP accumulations inversely correlate with neuronal density

(A) Sections of the somatosensory cortex of APP/PS1 immuno-labeled with NeuN and APP-Cter. Using NeuN labelling as a neuronal reporter, we counted neurons in 100 μ m-side squares, used as regions of interest (ROIs). The squares were placed in different regions along the sagittal to temporal axis containing or not one or several APP accumulations. (B) Analyses of pictures as in 'A' revealed an inverse relationship between the area covered by APP accumulations and neuronal density within the ROIs, the larger the area covered by the APP accumulations, the fewer neurons were present. (C) Pictures of APP/PS1 brain sections immuno-labeled with NeuN and APP-Cter. ROIs of the whole hippocampal molecular layer (ML) area and of the somatosensory cortex (SSC) where numbers of neurons were counted (results in 'D' and 'E') are highlighted by dashed lines. (D, E) Analyses of pictures as in 'C' revealed a neuronal loss in APP/PS1 at both 5 and 9 months-old in SC and in ML. The WT littermates do not present any neuronal loss.

Supplementary information

Table S1-3

Table S1- Clinical data from control patients

Control case number	Gender	Braak stage	Amyloid stage	Age of onset	Age of death	APOE	PMD (h)	Brain weight (g)	CSF PH	Brain bank
1	F	I	B	-	73	4:4	7	1444	-	NBB
2	F	II	O	-	76	3:3	7	1072	6,87	NBB
3	F	III	A	-	81	3:3	5	1192	6,77	NBB
4	F	II	A	-	84	3:3	5	1027	6,68	NBB
5	F	II	B	-	83	4:4	6	1030	6,6	NBB
6	F	II	B	-	82	3:3	3	1221	6,34	NBB
7	F	II	B	-	84	3:3	6	1266	7,65	NBB
8	F	II	A	-	93	3:3	5	1145	-	NBB
9	M	-	-	-	80	3:4	16	1200	-	KI
10	M	-	-	-	69	3:3	22	1350	-	KI
11	M	-	-	-	71	3:3	12	1300	6,40	KI
Average					79,6		8,54	1204	6,75	

M–male, F– female, F/M (73/27%) KI-Karolinska Institutet, NBB–Netherlands Brain Bank

Table S2 - Clinical data from SAD patients

SAD case number	Gender	Braak stage	Amyloid stage	Age of onset	Age of death	APOE	PMD (h)	Brain weight (g)	CSF PH	Brain bank
1	F	V	C	79	85	4:4	5	919	6,35	NBB
2	F	VI	C	87	90	3:3	4	1045	6,39	NBB
3	F	V	C	63	70	3:3	5	1245	6,73	NBB
4	F	V	C	73	91	4:4	6	1196	6,01	NBB
5	F	VI	C	56	70	3:3	6	824	6	NBB
6	F	VI	C	79	80	4:4	4	1112	6,26	NBB
7	F	VI	C	53	70	4:4	4	894	6,42	NBB
8	F	VI	C	78	86	3:3	5	1266	7,65	NBB
9	F	V	C	77	82	4:4	4	999	6,08	NBB
10	F	VI	-	-	78	3:4	12	1080	-	KI
11	M	V	-	65	71	3:4	12	1235	-	KI
12	F	V	-	84	99	-	66	923	-	KI
Average				72,1	81		11,08	1061	6,43	

M–male, F– female, F/M (92/8%) KI-Karolinska Institutet, NBB–Netherlands Brain Bank

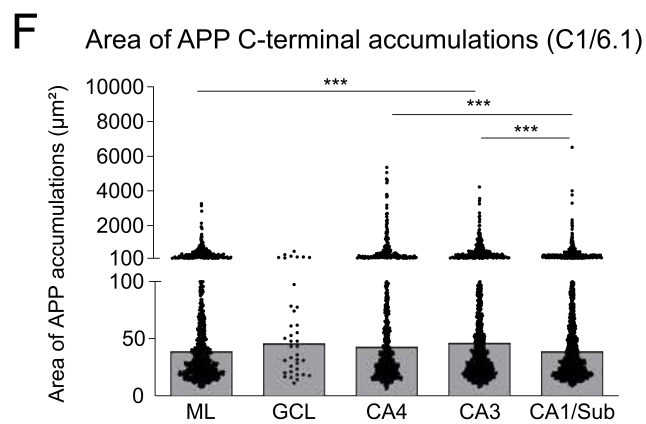
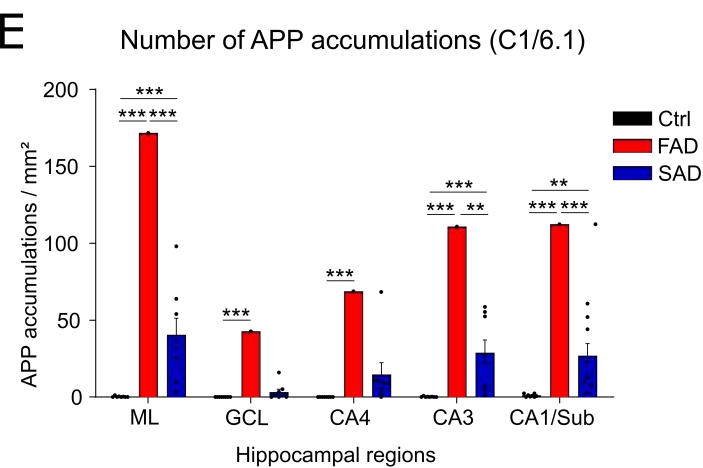
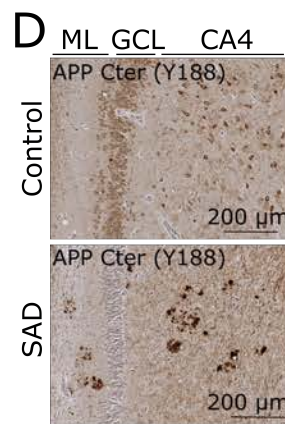
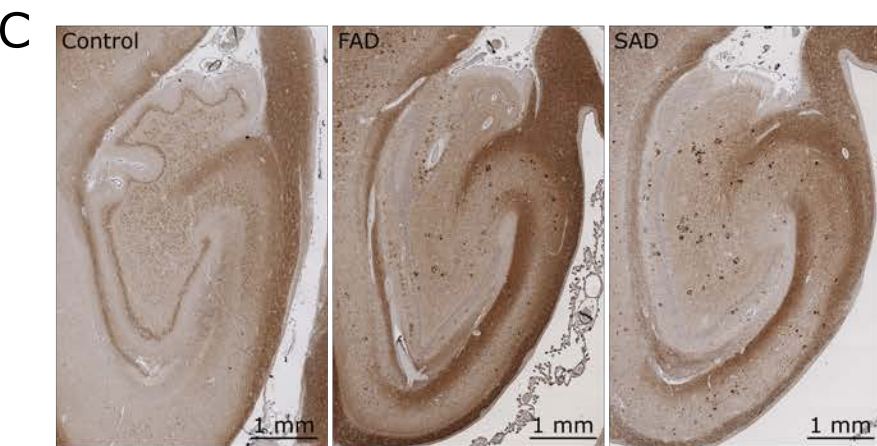
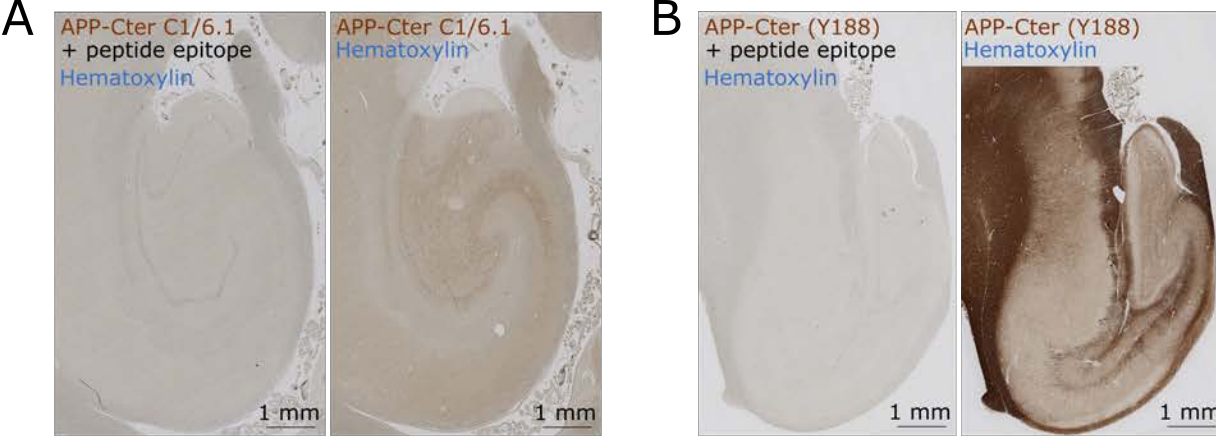
Table S3 - Clinical data from FAD patients

FAD case number	Gender	Braak stage	Amyloid stage	Age of onset	Age of death	APOE	PMD (h)	Brain weight (g)	CSF PH	Brain bank
PS1-G206A	F	VI	C	35	43	3:3	2	845	6,22	NBB
PS1-I143T	M	VI	-	37	45	3:3	-	1225	-	KI
PS1-I143T	F	VI	-	36	43	3:3	-	-	-	KI
PS1-H163Y	M	V	-	55	63	2:4	5	1234	-	KI
Average				40,7	48,5		3,5	1101	6,22	

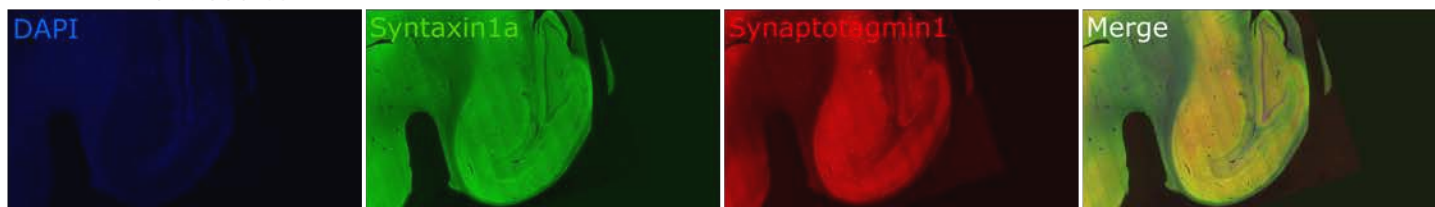
M–male, F– female, F/M (50/50%) KI-Karolinska Institutet, NBB–Netherlands Brain Bank

Supplementary figures

Figure S1

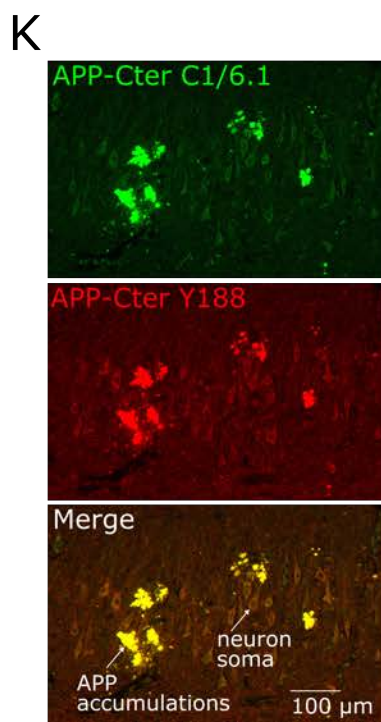
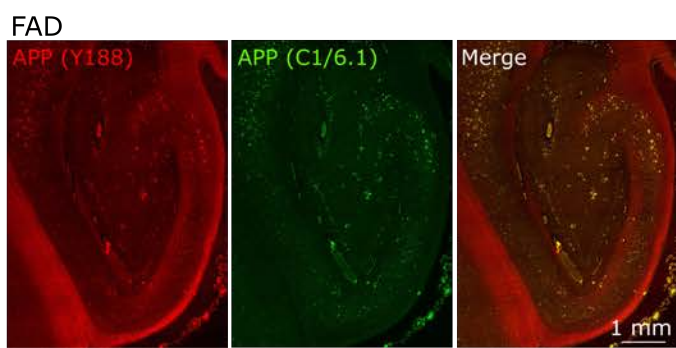
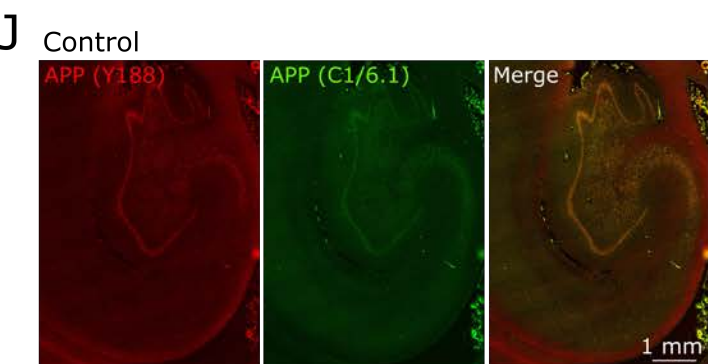
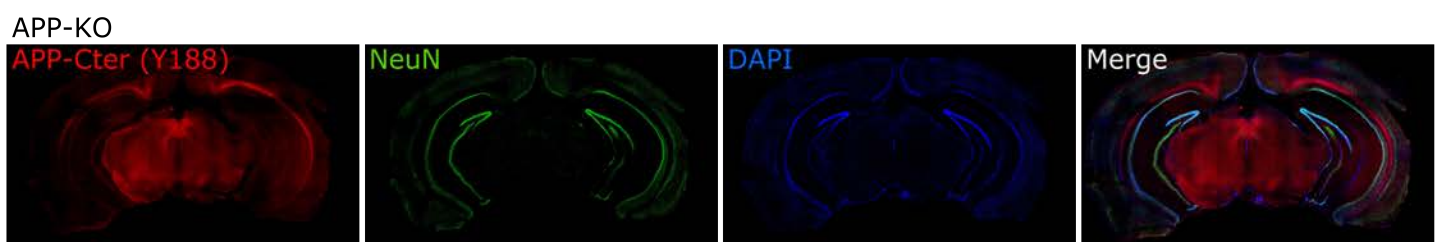
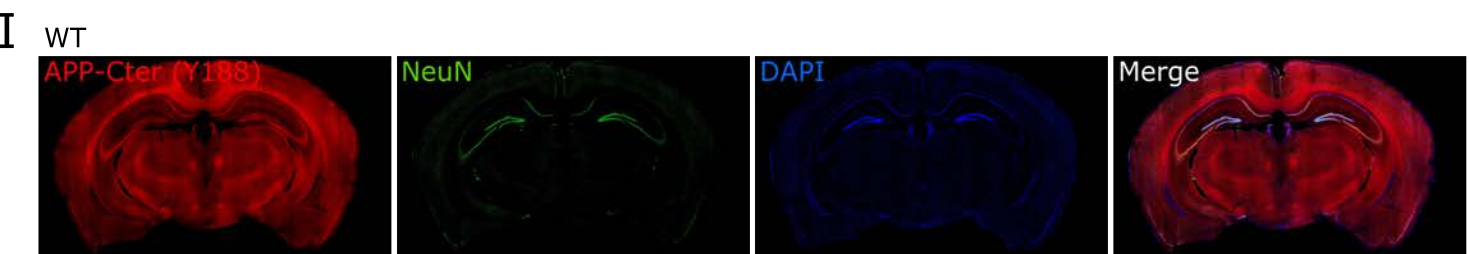
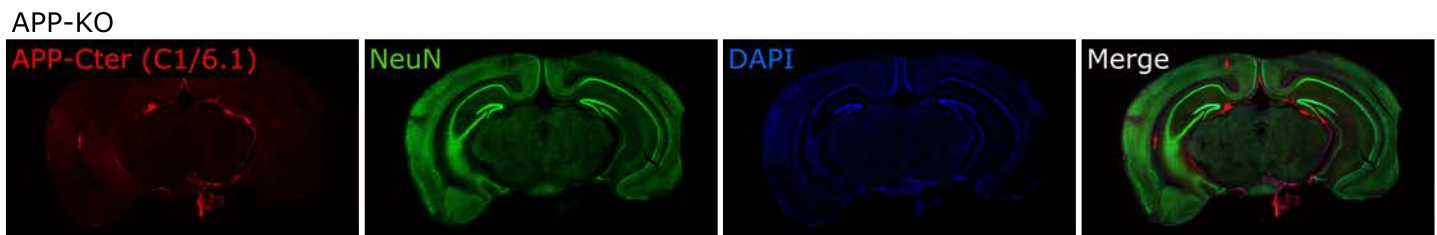
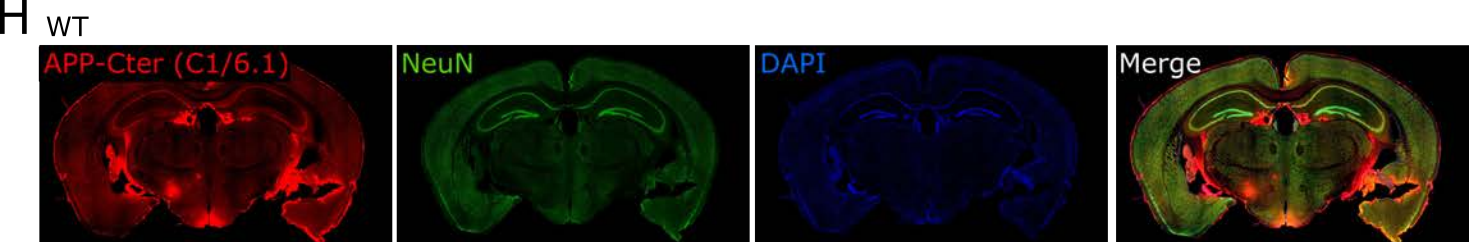


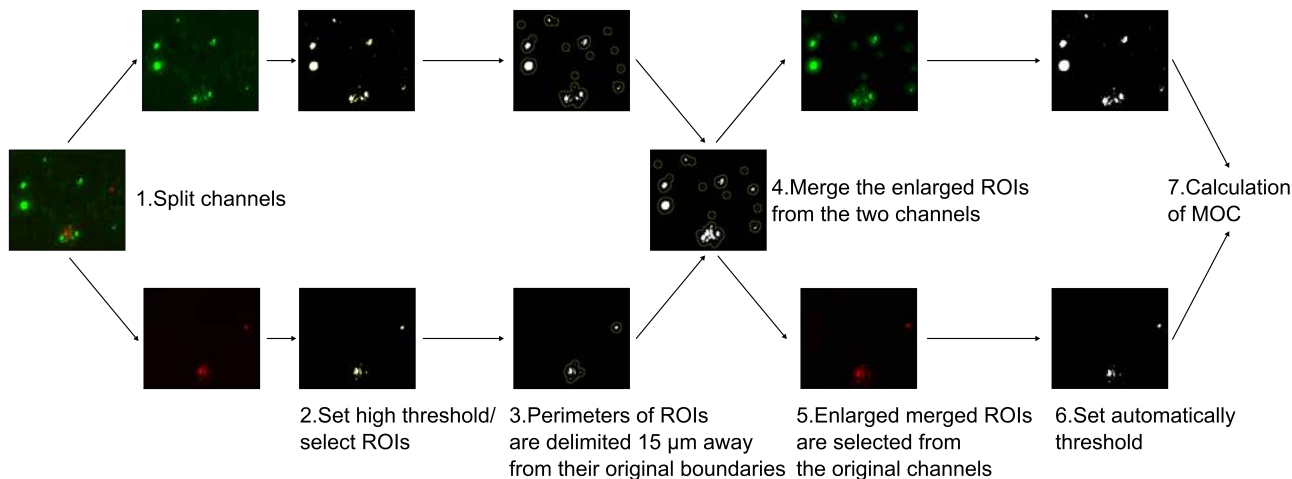
G I^{ary} + II^{ary} antibodies



II^{ary} antibodies only



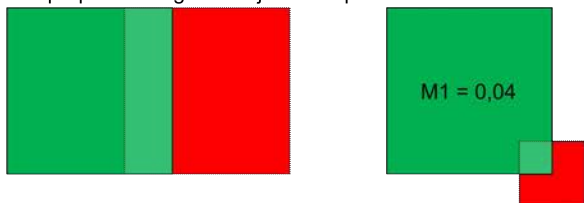




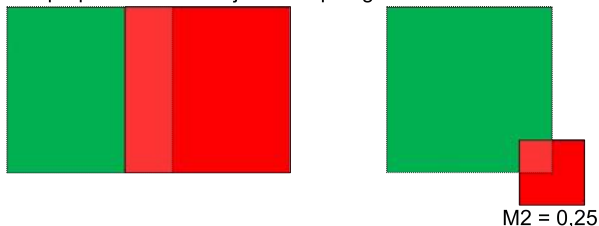
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Mander's Overall Coefficient: proportion of overlap of each channel with the other (from 0 to 1)

M1: proportion of green object on top of red



M2: proportion of red object on top of green



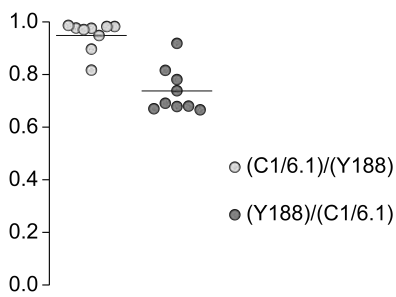
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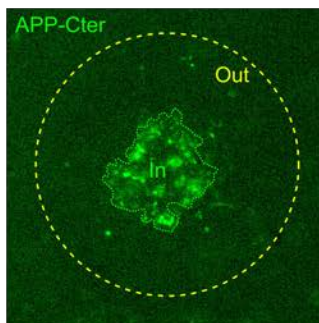


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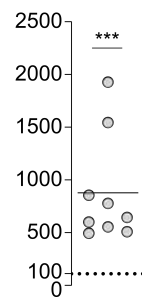
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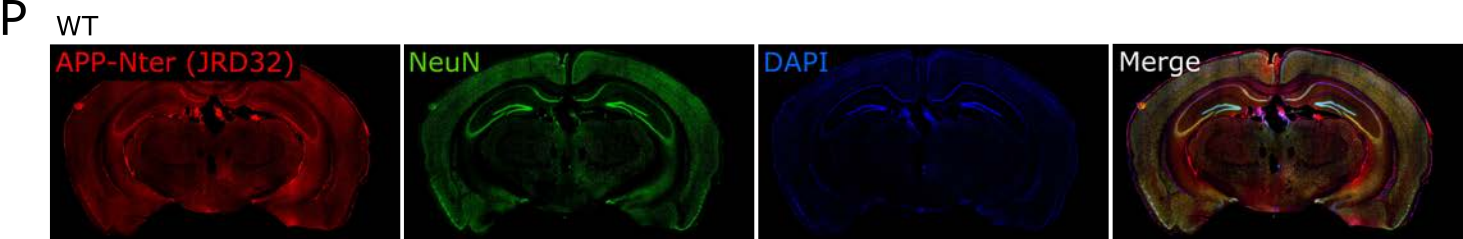


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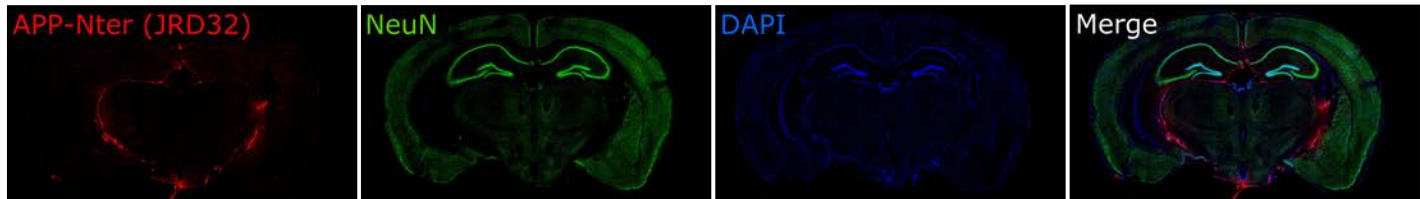


APP-Cter intensity inside APP accumulations

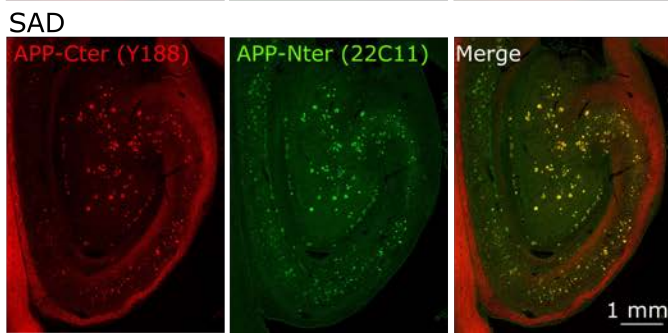
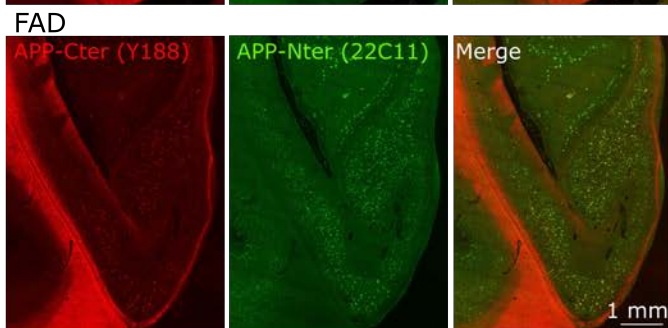
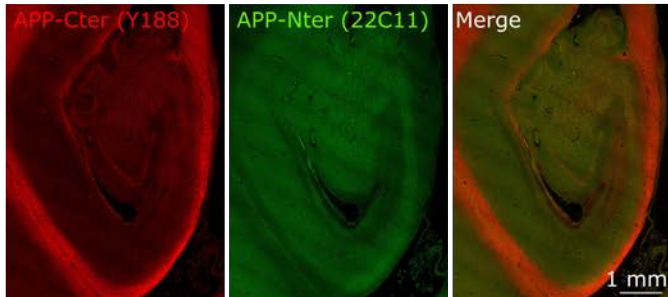




