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LOSING CONNECTIONS IN ALZHEIMER DISEASE - THE AMYLOID PRECURSOR PROTEIN PROCESSING MACHINERY AT THE SYNAPSE

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Losing connections in Alzheimer Disease - the amyloid precursor protein processing machinery at the synapse

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To all who have Alzheimer disease.

And to all who love someone with Alzheimer disease.

ABSTRACT

Synaptic degeneration is one of the earliest characteristics of Alzheimer disease (AD). The amyloid β-peptide (Aβ) plays a critical role in the pathology of AD and therefore thorough understanding of its production and functions is of outmost importance. Aβ is generated by sequential cleavage of the amyloid precursor protein (APP) by the β-secretase BACE1 and by the γ-secretase. In an alternative, non-amyloidogenic pathway, APP is cleaved by the α-secretase ADAM10 instead of BACE1, precluding Aβ formation. Increased synaptic activity has been associated with increased secretion of Aβ and since our lab had previously shown that Aβ can be produced at the synapse, we hypothesised that Aβ is produced inside synaptic vesicles and released through normal synaptic vesicle exocytosis. We found that small amounts of Aβ can be produced in synaptic vesicles, although these vesicles do not appear to be the main site of Aβ production. To study the secretion, synaptosomes (functional, pinched off, nerve endings) were isolated from rat brain and we could demonstrate that Aβ is continuously secreted from synapses in an activity-independent manner through a mechanism that is distinct from normal neurotransmitter release. While further investigating the highly pure synaptic vesicles, both ADAM10 and BACE1, as well as their cleavage products, APP C-terminal fragments (CTFs), were found to be greatly enriched in these vesicles compared to total brain homogenate. Yet, presenilin was the only enriched component of the γ-secretase complex. In addition, these Western blotting findings were confirmed by *in situ* proximity ligation assay (PLA) showing close proximity of both ADAM10 and BACE1 to the synaptic vesicle marker synaptophysin in intact mouse primary hippocampal neurons. Active γ-secretase, on the other hand, only gave rise to few PLA-signals, indicating that the first cleavage step in Aβ production takes place in synaptic vesicles while γ-secretase cleavage takes place elsewhere. Subsequently the synaptic location of the secretases was confirmed also in adult rat and human brain. Again using PLA, we could demonstrate that both ADAM10 and BACE1 were in close proximity to both synaptophysin and the postsynaptic density marker PSD-95 as well as to their substrate APP in both human and rat adult brain hippocampus and cortex. Also APP was in close proximity to both synaptophysin and PSD-95.

In addition to the known synaptotoxicity of Aβ, a number of studies have implied important and toxic roles for other APP-derived fragments, such as CTF-β or the synaptotoxic Aα-η. Alternative cleavage of APP by η-secretase gives rise to CTF-η, which is further cleaved to Aα-η by ADAM10. However, which of these fragments that is most abundant in AD brain had, to our knowledge, not been elucidated. When performing SDS-PAGE and Western

blotting we found that a 25 kDa CTF (likely corresponding to CTF-η) was abundant in human brain but present at much lower levels in rat and mouse brain. The 25 kDa CTF was also present in macaque and guinea pig brain but the levels of this fragment was not increased in the brain of a mouse model overexpressing the human APP gene with a Swedish/London mutation. This implies that it is the environment in the human brain, rather than the human APP gene itself, that determines whether the 25 kDa CTF is formed or not. Furthermore, we investigated whether AD patients have altered levels of the 25 kDa CTF in their brains but could not detect any significant differences between AD and control brain homogenate.

Altogether, this thesis has contributed with new knowledge about synaptic release of Aβ and the synaptic localisation of the APP processing enzymes. It has thus highlighted the complexity and species differences of APP processing and its regulation. Implementation of this knowledge may facilitate future development of more specific and efficient treatment strategies for AD.

POPULAR SCIENCE SUMMARY

English

Synapses are the chief components responsible for the communication between nerve cells. In Alzheimer disease the synapses, and eventually whole nerve cells, degenerate and die. This affects cognition and causes impairment in memory, disillusion and problems with coping with everyday tasks. The amyloid β-peptide (Aβ) is considered to have a pivotal role in the course of the disease and is formed by cleavage of the amyloid precursor protein (APP) by first the β-secretases BACE1 and then the γ-secretase complex. No Aβ is formed if APP is cleaved by the α-secretase ADAM10 instead of BACE1. In order to better understand Aβ production and possibly regulate it as treatment for Alzheimer disease, it is important to find out where the production takes place and thereby also where in the nerve cells the proteins involved in the production are located.

In two of the papers in this thesis the synaptic location of ADAM10 and BACE1 were examined and it was demonstrated that both of them are located both presynaptically (in the part of the nerve cell that sends out signals) and postsynaptically (in the part of the nerve cell that receives signals). This is somewhat controversial since it was previously assumed that ADAM10 is located mainly in the postsynaptic part and BACE1 mainly in the presynaptic part of the nerve cell. It was also demonstrated that active γ-secretase is present at much lower levels in the presynaptic vesicles in which we demonstrated that ADAM10 and BACE1 are enriched. This implies that, in order for Aβ to be produced, CTF-α and -β (the fragments produced by ADAM10 and BACE1 cleavage of APP) need to be transported from the synaptic vesicles to another (yet unknown) synaptic structure where γ-secretase is located.