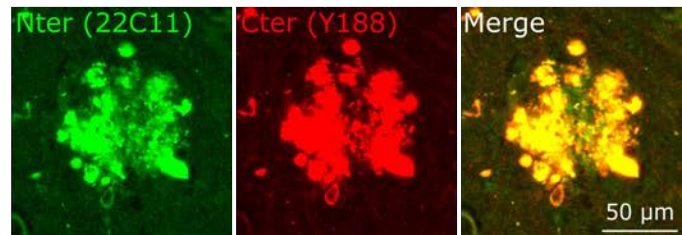
APP-KO



Q Control



R



S

MOC for APP-Cter (Y188) and Nter (22C11)

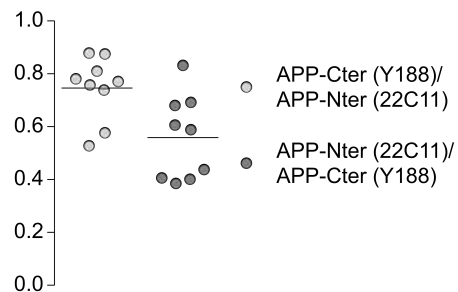
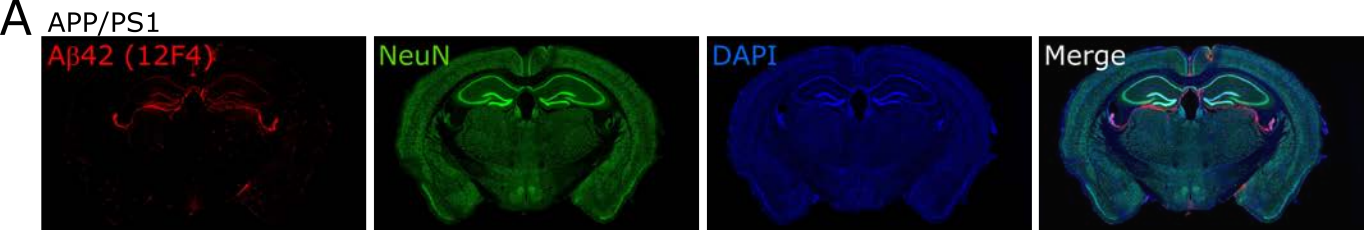


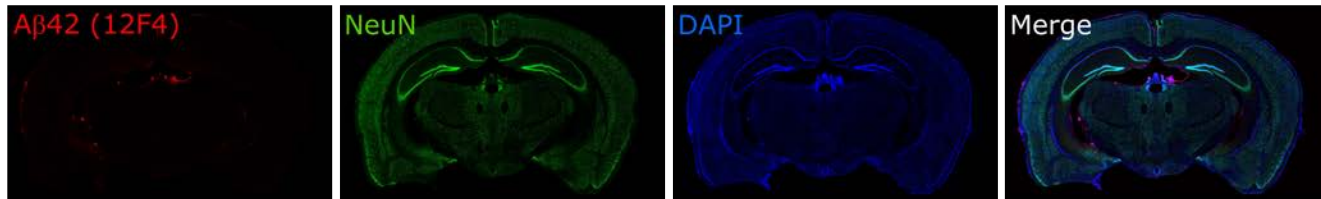
Figure S1. Controls and data supporting that full-length APP accumulates in the hippocampus of AD patients (A, B) Human hippocampal sections stained by DAB against APP-Cter with C1/6.1 antibody 'A' or with Y188 'B' and with hematoxylin. The control pictures are realized under the condition of labelling with a preadsorption step of the primary antibody with a recombinant peptide of the APP-Cter corresponding to the epitope. (C) Human hippocampal sections stained by DAB against the APP-Cter with the Y188 antibody. Cases from control, FAD and SAD groups are shown. In controls, APP staining is observed mostly in the soma of neurons in the dentate gyrus granule cell layer (GCL), CA4 and CA3. In addition, staining of the white matter tracts is visible; it is not specific 'I'. In both FAD and SAD cases, intense extra-somatic APP stainings are observable. (D) Magnifications in the dentate gyrus of pictures as in 'C'. In the SAD case, intense extra-somatic APP accumulations are observable in the molecular layer (ML), granule cells layer (GCL) and CA4 regions. (E) Histogram of the number of APP-Cter accumulations detected with the C1/6.1 antibody in different hippocampal regions in the control (Ctrl), FAD or SAD conditions. APP accumulations are barely detected in Ctrl whereas they are abundant in AD. (F) Histogram of the area of the APP accumulations detected with C1/6.1 antibody in different hippocampal regions. The CA4 and CA3 regions contain larger APP accumulations. (G) Controls of fluorescent immuno-labeling in human hippocampal sections. In the upper panels, the sections are stained for DAPI, syntaxin1a and Syt1. In the lower panels, only secondary antibodies were incubated in addition to DAPI. The fluorescent immuno-labelings are specific to the primary antibodies. (H, I) Mouse brain sections fluorescently immuno-labeled against APP-Cter with the C1/6.1 antibody 'H' or the Y188 antibody 'I' and for NeuN and DAPI. In WT, APP is labeled mostly in the soma of the neurons. For example, the different hippocampal layers are detectable in WT. In APP-KO mouse, this neuronal staining is lost. However, with the Y188 antibody, staining of white matter tracts remains in APP-KO revealing its non-specificity in white matter. (J) Human hippocampal sections fluorescently immuno-labeled against APP-Cter with the Y188 and C1/6.1 antibodies. In controls, APP labeled with both Cter antibodies is found mostly in the soma of the neurons. In FAD condition, intense extra-somatic APP stainings, co-labeled with the two antibodies are observable. (K) Close-up picture of staining as in 'J' showing APP accumulations co-labelled with the C1/6.1 and Y188 antibodies. (L) Diagram representing the method to calculate the Manders Overlap Coefficient (MOC) between objects in two different channels. (M) *Left*, Representation of the proportion of overlap between two channels and their calculated MOCs. *Right*, Description of the Costes test. (N) Scatter plot of MOCs between C1/6.1 and Y188 labelings. The analysis reveals a high colocalization between the two channels. (O) *Left*, Representation of an APP accumulation. The intensity of the APP staining is measured within ('In') the APP accumulation and normalized to the intensity outside the accumulation ('Out'),

i.e. the surrounding neuropil. *Right*, scatter plot of the intensity of APP-Cter labeling within APP accumulations. The data are normalized to the intensity outside the APP accumulations represented here by a dashed line. The analysis revealed that APP is enriched inside the APP accumulations. (P) Mouse brain sections fluorescently immuno-labeled against APP-Nter with the JRD32 antibody and for NeuN and DAPI. In WT, APP is labelled mostly in the soma of the neurons. In APP-KO mice, this neuronal staining is lost. (Q) Human hippocampal sections fluorescently immuno-labeled against APP-Cter with the Y188 antibody and APP-Nter with the 22C11 antibody. In controls, APP labeled with both Cter and Nter antibodies is mostly found in neuronal soma. In both FAD and SAD cases, intense extra-somatic APP stainings, co-labeled with the two antibodies are observable. (R) Close-up picture of an APP accumulation co-labeled with APP-Cter (Y188) and Nter (22C11). (S) Scatter plot of Manders overlapping coefficients (MOC) (Method described in FigS1L, M) between APP-Cter and Nter labelings. The analysis revealed a high colocalization between the two channels suggesting the presence of full-length APP in the accumulations.

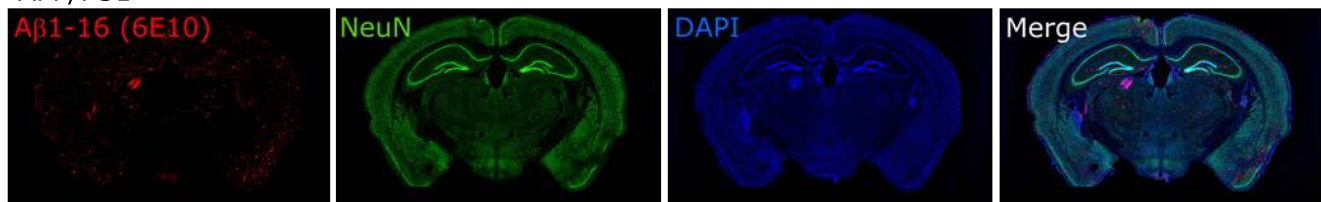
Figure S2



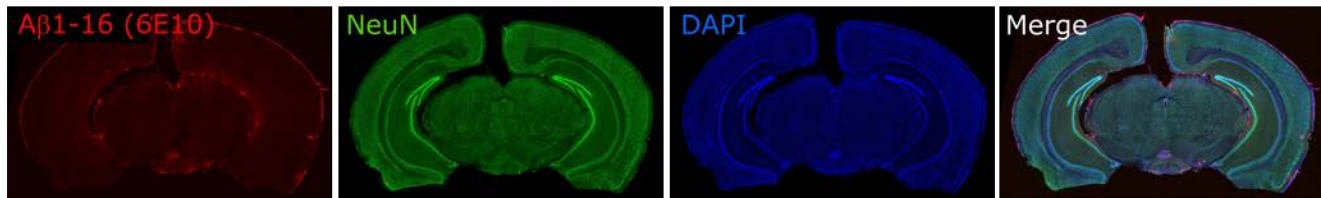
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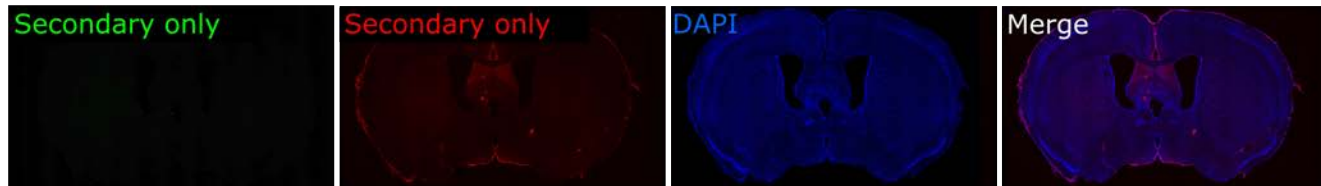
B APP/PS1



WT

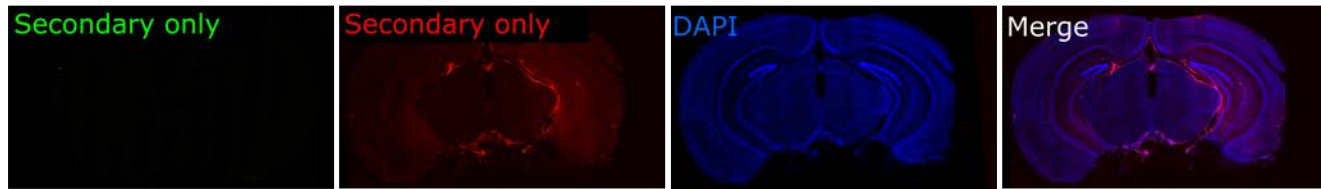


C WT

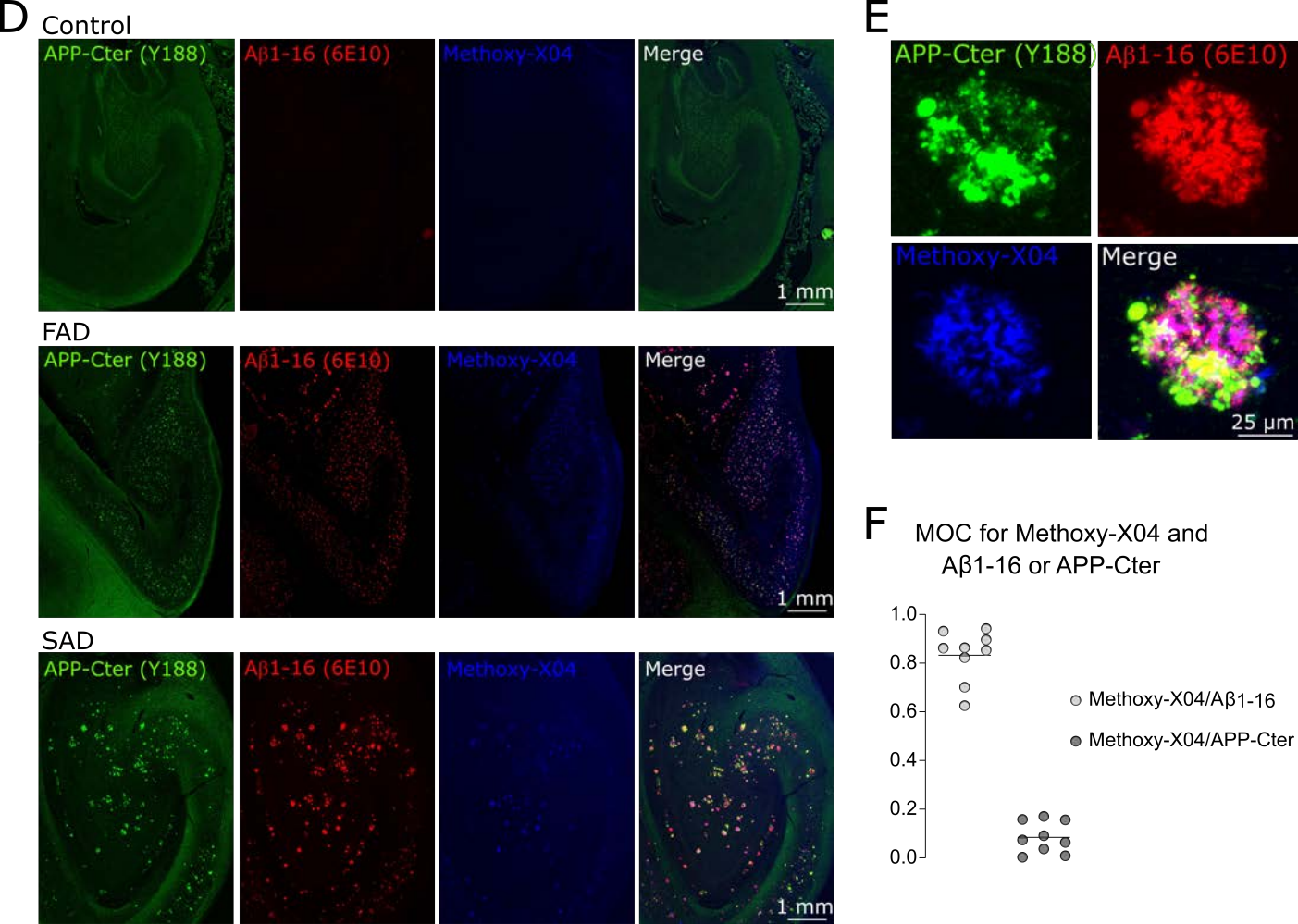


WT

APP-KO



APP-KO



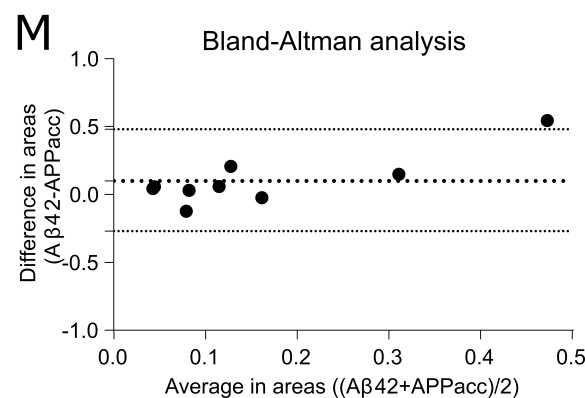
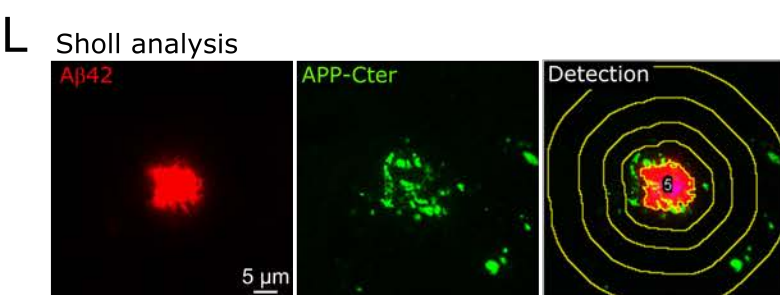
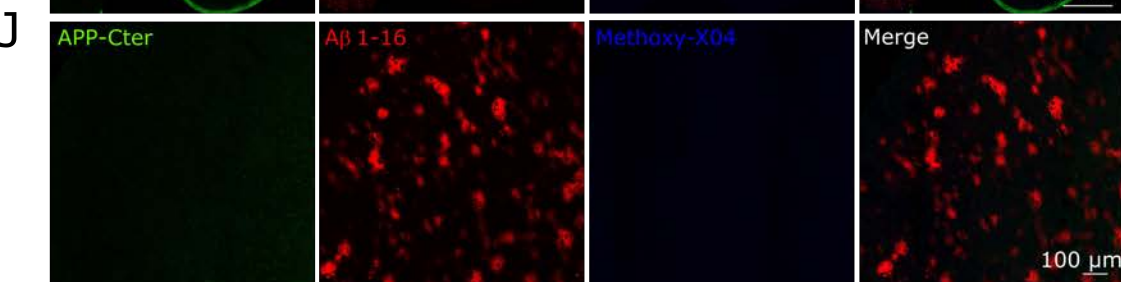
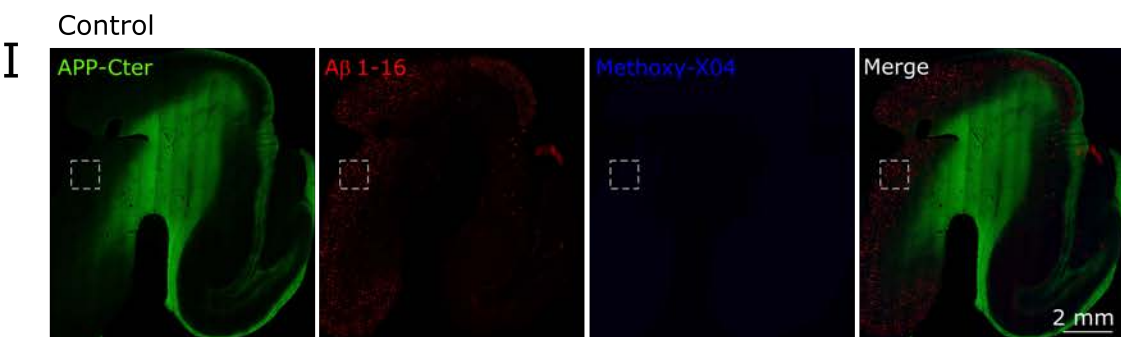
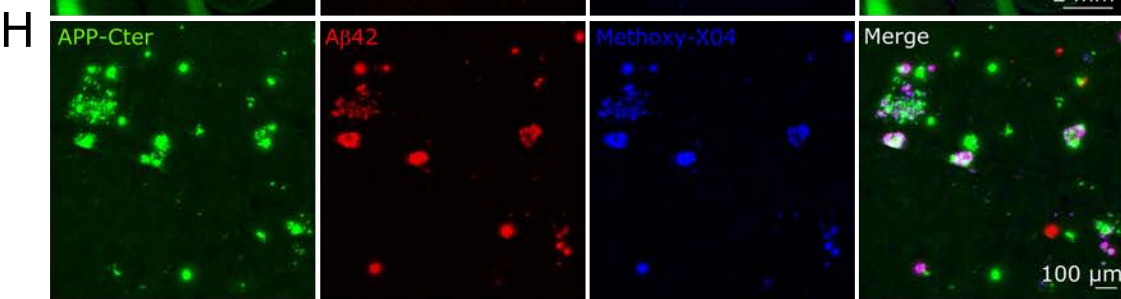
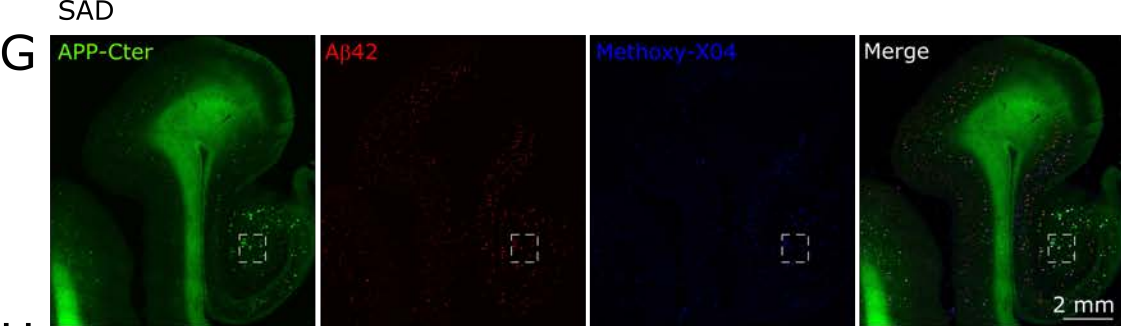
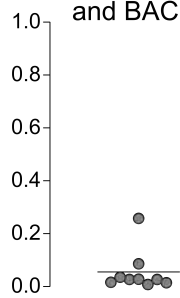
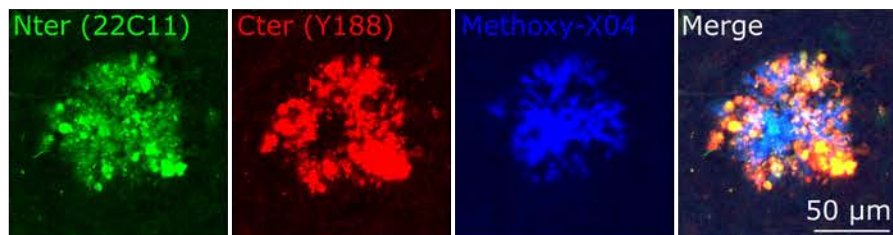
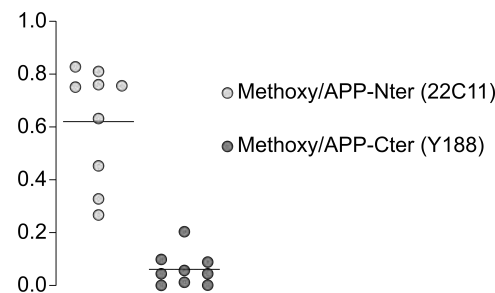
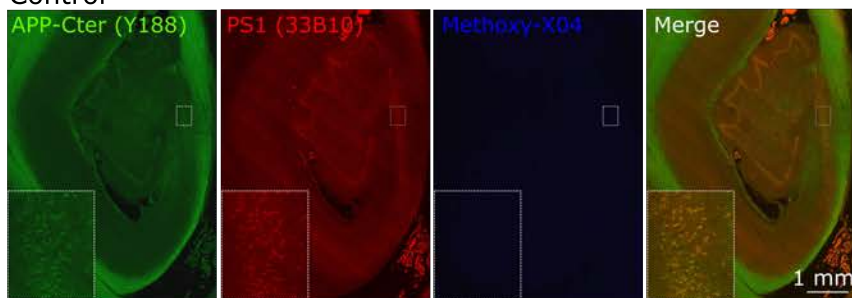


Figure S2. Controls and data supporting that APP accumulations surround dense-core amyloid plaques (A, B) Mouse brain sections fluorescently immuno-labeled against A β 42 with the 12F4 antibody 'A' or against A β 1-16 with the antibody 6E10 'B' and for NeuN and DAPI. In APP/PS1 mice, amyloid plaques are spread all over the brain. They are absent from WT littermate. (C) Staining as in 'A, B' but without primary antibody does not display significant fluorescent signal. (D) Human hippocampal sections fluorescently immuno-labelled against APP-Cter with the Y188 antibody and against amyloid plaques with the A β 1-16 antibody and methoxy-X04. In both FAD and SAD cases, intense A β 1-16 and methoxy-X04 labelings are observable in the proximity of APP accumulations whereas no staining is observable in control. (E) Close-up pictures of APP accumulations surrounding an amyloid plaque co-labeled with A β 1-16 and methoxy-X04. The A β 1-16 and methoxy-X04 stainings colocalize well together but not with the APP-Cter labeling. (F) Scatter plot of MOCs between methoxy-X04 and A β 1-16 or APP-Cter labelings. The analysis revealed a high colocalization between methoxy-X04 and A β 1-16 (amyloid plaques) and a low colocalization between methoxy-X04 and APP-Cter. (G, H) Pictures of a human brain section from an AD case containing the hippocampus and para-hippocampal cortex labeled against APP-Cter, A β 42 and methoxy-X04. The lower panels in (H) show hippocampal dense-core plaques (revealed by methoxy-X04 and A β 42) surrounded by APP accumulations. (I, J) Pictures of a human brain section from a control case containing the hippocampus and para-hippocampal cortex labeled against APP-Cter, A β 1-16 and methoxy-X04. The lower panels in (J) show cortical diffuse plaques labelled by A β 1-16 but not by methoxy-X04. They are not surrounded by APP accumulations. (K) Pictures as in (I) but labelled for A β 42. No dense-core amyloid plaques are observable in control cases. (L) Representation of a Sholl analysis to count APP accumulations around an amyloid plaque labeled with A β 42 in concentric annuli of increasing diameter (intervals of 5 μ m), up to 50 μ m away from the amyloid plaque. (M) Bland-Altman analysis of the areas covered by APP accumulations and A β 42-positive amyloid plaques. The difference in areas is plotted in function of the average in areas.

Figure S3

A MOC for methoxy-X04 and BACE1**B****C**

MOC for Methoxy and APP-terminals

**D** Control

SAD

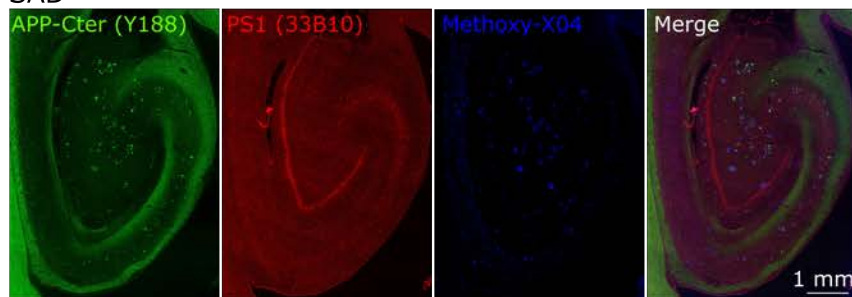
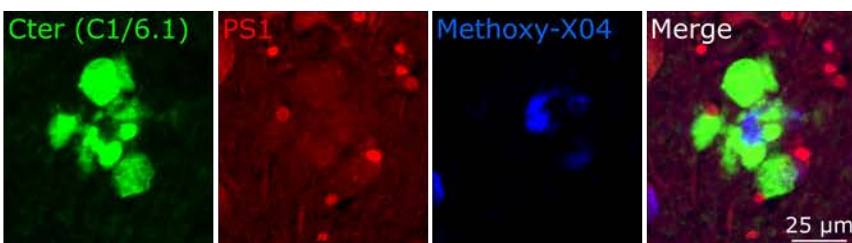
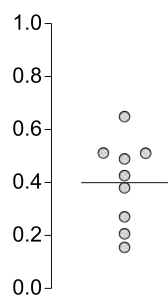
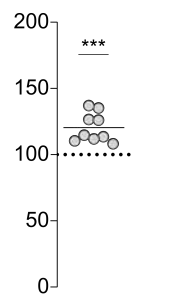
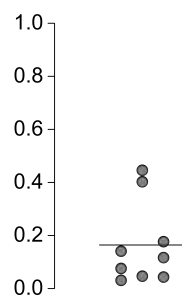
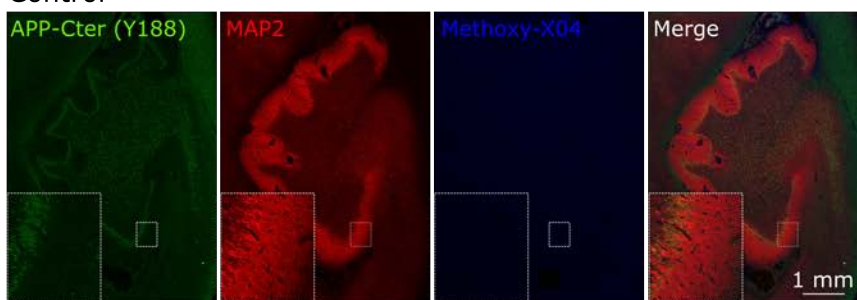
**E****F** MOC for APP-Cter and PS1**G** PS1 intensity inside APP acc**H** MOC for methoxy-X04 and PS1

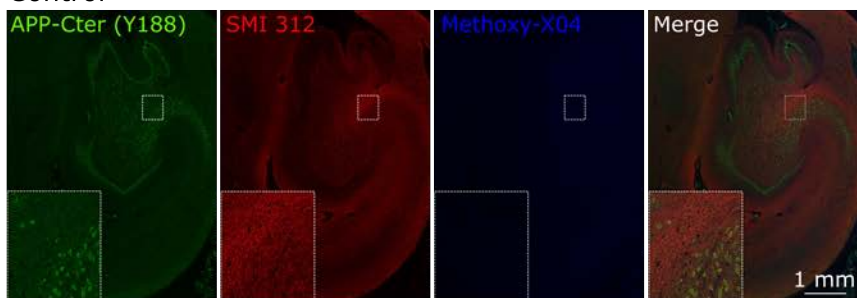
Figure S3. Controls and data supporting that dense-core plaques are surrounded by BACE1 and contain APP-Nter in their core (A) Scatter plot of the MOC between methoxy-X04 and BACE1. The analysis reveals a low colocalization between methoxy-X04 and β -secretase. (B) Close-up pictures of hippocampal amyloid plaques labeled at APP-Cter (Y188), Nter (22C11) and methoxy-X04 revealed a remarkable abundance of APP-Nter in the core of dense-core amyloid plaques surrounded by APP accumulations. (C) Scatter plot of the MOC between methoxy-X04 and APP-Nter or Cter. The analysis revealed a high colocalization between methoxy-X04 and APP-Nter (22C11) but not with APP-Cter (Y188) indicating that the colocalization between APP-Nter and the core of the plaques is specific of the Nter domain. (D) Human hippocampal sections immuno-labelled against APP-Cter, PS1 and with methoxy-X04. PS1 is prominently observed in neuronal soma reminiscent of the expression of APP, in addition to a fainter labeling of the neuropil. (E) Close-up pictures of stainings as in 'D' showing PS1 and APP accumulations colocalizing in the surrounding of an amyloid plaque labeled with methoxy-X04. (F) Scatter plot of the MOC between APP-Cter and PS1. (G) Scatter plot of the intensity of PS1 labeling within APP accumulations. The data are normalized to the intensity outside the APP accumulations represented here by a dashed line. The analysis revealed that PS1 is modestly enriched inside the APP accumulations. (H) Scatter plot of the MOC between methoxy-X04 and PS1. The analysis reveals a low colocalization between methoxy-X04 and PS1.

Figure S4

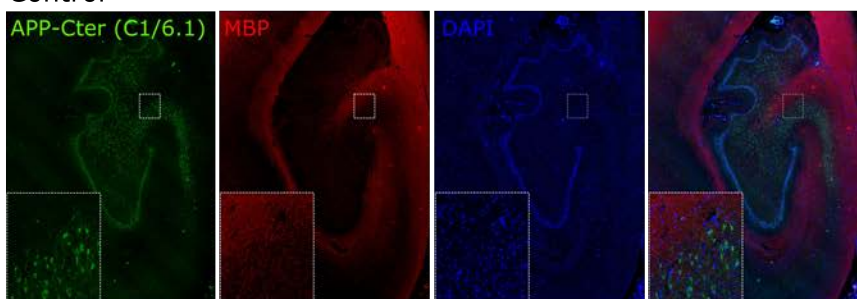
A Control



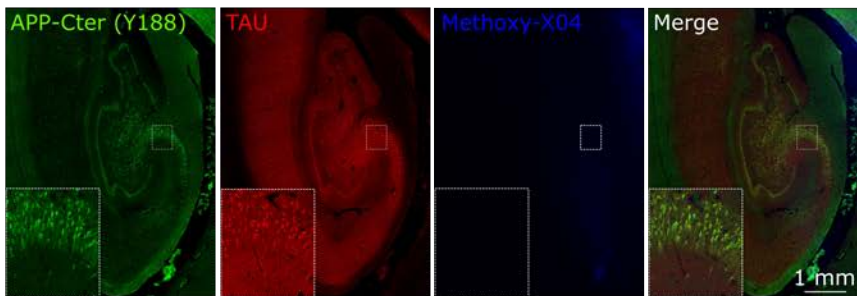
B Control



C Control



D Control



E Control

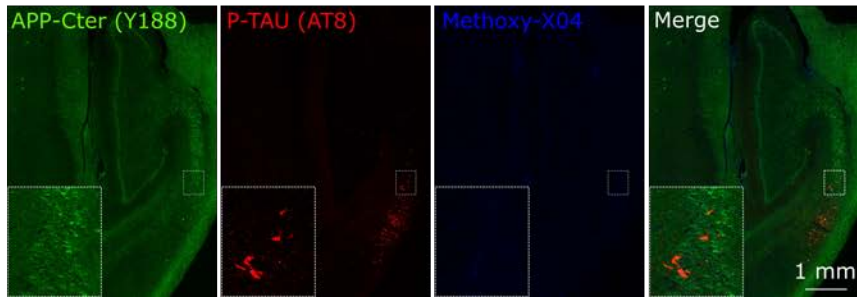


Figure S4. Neuritic markers in hippocampal sections from control cases (A-E) Human hippocampal sections stained against APP and methoxy-X04 (or DAPI in 'C') and with one of the following neuritic marker: MAP2 (A), SMI312 (B), MBP (C), TAU or P-TAU (E). Insets in the lower left corners display higher magnifications.

Figure S5

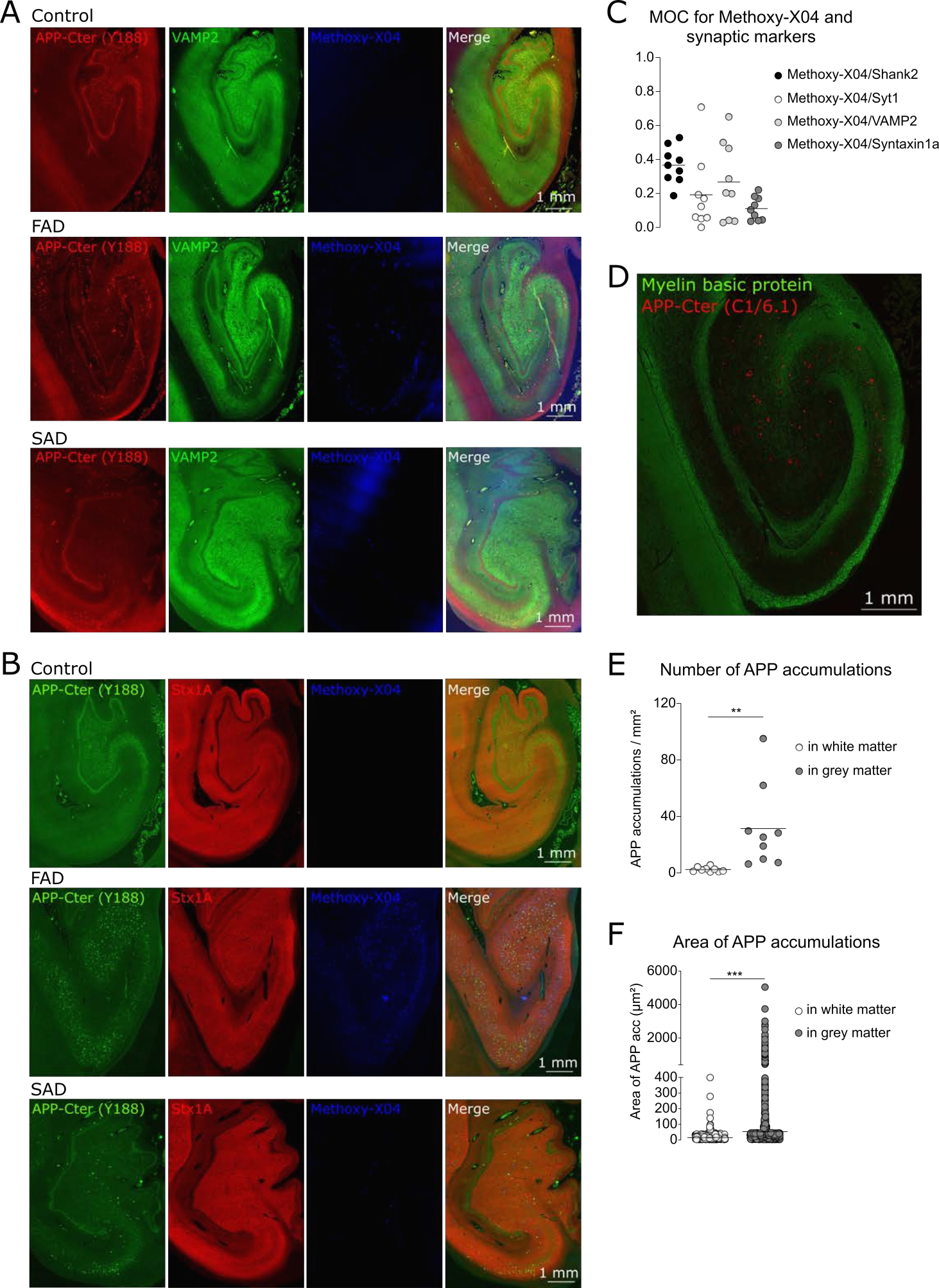
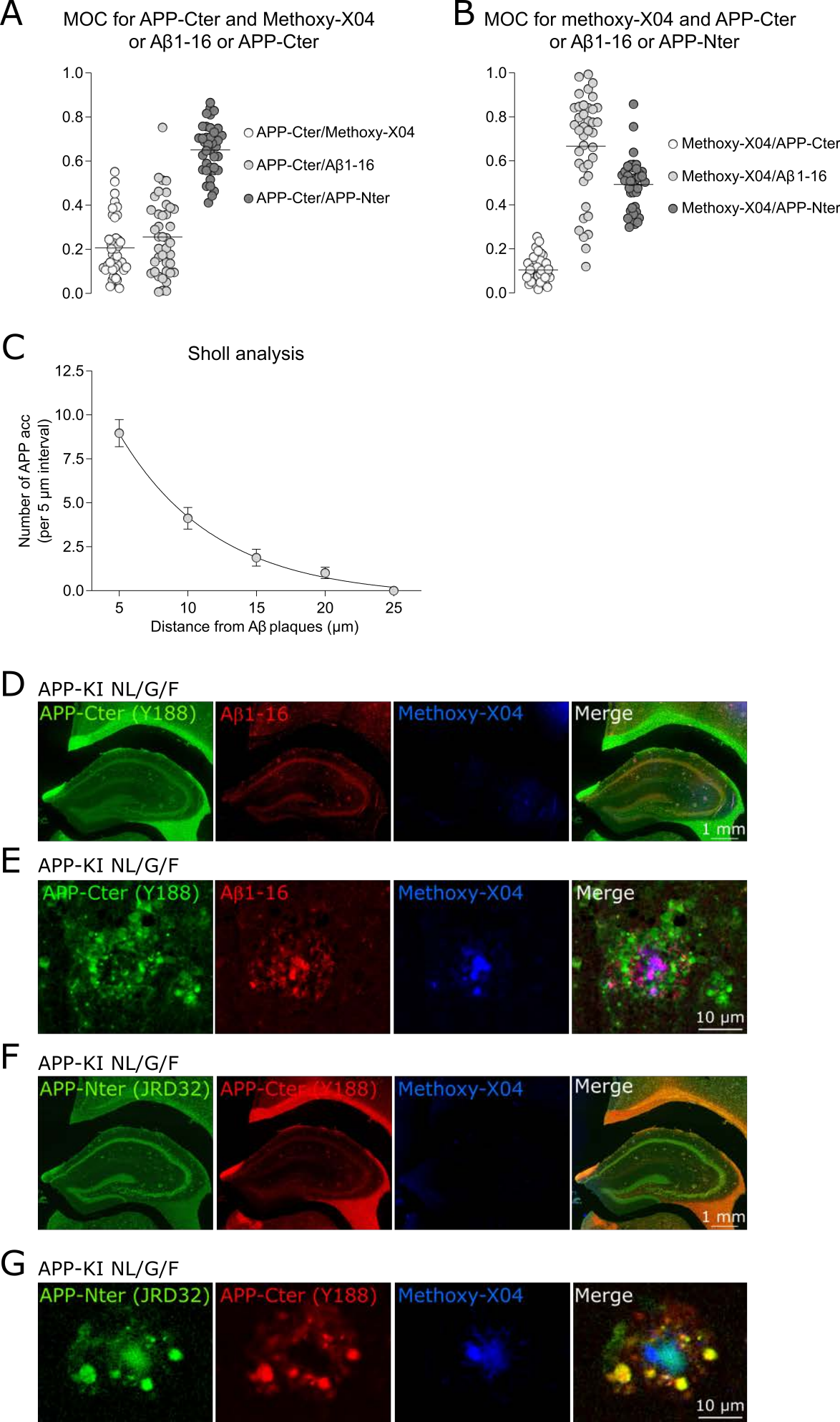


Figure S5. Synaptic vesicle proteins concentrate within APP accumulations in the grey matter (A, B) Human hippocampal sections fluorescently immuno-labeled against APP-Cter with Y188 and Vamp2 'A' or syntaxin1A (Stx1A) 'B'. Vamp2 staining is prominent in the neuropil, especially in the axonal terminals of CA4 and CA3; Stx1A staining is bright all over the neuropil. The two presynaptic proteins are present at the level of APP accumulations. (C) Scatter plot of the MOC between methoxy-X04 and synaptic markers (Shank2, Syt1, VAMP2, STX1A). The analysis revealed a low colocalization between methoxy-X04 and these markers. (D) Human hippocampal sections from an AD patient, fluorescently immuno-labeled against APP-Cter (C1/6.1) and myelin basic protein (MBP) enriched in white matter tracts. APP accumulations are mostly observable in MBP-negative regions. (E) Scatter plot of the number of APP accumulations in the white matter (regions MBP-positive) and in the grey matter (regions MBP-negative). The APP accumulations are more abundant in the grey matter. (F) Scatter plot of the area of APP accumulations in the white matter (regions MBP-positive) and in the grey matter (regions MBP-negative). The APP accumulations in the white matter are smaller.

Figure S6



H

APP/PS1

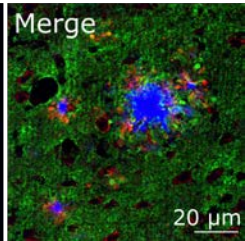
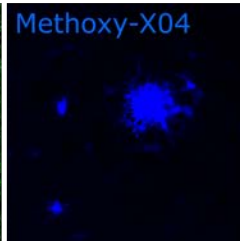
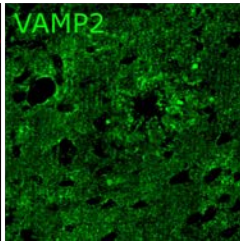
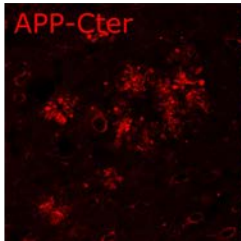


Figure S6. AD mouse models recapitulate the features of APP accumulations observed in human (A) Scatter plot of the MOC between APP-Cter and methoxy-X04 or A β 1-16 or APP-Nter. The analysis reveals a high colocalization between APP-Cter and Nter but not with A β 1-16 or methoxy-X04 indicating that amyloid plaques and APP accumulations are non-overlapping. (B) Scatter plot of the MOC between methoxy-X04 and APP-Cter or A β 1-16 or APP-Nter. The analysis reveals a high colocalization between methoxy-X04 and APP-Nter but not with APP-Cter indicating that amyloid plaques contain APP-Nter but not APP-Cter. (C) Graph of a Sholl analysis of the number of APP accumulations in function of the distance from A β 1-16 plaques indicating that APP accumulations are more numerous close to the amyloid plaques. (D, F) APP-KI NL/G/F mouse hippocampal sections immuno-labeled against APP-Cter and either A β 1-16 (D) or APP-Nter (F). (E, G) Close-up pictures of stainings as in 'D, F' showing that amyloid plaques containing APP-Nter are surrounded by APP accumulations as in human AD conditions. (H) Close-up pictures of stainings in APP/PS1 showing the presence of VAMP2 in APP accumulations surrounding amyloid plaques labeled with methoxy-X04.

3.3. Perspectives

The research performed in this chapter aimed to study the misdistribution of APP and its fragments by implementing novel analytical tools. But a major disadvantage of using postmortem fixed human tissue is that results can only be acquired at a specific time point of the most advanced stages of the disease, making the interpretation of early events very speculative and difficult. However, the use of *in vivo* mouse models together with MCI patients could be a possible solution to measure changes at different stages of the disease. *In vivo* 2-P microscopy in AD mouse models like the transgenic APP/PS1 would be a feasible approach to follow the cellular development of APP accumulations, together with A β plaques overtime during the disease. Therefore, while the labelling of plaques would be possible with periodic injections of Methoxy-X04⁷⁹, APP could be labeled by using the viral expression of APPfl tagged at the Cter with a fluorescent reporter. Another possibility to overcome the overexpression of APP would be to use mouse models that endogenously express the fluorescently labeled Cter or Nter domains of APP. These models would be very valuable to study the pathophysiological mechanisms of APP and its domains such as the abnormal distribution of APP Cter and Nter around A β plaques.

One of the main challenges in this project was to define if APP accumulations contained the full length protein or processed fragments. Regarding this aspect, the presence of different APP fragments in specific brain regions or cellular compartments could be achieved by combining laser microdissection with biochemical methods in mouse or human. By micro dissecting the area in and outside the core of the plaque we could measure the presence and relative quantity of each APP fragment. Although technically challenging, this could be performed in frozen samples where different anatomical regions could be laser-cut and analyzed using WB or Mass spectrometry. In addition, double immunolabeling with gold particles of different sizes would be another possible approach to observe with electron microscope (EM) the precise cellular location where APP Nter and Cter domains are found in pathological conditions.

The increased levels of APP within the accumulations could imply that APP is highly expressed within these structures and therefore the presence of the translating machinery might be a reality. The use of *in situ* hybridization could assess this hypothesis by detecting the presence of mRNA from APP or other proteins, suggesting that proteins could be locally expressed in pathological states. Importantly, the colocalization of APP accumulations with presynaptic proteins could also imply that part of these accumulations are degenerating synapses or damaged axons where proteins accumulate affecting cell activity and blocking the transport of other proteins. Therefore,

the effects of neuronal activity in the formation of APP accumulations are unknown and could be tested by the expression of designer receptor exclusively activated by designer drugs (DREADDs). This technique is used to modulate the neuronal cell activity by expressing a receptor that can only be activated by a synthetic ligand that is supplied to the animal. A possible strategy would be to inject viral constructions that express DREADDs that can excite or inhibit neurons at layer II of the entorhinal cortex. Since these cells project to the ML of the DG, our aim would be to study if the modulation of cell activity affects the number of accumulations in the ML. As the ML in humans and in the APP/PS1 mice contain a prominent number of APP accumulations, we think it would be an ideal location to study this phenomenon. Another interesting approach would be to co-infect cortical cells of the APP/PS1 with the genetically encoded calcium indicator GCaMP together with APP-mCherry to study the correlation between neuronal activity and the formation of APP accumulations.

Chapter 4 - Distinctive alteration of presynaptic proteins in the outer molecular layer of the dentate gyrus in Alzheimer's disease

4.1. Context and aims of the study

Synaptic dysfunction is one of the earliest pathogenic events in AD correlating with the cognitive deficits observed in patients. Studies in mouse models and in human tissue have provided experimental evidence that synaptic protein loss is an alteration that happens early in the disease progression^{297–300}. Furthermore, the molecular mechanisms that trigger synaptic failure are unknown but they affect pre- and post-synaptic proteins that play important functions in synapse integrity and function. In general, most AD studies reporting changes in synaptic proteins have been performed by using homogenates prepared from cortical and hippocampal tissue, which do not allow a detailed examination of the regions and synapse type affected. Importantly, certain types of synapses are more vulnerable than others in AD, indicating the importance of studying multiple anatomical regions in histopathological studies.

In this study, we measured the subregional changes of different synaptic proteins in the hippocampus of AD patients. By performing immunofluorescence labeling, we measured the pixel intensity of different pre- and post-synaptic proteins in different molecular and pyramidal cell layers of the DG, CA3 and CA1 regions. Interestingly, we detected a distinctive reduction of presynaptic proteins in the outer two-thirds of the ML of the DG but not in other MLs of the hippocampus. Furthermore, postsynaptic markers and neuritic markers were not affected in these regions suggesting that the deficit was specifically presynaptic and does not result from axonal loss. We further studied the lack of correlation between the AD hallmarks and the specific reduction of presynaptic proteins in the OML, reinforcing the idea of presynaptic failure in AD.

4.2. Manuscript

See PDF of the manuscript in the next page.

Distinctive alteration of presynaptic proteins in the outer molecular layer of the dentate gyrus in Alzheimer's disease

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Abstract

Synaptic degeneration has been reported as one of the best pathological correlates of cognitive deficits in Alzheimer's Disease (AD). However, the location of these synaptic alterations within hippocampal sub-regions, the vulnerability of the presynaptic versus postsynaptic compartments, and the biological mechanisms for these impairments remain unknown. Here, we performed immunofluorescence labelling of different synaptic proteins in fixed and paraffin embedded human hippocampal sections and report reduced levels of several presynaptic proteins of the neurotransmitter release machinery (complexin-1, syntaxin-1A, synaptotagmin-1 and synaptogyrin-1) in AD cases. The deficit was restricted to the outer molecular layer (OML) of the dentate gyrus whereas other hippocampal sub-fields were preserved. Interestingly, standard markers of postsynaptic densities (SHANK2) and dendrites (MAP2) were unaltered, as well as the relative number of granule cells in the dentate gyrus, indicating that the deficit is preferentially presynaptic. Notably, staining for the axonal components, myelin basic protein, SMI-312 and Tau, was unaffected, suggesting that the local presynaptic impairment does not result from axonal loss or alterations of structural proteins of axons. There was no correlation between the reduction in presynaptic proteins in OML and the extent of the amyloid load or of the dystrophic neurites expressing phosphorylated forms of Tau. Altogether, this study highlights the distinctive vulnerability of the OML of dentate gyrus and supports the notion of presynaptic failure in AD.