In the first paper of the thesis we attempted to determine how Aβ is secreted from synapses. Although we were unable to identify the mechanism behind Aβ secretion, we could demonstrate that Aβ is continuously secreted from neurons through a mechanism different from normal neurotransmitter release.

During the work with the first parts of this thesis, we found that a specific APP-derived fragment with a molecular weight of 25 kDa (“25 kDa CTF”) was abundant in human brain but not in rat brain. It is possible that this 25 kDa CTF is identical to the recently identified CTF-η. Since this fragment, together with Aβ, may have negative effects on synapses and brain function, we decided to examine it further. The 25 kDa CTF was always found in

human brain while it was hardly detectable in rat and mouse brain. However, people with Alzheimer disease did not have different levels of this fragment in their brains compared to control persons. We also found this fragment in the brain of macaque and guinea pig, which both are more evolutionary close to humans than rats and mice. Our results may possibly explain why humans, but not rats or mice, develop AD. When transferring results from animal experiments to human medicine, species differences need to be more carefully considered.

This thesis contributes with increased knowledge of the mechanisms behind APP cleavage and the fragments thereby produced. This is of great importance for future development of treatment which may delay, or even prevent, the cognitive impairment in Alzheimer disease.

Svenska

Synapser är grundstrukturen för kommunikation mellan nervceller och i Alzheimers sjukdom bryts både synapser och sedan hela nervceller ner och förtvinar. Detta påverkar den kognitiva förmågan och leder till försämrat minne, desillusion och problem med att klara dagliga sysslor. Amyloid β-peptiden (Aβ) antas ha en framträdande roll i sjukdomsförloppet och den bildas genom att amyloid prekursorproteinet (APP) klyvs på olika ställen av först β-sekretaset BACE1 och sedan av γ-sekretasenzymet. Om APP klyvs av α-sekretaset ADAM10 istället för av BACE1 bildas inget Aβ.

För att bättre förstå Aβ-produktionen och eventuellt kunna reglera den i medicinskt syfte är det viktigt att ta reda på var den sker och därmed var i nervcellerna de olika proteinerna som är involverade i produktionen finns. I två delarbeten i denna avhandling undersöker vi den synaptiska lokaliseringen av ADAM10 och BACE1 och visar att de finns både pre-synaptiskt (i den del av nervcellen som sänder ut signaler) och postsynaptiskt (i den del av nervcellen som tar emot signaler). I tidigare forskning har det antagits att ADAM10 återfinns i den postsynaptiska delen och BACE1 i den presynaptiska delen. Vi visar också att det aktiva γ-sekretaset till största del inte finns i de presynaptiska vesiklar där vi visade att ADAM10 och BACE1 finns. Detta tyder på att för att Aβ ska kunna bildas så måste CTF-α och -β (de fragment som bildas vid ADAM10- och BACE1-klyvning av APP) transporteras från dessa vesiklar till den (ännu okända) synaptiska struktur där γ-sekretaset finns.

I det första delarbetet undersökte vi hur Aβ utsöndras från synapser. Mekanismen bakom utsöndringen kunde inte påvisas men vi kunde visa att Aβ inte utsöndras från nervceller på samma sätt som vanliga signalsubstanser.

Under arbetet med de första delarbetena i denna avhandling upptäckte vi att ett specifikt APP-fragment med en molekyl-vikt på 25 kDa (”25 kDa CTF”) är anrikat i människohjärna men knappt finns i råtthjärna. Det är möjligt att detta 25 kDa CTF och det nyligen identifierade CTF-η är samma fragment. I det sista delarbetet undersökte vi förekomsten av detta 25 kDa CTF närmare eftersom detta fragment tillsammans med Aβ kan förorsaka negativa effekter på synapser och hjärnans funktion. 25 kDa CTF återfanns alltid i human-hjärna medan det knappt gick att detektera i hjärna från råtta och mus. Dock kunde vi inte urskilja skillnader i nivåerna av detta fragment mellan kontrollpersoner och personer med Alzheimers sjukdom. Även i hjärnan hos makaker och marsvin, som båda evolutionsmässigt är närmare människan än råtta och mus, återfanns 25 kDa CTF. Artskillnader i nivåerna av detta fragment kan vara en förklaring till varför människor men inte råttor och möss får Alzheimers sjukdom. Kunskapen om denna artskillnad är viktig att ta i beaktande vid läke-medelsutveckling som baseras på djurförsök.

Denna avhandling bidrar till ökad förståelse av mekanismerna bakom APP-klyvning och de olika fragment som därmed kan bildas. Detta är av stor betydelse för framtida utveckling av medicin som kan fördröja eller rentav förhindra uppkomsten av den kognitiva nedsättningen i Alzheimers sjukdom.