Key words

Alzheimer's Disease, Postmortem human brain, Hippocampus, Outer molecular layer of Dentate Gyrus, Perforant path, Presynaptic impairment, Immunofluorescence

Abbreviation list

AD: Alzheimer's disease
CA: Cornu ammonis
CPLX1: Complexin-1
CPLX2: Complexin-2
EC: Entorhinal cortex
IML: Inner molecular layer
LM: Stratum lacunosum-moleculare
IR: Immuno-reactivity
LUC: Stratum lucidum
MAP2: Microtubule-associated protein 2
MBP: Myelin basic protein
MOPP: Molecular layer perforant path-associated cells

NEUN: Neuronal nuclei

OML: Outer molecular layer

PMI: Postmortem interval

RAD: Stratum radiatum

SHANK2: SH3 and multiple ankyrin repeat domains protein 2

SNARE: Soluble NSF-attachment protein receptor

STX1A: Syntaxin-1A

SYNGR1: Synaptogyrin-1

SYT1: Synaptotagmin-1

VAMP2: Vesicle-associated membrane protein 2

Introduction

Synaptic dysfunction and degeneration are early features in Alzheimer's Disease (AD) pathogenesis and correlate well with measures of cognitive decline [11, 29, 53]. The hippocampus, a brain region which is essential for episodic memory formation is severely impaired in AD. The hippocampus consists of different anatomical regions including the dentate gyrus, the cornu ammonis (CA) regions, and the subiculum [33] (Fig. 1a). The main input to the hippocampus comes from the entorhinal cortex (EC) through the perforant path [9] (Fig. 1a, c). The neurons from the layer II of the EC project to the outer two-thirds of the molecular layer of the dentate gyrus (hereafter referred as to outer molecular layer, OML; Fig. 1c) as well as to CA3, whereas the neurons from layer III project to CA1 and subiculum regions (Table 1). The hippocampus also receives major modulatory input from the serotonergic, noradrenergic, and dopaminergic transmitter systems as well as from the medial septal nucleus (cholinergic neurons) via the fimbria/fornix pathway [47]. The perforant path has been proposed to be vulnerable in AD pathogenesis due to (i) synaptic loss observed in the OML of AD and mild cognitive impairment cases [43, 44]; (ii) the presence of AD-hallmarks, such as amyloid plaques and neurofibrillary tangles, both in the EC [2, 6, 54] and in the OML [22, 54]; and (iii) substantial loss of EC neurons in AD cases, particularly of layer II [16, 24, 36], as well as loss of cholinergic input to the hippocampus [17].

In order to further delineate the synaptic pathology of OML in AD brain, we recently performed a proteomic study of microdissected OML from five AD (Braak IV and amyloid C) and five control cases [18]. We found that a large number of presynaptic proteins were significantly decreased in the OML of AD brains, whereas postsynaptic proteins were relatively spared. In addition, extensive pathway analysis indicated that synaptic vesicle cycling and exocytosis as prominently affected pathways in the OML of AD brains. The current work is an extension of this earlier publication, where we investigate the specificity and the mechanisms leading to the alteration of presynaptic proteins by performing immunofluorescent stainings in hippocampal sections from human AD and control cases. We selected five synaptic proteins: complexin-1 (CPLX1), synaptotagmin-1 (SYT1), syntaxin-1A (STX1A), synaptogyrin-1 (SYNGR1) and vesicle-associated membrane protein 2 (VAMP2), based on the extent of their reduction determined in our proteomic assay [17], their presynaptic function and the availability of validated antibodies.

STX1A, SYT1 and VAMP2 are all part of the core synaptic vesicle-membrane-fusion machinery and thus play important roles in neurotransmission [49]. STX1A together with the synaptic vesicle protein VAMP2 and the plasma membrane protein SNAP-25 form the soluble NSF-attachment protein receptor (SNARE) complex [48, 50], which is the key component of membrane fusion machinery in exocytosis [49]. SYT1 is a synaptic vesicle protein that acts as a Ca^{2+} sensor [15]; increased levels of Ca^{2+} in the presynaptic terminal triggers an interaction between SYT1 and the plasma membrane protein STX1A that is important for synaptic vesicle exocytosis [45]. Moreover, complexins, such as CPLX1, bind to SNARE complexes [31], participate in Ca^{2+} -triggered exocytosis [38], and therefore are essential for neurotransmitter release [28]. Among these proteins, the synaptic vesicle protein SYNGR1 is the least

studied. Although its exact function still remains unknown, earlier studies suggest that SYNGR1 is most likely not essential for exocytosis, but it may play a regulatory role in synaptic plasticity, and therefore, neurotransmitter release [23].

Although decreased mRNA and/or protein levels of these five presynaptic markers have been previously detected in AD brain [4, 7, 32, 51], these studies have so far been performed on homogenates prepared from cortical or hippocampal bulk tissue, thus not allowing any detailed examination of different hippocampal sub-regions. Here, we investigated the distribution of presynaptic proteins in 10 hippocampal sub-regions of AD and control cases using immunofluorescence. We observed a reduction of presynaptic protein staining densities in the OML consistent with the reduction determined in our proteomic approach. Interestingly, this reduction appeared to be specific to the OML since all others hippocampal sub-regions examined, displayed a similar expression level between controls and AD cases. In order to depict what underlying causes might be responsible for the observed changes in the presynaptic proteins in AD OML, we further investigated both post- and presynaptic compartments by assessing: (1) staining density of postsynaptic protein and a dendritic marker, (2) the relative number of dentate granule cells, and finally (3) the staining densities of axonal markers. Finally, we investigated a possible mechanism involving the major AD hallmarks, amyloid plaques and phosphorylated-Tau.

Results

We investigated the specificity and the mechanisms leading to the alteration of presynaptic proteins in AD by comparing hippocampus sections from AD (n=8) and control (n=7) patients from the Netherlands Brain Bank. There were no statistically significant differences in gender, age, postmortem interval (PMI), ApoE status, or brain pH between the two groups, while Braak and amyloid stages showed statistically significant differences, as expected (Table 2).

We used an immunofluorescence approach combining an antigen retrieval and autofluorescence quenching methods (see absence of auto-fluorescence in controls; Supplementary Fig. 1a) to label synaptic proteins in fixed, paraffin embedded sections of human brain tissue. The sections were imaged with a Nanozoomer 2.0HT slide scanner (Hamamatsu) allowing the acquisition of full sections without mosaicism and a resolution of 454 nm/pixel (55947 DPI). The thickness of the sections of 5 μ m was not suitable to stereological approaches. Taking advantage of the linearity of the fluorescent signal, we measured the mean fluorescent pixel intensities of synaptic proteins in different anatomical regions to evaluate the distribution of synaptic alterations in AD.

Marked reduction of presynaptic proteins levels in the OML in AD

The fluorescence pixel intensities of the five presynaptic proteins CPLX1, STX1A, SYT1, SYNGR1 and VAMP2 were measured in the OML and inner molecular layer (IML) of the dentate gyrus. Interestingly, we found that these proteins displayed different spatial distribution between IML and OML. In a control brain, staining with antibodies directed against CPLX1, STX1A and SYT1 gave rise to a stronger immuno-reactivity (IR) in OML than in IML (Fig. 1d-i), while SYNGR1 and VAMP2 appeared to be more abundantly expressed in IML than in OML (Fig. 1j-m). The region-specific distribution of these presynaptic proteins created a visible line in the molecular layer, thus allowing us to easily separate IML and OML by gross visualization (Figure 1).

Semi-quantitative densitometric analysis showed that the staining densities of CPLX1 (44 % decrease, p-value = 0.0051, Fig. 1d, e), STX1A (42 % decrease, p-value = 0.016, Fig. 1f, g), SYT1 (29 % decrease, p-value = 0.031, Fig. 1h, i), and SYNGR1 (34 % decrease, p-value = 0.042, Fig. 1j, k) were significantly reduced in OML in AD compared to control cases, whereas there was a non-significant decreased tendency in the same direction for VAMP2 staining (45 % decrease, p-value = 0.068, Fig. 1l, m).

Interestingly, we observed no profound changes between the two groups in the levels of these presynaptic proteins in the IML which receives afferents from the medial septum [4], the thalamus [6] and the hilus of the dentate gyrus [2] (Fig. 1d-m). When the mean pixel intensity of a synaptic protein in OML was normalized to the intensity of the same protein in IML, the ratio was significantly decreased for CPLX1, STX1A, SYT1 and SYNGR1 but not for VAMP2 (Supplementary Fig. 1) giving further support to the notion of a specific impairment of OML compared to IML.

Presynaptic protein staining reduction in AD hippocampus specific to the OML

The marked difference in the reduction of presynaptic proteins in OML compared to IML indicates that the synaptic alterations depend on the afferent inputs within a brain sub-region. This led us to investigate

whether other hippocampal sub-regions could be affected – which might be innervated by other modalities. The OML receiving abundant afferents from the EC, we hypothesized that other hippocampal sub-regions receiving EC projections (Table 1) could be affected. Thus, in addition to the OML and IML, we analyzed the fluorescence pixel intensities of CPLX1, STX1A, SYT1, SYNGR1 and VAMP2 in 8 different sub-regions of the hippocampus visible on the same sections, categorized into two groups: (i) molecular layers, enriched in synapses, consisting of CA3-LUC, CA3-RAD, CA3-LM, CA1-RAD and CA1-LM, and (ii) neuronal layers, consisting of CA4, CA3 and CA1 neuronal layers. Despite the observed reduction in CPLX1, SYT1, SYNGR1 levels in AD OML, no significant reductions were detected in any other investigated molecular layers in AD compared to control cases (Fig. 2a-j) or between neuronal layers (see pictures in Fig. 1 and quantifications in Supplementary Fig. 2). Interestingly, the cell-surface SNARE protein STX1A exhibited a markedly different staining profile in AD than the vesicular or cytosolic presynaptic proteins described above. Indeed, a marked increase in STX1A staining was observed in the CA4 neuronal layer of AD cases compared to controls (36 % increase, p-value = 0.048, Supplementary Fig. 2b). Moreover, non-significant trends towards increased levels of STX1A were detected also in the CA3 neuronal layer (52 % increase, p-value = 0.0506), CA3-LUC (55 % increase, p-value = 0.08), CA3-RAD (34 % increase), CA1 neuronal layer (18 % increase), and CA1-RAD (31 % increase, p-value = 0.09) (molecular layers in Fig. 2b; neuronal layers in Supplementary Fig. 2b).

Preserved postsynaptic compartment in OML of AD cases

The observation of reduced levels of presynaptic proteins in OML raised the question of whether the postsynaptic proteins were also reduced, as expected from the hypothesis of an overall synaptic loss. We assessed the levels of the standard markers of postsynaptic density and dendrites, respectively SHANK2 and MAP2. Despite the presence of severe Braak and amyloid stages among the AD cases, the levels of SHANK2 and MAP2 were not reduced in AD compared to controls (Fig. 3a-d). Furthermore, we investigated the density of dentate granule cells, the dendrites of which are located at the OML forming synapses with the perforant path termini and noted that there was no obvious reduction in granule cells density in AD compared to control cases (Fig. 3e, f). Together, these results advocate for a distinctive impairment of presynaptic proteins in OML synaptic inputs rather than an overall synaptic loss.

Evidence for maintained axonal projections in AD OML

We next investigated potential mechanisms involved in alterations of presynaptic proteins in OML in AD. One potential mechanism would be if a reduction of axonal projections to the OML would proportionally lead to a reduced amount of presynaptic proteins in this region. Consecutive hippocampal sections from AD and control cases were stained with three different axonal markers: myelin basic protein (MBP; for myelinated axons), SMI-312 (for phosphorylated neurofilaments M and H) and the commonly used axonal marker total Tau. Semi-quantitative densitometric assessment showed no difference in the levels of above-mentioned axonal markers between the two groups (Fig. 4a-f) suggesting that biological mechanisms other than a reduction in afferent fibers density are at play in the alterations in presynaptic proteins in OML.

Relationship of presynaptic failure with AD pathology

To further explore the mechanisms behind the specific impairment in presynaptic proteins in OML, we investigated the presence of AD-related pathological hallmarks in the different hippocampal sub-regions of interest. We immuno-labelled amyloid plaques with an antibody against A β peptides (clone 6E10) and pathological forms of Tau with an antibody against its phosphorylated isoforms (AT8). Dense core amyloid plaques were distinctly observable in AD cases whereas they were absent from the controls (Fig. 5a). The extent of the amyloid burden defined as the total surface covered by A β peptides normalized to the surface of the sub-regions analyzed was not significantly different between OML and the other hippocampal regions (Fig. 5b). Besides, within OML there was no correlation between amyloid load and the extent of presynaptic proteins reduction among the different AD patients (Fig. 5c). Together, these data indicate that the distinctive reduction in presynaptic proteins in OML cannot be explained by a larger amyloid burden in this sub-region.

The antibody against phosphorylated Tau (AT8) labelled numerous soma in AD cases, in the different CA regions and in the dentate gyrus (Fig. 5d). Strikingly, the abundance of Tau (AT8)-positive neurites was massively higher in AD compared to controls (Fig. 5d). We hypothesized that a higher density of dystrophic neurites, as revealed by phosphorylated Tau (AT8) immunogenicity, could be related to the presynaptic impairment. However, the extent of the burden in dystrophic neurites, i.e. the total surface covered by dystrophic neurites positive for phosphorylated Tau (AT8), normalized to the surface of the sub-region analyzed was not significantly different between OML and the other hippocampal molecular layers (Fig. 5e). Finally, within OML there was no correlation between the burden in dystrophic neurites and the extent of presynaptic proteins reduction among the different AD patients (Fig. 5f).

Discussion

Synaptic dysfunction, as revealed by reduction in synaptic proteins [56] as well as loss of synapses [41], have been observed in early stages of AD. To have a better insight into the molecular mechanisms involved, we previously performed a proteomic analysis of the OML, one of the terminal zones of the perforant path, the main afferent pathway innervating the hippocampus. This approach revealed an impairment of OML proteome in AD characterized by a reduced level of presynaptic proteins whereas the level of the post-synaptic proteins were unchanged [18]. Here, we aimed to expand that study and further explore the distribution of presynaptic proteins across the entire human hippocampus and to investigate the possible causes of the proteomic impairment in OML. We selected five presynaptic proteins, i.e. CPLX1, STX1A, SYT1, SYNGR1 and VAMP2, which play important roles in neurotransmission. We performed immunofluorescent staining to assess their expression level assessed across 10 different sub-regions of the hippocampus, categorized into molecular and neuronal layers.

Heterogeneous distribution of key presynaptic proteins in healthy controls

All selected antibodies showed a strong IR in the neuropil but not in the cell soma such as the densely packed dentate granule cell layer, confirming the enrichment of these proteins in synapses. Interestingly, the five proteins displayed a region-specific distribution which allowed a clear visualization of the OML/IML border: CPLX1, STX1A and SYT1 displayed a stronger IR in the OML whereas SYNGR1- and VAMP2-IR appeared to be more abundant in the IML (Fig. 1). Ramos-Miguel et al. have also detected higher levels of CPLX1 in the OML than in the IML [37]. Albeit both IML and OML contains mainly excitatory inputs to GC, the afferent input is, to a large extent distinct and arises from mossy cells of the CA4 (called associational/commissural projections) and EC layer II, respectively. Thus, it is plausible that the distinctive spatial distribution of the presynaptic proteins occurs as a result of different synapses exhibiting different molecular compositions [34].

OML-specific presynaptic failure within the hippocampus

The labelling intensity of CPLX1, STX1A, SYT1 and SYNGR1 was significantly reduced in the OML of AD compared to control cases (Fig. 1), corroborating our previous proteomics findings [18]. Interestingly, these reductions were specific to the OML region since all nine other molecular and neuronal sub-regions we examined were preserved, at least in the AD cases examined herein (Fig. 2). Previous studies based on immunohistochemistry reported a decrease in synapsin and synaptophysin in OML, however, detailed analyses of the distribution of these presynaptic proteins between hippocampal sub-regions were missing [21, 25]. In agreement with our study, a decreased ratio of OML/IML levels of synaptic proteins has previously been reported in AD brain, although the potential changes in staining intensity changes in the IML itself was not clearly stated [30, 39]. This specificity is intriguing when considering that our cohort consists of AD cases with late stages of AD pathology (Braak V-VI and amyloid C; Table 2) and that perforant path afferent input from the EC does not solely project to the OML but also to CA fields. Thus, more than a global failure of the perforant path, our results highlight the specific vulnerability of the pathway projecting to the OML in AD, with specific biological mechanisms for this selective vulnerability not yet revealed.

Possible homeostatic compensation in AD hippocampus

While no profound alterations were detected in the levels of CPLX1, SYT1, SYNGR1 and VAMP2 in any of the other studied hippocampal sub-regions of AD patients, the plasma membrane protein STX1A showed increased immunostaining in the CA4 (Fig. 2). A possible explanation for this finding could be that STX1A levels undergo a compensatory increase in these regions (possibly via increased collateral branching or sprouting) in order to compensate for the reduced input that dentate granule cells receive from the EC layer II, which is evident by the reduced STX1A level in the OML. As opposed to our findings, in a few proteomics study, decreased levels of STX1A were reported in AD brains [7, 32, 58]. However, these studies have used homogenates prepared from bulk tissue of AD brains, and would not be able to tease out cell- and layer-specific discrete changes as the ones observed in sub-hippocampal regions in the current study. The increase in STX1A staining in the AD cases is clearly visible (Fig. 2C) but only extends through one layer, and therefore would be hard to discern using whole tissue homogenates.

Specific alteration of OML neurotransmitter release proteins in AD

We next investigated whether the post-synaptic compartment was also altered as expected in the hypothesis of full synapses collapse. When the components of the postsynaptic compartment were investigated, we did not find any alterations in the levels of the postsynaptic density protein SHANK2 and the dendritic marker MAP2 in AD (Fig. 3), which were in line with our previous proteomic findings [18]. Furthermore, the density of dentate granule cells, using NeuN staining, showed no loss of NeuN staining in AD compared to controls. Taken together, our findings suggest that postsynaptic compartments in the OML are rather intact and the deficit is specifically presynaptic.

However, quantification of post-synaptic densities using structural methods such as electron microscopy indicated that they decrease in AD condition [40, 42, 44]. Differences between the amount of synaptic protein amounts (as assessed in the present study) and the number of structurally defined synaptic compartments (as assessed by EM) could account for the divergence in results. Indeed, structural analyses have reported that synapse loss is associated with an increase in the length of synaptic appositions (indicating larger synapses) [44], possibly as a compensation by a synaptic homeostasis mechanism. Therefore, the total amount of synaptic proteins may not change in most synaptic fields. This is however not the case of the OML of the DG, where the decrease in the IR of presynaptic proteins, together with the reduction assessed by our previous proteomic approach [18], advocates for a marked reduction specific to presynaptic proteins. This gives support to the notion of a preferential presynaptic failure in OML of AD brain [3].

Among the proteins we investigated, CPLX1 was the one with the lowest AD/Control ratio both in the present study and in our previous proteomic study [18] performed on different human cohorts, emphasizing the importance of this protein in AD pathogenesis. In agreement with this, previous reports showed that higher levels of specific presynaptic proteins, including CPLX1, were associated with better cognitive function [20, 57]. Before, or in addition to the neurodegeneration occurring in AD, specific

alterations in the metabolism of proteins involved in presynaptic plasticity may well contribute to the cognitive impairment.

Neurodegeneration versus retrograde degeneration

The question of the order of the different pathological events occurring during AD progression is important and has not yet been fully answered. For example, to know whether the presynaptic failure occurs before or as a consequence of neurodegeneration is crucial because it will impact the design of future therapeutic approaches. Compelling neuropathological evidence has shown a dramatic and early loss of EC neurons in AD, particularly in layer II, which correlates with cognitive impairment in AD [16, 24]. Hence, one theory proposes that the degeneration of EC neurons leads to the loss of perforant path afferents and therefore to synaptic loss and reduced synaptic protein-IR in the terminal zone of the perforant path. Supporting this notion, it has been demonstrated that following an EC lesion synaptophysin-IR was reduced in the OML in rat brain [8]. Unfortunately, we were not able to investigate whether the reduction in presynaptic proteins correlated with a loss of EC neurons in layer II since the brain sections in our cohorts were too posterior with neurons in the para-hippocampal cortex not distributed in islands as expected for EC layer II.

We could nevertheless test if the presynaptic changes at the OML were related to axonal degeneration whether this depends or not on somatic degeneration. We assessed possible structural alteration of the axonal projections in OML by investigating the levels of the MBP (for myelinated axons), SMI-312 (for phosphorylated neurofilaments M and H) and the commonly used axonal marker Tau. Despite the advanced stage of the AD cases, we did not find significant alterations in the levels of these three markers in the OML (Fig. 4). This observation is apparently inconsistent with a massive degeneration of EC neurons from layer, a scenario in which the number of projecting afferents should be reduced proportionally to the neuronal loss. One possible explanation is that the afferent input from EC only represent a minority of axons passing through the OML or that axonal projections coming from non-degenerated areas compensate for the loss of axons within the perforant path.

An alternative hypothesis is that axonal projections from EC layer II are not massively degenerated. This possibility offers a straightforward explanation for the unchanged labelling intensities of the axonal markers between AD and controls. It also clarifies why all presynaptic proteins are not altered at the same extent between AD and controls [18]. Indeed, if the reduction in presynaptic proteins was directly resulting from a loss of afferent inputs to the OML, similar AD/Control ratio between the presynaptic proteins detected would have been expected. As previously discussed by Terry et al [52, 53], the loss of neocortical synapses correlates with cognitive decline much better than cell counts and this synaptic loss occurs earlier than death of the neuronal soma. This observation is the basis for the hypothesis of retrograde degeneration which states that synaptic dysfunction precedes axonal degeneration and neuronal death. This hypothesis is supported by a recent observation that changes in synaptic proteins precede neurodegeneration markers in prodromal AD [27]. The data that we collected here are consistent with a retrograde degeneration in which presynaptic proteins are reduced but the axons are not yet degenerated and which, within the hippocampus, would specifically start in the OML.

A retrograde degenerative mechanism could also explain the intriguing observation that the genetic deletion, or the loss of function (using neurotoxins), of several presynaptic proteins leads to neurodegeneration. For example, loss of Munc18 that prevents neurotransmitter release leads to a rapid neurodegeneration [13, 19, 55]. In a similar way, genetic deletion of CSP α revealed that it cooperates with α -synuclein and SNAP25 to prevent neurodegeneration [10, 14, 46] and use of botulinum neurotoxins revealed a direct role of STX1A and SNAP-25 in neuron survival [5, 35]. Thus, there is a possibility that massive reduction in presynaptic proteins, as seen in the perforant path projecting to the OML, leads to the loss of the projecting neurons in the entorhinal cortex. The resolution of the cause-consequence relationship between presynaptic failure and somatic degeneration will be one of the next challenges for research on AD, using studies in populations who are vulnerable for AD.

Homogeneous burden in amyloid plaques and dystrophic neurites across the hippocampus

We searched for a possible pathological correlate of the presynaptic alteration in OML by investigating the extent of the AD-hallmarks in this region compared to the other hippocampal sub-regions. We labelled amyloid plaques with an antibody against A β 1-16 and observed neuritic and diffuse plaques in all hippocampal regions in AD whereas amyloid plaques were absent in controls (Fig. 5). Notably, the area covered by amyloid plaques was not larger in the OML compared to other molecular layers of the hippocampus (Fig. 5b). In OML, the presynaptic alterations were not proportional to the amyloid burden (Fig. 5c).

In an effort to specially search for a cause of an axonal or presynaptic impairment we focused our attention on the presence of neurites immuno-reactive for phosphorylated-Tau. Indeed, a role of pathological form of tau in the impairment of presynaptic proteins has been recently exemplified by the observation that pathological Tau binds to synaptic vesicles [26, 59] leading to a smaller pool of active vesicles and to decreased synaptic transmission [59]. We observed that neurites expressing phosphorylated-Tau were remarkably more abundant in AD compared to controls (Fig. 5d). Interestingly, neurites expressing phosphorylated-Tau were arranged sometimes in islands, for example in the IML (Fig. 5d) likely corresponding to dystrophic neurites around senile plaques [1]. The area covered by neurites expressing phosphorylated-Tau was not larger in OML compared to other molecular layers of the hippocampus (Fig. 5e). Moreover, in OML, the presynaptic alterations were not proportional to the burden in phosphorylated-Tau (Fig. 5f). Thus, with the limitations inherent to our study (i.e. incompatibility with stereological investigations) in mind, our data suggest a lack of correlation between the presynaptic alterations observed in OML and the two AD-related hallmarks we investigated.

In summary, our investigation distinctly highlights the projections to the OML, i.e. the perforant path from EC layer II to dentate granule cells, as a specific vulnerable pathway in AD. Our findings also emphasize that postsynaptic compartments in the OML are rather intact, giving support to the notion of specific presynaptic failure in AD. These findings might lead to novel and more targeted therapy options for the early stages of AD.