LIST OF SCIENTIFIC PAPERS

I. **Lundgren J. L.**, Ahmed S., Winblad B., Gouras G. K., Tjernberg L. O. and Frykman S. (2014) Activity-independent release of the amyloid-β peptide from rat brain nerve terminals. *Neuroscience Letters* 566:125-130

II. **Lundgren J. L.**, Ahmed S., Schedin Weiss S., Gouras G. K., Winblad B., Tjernberg L. O. and Frykman S. (2015) ADAM10 and BACE1 are localized to synaptic vesicles. *Journal of Neurochemistry* 135: 606-615

III. **Lundgren J. L.**, Vandermeulen L., Sandebring-Matton A., Ahmed S., Winblad B., Di Luca M., Tjernberg L. O., Marcello E. and Frykman S., Similar pre- and postsynaptic distribution of ADAM10 and BACE1 in rat and human adult brain. *Manuscript*

IV. Haytural H.*, **Lundgren J. L.***, Jorda T., Seed Ahmed M., Winblad B., Årsland D., Graff C., Barthet G., Tjernberg L. O. and Frykman S, A 25 kilodalton amyloid precursor protein C-terminal fragment is abundant in human but not in mouse or rat brain. *Manuscript*

* These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
[^{18}F]FDG	2-[^{18}F]fluoro-2-deoxy-D-glucose
Aβ	Amyloid β-peptide
AD	Alzheimer disease
ADAM10	A disintegrin and metalloproteinase 10
AICD	Amyloid precursor protein intracellular domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Aph-1	Anterior pharynx defective-1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Atg7	Autophagy-related gene 7
BACE1	β-site amyloid precursor protein cleaving enzyme
CHO cells	Chinese hamster ovary cells
CLU	Clusterin
CR1	Complement receptor 1
CSF	Cerebrospinal fluid
CT	Computed tomography
CTF	C-terminal fragment
DIAN-TU	Dominantly Inherited Alzheimer Network Trials Unit
DIV	Days *in vitro*
DPBS	Dulbecco´s phosphate buffered saline
ER	Endoplasmic reticulum
FAD	Familial Alzheimer disease
FDD	Familial Danish dementia
FL-APP	Full length amyloid precursor protein
GSI	γ-secretase inhibitor
GSK3β	Glycogen synthase kinase 3β
GSM	γ-secretase modulator

iPSC	Induced pluripotent stem cell
KCl	Potassium chloride
kDa	Kilodalton
LTD	Long-term depression
LTP	Long-term potentiation
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartic acid
NFTs	Neurofibrillary tangles
Pen-2	Presenilin enhancer-2
PET	Positron emission tomography
PICALM	Phosphatidyl-inositol binding clathrin assembly protein
PLA	Proximity ligation assay
PS	Presenilin
PSD	Postsynaptic density
ROS	Reactive oxygen species
sAPP	Soluble amyloid precursor protein fragment
SCI	Subjective cognitive impairment
siRNA	Short/small interfering ribonucleic acid
SORL1	Sortilin-related receptor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV	Synaptic vesicle
WHO	World Health Organization

1 INTRODUCTION

1.1 ALZHEIMER DISEASE AND DEMENTIA

Alzheimer disease (AD) is the most common neurodegenerative disorder and cause of dementia, comprising up to 70 % of all dementia cases (Prince *et al.* 2013). Vascular dementia, Frontotemporal dementia, Dementia with Lewy Bodies and Parkinson's disease dementia are other common types of dementia, all of which are associated with abnormal accumulation and deposition of toxic protein aggregates in the brain. The appearance of these aggregates often precede the clinical symptoms by many years (Ross and Poirier 2004). The clinical manifestations of dementia are chronic and progressive worsening of cognitive function. After early and subtle impairments in memory formation and recall, the symptoms gradually worsen and begin to affect also other cognitive functions, such as behaviour, orientation, language and executive functions including planning, judgement and problem solving (Prince *et al.* 2013).

Around 50 million people suffer from dementia and there are almost 10 million new cases each year (WHO, 2017). The main reason for this dramatic increase in dementia is the aging population in the world, especially in low- and middle-income countries (WHO, 2017; Prince *et al.*, 2013). Accordingly, dementia is a huge socioeconomic burden, costing the society 818 billion USD in 2015, which is 1.1 % of the world's gross domestic product (Winblad *et al.*, 2016; WHO, 2017). The cost of dementia is unevenly distributed and affects low- and middle-income countries most. Consequently, the unequal burden of dementia will largely be affecting countries that have less awareness of the disease and lower capacity to handle it. However, policymakers in the West are starting to appreciate the dramatic effect of dementia on society and focus resources on combating dementia from different angles. To raise awareness of dementia as well as to encourage and promote national and international action, the World Health Organization (WHO), together with Alzheimer's Disease International, has published the report "Dementia: a public health priority" (Winblad *et al.*, 2016; WHO, 2017). The fact that WHO has declared dementia a public health priority is a welcomed progression and will hopefully have noticeable effects in society. However, taking the suffering and psychological burden of the patients and their family and caregivers into account, focusing on preventing and treating dementia is, evidently, not primarily of economic interest.

1.1.1 Stages of Alzheimer disease

Individuals who experience cognitive decline or impaired memory although they cannot be clinically diagnosed are classified as having subjective cognitive impairment (SCI). Mild cognitive impairment (MCI), on the other hand, is a pre-stage of dementia, yet not all MCI patients develop dementia or AD. The cognition state between dementia and normal aging are described as MCI. MCI patients display objective cognitive decline, yet they still function in their everyday lives and usually do not need much extra assistance or care (Knopman and Petersen 2014).