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Author contributions

H.H., T.J.S., S.F. and G.B. designed the research. T.J.S. performed immunofluorescence experiments. H.H. did densitometry measurements and data analysis. H.H., S.F., T.J.S and G.B. wrote the manuscript. C.M., A.-C.G., L.T.O. and B.W. contributed to the writing of the manuscript. The manuscript was read, edited and approved by all authors.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Materials and methods

Post-mortem human brain tissues

Formalin-fixed paraffin-embedded hippocampal sections (5 µm thick) from eight sporadic AD and seven non-demented control cases were obtained from the Netherlands Brain Bank. AD cases were clinically diagnosed according to previously published research criteria [12] and pathologically confirmed by the presence of amyloid plaques and neurofibrillary tangles. On the other hand, control cases showed no sign of dementia and presented no or little pathological alterations including a few plaques and tangles. The demographic characteristics of AD and control cases are shown in Table 2. All donors or their next-of-kin gave informed consent. The establishment of the Netherlands Brain Bank was approved by the Independent Review Board of VU University Medical Center in Amsterdam, the Netherlands (2009/148).

Immunofluorescence

Hippocampal sections were first deparaffinized in xylene ($\geq 98.5\%$, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent) and then rehydrated using the following wash steps: 99 %, 95 % and 70 % ethanol, and distilled water. Subsequently, heat-induced antigen retrieval was done using citrate buffer (10 mM citric acid (Sigma Aldrich), with 0.05 % Tween-20 (Sigma Aldrich), pH 6) at 110 °C for 30 min inside a pressure cooker (Biocare Medical). Slides were then washed in PBS with 0.05% Tween-20 (PBS-T) and were subsequently blocked with normal goat serum (Thermo Fisher Scientific) for 20 min at room temperature (RT). Slides were incubated with the primary antibodies listed in Table 3 for 2 hours at RT. In order to reduce autofluorescence, slides were then incubated with 1x TrueBlack® Lipofuscin Autofluorescence Quencher (Biotium), which was diluted in 70% ethanol, for 5 min at RT. Subsequently, sections were washed with 1x PBS for three times, which was followed by the incubation with the secondary antibodies (anti-rabbit, anti-mouse and anti-guinea pig IgG conjugated to Alexa 488 or Alexa 568, Thermo Fisher Scientific, 1:500 dilution) for 2 hours at RT. Sections were washed with 1x PBS for three times and incubated with either 5 µM of Methoxy X04 (Tocris Bioscience) for 20 min at RT or 300 nM of DAPI for 10 minutes at RT. Finally, sections were washed in 1x PBS three times and in distilled water, cover-slipped using Fluoromount™ (Southern Biotech) and air-dried overnight at RT. In order to avoid inter-experimental variability, sections from all AD and control cases were stained with a certain antibody at the same time.

Additionally, we assessed the specificity of the fluorescent signal coming from the secondary antibodies by performing several control experiments. In the first experiment, we tested the presence of non-specific fluorescence signal that can be caused by the secondary antibody itself, and incubated consecutive hippocampal sections from a healthy control case with either primary (i.e. STX1A and SYT1) and secondary antibodies (i.e. Alexa 488 and Alexa 568) or only secondary antibodies. In the second experiment, we tested the efficiency of TrueBlack to remove the endogenous autofluorescence coming from the tissue (Jordá-Siquer et al. unpublished results).

Imaging and image analysis

Slides were loaded into the semi-automated Nanozoomer 2.0HT slide scanner (Hamamatsu) and scanned using FITC (green), TRITC (red) and DAPI channels at 20x magnification and fixed additional

lens 1.75X (resolution of 454 nm/pixel). Each human case was represented by one hippocampal section. Image acquisitions and settings were kept constant for all sections stained with a certain antibody. Additionally, to avoid biases in fluorescence intensity, all AD and control cases were also scanned together. Gamma correction of 1 was applied to all images in order to reach linearity in fluorescence signal and images were then exported as tiff files using the NDP.view2 software (Hamamatsu) and analyzed using ImageJ Fiji (NIH).

Semi-quantitative assessment of fluorescence pixel intensity of CPLX1, STX1A, SYT1, SYNGR1 and VAMP2 stainings was carried out in the following sub-regions of the hippocampus: molecular layers of the dentate gyrus (IML and OML), molecular layers of CA3 (CA3-LUC, CA3-RAD, CA3-LM), molecular layers of CA1 (CA1-RAD and CA1-LM) as well as the neuronal layers like CA4, CA3 and CA1. One image per subfield per case was exported and the mean pixel intensity of each subfield was measured blindly as the average from 5 ROIs. The background was measured from areas outside the tissue for each case and each synaptic marker using the same settings (same magnification and gamma correction of 1. The mean pixel intensity was measured from 2 ROIs and the average was then subtracted from the actual density readings of synaptic markers.

In order to explore changes occurring in the presynaptic and postsynaptic compartments, we did densitometric analysis of three axonal markers (MBP, SMI-312 and Tau) as well as of a postsynaptic density marker (SHANK2) and a dendritic marker (MAP2) in the OML and the adjacent IML. Additionally, we measured the neuronal density of the dentate granule cells, which were normalized to the total surface area (mm²), by using NeuN and DAPI stainings in a blinded fashion. Similarly, amyloid and phosphorylated Tau burden were determined by measuring the mm² area in each of the synaptic regions of interest normalized to the total surface of the analyzed region.

Statistical analysis

Demographic characteristics between AD and control groups as well as semi-quantitative densitometric assessments were analyzed in GraphPad PRISM 7.0 (San Diego, CA). Two-tailed Student's t-test was used to assess whether age, brain pH and PMI significantly differ between control and AD groups, while Mann-Whitney test was used to assess the statistical significance, ApoE status, Braak and amyloid stages between the two groups. Statistical analysis of semi-quantitative densitometric assessment of fluorescence pixel intensity was done using either two-tailed Student's t-test or Mann-Whitney test, depending on whether the data follows a normal distribution or not, which was checked by Kolmogorov-Smirnov test. The presence of outliers was also checked by the ROUT (default) method. In all statistical comparisons between AD and control groups, p-value < 0.05 was considered statistically significant.

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Tables

Table 1. Summary of afferents and efferents of the hippocampus.

Afferents		Efferents	
OML	¹ Perforant path fibers from entorhinal cortex layer II	Granule cell layer	¹ CA3 via mossy fibers
IML	¹ Associational/Commissural fibers from hilus ² CA3 collaterals ³ Septal fibers ⁴ Fibers from thalamus		
CA3-LUC	¹ Mossy fibers from hilus ² Fibers from locus coeruleus	CA3	¹ CA1 via Schaffer collaterals
CA3-RAD	¹ CA3 associational fibers ² Septal fibers		
CA3-LM	¹ Perforant path fibers from entorhinal cortex layer II ² Septal fibers ³ Fibers from locus coeruleus		
CA1-RAD	¹ Schaffer collaterals from CA3 ² Recurrent collaterals from CA1 ³ Fibers from amygdaloid complex	CA1	¹ Entorhinal cortex layer V via Subiculum
CA1-LM	¹ Perforant path fibers from entorhinal cortex layer III ² Fibers from thalamus ³ Fibers from amygdaloid complex		

Table 2. Clinical and neuropathological details of cases.

Case	Diagnosis	Gender	Age	PMI (h)	Brain pH	APOE status	Braak tau stage	Amyloid stage
1	C	F	73	7.5	n/a	44	1	B
2	C	F	84	6	7.65	33	2	B
3	C	F	76	7	6.87	33	2	O
4	C	F	81	5.5	6.77	33	3	A
5	C	F	84	5.5	6.68	33	2	A
6	C	F	83	6	6.6	44	2	B
7	C	F	82	5	6.34	33	2	B
8	AD	F	85	6	6.35	44	5	C
9	AD	F	90	4.5	6.39	33	6	C
10	AD	F	70	5	6.73	33	5	C
11	AD	F	91	6.5	6.01	44	5	C
12	AD	F	70	6	6	33	6	C
13	AD	F	80	4	6.26	44	6	C
14	AD	F	70	4	6.42	44	6	C
15	AD	F	86	5	6.2	33	6	C
p-value:		NS	NS	NS	NS	NS	0.0002	0.0002

The clinical and pathological characteristics were compared between AD and control groups using either Student's t-test (age, PMI and brain pH) or Mann Whitney test (ApoE status, Braak), and p-value < 0.05 considered statistically significant.

Table 3: The list of primary antibodies used in this study.

Antibodies	Source	Dilution
CPLX1 antibody, rabbit polyclonal	#10246-2-AP, Protein Tech	1:500
STX1A antibody, rabbit polyclonal	#ANR-002, Alomone	1:1000
SYNGR1 antibody, mouse monoclonal	#103011, Synaptic Systems	1:500
SYT1 antibody, mouse monoclonal	#105011, Synaptic Systems	1:500
VAMP2 antibody, mouse monoclonal	#104211, Synaptic Systems	1:500
MBP antibody, rabbit monoclonal	#ab216668, Abcam	1:500
A β 1-16 antibody (DE2), mouse monoclonal	#MAB5206, Sigma Aldrich	1:500
Phospho-Tau antibody, mouse monoclonal	#MN1060 Thermo fisher scientific	1:500
SMI-312 antibody, Purified anti-neurofilament marker, mouse polyclonal	#837904, BioLegend	1:500
SHANK2 antibody, guinea pig polyclonal	#162204, Synaptic Systems	1:500
Tau-1 antibody, mouse monoclonal	#MAB3420, Sigma-Aldrich	1:500
MAP2 antibody, mouse monoclonal	#M4403, Sigma-Aldrich	1:500
NeuN antibody, rabbit polyclonal	#ABN78, Merck	1:500

Main figures

Figure 1

Figure 1

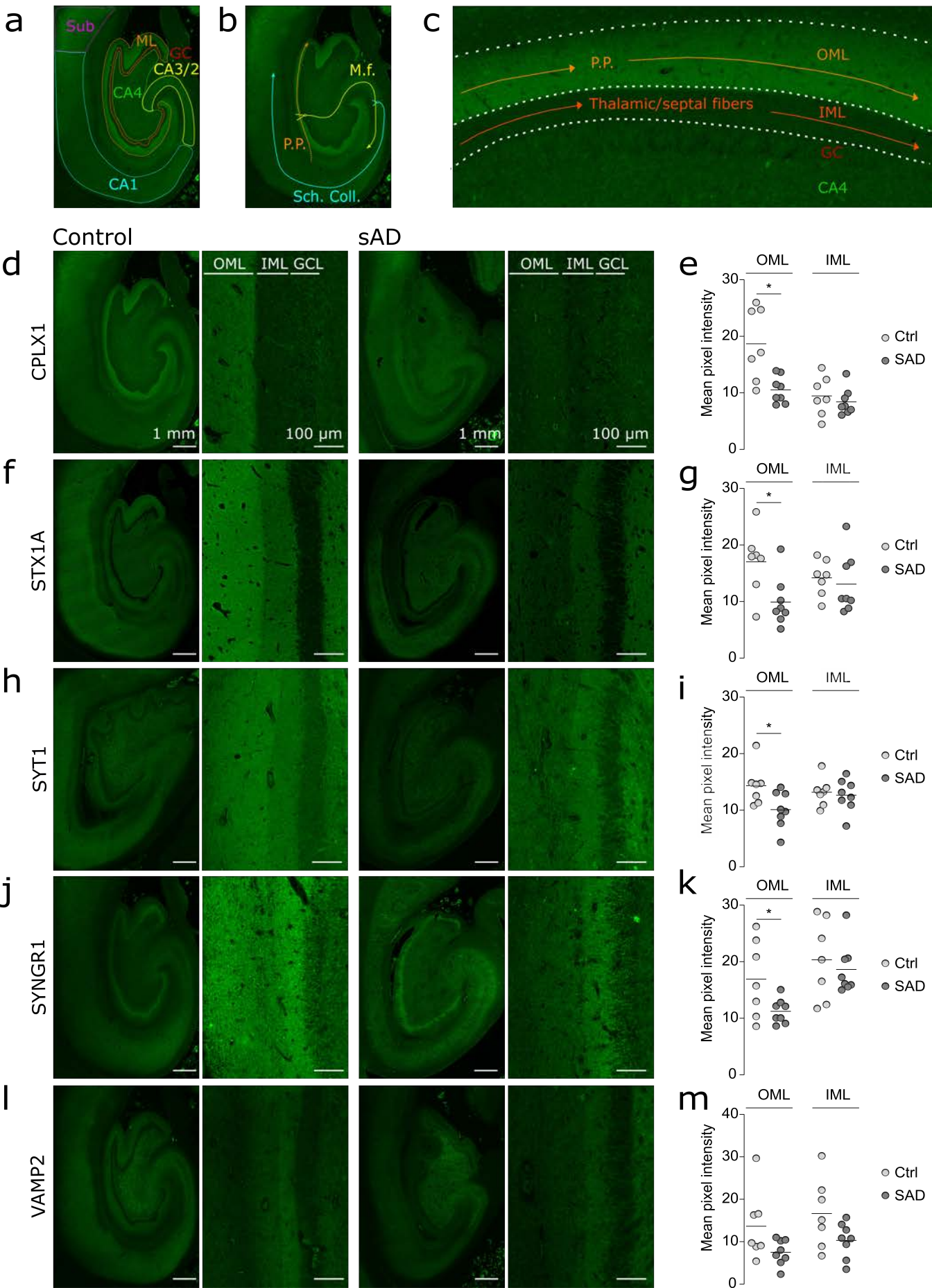


Figure 1. OML-specific reduction in presynaptic proteins (a) Schematic diagram showing the main hippocampal sub-regions in a section labelled for CPLX1 in a healthy hippocampus. (b) Schematic diagram showing the hippocampal trisynaptic circuit. (c) Schematic diagram showing the main afferents projections in OML and IML. (d, f, h, i, j) Representative images of the whole hippocampus and zoom-in pictures of the molecular region of the DG in controls (left) and AD (right) brain sections labelled respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The regions of interests are indicated by outer (OML) and inner (IML) molecular layers, which are located right next to the granule cell layer (GCL). (e, g, i, k, m) Scatter plots of the mean pixel intensity of the fluorescent labelling in OML and IML respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The fluorescence pixel intensities were significantly decreased in AD cases by 44% for CPLX1 (c, p-value = 0.0051), 42 % for STX1A (d, p-value = 0.016), 29 % for SYT1 (e, p-value = 0.031) and 34% for SYNGR1 (f, p-value = 0.042). In contrast, the levels of these proteins were not altered in the IML of AD cases. (m) The levels of VAMP2 showed a non-significant reduction by 45% (p-value = 0.068) and 38% (p-value = 0.075) both in the OML and the IML, respectively. Note that in a healthy brain, CPLX1, STX1A and SYT1 displayed a stronger IR in the OML with respect to IML (d, f, h), while SYNGR1- and VAMP2-IR was more prominent in the IML than the OML (j, l). Scale bar is 1 mm (low magnification) and 100 μ m (high magnification).

Figure 2

Figure 2

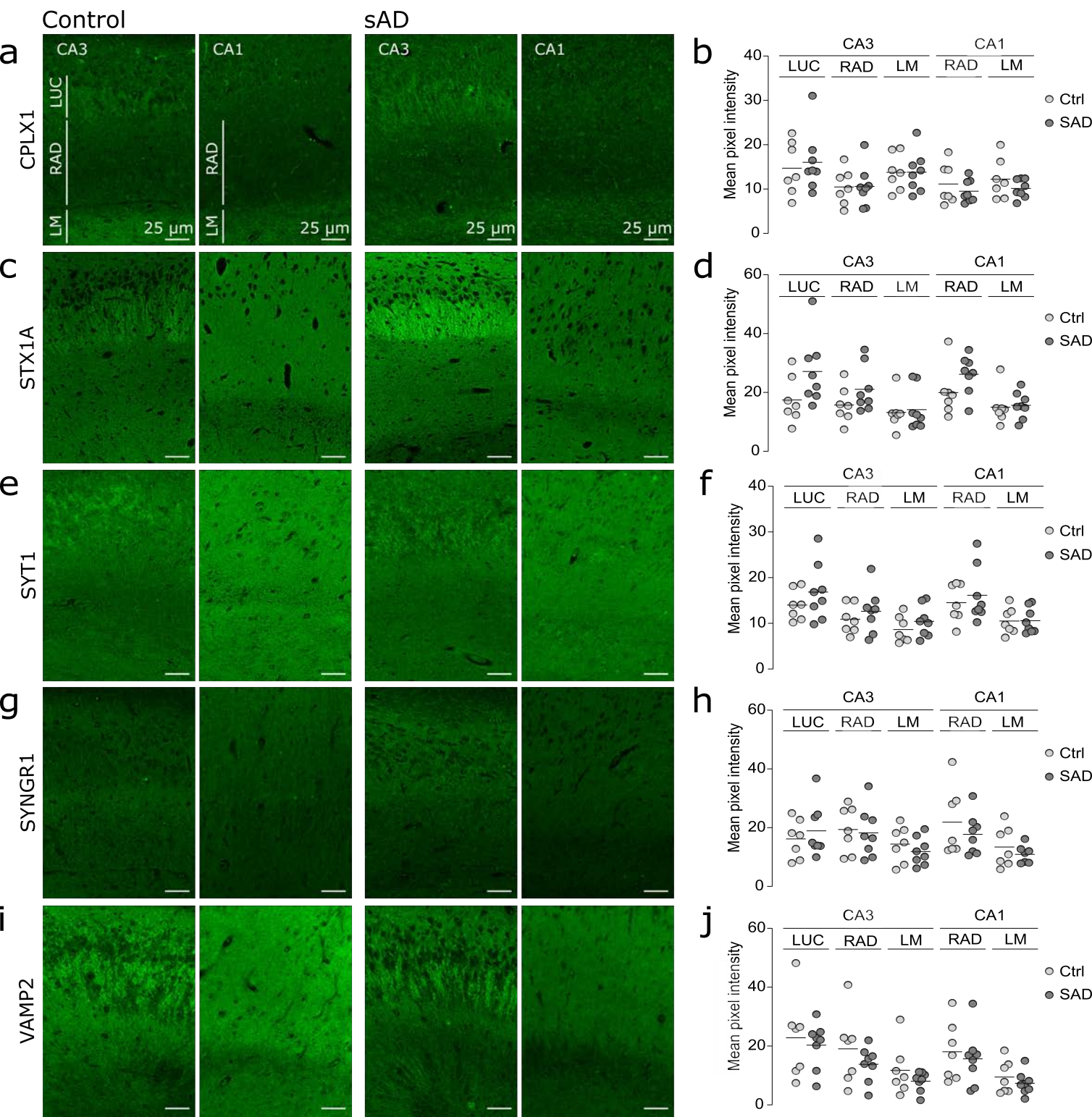


Figure 2. Preserved level of presynaptic proteins in various molecular layers of the hippocampus

(a, c, e, g, i) Representative images of the CA3 and CA1 regions in controls (left) and AD (right) brain sections labelled respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The region of interests stratum lucidum (LUC), radiatum (RAD) and lacunosum moleculare (LM) are indicated. (b, d, f, h, j) Scatter plots of the mean pixel intensity of the fluorescent labelling in OML and IML respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The mean pixel intensities were not altered between AD and control groups in the molecular layers of the CA3 and CA1. Scale bar is 25 μ m.

Figure 3

Figure 3

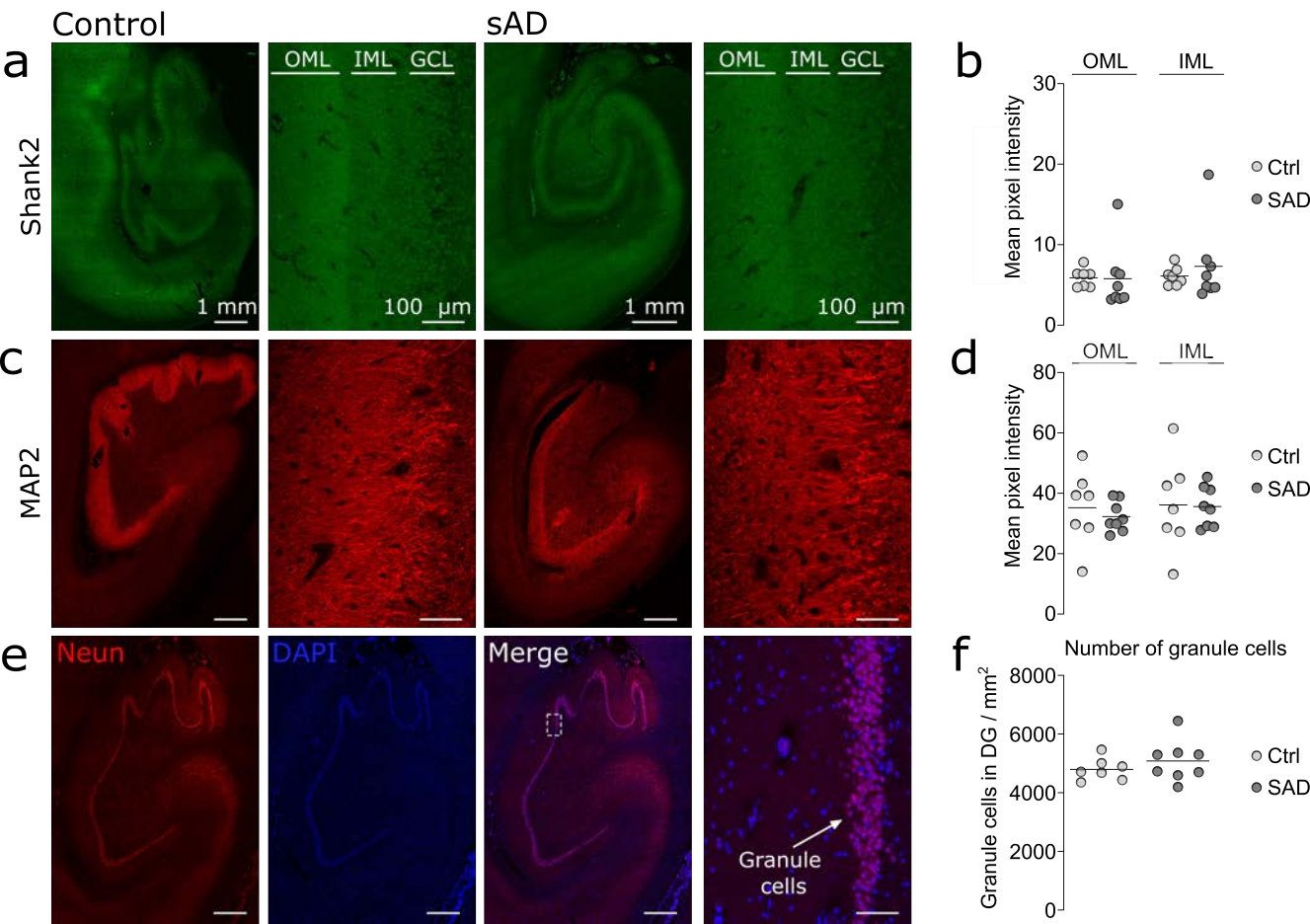


Figure 3. Preserved postsynaptic compartment in AD OML (a, c, e) Representative images of the whole hippocampus and zoom-in pictures of the molecular region of the DG in controls (left) and AD (right) brain sections labelled respectively for Shank2, MAP2 and colabelled for NeuN and DAPI. The regions of interests are indicated by inner (IML) and outer (OML) molecular layers, which are located right next to the granule cell layer (GCL). Scale bars are 1 mm (low magnification) and 100 μ m (high magnification). (b, d) Scatter plots of the mean pixel intensity of the fluorescent labellings in OML and IML respectively for Shank2 and MAP2. The levels of SHANK2 and MAP2 were not altered. (f) Scatter plots of the mean pixel intensity of the neuronal density (number of dentate granule cells per mm²) did not show a difference between AD and control groups.

Figure 4

Figure 4

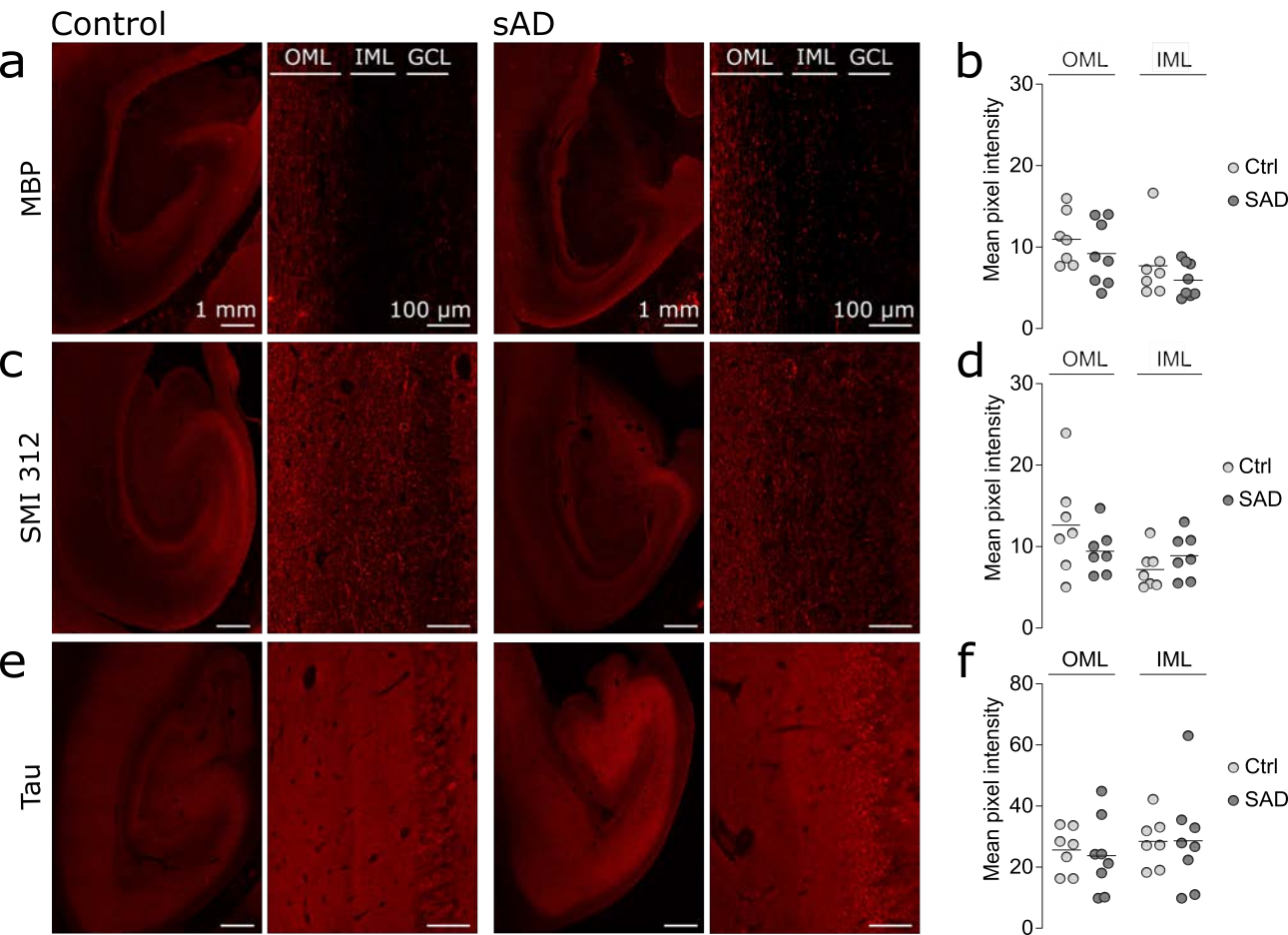
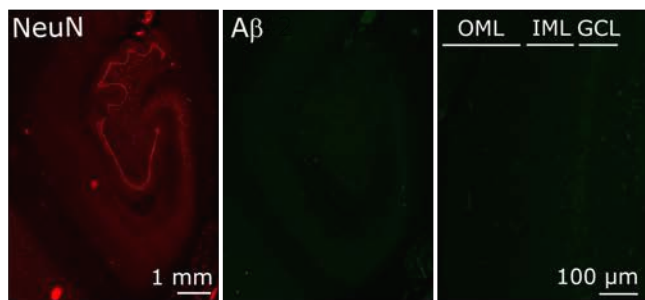


Figure 4. Evidence for maintained axonal projections in AD OML (a, c, e) Representative images of the whole hippocampus and zoom-in pictures of the molecular region of the DG in controls (left) and AD (right) brain sections labelled respectively for MBP, SMI 312 and Tau. The regions of interests are indicated by outer (OML) and inner (IML) molecular layers, which are located right next to the granule cell layer (GCL). Scale bars are 1 mm (low magnification) and 100 μ m (high magnification). (b, d, f) Scatter plots of the mean pixel intensity of the fluorescent labelling in OML and IML respectively for MBP, SMI 312 and Tau. The levels of MBP, SMI 312 and Tau were not altered.

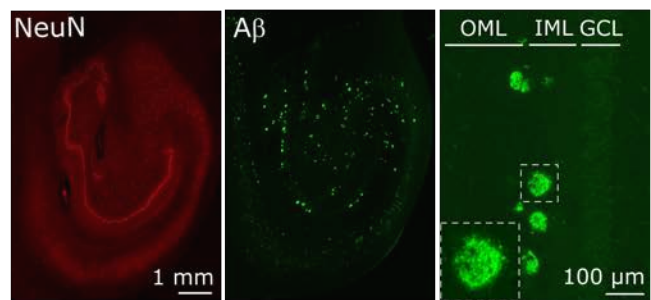
Figure 5

Figure 5

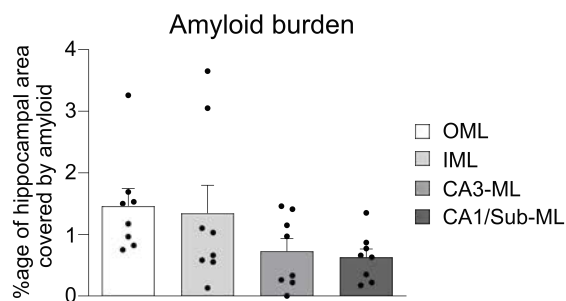
a Control



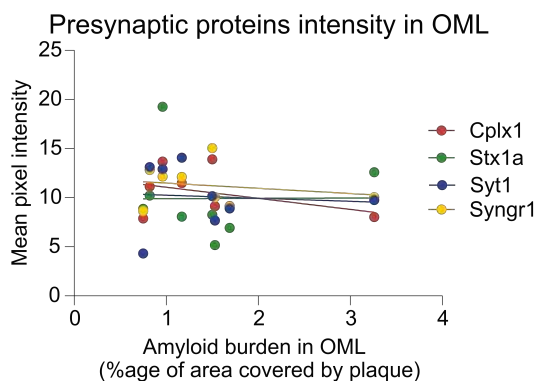
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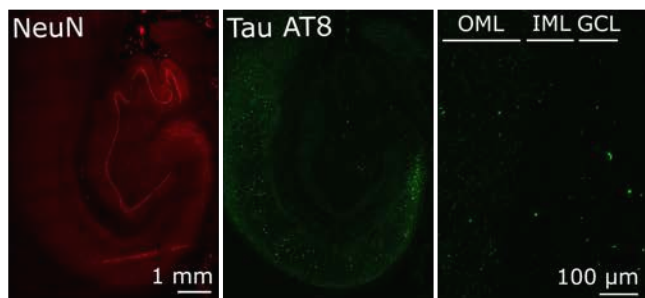
b



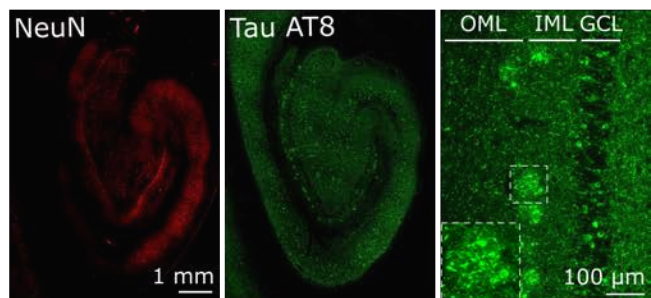
c



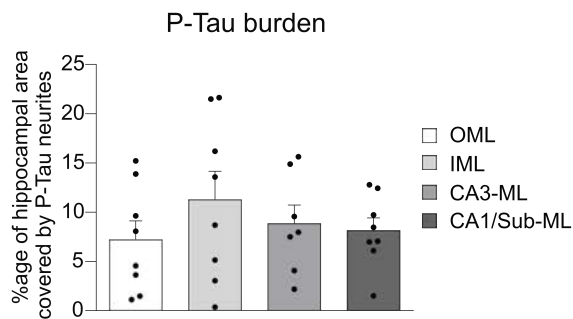
d Control



sAD



e



f

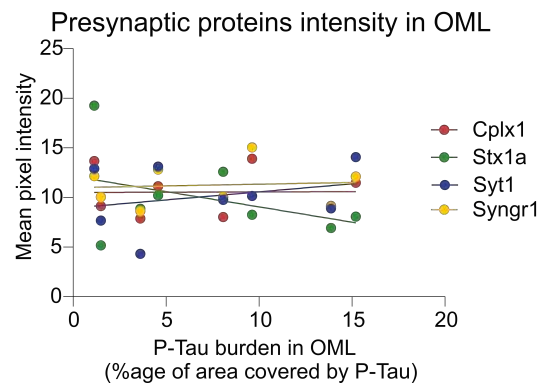


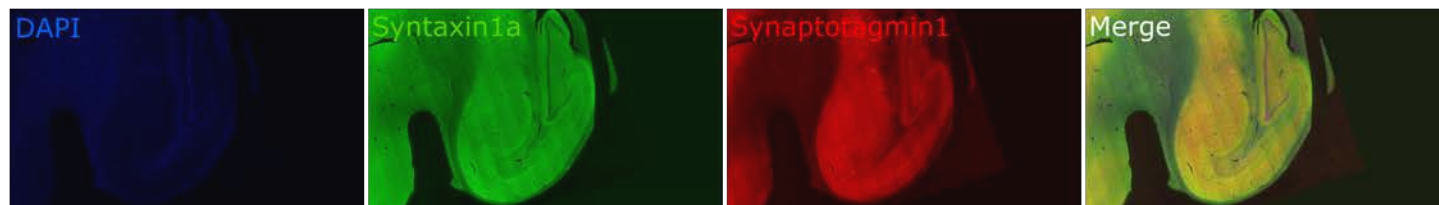
Figure 5. AD-related hallmarks relationship to presynaptic proteins decrease (a) Representative images of the whole hippocampus and zoom-in pictures of the molecular region of the DG in controls (left) and AD (right) brain sections labelled respectively for NeuN and A β 1-16. The regions of interests are indicated by outer (OML) and inner (IML) molecular layers located next to the granule cell layer (GCL). Scale bars are 1 mm (low magnification) and 100 μ m (high magnification). (b) Scatter plots with bars of the mean percentage of area covered by amyloid staining in different hippocampal molecular layers (OML, IML, CA3-ML, CA1-ML). The amyloid burden is not significantly different between the regions. (c) Graph of the mean pixel intensity of the presynaptic protein CPLX1, STX1A, SYT1 and SYNGR1 in OML in function of the amyloid burden. Each dot represents the value of one AD case. There was no correlation between the amyloid burden and the extent of the presynaptic proteins reduction. (d) Representative images of the whole hippocampus and zoom-in pictures of the molecular region of the DG in controls (left) and AD (right) brain sections labelled respectively for NeuN and phosphorylated-Tau (AT8). The regions of interests are indicated by outer (OML) and inner (IML) molecular layers located next to the granule cell layer (GCL). Scale bars are 1 mm (low magnification) and 100 μ m (high magnification). (e) Scatter plots with bars of the mean percentage of area covered by neurites positive for phosphorylated-Tau (AT8) in different hippocampal subregions (OML, IML, CA3, CA1). The dystrophic neurites burden is not significantly different between the regions. (f) Graph of the mean pixel intensity of the presynaptic protein CPLX1, STX1A, SYT1 and SYNGR1 in OML in function of the burden in AT8-positive dystrophic neurites. Each dot represents the value in one AD case. There was no correlation between the burden in AT8-positive dystrophic neurites and the extent of the presynaptic proteins reduction.

Supplementary figures

Figure S1

Supplementary Figure 1

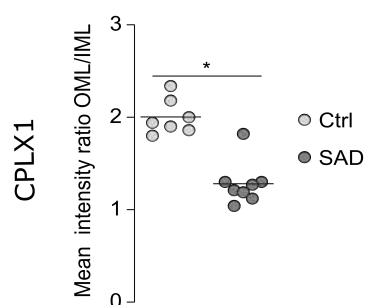
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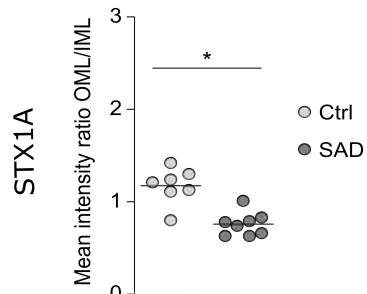
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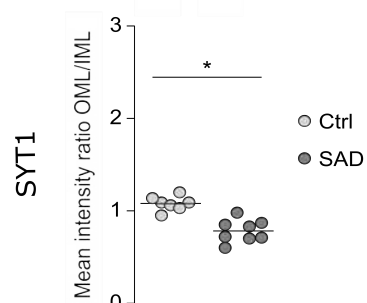
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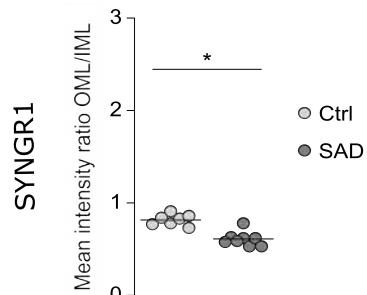
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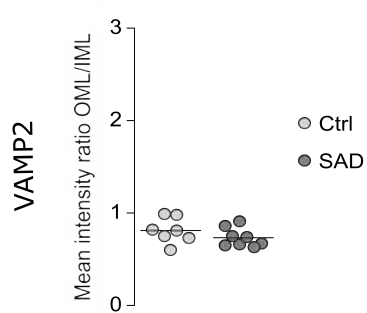
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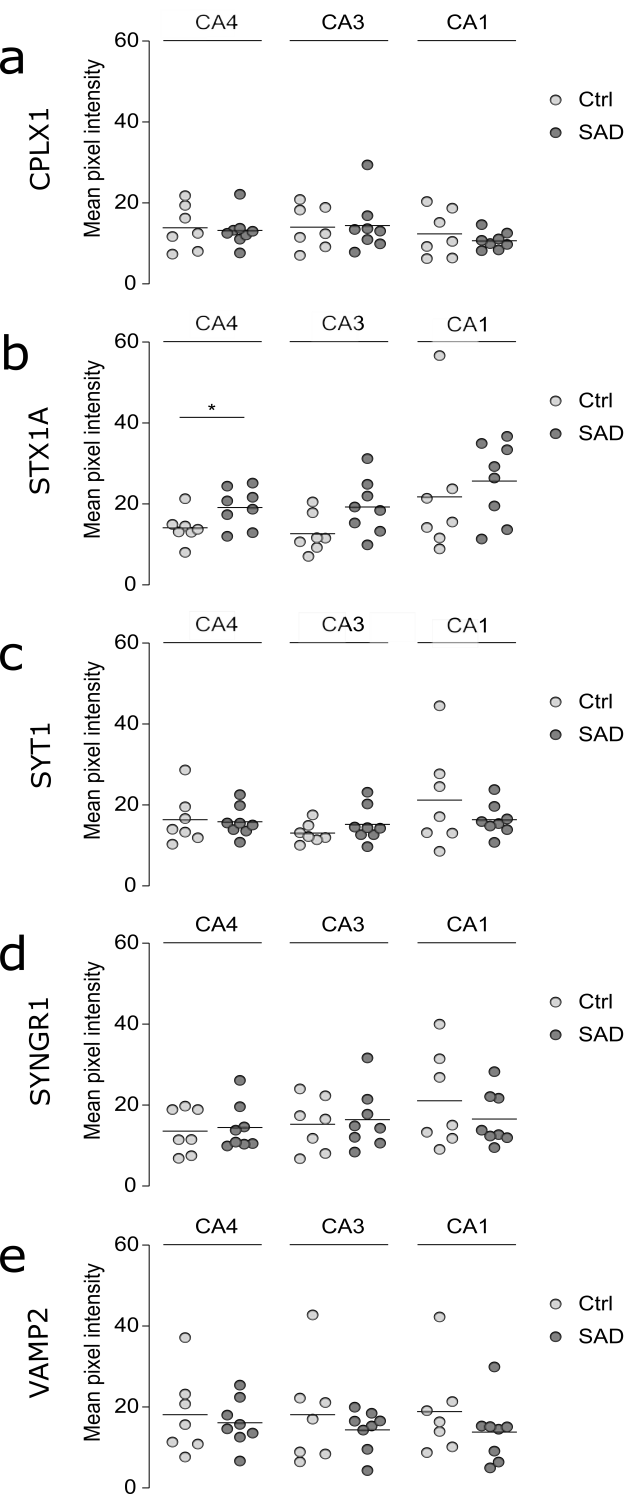


f



Supplementary Figure 1. Impaired OML/IML intensity ratio for presynaptic proteins (a) Controls of fluorescent immuno-labelling in human hippocampal sections. In the upper panels, the sections are stained for DAPI, syntaxin1a and Syt1. In the lower panels, only secondary antibodies were incubated in addition to DAPI. The fluorescent immuno-labellings are specific to the primary antibodies. (b-f) Scatter plots of the mean pixel intensity OML/IML ratio of the fluorescent labellings respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The ratios were significantly decreased in AD cases by 36 % for CPLX1 (t-test, $p < 0.0001$), 35 % for STX1A ($p = 0.0003$), 28 % for SYT1 ($p = 0.0001$) and 25 % for SYNGR1 ($p < 0.0001$). The ratio for VAMP2 showed was unaltered.

Supplementary Figure 2



Supplementary Figure 2. Preserved level of presynaptic proteins in various neuronal layers of the hippocampus (a-e) Scatter plots of the mean pixel intensity of the fluorescent labellings in CA3, CA1 and CA4 respectively for CPLX1, STX1A, SYT1, SYNGR1, VAMP2. The fluorescence pixel intensities were not altered in these regions except for STX1A which was increase in AD CA4.

4.3. Perspectives

The research carried in this chapter focused in studying the decrease of synaptic proteins in different anatomical regions of the human hippocampus. We showed that the OML of the DG is a distinctive affected region, where presynaptic markers are decreased. Previously, the use of brain homogenates and stereological semi-quantifications with DAB stainings were some of the most commonly used methods to study the synaptic decrease. However, the technical advances in immunofluorescence and imaging methods constituted a remarkable step forward in the qualitative and quantitative data obtained from protein stainings at different anatomical areas. The combination of methods that allow the 3D imaging and reconstruction of neuronal circuits in the human brain would be ideal to study neurodegenerative diseases like AD. Furthermore, many of the hypotheses discussed in this chapter (see article) such as the cell loss in layer II of the EC could be tested using clarifying techniques such as clarity or iDISCO, which would enable the 3D visualization of the perforant path input into the OML of the DG. Thus, this approach would allow us to study at an unprecedented detail the most affected cell compartments and circuits in the AD hippocampus and other brain areas. Furthermore, the growing interest in synaptic failure has driven the development of high resolution techniques like array brain tomography that provides ultrastructural information for the study of clinico-pathological correlations at the synapse level. Thus, this technique could be also used in our studies to characterize the synaptic morphology of pre and postsynaptic compartments at the OML and assess the correlation with P-tau and A β plaques.

CONCLUSIONS

From animal models to human patients: A strategy to study AD mechanisms

Mouse models have been one of the most useful approaches to understand the physiological and pathophysiological mechanisms involved in AD. Early in the days, the development of KO and transgenic mouse models expressing FAD or Tau mutations, constituted a milestone that expanded the knowledge of every discipline in AD research. Nevertheless, the failure of clinical trials in humans suggests that there has been a premature translation from the positive results obtained in AD mouse models into humans. Consequently, despite its importance, animal models only recapitulate certain characteristics of the disease, overlooking others that might explain the discrepancies between results obtained in mice and humans. Importantly, the progress and the knowledge gained in AD will depend on understanding the neuropathological limitations of each model and comparing new findings with experimental data acquired in human AD patients when possible⁴²⁵. Therefore, the design of studies with a significant cohort of patients and proper methodological controls are critical to understand and translate important discoveries directly into the clinical field of AD research.

The work performed in this thesis is the result of merging fundamental and histopathological studies in mouse and humans with the final aim of understanding some of the mechanisms involved in AD. In each of the projects I combined the use of cutting edge techniques through different models in order to characterize the function of proteins that have a synaptic role and are directly involved in AD. In addition, while writing this thesis I learnt a lot on the history of AD. I have gained a better perspective of how and why we got here but most importantly where are we going now.

In the last decades of research, AD has been defined as a multifactorial disease caused by the influence of genetic and environmental risk factors, which trigger the start of the disease. However, the research related to the amyloid hypothesis focused a lot of attention, suggesting A β plaques as the main cause of the disease that could be used as a therapeutic target. Despite the significant efforts made to turn the hypotheses into a theory, the failure of clinical trials targeting A β has been an unexpected reverse, which exposed the limitations of targeting a multifactorial disease by one target. As a consequence, several pharmaceutical companies drop out of the race to develop AD therapies to the point of even scaling down their neuroscience teams.

One positive aspect among these negative outcomes is that alternative research lines have started to emerge giving evidence of the complexity of the disease. Neuroinflammation, Tau

pathology, microorganism infection, or presynaptic degeneration are some of the fields that have started to explain other risk factors rather than A β pathology. In addition, the physiological functions of proteins directly involved in the disease will have to be investigated with modern research tools, which can provide a new view of how these proteins trigger the pathological mechanisms of the disease.

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ANNEXES



Non-specific Detection of a Major Western Blotting Band in Human Brain Homogenates by a Multitude of Amyloid Precursor Protein Antibodies

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The use of human post-mortem brain material is of great value when investigating which pathological mechanisms occur in human brain, and to avoid translational problems which have for example been evident when translating animal research into Alzheimer disease (AD) clinical trials. The amyloid β ($A\beta$)-peptide, its amyloid precursor protein (APP) and the intermediate APP-c-terminal fragments (APP-CTFs) are all important players in AD pathogenesis. In order to elucidate which APP CTF that are the most common in brain tissue of different species and developmental stages, and whether there are any differences in these fragments between AD and control brain, we investigated the occurrence of these fragments using different APP c-terminal antibodies. We noticed that whereas the conventional APP-CTF α and CTF β fragments were most prominent in rat and mouse brain tissue, the major western blotting band detected in human, macaque and guinea pig was of approximately 20 kDa in size, possibly corresponding to the newly discovered APP-CTF η . However, this band was also intensely stained with a total protein stain, as well as by several other antibodies. The staining intensity of the 20 kDa band by the APP antibodies varied considerably between samples and correlated with the staining intensity of this band by the total protein stain. This could potentially be due to non-specific binding of the antibodies to another protein of this size. In-gel digestion and mass spectrometry confirmed that small amounts of APP were present in this band, but many other proteins were identified as well. The major hit of the mass spectrometry analysis was myelin basic protein (MBP) and a myelin removal protocol removed proportionally more of the 20 kDa APP band than the full-length APP and APP-CTF α/β bands.

Abbreviations: $A\beta$, amyloid β -peptide; AD, Alzheimer disease; ADAM10, a disintegrin and metalloproteinase 10; AICD, amyloid precursor protein intracellular domain; APP, amyloid precursor protein; BA9, Brodmann area 9; BACE1, β -site amyloid precursor protein cleaving enzyme 1; CSF, cerebrospinal fluid; CTF, C-terminal fragment; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; FL-APP, full length APP; GC, Vitamin D binding protein; H, homogenate; HRP, horseradish peroxidase; KEAP1, Kelch-like ECH-associated protein 1; PMD, post-mortem delay; PVDF, polyvinylidene difluoride; RIPA, radio immunoprecipitation assay; sAPP, soluble amyloid precursor protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

However, the signal could not be immunodepleted with an MBP antibody. In summary, we report on a potentially non-specific western blotting band of approximately 20 kDa and call for precaution when analyzing proteins of this size in human brain tissue.

Keywords: amyloid precursor protein, human brain, western blotting, cross-reactivity, η -secretase

INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia and causes extensive suffering for millions of patients and their relatives. Unfortunately, the only medication available gives just subtle symptomatic relief, and a large number of clinical trials of disease-modifying drugs have failed in the last decade. This is partially due to problems with translation, i.e., compounds that work well in mouse models fail in clinical trials. Thus, it is of utmost importance to study the alterations in human AD brain.

The synaptotoxic amyloid β -peptide (A β) plays a critical role in the pathophysiology of AD (Selkoe, 2011; Prince et al., 2013). A β is generated by the cleavage of the 695–770 amino acid long amyloid precursor protein (APP) by first β -secretase and then the γ -secretase complex. After the first cleavage by β -site APP cleaving enzyme 1 (BACE1), the main β -secretase of neurons, the 99 or 89 amino acid long c-terminal fragment (CTF β) remains in the membrane while the soluble APP β (sAPP β) is released. The CTF β is further cleaved by the protein complex γ -secretase, generating A β and the APP intracellular domain (AICD; Selkoe, 2011). In a separate, non-amyloidogenic pathway, APP is cleaved by the α -secretase a disintegrin and metalloproteinase 10 (ADAM10) instead of BACE1. This cleavage produces the 83 amino acids long CTF α which can also be cleaved by the γ -secretase complex, thus precluding A β production since the α -cleavage site is situated within the A β sequence (Postina et al., 2004).

APP can also be cleaved by a number of other proteases, such as caspases (Galvan et al., 2002), asparagine endopeptidase (δ -secretase; Zhang et al., 2015), the recently identified η -secretase (Wang et al., 2015; Willem et al., 2015), or other unknown proteases (Nikolaev et al., 2009), to produce toxic fragments and/or influence further processing. The situation is further complicated by the fact that also CTF β and the fragment derived from sequential cleavage by η - and α -secretase (A η - α) have been shown to be neurotoxic and may therefore be involved in the pathology of AD (Oster-Granite et al., 1996; McPhie et al., 1997; Bittner et al., 2009; Jiang et al., 2010; Lauritzen et al., 2012; Willem et al., 2015). However, which of these fragments that are most abundant in human brain, and whether the processing is the same in different species, or at different developmental stages, has to our knowledge not been carefully investigated.