A family history of dementia is linked to increased risk of developing AD. Genetically autosomal dominant inherited, familial AD (FAD) is caused by mutations in three genes (*APP*, *PSEN1* and *PSEN2*), all of which influence the processing of the amyloid precursor protein (APP) into the amyloid β-peptide (Aβ) which aggregates and make up the amyloid plaques which are characteristic hallmarks in the brains of AD patients. AD-associated mutations in these genes lead to aggregation of Aβ and aggressive early onset of the disease. FAD accounts for around 5 % of the AD cases while the rest are classified as sporadic AD (Bagyinszky *et al.* 2014; Winblad *et al.* 2016).

1.1.2 Neuropathology

AD is characterised by the presence in the brain of extracellular amyloid plaque depositions and intraneuronal tangles (Fig 1). However, these pathological hallmarks are often preceded by synaptic dysfunction and degeneration, which is one of the earliest events in the pathology of AD (Knobloch and Mansuy 2008). The amyloid plaques in the brains of AD patients are made up of aggregated, fibrillar forms of synaptotoxic Aβ while the neurofibrillary tangles (NFTs) consists of hyperphosphorylated tau protein (Selkoe 2011). Degeneration of neurites and eventually neuronal death cause brain atrophy which often is prominent at later stages of the disease progression (Knobloch and Mansuy 2008). Since the correlation between amyloid plaque

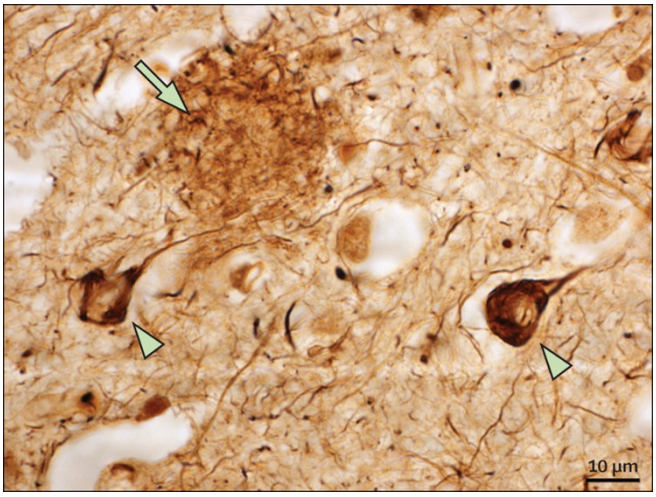

Figure 1. The neuropathological hallmarks of Alzheimer disease. Silver staining of a *post mortem* AD brain showing amyloid plaques (arrows) and neurofibrillary tangles (arrow-heads). Adapted from Winblad *et al.* (2016).

density and cognitive impairment is poor, it is evident that also other pathological features are involved (Soldano and Hassan 2014). The best correlation to cognitive decline in AD is synaptic loss (Terry *et al.* 1991), yet, the mechanisms behind the synaptic degeneration are still unclear.

1.1.2.1 Tau

As mentioned above, NFTs are pathological hallmarks of AD alongside the amyloid plaques. These intraneuronal NFTs consist of aggregated fibrillar forms of the microtubule-associated protein tau (Nisbet *et al.* 2015). Under normal physiological conditions tau stabilises microtubules, regulates axonal transport and functions as a scaffolding protein by interacting with a variety of proteins. However, during the pathology of AD, tau becomes hyperphosphorylated, loses its physiological function and starts to aggregate (Brandt and Leschik 2004). Misfolded, aggregated and hyperphosphorlylated tau is unable to exert its role as a transport protein and may even disrupt the overall cellular trafficking. This impairs the transport of essential receptors and mitochondria to the synapses, with detrimental effects on synaptic function (Ebneth *et al.* 1998; Kanaan *et al.* 2011). Hyperphosphorylated, dysfunctional tau also redistributes from the axons into somatodendritic compartments (Götz *et al.* 1995). However, analysing synaptoneurosome preparations, Tai *et al.* (2012) demonstrated both pre- and postsynaptic localisation of tau in both AD and control human brain.

Some of the toxic effects of Aβ are believed to be exerted via tau and there is considerable crosstalk between the toxic versions of these two proteins. Aβ and tau have both separate and common, synergistic pathological functions in AD (Nisbet *et al.* 2015). Injection into rat brain of Aβ extracted from human plaque cores caused tau pathology and neuronal loss (Frautschy *et al.* 1991). Reducing endogenous levels of tau in AD transgenic mice, without altering Aβ levels, reversed memory impairment, reduced early mortality and protected against excitotoxicity (Roberson *et al.* 2007). Moreover, double transgenic mice with both APP and tau mutations exhibit more tau pathology than the single transgenic mice, although tau levels are not increased (Lewis *et al.* 2001). The same is true for transgenic mice with mutated APP, presenilin and tau which also show increased neuronal loss in the hippocampus (Héraud *et al.* 2014). Consequently, it appears as if Aβ toxicity is tau-dependent. However, tau may also sensitise synapses to Aβ toxicity which is further amplified by the effect of Aβ on tau, causing a vicious cycle (Ittner and Götz 2011).