Here, we studied the occurrence of different APP-derived fragments in human as well as in other species including macaque, guinea pig, rat and mouse brain using western blotting. Using several APP-antibodies we detected a major band of approximately 20 kDa in adult human, macaque and guinea pig brain. However, more careful investigation using total protein stain, mass spectrometry and immunoprecipitation indicated

that this band was unspecific and was also recognized by several other antibodies.

MATERIALS AND METHODS

Human Post-mortem Material

The use of human brain material in this study was conformed to the Declaration of Helsinki and approved by the regional ethical review board of Stockholm (2015/1803-31/2 and 2007/1477-3). Brain tissues from frontal cortex Brodmann area 9 (BA9) of 10 sporadic AD and 10 control cases were obtained from Brains for Dementia Research, London, UK, while the human brain tissue (mixed cortex) used for the experiments with the different antibodies, immunoprecipitation and different species was obtained from the Brain Bank at Karolinska Institutet, Stockholm, Sweden. All patients were clinically and pathologically diagnosed and the characteristics of the patients are described in **Table 1**. Cortical brain tissue from human fetuses, post-conception age 7–11 weeks, was obtained from clinical routine abortions through the Developmental Tissue Bank at Karolinska Institutet. All participants or their consultees (for participants that lacked capacity) gave informed consent to the donations and all procedures were approved by ethical review boards.

Animals

Male Wistar rats (Charles River) were killed by carbon dioxide treatment while female C57BL/6 mice (bred at Karolinska Institutet) were killed by cervical dislocation. The animals used in this study were handled according to the Karolinska Institutet guidelines, Swedish national guidelines and current European Law (Directive 2010/63/EU). The use of rat brains was approved by the animal research ethical committee of southern Stockholm (S21-14) while the use of mouse brain was approved by Linköping ethical committee (ID156). Brain lysates from guinea pig and macaque was purchased from Novus Biologicals who ensure that the animals have been handled according to the United States Department of Agriculture (USDA) animal welfare act as well as the National Institutes of Health (NIH), Office of Laboratory Animal Welfare (OLAW) and the Public Health Service (PHS) policy on humane care and use of laboratory animals. No experiments were performed on live animals.

TABLE 1 | Characteristics of cases for post-mortem human brain samples Brodmann area 9 (BA9) obtained from the Brains for Dementia Research.

	Age (years)	Gender	PMD (h)
Sporadic AD (<i>n</i> = 10)	88.7 $\pm$ 6.6	70% F	34.0 $\pm$ 23.3
Control (<i>n</i> = 10)	82.3 $\pm$ 7.4	70% F	35.7 $\pm$ 20.2

F, female; PMD, post-mortem delay.

Brain Homogenization

Brain homogenates derived from different sources were homogenized in slightly different buffers and with slightly different protocols. For all samples except the ones mentioned specifically below the following method was used: homogenization of the cortical tissue from human, rat and mouse was carried out in four volumes of cold homogenization buffer [20 mM HEPES, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0] with Complete Protease Inhibitor Cocktail (Roche) by eight strokes at 800 rpm using a mechanical glass-teflon homogenizer. Brain tissues from human and mouse embryo were then sonicated. AD and control samples from the Brains for Dementia Research were homogenized in 50 mM Tris-HCl, 5 mM EGTA, 10 mM EDTA and Sigma protease inhibitor cocktail. The guinea pig and macaque homogenates were delivered as ready lysates from Novus Biologicals. Protein determination was done by PierceTM BCA Protein Assay Kit (Thermo Fisher). The brain homogenates were stored at -80°C .

Antibodies

All primary antibodies used in the study are described in Table 2 and the epitopes of the APP antibodies are delineated in Figure 1A. The secondary antibodies IRDye 800CW donkey anti-mouse IgG and IRDye 680RD donkey anti-rabbit IgG were purchased from LI-COR[®]. For the in-gel digestion, we instead used a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (GE-Healthcare).

SDS-PAGE and Western Blotting

Equal amounts of protein (30–40 μg depending on experiment) from each sample were denatured in either 4 $\times$ of protein sample loading buffer (LI-COR) containing 2-mercaptoethanol, or 4 $\times$ NuPAGE LDS sample buffer containing dithiothreitol (DTT), or 2 $\times$ Laemmli buffer and boiled at 95°C for 5 min. Samples and 7 μl of the Chameleon duo pre-stained protein ladder (LI-COR) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–12% polyacrylamide bis-tris gels (Invitrogen) or 16% polyacrylamide tricine gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare). As a reference, a recombinant C99-FLAG peptide (a gift from Dainippon Sumitomo Pharma) was loaded. After transfer, nitrocellulose membranes were blocked in Odyssey blocking buffer (TBS) for up to 1 h at room temperature, followed by incubation with primary

antibodies at 4°C overnight. Membranes were washed with TBS-T and then incubated with fluorescently labeled IRDye secondary antibodies (LI-COR) for 1 h at room temperature. After TBS-T washes, membranes were washed in TBS for one last time, and digital fluorescent visualization of signals was detected at 700 and/or 800 nm channels using the Odyssey[®] CLx Imaging System (LI-COR). All primary and secondary antibodies were diluted in Odyssey blocking buffer (TBS). In some experiments, membranes were stained with a total protein stain [REVERTTM Total Protein Stain (LI-COR)] especially for normalization of the signal (Figures 3A,B). In order not to interfere with the signal that could arise from antibodies, this step was done right after transfer. Once membranes were incubated with total protein stain and the signal was immediately detected at the 700 nm channel. After removal of the stain using reversal buffer, the membrane was incubated with Odyssey blocking buffer (TBS) and incubation with primary and secondary antibodies was done as mentioned above. Quantitation of protein (20 kDa band and total protein) was done using Image Studio Lite v5.2 (LI-COR). In order to assure linearity of the signals three different concentrations of a standard sample (A1) was loaded on all gels and a standard curve was created which was used for quantification. Statistical analysis of the comparison of the levels of the 20 kDa band in AD and control was performed using Student's *t*-test.

For the in-gel digestion experiment, equal amounts of samples were loaded onto the 4%–12% polyacrylamide bis-tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Following primary antibody incubation, membranes were incubated with secondary antibodies coupled to HRP (GE-Healthcare). SuperSignal West Pico enhanced chemiluminescent reagent (Pierce) was then used to visualize the signals which were exposed to film (GE-Healthcare).

In-Gel Digestion and Mass Spectrometry

Protein bands were excised manually from a bis-tris 4%–12% gel, using western blotting as a location reference, and in-gel digested using a MassPREP robotic protein-handling system (Waters). Gel pieces were destained twice with 100 μl 50 mM ammonium bicarbonate (Ambic) containing 50% acetonitrile at 40°C for 10 min. Proteins were reduced with 10 mM DTT in 100 mM Ambic for 30 min at 40°C and alkylated with 55 mM iodoacetamide in 100 mM Ambic for 20 min at 40°C followed by

TABLE 2 | List of primary antibodies.

Antibody name	Company and product number	Dilution (WB)	Final concentration ($\mu\text{g/ml}$)
Anti-amyloid beta precursor protein (Y188)	Abcam (ab32136)	1:5,000	0.08
Anti-APP c-terminal fragment (C1/6.1)	BioLegend (802801)	1:5,000	0.2
Anti-amyloid beta (6E10)	BioLegend (803001)	1:1,000	1
Anti-amyloid beta precursor protein (A8717)	Sigma (A8717)	1:2,000	5
Anti-beta amyloid (7N22)	Invitrogen (AHB0272)	1:5,000	0.1
Anti-amyloid beta precursor protein (9478)	Gift from Dr. Willem	1:500	4
Anti-myelin basic protein (MBP)	Abcam (ab216668)	1:500	0.1
Anti-kelch-like ECH-associated protein 1 (KEAP1)	Protein Tech (10503-2-AP)	1:5,000	0.07
Anti-vitamin D-binding protein (GC)	AbFrontier (LF-MA0147)	1:20,000	0.025

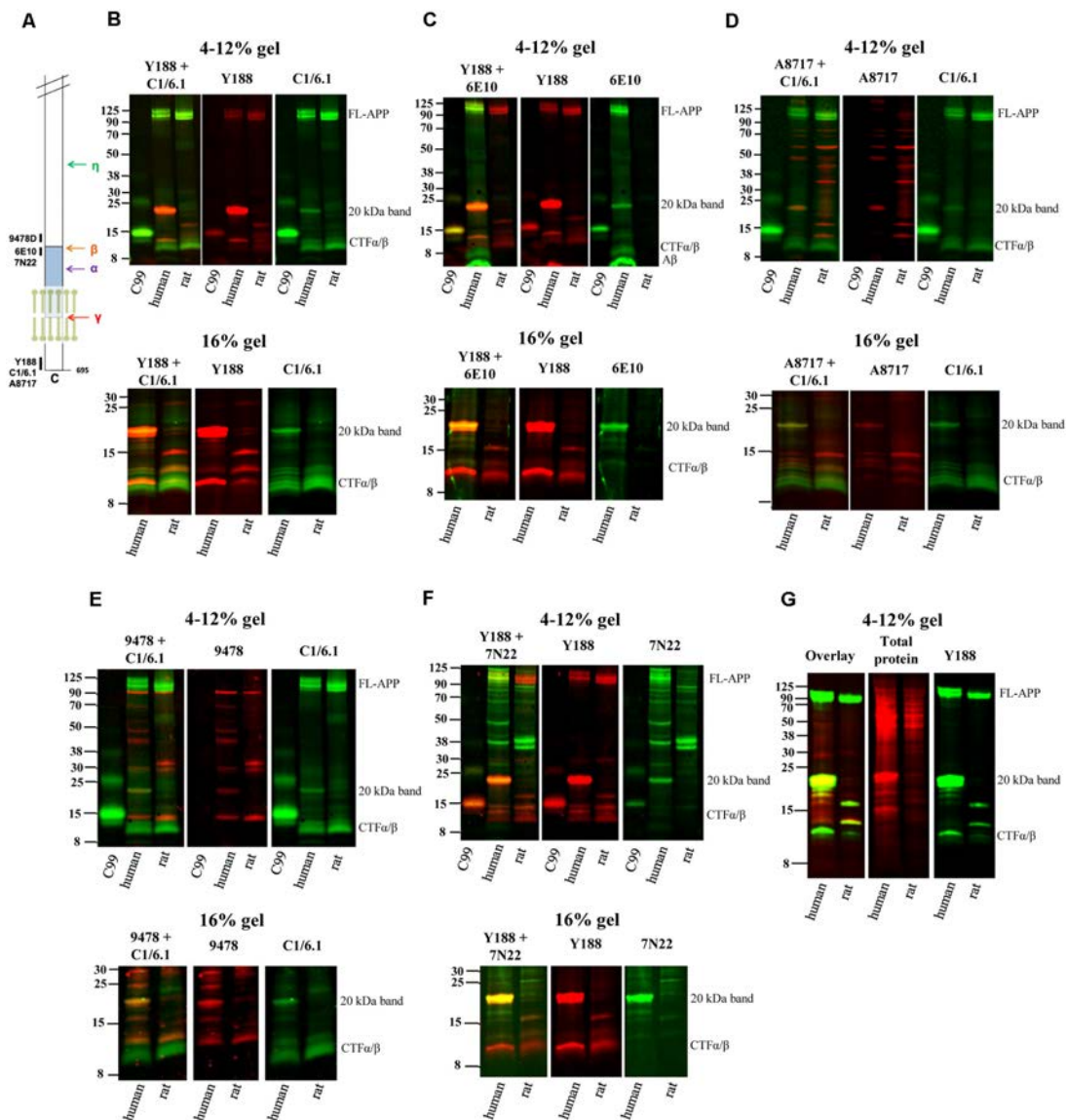


FIGURE 1 | Elucidating the pattern of bands detected by different amyloid precursor protein (APP) antibodies in rat and human brain. **(A)** The APP protein, indicating the epitopes of the antibodies used in the study. **(B–F)** Equal amounts of homogenates of human Alzheimer disease (AD) and adult rat brain were loaded on 4%–12% (upper panels) or 16% (lower panels) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to western blotting using the Odyssey digital fluorescent system with detection by primary antibodies **(B)** Y188 and C1/6.1, **(C)** Y188 and 6E10, **(D)** A8717 and C1/6.1, **(E)** 9478 and C1/6.1 or **(F)** Y188 and 7N22. A recombinant C99-FLAG peptide was loaded on the 4%–12% gels to indicate the approximate position of c-terminal fragments (CTF)-β (minus the 1 kDa FLAG-tag). The figures are representative figures and four additional rat brain samples and five additional human brain samples showed a similar pattern using the Y188 and C1/6.1 antibodies (see also **Figure 5**). **(G)** Equal amounts of homogenates of human AD and adult rat brain were loaded on a 4%–12% SDS-PAGE gel and subjected to western blotting using the Odyssey digital fluorescent system with detection by primary antibody Y188 together with a total protein stain. A major band was detected around 20 kDa by all antibodies as well as by the total protein stain.

in-gel digestion with 0.3 μg Trypsin (Sequence grade, Promega) in 50 mM Ambic for 5 h at 40°C. The tryptic peptides were extracted with 1% formic acid in 2% acetonitrile, followed by 50% acetonitrile twice. The liquid was evaporated to dryness and the peptides were separated on an EASY-spray column connected to an EASY-nLC 1000 system (Thermo Scientific). The peptides were eluted in a 60 min gradient (from 5% to 26% of buffer B (2% acetonitrile, 0.1% formic acid) in 55 min and up to 95% of

buffer B in 5 min) at a flow rate of 300 nL/min and analyzed on a Fusion Orbitrap mass spectrometer (Thermo Scientific). The spectra were analyzed using the Mascot search engine v.2.4 (Matrix Science Limited).

Immunoprecipitation

For immunoprecipitation with the Y188 and A8717 antibodies, antibodies or a rabbit IgG (used as a negative control) were

bound to PureProteome™ Protein A/G mix magnetic beads (Millipore). Brain homogenates from AD cortex were solubilized in 1× RIPA lysis buffer without NP-40 (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris/HCl, pH 8.0) containing Complete Protease Inhibitor Cocktail (Roche). The sample was precleared with magnetic beads for 2 h at 4°C in order to remove any potential non-specific binding. Twenty microliter of sample was collected as input for western blotting, whereas the remaining sample was incubated with the antibody coupled beads overnight at 4°C. After immunoprecipitation, 20 µl of sample was collected to detect unbound proteins. Precipitated proteins were washed with PBS and eluted in 20 µl of 1× NuPAGE LDS sample buffer (Invitrogen) and boiled at 95°C for 5 min. The eluates were transferred to new microcentrifuge tubes and subjected to western blotting as described above.

For myelin basic protein (MBP) immunodepletion, brain homogenate from AD cortex was solubilized in 1× RIPA lysis buffer as mentioned above, and the sample was precleared with magnetic beads for 30 min at room temperature in order to remove any potential non-specific binding. Twenty microliter of sample was collected as inputs before and after pre-absorption with beads, respectively. The remaining sample was incubated with the MBP antibody for 1 h at room temperature to create antibody-antigen complex, which was followed by incubation with the magnetic beads for 30 min at room temperature. After immunoprecipitation, 20 µl of the unbound sample was collected and the sample bound to the beads was washed three times in 1× RIPA. Precipitated proteins were eluted in 40 µl of 1× Protein Sample Loading buffer (LI-COR) and boiled at 95°C for 5 min. The eluates were transferred to new microcentrifuge tubes and subjected to western blotting as described above.

Myelin Removal

Myelin Removal Beads II (Miltenyi Biotec) was used according to the manufacturer's protocol with some modifications. The protocol builds on binding of myelin to a specific but confidential antibody coupled to the beads. Sixty microliter beads were added to 500 µl of AD brain homogenate in 0.5% BSA in PBS (1.5 µg/µl) and incubated for 15 min, rotating at room temperature. The sample was then further diluted 5.5 times with PBS/BSA and centrifuged at 100,000× *g*, 30 min and resuspended in 1 ml of PBS/BSA in order to first wash and then concentrate the membranes. The sample was then passed through a magnetic LS column and the flow-through (myelin-depleted sample) was collected.

RESULTS

A Multitude of APP Antibodies Labels a 20 kDa Predominant Band in Human Brain

Using different APP antibodies, we analyzed APP fragments in human AD and rat brain homogenates after separation on BisTris 4%–12% polyacrylamide gels or tricine 16% polyacrylamide gels. The use of the LI-COR detection system, which allows simultaneous detection with two different antibodies, enabled us to visualize whether the same band was stained with the different antibodies. Surprisingly, we found

that in human brain, a predominant band of approximately 20 kDa was detected by all antibodies tested, including Y188 (Figures 1B,C,F), C1/6.1 (Figures 1B,D,E), 6E10 (Figure 1C), A8717 (Figure 1D), 9478 (Figure 1E) and 7N22 (Figure 1F). From now on, we will refer to this band as the 20 kDa band although the molecular weight varies slightly depending on gel systems and molecular weight markers, and the exact size has not been determined. In contrast to human brain, the 20 kDa band was not detected in rat brain homogenates by any of the antibodies. The 20 kDa band corresponds to the expected weight of the proposed APP-CTF η , generated by η -secretase cleavage at N504 in the APP695 sequence (Willem et al., 2015). When using the Y188 antibody and 16% gels, which effectively separate proteins of lower molecular weight, we observed that the 20 kDa band is actually a double band (Figures 1B,C,F) in agreement with the double CTF- η band detected by Wang et al. (2015).

ADAM10 cleavage of APP gives rise to the 83 amino acids long CTF α , while CTF β is either 99 or 89 amino acids long depending on where APP is cleaved by BACE1. In addition, post-translational modifications of APP, such as phosphorylation and glycosylation may give rise to even more CTF bands using western blotting. Thus, several bands between 8 and 14 kDa were detected, most clearly demonstrated with the C1/6.1 antibody. Using this antibody, the staining intensity for the CTF α and - β bands were similar between human and rat homogenates (Figures 1B,D,E). The 9478 antibody is directed to the N-terminal part of the CTF η sequence and is not expected to detect CTF α or - β . Instead, the band of approximately 12 kDa detected by 9478 could be the proposed A α - η peptide (Figure 1E). A major human-specific band of around 11 kDa was detected with the Y188 antibody. On 16% gels, this band migrates as one of the lower conventional CTF bands (Figures 1B,C,F, lower panels). Since this band does not overlap with bands detected by 6E10, we can conclude that it does not include the first eight amino acids in the A β sequence and is thus not identical to C99. Y188 also detected a major 15 kDa fragment in rat brain, possibly reflecting CTF δ (Zhang et al., 2015).

In addition, the expected immature (non-glycosylated) and mature (glycosylated) forms of full-length APP [FL-APP, approximately 100 kDa (Weidemann et al., 1989)] were clearly detected with the Y188, C1/6.1 and 6E10 antibodies (Figures 1B–F), whereas the A8717 did not detect these bands (Figure 1D), 9478 detected a band of slightly smaller molecular weight (Figure 1E) and 7N22 only detected the lower FL-APP band in human brain (Figure 1F). In general, the A8717, 9478 and 7N22 antibodies detected a multitude of bands of unexpected molecular weight, possibly due to non-specific or cross-reactive binding. The staining intensity of FL-APP and APP-CTF α and - β were similar between human and rat when detected with the Y188 or C1/6.1 antibodies. Since 6E10 is a human-specific antibody that recognizes the first amino acids in the APP-CTF β sequence, no bands were detected in rat brain homogenate using this antibody and the lower CTF α / β bands, corresponding to CTF α were not detected (Figure 1D).

Peculiarly, the ratio between the different western blotting bands varied between the different antibodies. Whereas the 20 kDa band gave rise to the strongest signal of all APP-derived

fragments in human brain using the Y188 or 6E10 antibodies (Figures 1B,C,F), the signal from this band was about as intense as the signal from the other CTFs when using the C1/6.1 antibody (Figures 1B,D,E). However, the 20 kDa band was always stronger in human than in rat brain samples. We also noticed that a band stained intensively with a total protein stain, overlapped with the 20 kDa band (Figure 1G).

In summary, using six different APP antibodies (Y188, C1/6.1, 6E10, A8717, 9478 and 7N22), we detected a 20 kDa band in human AD brain homogenate which was hardly detectable in rat brain homogenate. In the subsequent experiments, we chose to focus on the combination of Y188 and C1/6.1 antibodies. It should also be noted that although all experiments in Figure 1 are performed using the same human brain homogenate, the 20 kDa band was present in almost all human brain samples tested (see Figure 5) while being non-detectable or present in very low levels in five additional rat brain samples (data not shown). The presence of the 20 kDa band was not dependent on sample treatment, since it was present independently on which loading buffer that was used (data not shown) or if the samples were treated with RIPA buffer.

The 20 kDa Band Is Also Present in Guinea Pig and Macaque Brain but Not in Mouse Brain or Human Embryonic Brain

Since the staining pattern of the different bands detected by APP antibodies was so different in human and rat brain (Figure 1), we decided to examine these bands in brain homogenates from a few more animal species. We found that the 20 kDa band was present also in brain homogenates from guinea pig, and to less extent in macaque, but not in mouse brain homogenates (Figure 2A). However, a faint band slightly above the prominent 20 kDa band was detected by C1/6.1 in all species. Again, the intensity ratios between 20 kDa band and the CTF α and - β bands were different depending on whether the Y188 or the C1/6.1 antibody was used for detection (Figure 2A).

To investigate whether the presence of the 20 kDa band is developmentally regulated, we next compared the expression pattern of APP-CTFs in brain homogenates prepared from embryo and adult (Figure 2B). Interestingly, the pattern of CTFs in brain homogenates from human fetus was clearly different from that in human adult brain, with undetectable levels of the 20 kDa band (Figure 2B). This pattern was further confirmed in three additional human fetus brain homogenates (data not shown). The pattern of CTFs in brain homogenates from mouse embryo was similar to that from adult mouse (Figure 2B).

In summary, the 20 kDa was present in adult brain from human, macaque and guinea pig, but was not present at detectable levels in adult brain from rat or mouse, nor in human fetal or mouse embryonic brain.

Large Variation in Staining Intensity of the 20 kDa Band in Human Brain Samples

In order to determine whether there were any differences in the levels of the 20 kDa band between AD and control brain, we loaded equal amounts of homogenate from BA9 of the frontal

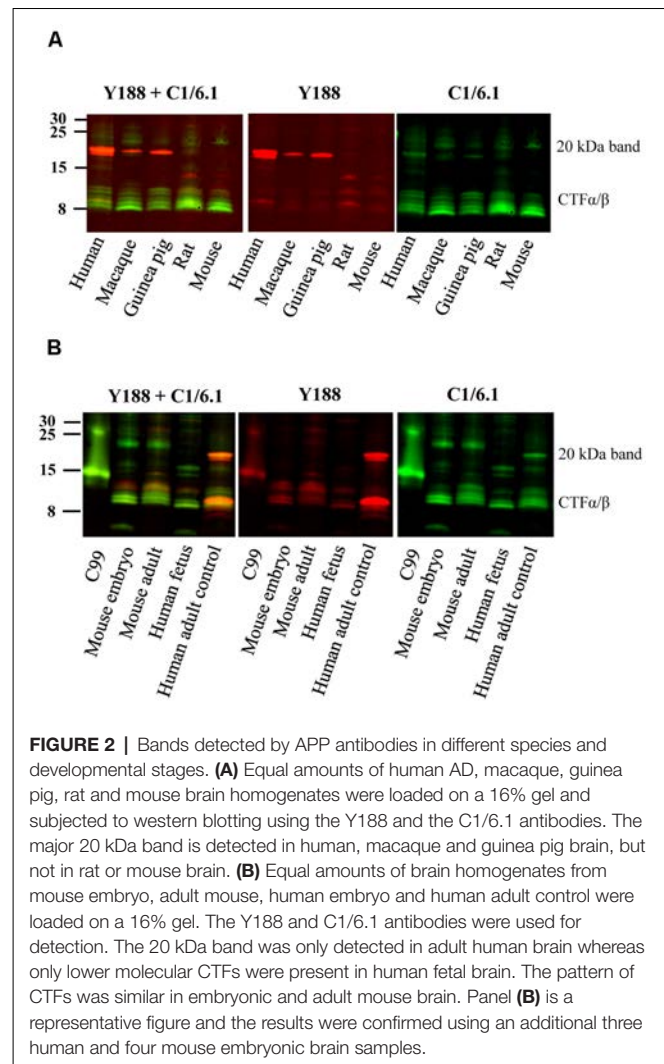


FIGURE 2 | Bands detected by APP antibodies in different species and developmental stages. **(A)** Equal amounts of human AD, macaque, guinea pig, rat and mouse brain homogenates were loaded on a 16% gel and subjected to western blotting using the Y188 and the C1/6.1 antibodies. The major 20 kDa band is detected in human, macaque and guinea pig brain, but not in rat or mouse brain. **(B)** Equal amounts of brain homogenates from mouse embryo, adult mouse, human embryo and human adult control were loaded on a 16% gel. The Y188 and C1/6.1 antibodies were used for detection. The 20 kDa band was only detected in adult human brain whereas only lower molecular CTFs were present in human fetal brain. The pattern of CTFs was similar in embryonic and adult mouse brain. Panel **(B)** is a representative figure and the results were confirmed using an additional three human and four mouse embryonic brain samples.

cortex from 10 sporadic AD and 10 control subjects on 16% SDS-PAGE gels. Using both the Y188 and the C1/6.1 antibody for detection, we found that the 20 kDa was present in the majority of the brain homogenates from AD and control subjects (Figure 3, representative figure). The levels of the 20 kDa band varied considerably between individuals, and no significant differences could be detected between AD and control subjects. In addition, no obvious correlations could be found between levels of the 20 kDa band and parameters such as gender, age, post-mortem delay or storage time (data not shown). In order to normalize the levels to the total protein content, we also performed a total protein stain of the membrane. As in our initial experiments shown in Figure 1G, we noticed that a band that was intensively stained with the total protein stain overlapped in size with the 20 kDa band detected by the APP antibodies. Furthermore, the staining intensity of this band by the total protein stain correlated well with the intensity of the 20 kDa APP band.