1.1.2.2 Inflammation

Chronic or abnormally activated inflammation can be detrimental and many neurodegenerative disorders, e.g. multiple sclerosis, Parkinson's disease, and AD, are associated with neuroinflammation (Graeber *et al.* 2011). In the AD brain, amyloid plaques as well as NFTs are surrounded by activated microglia and reactive astrocytes (Beach *et al.* 1989; Itagaki *et al.* 1989; Masliah *et al.* 1991) and both the complement and the innate immune system are activated in AD brains (Pimplikar 2014). Systemic inflammation has also been linked to AD (Pimplikar 2014) and many of the AD-associated genes identified by genome-wide association studies are involved in the inflammatory response (Tosto and Reitz 2013). In addition, several studies have shown that receiving anti-inflammatory treatment is associated with lower prevalence of AD (Rich *et al.* 1995; Etminan *et al.* 2003; McGeer *et al.* 2017) though other studies imply that the prevalence of AD is not affected by anti-inflammatory treatment (Gupta *et al.* 2015). Nevertheless, whether inflammation is a risk factor and potential cause of AD or a consequence of the pathological changes in AD is not clear.

1.1.2.3 Energy metabolism, oxidative stress and mitochondria

Oxidative stress is a result of imbalance in the production of reactive oxygen species (ROS) and the ability of the cell to clear them. Oxidative stress is a common feature of dysfunctional neurons already at an early stage of AD. This is considered to be caused by impaired mitochondrial function (Zhu *et al.* 2006) which is detrimental to neurons since the mitochondria, which are particularly abundant at the presynapse, provide the energy needed for neuronal function, especially neurotransmission. Positron emission tomography (PET) reveals evident decrease in the uptake of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) in the brains of AD patients compared to controls, indicating decreased energy consumption in specific, pathology affected regions of their brain (Heiss *et al.* 1991; Frisoni *et al.* 2013). Mitochondrial dysfunction leads to decreased ATP production, increased production of ROS as well as reduced Ca^{2+} buffer capacity which affects neurotransmission (Bhat *et al.* 2015; Zhu *et al.* 2006; Ankarcrona *et al.* 2010). In addition, inappropriately activated apoptosis in the AD brain may be caused by death factors released from damaged mitochondria (Dawson and Dawson 2017).

Mitochondrial dysfunction in AD is associated with Aβ (Zhu *et al.* 2006; Ankarcrona *et al.* 2010). Aβ accumulates in mitochondria both in AD patients and transgenic animal models of AD (Lustbader *et al.*, 2004; Manczak *et al.*, 2006). The γ-secretase components are

found in mitochondria (Hansson *et al.* 2004) and Aβ can be produced at mitochondria-endoplasmic reticulum (ER) contact sites (Schreiner *et al.* 2015). Aβ interacts with several mitochondrial proteins, thereby directly affecting mitochondrial function and subsequently contributing to oxidative stress (Lustbader *et al.*, 2004; Manczak *et al.*, 2006; Ankarcrona, Mangialasche and Winblad, 2010).

1.1.3 APP processing and the amyloid β-peptide

Aβ is a proteolytic product of APP. β- and γ-secretase generate Aβ by sequential cleavage of APP. After β-secretase cleavage, soluble APPβ (sAPPβ) and a C-terminal fragment of APP (CTF-β) are produced. CTF-β is further cleaved by γ-secretase, yielding Aβ and the intracellular domain AICD (Fig 2) (Vassar *et al.* 2009; Selkoe 2011). In a non-amyloidogenic pathway, APP is cleaved by α-secretase instead of β-secretase, generating CTF-α and sAPPα. CTF-α will be cleaved into a P3 fragment by the γ-secretase complex (Fig 2). In contrast to Aβ, P3 is not prone to aggregate and have no known toxic effect (Postina *et al.* 2004; Kuhn *et al.* 2010).

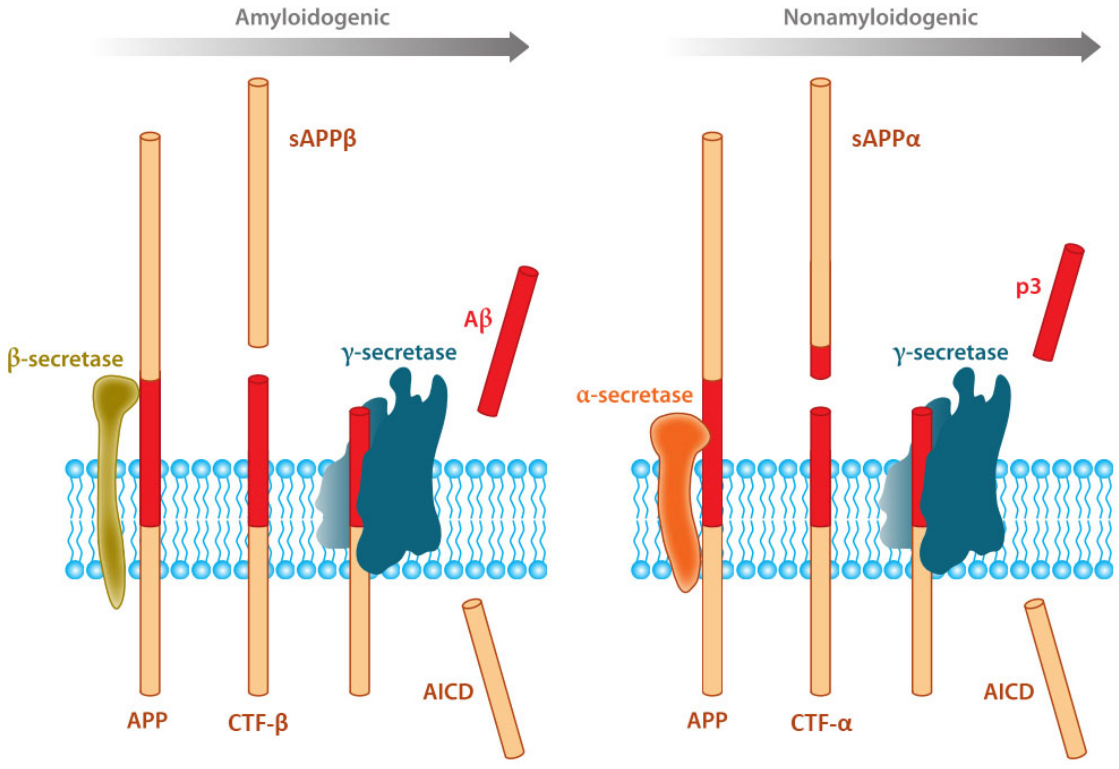

Figure 2. Processing of the amyloid precursor protein (APP). The first cleavage of APP by α- or β-secretase generates soluble APP fragments (sAPPα and sAPPβ) and the APP C-terminal fragments CTF-α and CTF-β. The CTFs are further cleaved by γ-secretase generating the APP intracellular domain (AICD) as well as Aβ (in the amyloidogenic pathway, left) or the P3 fragment (in the non-amyloidogenic pathway, right). (Adapted from O'Brien and Wong 2011).

APP is a single-pass transmembrane protein which under physiological conditions mainly is processed in the non-amyloidogenic pathway. APP and sAPPα are important for the regulation of neural development, neurite outgrowth and guidance and for synapse formation (Soldano and Hassan 2014).

Since APP is processed by γ-secretase in a stepwise manner, the Aβ-peptide may be of varying length, Aβ40 being the most common while Aβ42 is more toxic and prone to aggregate. However, other species, such as Aβ43 or N-terminally truncated Aβ, also exist (Vetrivel and Thinakaran 2006). Soluble, oligomeric forms of Aβ contribute more to AD pathology than fibrillar forms and also correlate better with cognitive decline than plaque-associated Aβ (McLean *et al.* 1999; Näslund *et al.* 2000). Although extracellular amyloid plaques are hallmarks of AD, intracellular accumulation of Aβ, especially in neurites and synapses, are more toxic and cause synaptic dysfunction (Takahashi *et al.* 2002; Bayer and Wirths 2010; Tampellini and Gouras 2010; Gouras *et al.* 2012). Neurons from both sporadic AD and FAD patients are enriched in intraneuronal Aβ42 and have increased Aβ42/40 ratio (Aoki *et al.* 2008).

1.1.4 The APP processing secretases

The major β-secretase of neurons is the β-site APP cleaving enzyme 1 (BACE1) which is an aspartic protease active in acidic environments, mainly endosomes and the trans-golgi network (Kinoshita *et al.* 2003; Yan and Vassar 2014; Kandalepas and Vassar 2014). Apart from BACE1, other enzymes with β-secretase activity have also been suggested, such as Cathepsin D (Schechter and Ziv 2008) and Cathepsin B (Hook *et al.* 2005). Yet BACE1 activity on APP is most relevant for AD pathology since BACE1 knock-out animals do not display amyloid pathology and have no production of Aβ in their brain (Kandalepas and Vassar 2014). Synaptic BACE1 is primarily considered to be localised to the presynaptic compartment and BACE1 in enriched presynaptically at the mossy fibres in the stratum lucidum in the CA3 region of the hippocampus (Kandalepas *et al.* 2013). Del Prete *et al.* (2014) have found BACE1 also in synaptic vesicles (SVs).

γ-secretase is a transmembrane complex consisting of presenilin1 (PS1) or PS2, nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) and it cleaves its substrates within the membrane. The γ-secretase complex cleaves numerous type I transmembrane proteins in addition to APP, such as Notch, N-cadherin and ephrinB (Gertsik *et al.* 2015). The active γ-secretase complex has been observed to localise both pre- and postsynaptically (Schedin-Weiss *et al.* 2016). Inhibiting γ-secretase cleavage of APP

would reduce Aβ levels and consequently inhibitors and modulators of γ-secretase have been developed as potential AD therapy. Still, no γ-secretase inhibitors (GSIs) or γ-secretase modulators (GSMs) have had success in in clinical trials, likely due to lack of effect or adverse effects caused by inhibition of the cleavage of other γ-secretase substrates, mainly Notch (Mikulca *et al.* 2014; Bachurin *et al.* 2017). Inhibition of γ-secretase will also lead to accumulation of CTF-β which probably has toxic effects on neurons (see section 1.1.6), possibly contributing to the adverse effects caused by γ-secretase inhibition.