Thus, the 20 kDa band was present in all human brain homogenates studied but that the levels varied considerably between samples and no significant differences in the levels of this CTF between AD and control cases. In addition, the

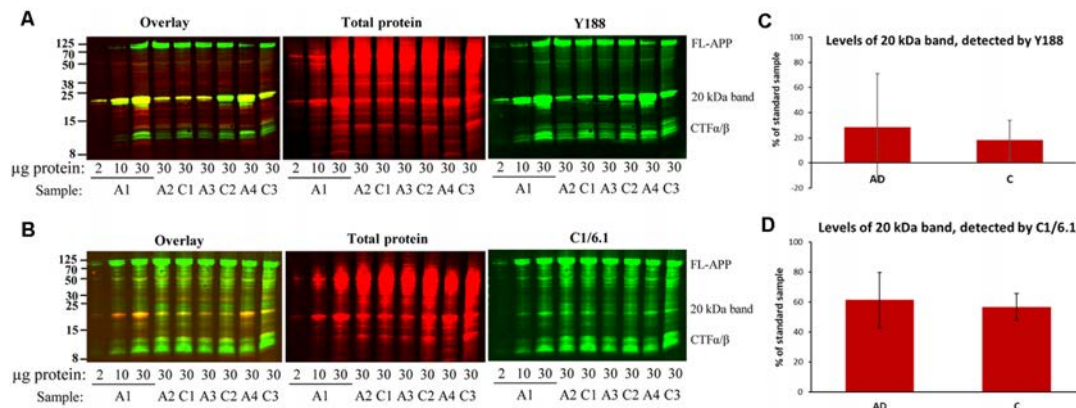


FIGURE 3 | Bands detected by APP antibodies in human AD and control brain homogenates. Equal amounts of homogenates from AD and control brain were loaded on 16% gels. In order to assure linearity of the signals, three different concentrations of a standard sample (A1) was loaded on all gels and a standard curve was created which was used for quantification. The gels were subjected to SDS-PAGE and western blotting using (A) Y188 antibody together with a total protein stain or (B) C1/6.1 antibody together with a total protein stain. Representative gels are shown with age- and gender-matched pairs of AD and control cases loaded beside each other. (C,D) Densitometric analysis of the 20 kDa band using the Y188 (C) or the C1/6.1 antibody (D). All values were normalized to the total protein stain and expressed as % of the standard sample. The major 20 kDa APP band varies considerably between cases and correlates with the size and intensity of a major total protein stained band. No difference in the levels of the 20 kDa band could be detected between AD and control brain. A, AD; C, control.

band overlapped in both size and intensity with a major total protein band.

Mass Spectrometry Confirms That the 20 kDa Band Contain APP but That Many Other Proteins Are Present in This Band as Well

The overlap of the 20 kDa APP band with a major total protein band raised our concern whether the antibodies studied cross-react or bind non-specifically to constituents of this band. In order to further elucidate the identity of this band, we therefore loaded human brain homogenate on a gel and cut out the bands corresponding to the size of full-length APP (as a positive control) and the 20 kDa band (as indicated by detection by western blotting using the Y188 antibody of parallel lanes of the same gel). The gel pieces were destained and alkylated, and in-gel digestion was performed. The digested band corresponding to full-length APP gave rise to a number of spectra of high quality whereof one corresponded to the LVFFAEDVGSNK peptide ($A\beta$ 17–28, **Figure 4**, upper panel). This peptide was also identified in the digested 20 kDa band with a similar spectrum and retention time (**Figure 4**, lower panel), showing that this band indeed is partially derived from APP. However, the score of the APP-derived peptide was low and the digested gel piece also contained a large number of other proteins (**Supplementary File S1**). The protein with the highest score was MBP for which several of the major isoforms have the correct size of the digested band (17–21.5 kDa¹). Other major hits included glutathione S-transferase P as well as different isoforms of tubulin beta and alpha. The peptides used for identifying the different tubulin isoforms were to a large degree overlapping.

¹www.uniprot.org/uniprot/P02686

The 20 kDa Band Cannot be Efficiently Immunoprecipitated From Human Brain Using APP Antibodies and Is Depleted by Myelin Removal Beads but Not by Immunodepletion With an MBP Antibody

Next, we tested whether the 20 kDa band could be immunoprecipitated using the Y188 and the A8717 antibodies. To our surprise, immunoprecipitation of human AD brain

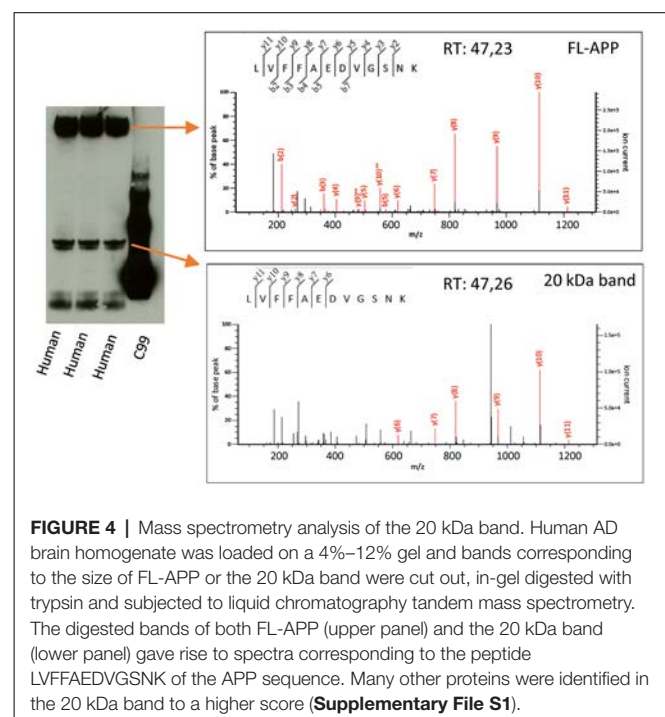


FIGURE 4 | Mass spectrometry analysis of the 20 kDa band. Human AD brain homogenate was loaded on a 4%–12% gel and bands corresponding to the size of FL-APP or the 20 kDa band were cut out, in-gel digested with trypsin and subjected to liquid chromatography tandem mass spectrometry. The digested bands of both FL-APP (upper panel) and the 20 kDa band (lower panel) gave rise to spectra corresponding to the peptide LVFFAEDVGSNK of the APP sequence. Many other proteins were identified in the 20 kDa band to a higher score (**Supplementary File S1**).

homogenates with these antibodies immunoprecipitated only low amounts of the 20 kDa band and not significantly more than the IgG control (**Figure 5A**). In contrast, FL-APP, as well as the lower molecular weight APP-CTFs detected by the C1/6.1 antibody were very efficiently immunoprecipitated using either of the antibodies (**Figure 5A**). The large variation of the intensity of the 20 kDa APP-reactive band in human samples, and the correlation with the intensity of the same band visualized with a general protein stain, could potentially be explained by different amounts of white matter in these samples. In line with this, the levels of the 20 kDa APP band were undetectable in early human fetal stages (**Figure 2B**), where myelin is absent (Dubois et al., 2014). These observations, combined with the fact that MBP was the most abundant protein in the 20 kDa band, made us suspect that the labeling of the band was due to non-specific binding to myelin and/or cross-reaction of antibodies to the MBP protein. We, therefore, co-stained the immunoblots with the C1/6.1 and MBP antibodies. Several MBP isoforms between 14–21 kDa have been identified (Barbarese et al., 1977) and we detected several bands using the MBP antibody (**Figure 5B**). A major MBP-reactive band overlapped

perfectly with the 20 kDa band detected with the C1/6.1 antibody (**Figure 5B**).

We also performed a myelin removal protocol using Myelin removal beads from Miltenyi Biotec. This product is developed to remove myelin from single-cell suspensions, not from tissue homogenates, and we experienced large loss of material during the procedure. However, proportionally more of the 20 kDa band was lost compared to the APP-FL and APP-CTF α and - β bands (**Figure 5C**). Thus, although we cannot be sure that this is due to the removal of myelin, the proteins represented by the 20 kDa band appear to bind more to the myelin removal beads (specifically or non-specifically) than the other APP bands do. An indication that myelin indeed was removed by this protocol was that both of the major MBP bands were also depleted. The intensity of the staining of the 20 kDa band with a total protein stain was also decreased after myelin depletion (**Figure 5C**).

To further elucidate whether the detection of the 20 kDa band by APP antibodies is due to cross-reactivity to MBP, we immunodepleted the homogenates from MBP using an MBP antibody. However, whereas an approximately 15 kDa band detected by the MBP antibody was efficiently depleted from the homogenates, and detected in the immunoprecipitates,

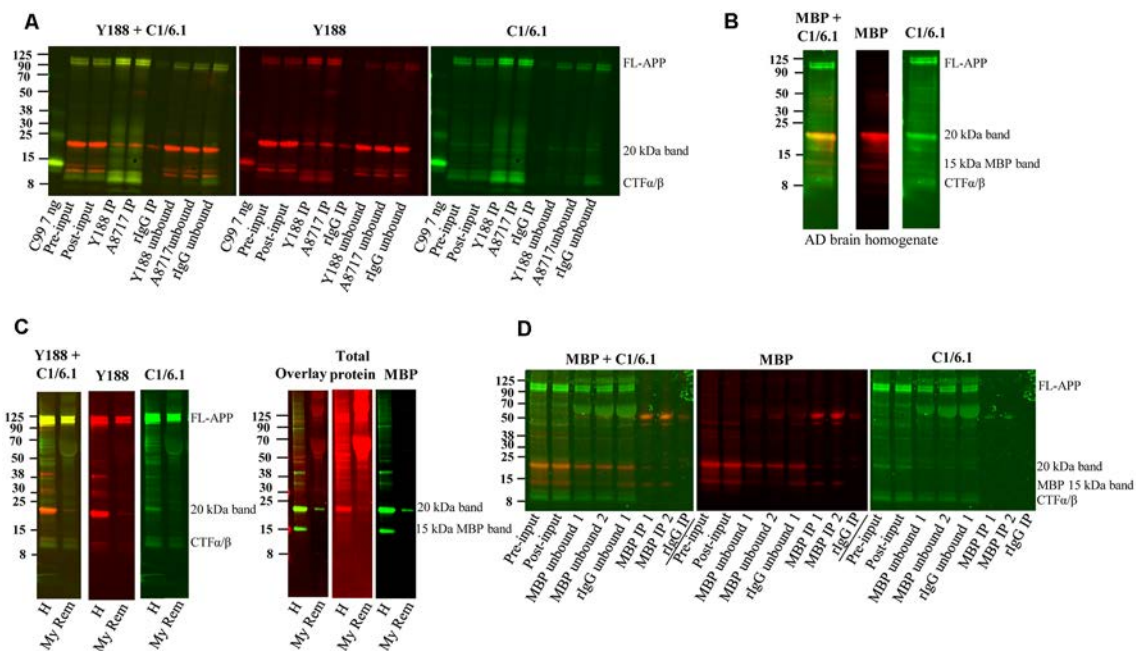
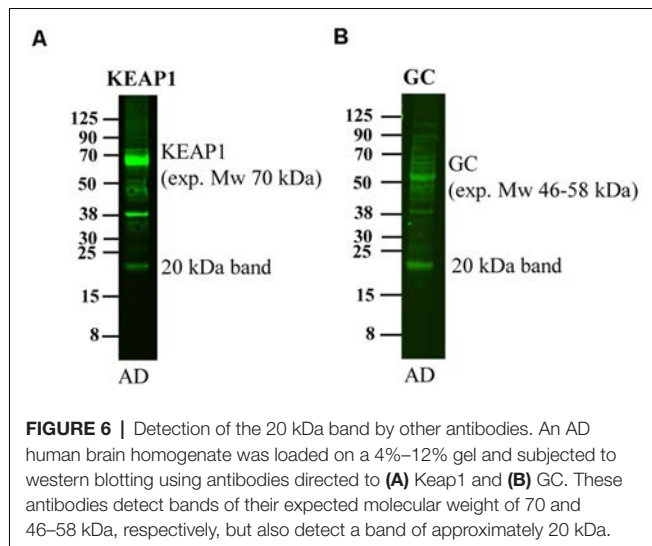


FIGURE 5 | Immunoprecipitation of APP and depletion of myelin and myelin basic protein (MBP). **(A)** Western blot of immunoprecipitation of human AD brain homogenate using the Y188, the A8717 antibody or a rabbit immunoglobulin antibody (rIgG) as negative control. A recombinant C99-FLAG peptide was loaded as a size indicator. The 20 kDa band could not be immunoprecipitated to a higher level with the specific antibodies Y188 and A8717 than with the control rIgG, and the majority of the 20 kDa remained in the unbound fraction. On the contrary, full length (FL) APP and some of the lower molecular weight CTFs, were successfully immunoprecipitated. Fifteen percentage of the brain homogenate was collected before immunoprecipitation and loaded as input. This is a representative figure of eight experiments. **(B)** Western blotting of a human brain AD homogenate using the MBP and the C1/6.1 antibody shows overlap of the signal in the 20 kDa band. **(C)** A human brain AD homogenate was subjected to myelin removal beads (My Rem) or kept on ice (H), loaded on a gel and subjected to western blotting using Y188, C1/6.1 and MBP antibodies as well as a total protein stain. The loaded My Rem sample corresponded to 25 times more of the input than the loaded H sample. The 20 kDa band and the 15 kDa MBP band was depleted proportionally more than the FL-APP and APP-CTF α / β bands. **(D)** Immunodepletion of a human AD brain using an MBP antibody was performed using 2.5 μ g (IP 1) or 5 μ g (IP 2) MBP antibody or a rabbit IgG (rIgG) as negative control. Five percentage of input before (pre-input) and after (post-input) pre-clearing with beads only, as well of immunodepleted samples were loaded. Whereas the 15 kDa MBP band was efficiently and specifically depleted, the 20 kDa band detected by C1/6.1 was not depleted more by the MBP antibody than by the rIgG.



the 20 kDa band detected by the C1/6.1 antibody was not specifically immunodepleted or immunoprecipitated using the MBP antibody. The 20 kDa band (as detected either by C1/6.1 or the MBP antibody) was, to a certain degree, immunodepleted both by the MBP antibody and the negative control rabbit IgG. In contrast, no differences in staining intensity could be observed before and after pre-clearing of the homogenates with beads only (Figure 5D).

Thus, both APP antibodies and the MBP antibody detects a 20 kDa band in human brain but the same antibodies cannot specifically immunoprecipitate this band, indicating that the detection is non-specific also for MBP. However, the protein(s) responsible for this staining can, to a certain degree, bind to any antibody-bead complexes (also negative control rabbit IgG) or to myelin removal beads but not to the magnetic beads alone.

Several Other Antibodies Also Recognize a 20 kDa Band in Human Brain

Upon running western blotting experiments of human brain homogenates for other proteins in our laboratory, we noted that also these antibodies detected a band of approximately 20 kDa although the expected molecular weight was of a different size. These antibodies include Kelch-like ECH-associated protein 1 (Keap1, expected molecular weight 70 kDa, Figure 6A), and Vitamin D-binding protein (GC, expected molecular weight 46–58 kDa, Figure 6B). Thus, a multitude of antibodies, including APP antibodies, an MBP and other antibodies recognize this 20 kDa band, strongly indicating that the binding is at least partially non-specific.

DISCUSSION

Using western blotting to characterize the different APP-CTFs in brain homogenates, we here demonstrate that a band of approximately 20 kDa is abundant in human brain but not in rat or mouse brain. We performed a detailed investigation of this band in brain tissue from different species and developmental

stages, AD patients and healthy controls, using a range of antibodies, mass spectrometry and immunoprecipitation.

Several studies have shown that bands that are detected by APP antibodies and migrate between 20 kDa and 30 kDa on SDS-PAGE gels are present in human brain (Estus et al., 1992; Haass et al., 1992; Tamaoka et al., 1992), but the identity of these bands has not been thoroughly investigated. However, recently Wang et al. (2015) identified an APP-band of similar size in human cell-lines and named it APP-CTF η . This band was further characterized by Willem et al. (2015) who also showed that subsequent cleavage of APP-CTF η by α -secretase resulted in a synaptotoxic A η - α peptide. The detection of the APP-CTF η in mouse brain was specific since it was not detected in APP knock-out mice (Willem et al., 2015).

The 20 kDa band that we detect in human, guinea pig and macaque brain was much more prominent than any bands of similar size in mouse or rat brain, although we could detect a weak band of slightly higher molecular weight in mouse brain homogenates using the C1/6.1 antibody. In addition, the 20 kDa band was not present in human embryonic brain. Given the similarity in size between the 20 kDa band and the APP-CTF η band, we investigated whether the 20 kDa band could indeed be APP-CTF η . If so, the APP-CTF η levels would be much higher in human, guinea pig or macaque brain than in mouse or rat brain and thus reveal important species and developmental differences in APP processing. However, several facts indicate that the detection of the binding of APP antibodies to the 20 kDa band is partially non-specific:

- (1) The binding of the Y188 and C1/6.1 to the 20 kDa band correlates both in molecular weight and intensity with a major band in a total protein stain. The total protein stain of this band is not likely to be due to staining of APP since APP was only detected with a low score in mass spectrometry analysis of this band. It should be noted, however, that APP is present in this band as well, albeit at low levels.
- (2) The pattern of bands varied considerably depending on which APP antibody that was used. For example, the Y188 antibody binds proportionally more of the 20 kDa band than the APP-CTF α/β and APP-FL bands, despite the fact that the epitope of the antibodies should be present in all these fragments.
- (3) Several other antibodies also detect the 20 kDa band although their expected molecular weight is not 20 kDa.
- (4) It was not possible to specifically immunoprecipitate the 20 kDa band either with APP antibodies or with an MBP antibody even though other APP and MBP bands were efficiently immunoprecipitated.
- (5) The 20 kDa band was proportionally more depleted than other APP fragments using a myelin removal protocol. Although this protocol is not designed for brain homogenates and we thus cannot exclude that proteins were non-specifically depleted, MBP was also depleted to a higher degree than APP-FL and APP-CTF α/β .

To conclude, these results indicate a non-specific binding of a multitude of antibodies to a 20 kDa band that also correlates to a band stained by a total protein stain. Regarding the proportional

differences in binding capacity of different APP antibodies to the 20 kDa band compared to other APP fragments, as well as the inability to immunoprecipitate the 20 kDa band, we cannot totally rule out that this is due to a conformational change in the proposed APP-CTF η but we find this explanation quite unlikely.

The large variation of intensity of the 20 kDa band (detected by APP antibodies and a total protein stain) between different human brain samples could possibly be due to variation in white matter content. This note, together with the selective depletion of the 20 kDa band using a myelin removal protocol and the fact that MBP was the top hit in the MS analysis of the band, speak for that the binding was due to myelin. Using immunohistochemistry of human brain sections, the Y188 antibody also stained axonal fibers, more strongly than the C1/6.1 antibody (Jordá-Siquer et al. unpublished results), in line with the more prominent detection of the 20 kDa band with the Y188 than with the C1/6.1 antibody, further indicating binding to myelin. In addition, a previous study has shown non-specific immunohistochemical labeling of IgMs to myelin in human brain sections (Perentes and Rubinstein, 1986).

We could not, however, specifically immunodeplete the 20 kDa band detected by the C1/6.1 antibody using an MBP antibody, suggesting that the detection of this band is due to non-specific binding to a component in the 20 kDa band rather than cross-reactivity of these antibodies to MBP. The fact that we could non-specifically immunodeplete the C1/6.1-reactive 20 kDa band using a control rabbit IgG, as well as the detection of this band by a multitude of antibodies, further supports this conclusion. The myelin-removal beads technology also builds on immunodepletion but we cannot conclude whether this depletion is specific or not.

Although the major 20 kDa band, which is most prominently stained band by the Y188 antibody, is most probably to a large degree non-specific, we still detected an APP peptide in this band using in-gel digestion and mass spectrometry. Although the score of this peptide was low, it showed a similar spectrum and identical retention time as a peptide from the in-gel-digested FL-APP band, indicating that an APP-derived fragment of approximately 20 kDa indeed exists. In line with this, Willem et al. (2015) could detect the APP-CTF η in wild-type but not in APP knock-out mice, which strongly indicate the presence of a specific APP fragment of approximately 20 kDa in mouse brain. The levels of APP-CTF η in mouse brain and N2a cells were generally low but were increased upon BACE1 inhibition (Willem et al., 2015). In line with this, using the C1/6.1 antibody, we could detect a weak band migrating slightly above the major 20 kDa band in human brain. The fact that Willem et al. (2015) can detect low levels of APP-CTF η also using the Y188 antibody, whereas we cannot, is most probably due to slight differences in the detection limit between our protocols. Thus, this upper band might be a specific APP band, but in human brain this signal is hidden by the major potentially non-specific 20 kDa band. This fact makes it difficult to interpret results intended to rule out differences in levels of this band in different species, developmental stages or between AD and control brain.

García-Ayllón et al. (2017) reported on a 25 kDa fragment, presumably corresponding to APP-CTF η with increased levels

in cerebrospinal fluid (CSF) of AD and Down syndrome patients compared to healthy controls. Since we do not know whether the major band detected by the total protein stain is present in CSF, we cannot determine whether the detection of the APP fragment in CSF is specific. In contrast to our results, García-Ayllón et al. (2017) succeeded in immunoprecipitating the 25 kDa band using Y188 or A8717 antibodies, which supports that the antibody binding to the band they detect is specific.

In summary, our results strongly indicate non-specific binding to a 20 kDa band in human brain by a multitude of APP and other antibodies. We call for precaution when analyzing proteins of a similar size in human brain. However, APP is present in this 20 kDa band and there is good evidence that a specific APP band of a similar size is present in mouse brain and possibly also in CSF.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the regional ethical review board of Stockholm (Regionala etikprövningsnämnden i Stockholm). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the animal research ethical committee of southern Stockholm (Stockholm södra djurförsöksetiska nämnd) and Linköping ethical committee (Linköpings djurförsöksetiska nämnd).

AUTHOR CONTRIBUTIONS

JL, SF and HH planned the experiments. JL and HH performed the major part of the experiments. TK, TJ-S and MS performed the analysis of the different human brain samples. ES provided the human fetal tissue. MK performed the western blotting with Keap1 and GC antibodies. HH, JL, SF, LT, GB, ES and BW analyzed the data and contributed to the interpretation of the data. JL, SF and HH wrote the manuscript and all authors proof-read the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnagi.2019.00273/full#supplementary-material>.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Physiological and pathological roles of the amyloid precursor protein at the presynapse

Alzheimer's disease (AD) is a progressive neurodegenerative disease which affects 47 million people worldwide, being the most prominent type of dementia. The etiology of the disease is unknown but genetic evidence from the familial form of the disease indicates that the amyloid precursor protein (APP) plays a key role in the pathology. Importantly, APP is the substrate in the proteolytic reaction producing A β peptides which compose the amyloid plaques, one of the main pathological hallmarks in AD brain. In addition, APP is ubiquitously expressed by neurons where it interacts with multiple presynaptic proteins but the role of these interactions is elusive.

The aim of my thesis was to study the physiological and pathological functions of APP related to its location at the presynapse. First, we studied the consequences on presynaptic mechanisms of the genetic deletion of presenilin, the catalytic subunit of γ -secretase, the intramembrane protease which cleaves APP. We observed that in absence of presenilin, APP accumulates in axons. By combining optogenetic to electrophysiology, we assessed synaptic transmission and plasticity in the CA3 region of the hippocampus. The presynaptic facilitation, the increase in synaptic vesicle release during repetitive stimulation, was altered whereas the basal neurotransmission was not. The impairment of presynaptic mechanisms was due to the accumulation of APP Cter, which decreases the abundance of synaptotagmin-7, a calcium sensor essential for facilitation. Using a similar approach, we investigated the consequences of the genetic deletion of APP itself and observed again an impairment of presynaptic facilitation. Together, these results demonstrate the importance of APP homeostasis in presynaptic plasticity.

I then investigated possible alterations of APP, other than the amyloid peptides, in the AD brain. I discovered that APP dramatically accumulates together with presynaptic proteins around dense-core amyloid plaques in human AD brain. In addition, the Nter domain, but not the Cter domain of APP is enriched in the core of amyloid plaques uncovering a potential pathological role of the secreted APP Nter in dense-core plaques. Ultrastructural analysis of APP accumulations reveals abundant multivesicular bodies containing presynaptic vesicle proteins and autophagosomal built-up of APP. Finally, we observed that outside the APP accumulations, presynaptic proteins were downregulated, in the neuropil area of the outer molecular layer of the dentate gyrus. Altogether, the data I collected during my thesis supports a role of presynaptic APP in physiology and in AD pathology and highlights APP accumulations as a pathological site where presynaptic proteins are mis-distributed.

Keywords: Alzheimer's disease, amyloid precursor protein, presynaptic mechanisms, hippocampus, presenilin, synaptotagmin-7, γ -secretase, hallmark, presynaptic failure.