The main α-secretase of neurons is a disintegrin and metalloproteinase 10 (ADAM10) which cleaves APP within the Aβ sequence, precluding Aβ formation. ADAM10 is localised to the plasma membrane, postsynaptic density and the trans-Golgi network (Lammich *et al.* 1999; Gutwein *et al.* 2003; Marcello *et al.* 2007). Activity-dependent synaptic plasticity regulates ADAM10 interaction with some of its binding partners which further regulates ADAM10 insertion into synaptic membranes (Musardo and Marcello 2017). Enhancing ADAM10 and APP co-localisation within neurons might be beneficial since less APP would be cleaved by BACE1 and thereby less Aβ would be formed. Consequently, steering ADAM10 trafficking to cellular compartments where it may cleave APP might have potential as AD therapy.

1.1.5 The amyloid cascade hypothesis

A shift towards more amyloidogenic processing of APP and abnormal Aβ metabolism has been proposed to be the cause of AD. Although Aβ has physiological functions, e.g. as chelator and antioxidant, it appears to easily gain toxicity mainly by aggregating into oligomers (Atwood *et al.* 2003; Carrillo-Mora *et al.* 2014). In 1991, Hardy and Allsop published the amyloid cascade hypothesis which states that excessive accumulation of Aβ initiates and drives the pathological changes in AD (Carrillo-Mora *et al.* 2014; Hardy and Allsop 1991; Hardy and Selkoe 2002).

There is genetic support for the amyloid cascade hypothesis. The A673T mutation close to the β-secretase cleaving site of APP leads to a 40 % reduction in Aβ production and protects against AD (Jonsson *et al.* 2012) while all known genes causing FAD are related to APP or its processing, causing excessive Aβ production. In sporadic AD, on the other hand, the accumulation and aggregation of Aβ is considered a result of failure to effectively degrade and clear Aβ from the brain (Selkoe 2011; Selkoe and Hardy 2016).

Amyloid plaques are present in the brains of all AD patients. Transgenic animals with mutations in APP or the secretases involved in its processing, develop AD-like symptoms as well as several of the pathological hallmarks. This further supports the amyloid cascade hypothesis (Carrillo-Mora *et al.* 2014). Moreover, tau models with NFTs do not entirely mimic AD pathology (Hardy and Selkoe 2002).

Yet, Aβ depositions are not specific to the brains of AD patients but may appear in subjects with other neurodegenerative diseases and even in cognitively normal and healthy elderly, making the amyloid cascade hypothesis debatable. However, the amyloid plaques which are not associated with AD are diffuse and lack some of the characteristics of the amyloid plaques of AD patients, such as surrounding inflammation and glial activation (Hardy and Selkoe 2002; McLean *et al.* 1999; Näslund *et al.* 2000).

1.1.6 Toxic effects of other APP-derived fragments

There are indications that APP-CTFs are more toxic than previously believed and may be involved in the pathology of AD (Oster-Granite et al. 1996; McPhie et al. 1997; Kim et al. 2003; Jiang et al. 2010; Lauritzen et al. 2012). Inhibition of γ-secretase causes a reduction in the number of dendritic spines in the brains of wild type but not APP knock-out mice, suggesting that accumulation of CTFs is likely to contribute to the adverse effects of γ-secretase inhibition (Bittner *et al.* 2009). GSIs have been withdrawn from clinical trial due to lack of effect and increased cognitive decline was reported for some of the participants (Imbimbo and Giardina 2011). Deletion of the γ-secretase component presenilin leads to AD-like neurodegeneration (Saura *et al.* 2004). β-secretase inhibitors, but not γ-secretase inhibitors, can rescue synaptic and memory deficits in a model of familial Danish dementia (FDD) (Tamayev *et al.* 2012). Also phosphorylation of tau and over-activation of glycogen synthase kinase 3β (GSK3β), which is prominent in the AD brain, can be inhibited in induced pluripotent stem cells (iPSCs) by β-secretase inhibitors but not by γ-secretase inhibitors (Israel *et al.* 2012). In agreement with all this, CTF-β is the first APP-derived fragment to accumulate in animal models of severe AD (Lauritzen *et al.* 2012).

Other APP-derived fragments, such as caspase cleaved APP and an N-terminal fragment, have also been shown to be toxic (Galvan *et al.* 2002; Xu *et al.* 2015). Also CTF-η, the product of a newly identified, η-secretase processing of APP has been proposed to be toxic (Willem *et al.* 2015; Wang *et al.* 2015). CTF-η can be further processed by ADAM10 or BACE1 to generate the shorter fragments Aη-α and Aη-β. The levels of CTF-η and Aη are enriched in the brains of APP transgenic mice compared to wild type mice and in the

cerebrospinal fluid (CSF) from human AD patients where the levels of Aη exceed that of Aβ. Moreover, a 25 kDa CTF, probably identical to CTF-η, is more abundant in the CSF of AD patients and demented individuals with Downs syndrome than in control subjects (García-Ayllón *et al.* 2017). According to Willem *et al.* (2015) the proposed synaptotoxicity of Aβ dimers might actually be caused by Aη-α. When applied on hippocampal slice cultures, both synthetic Aη-α and Aη-α secreted from Chinese hamster ovary (CHO) cells caused a reduction in long term potentiation (LTP) ta a similar degree as synthetic Aβ dimers (Willem *et al.* 2015). In addition, inhibition of BACE1 leads to an increase in the levels of CTF-η and Aη-α at the same time as LTP is reduced (Willem *et al.* 2015). This should be taken into consideration when designing clinical trials with BACE1 inhibitors.

1.1.7 Biomarkers and diagnosis

Of all people living with dementia, only 20-50 % have been diagnosed in primary care (Winblad *et al.* 2016). Early and correct diagnosis is necessary in order to obtain support services and for the initiation of treatment available for symptomatic relief. Development of new, effective treatment also relies on early diagnosis since clinical trials seem to constantly fail when initiated at a stage when clinical symptoms of dementia are already too severe (Winblad *et al.* 2016).

A combination of clinical and neuropathological examinations is used in the diagnosis of AD, which today can be diagnostically classified as “possible”, “probable” or “definite” AD. People with AD and people without dementia can be distinguished with fairly high specificity using the clinical diagnostic criteria of today. However, the distinction between AD and other dementias is less accurate. Therefore, combining clinical assessment with computed tomography (CT) scan, magnetic resonance imaging (MRI) and other biomarker assessments is generally used for diagnosis (Reitz and Mayeux 2014; Ballard *et al.* 2011).

Different isoforms of Aβ as well as total and phosphorylated tau in the CSF are used as biomarkers for AD. Low levels of Aβ42 in the CSF and high levels of total tau and hyperphosphorylated tau distinguish AD patients from patients with other dementias. Combining different CSF biomarkers increases the specificity and sensitivity of the diagnosis (Hansson *et al.* 2006; Ballard *et al.* 2011). CSF biomarkers as well as imaging techniques are also used in research to predict conversion of MCI into AD. Markers of inflammation, oxidative stress and synaptic degeneration have potential as biomarkers and are currently being investigated (Ballard *et al.* 2011; El Kadmiri *et al.* 2017). Retrieving and analysing blood is less invasive, more cost effective and easier to perform in primary care

compared to CSF assessment. Consequently, blood biomarkers, e.g. different isoforms of Aβ in plasma, have high potential and are currently under investigation (Ballard *et al.* 2011; El Kadmiri *et al.* 2017). Unfortunately, blood biomarkers are still less specific and less accurate than are CSF biomarkers (El Kadmiri *et al.* 2017).

Effective and reliable biomarkers as well as accurate diagnosis are essential in order to monitor and predict AD. In addition, biomarkers are of outmost value for assessment of the outcome when developing and testing disease modifying treatments.

1.1.8 Risk and protective factors

AD is a multi-factorial disease with no known direct cause. The main risk factor for AD is advanced age and the prevalence is increasing with age, being 1 % at the age of 65 and up to 50 % at the age of 85 (Prince *et al.* 2013). Dietary and life-style factors contribute to the progression of AD which shares many risk factors with cardiovascular disorders, such as smoking, hypertension, obesity, hypercholesterolemia and diabetes. Depression and traumatic brain injuries also increase the risk of developing AD (Reitz and Mayeux 2014; Rakesh *et al.* 2017). Advanced depression may cause brain atrophy, reduced levels of neurotrophins and increased inflammatory responses in the brain; symptoms which are common also in AD. Consequently, treating depressive disorders will also reduce the risk of developing AD and dementia, as will treatment of cardiovascular disorders and diabetes (Rakesh *et al.* 2017). Long-term treatment with e.g. non-steroidal anti-inflammatory drugs (NSAIDs), cholesterol-lowering drugs, oestrogen and vitamin E have been shown to reduce the risk of AD (Silvestrelli *et al.* 2006).

Since higher education is associated with reduced risk of developing AD, cognitive reserve has been proposed to be protective (Stern 2012). Cognitive reserve refers to the brain's capacity to use pre-existing cognitive processes and compensation mechanisms to circumvent pathology. Cognitive reserve would therefore increase with education as a result of the high brain activity and could in this way contribute to delay cognitive decline in AD (Stern 2012).

Diet and lifestyle changes can greatly decrease the individual risk of developing AD by ameliorating the effect of the modifiable risk factors, such as those in common with cardiovascular disorders (Fig 3) (Reitz and Mayeux 2014; Rakesh *et al.* 2017). A Mediterranean diet reduces the risk of AD, proposedly since it to a large extent consists of plant-based food, olive oil and fish, which are high in essential nutrients and poly-

unsaturated fat (Gu *et al.*, 2010; Barnard *et al.*, 2014). Increased physical activity is also beneficial and the amount of activity appears to be more important for the prevention of AD than the type of activity (Groot *et al.* 2016; Chu *et al.* 2015; Rakesh *et al.* 2017). Yet, the molecular mechanisms behind this effect remain unknown but increased vascularisation, improved plasticity and elevated levels of neurotrophins have been suggested (Rakesh *et al.* 2017; Chu *et al.* 2015). Even later life physical exercise may protect against AD and improve cognition. An 8-year longitudinal study showed that exercise of at least 30 minutes reduced the risk of cognitive decline regardless of physical or mental health and previous cognitive or social activity (Chu *et al.* 2015). In addition, physical activity can improve cognition for MCI patients (Nagamatsu *et al.* 2013; Groot *et al.* 2016). A large-scale long-term randomised controlled trial demonstrated that multi-domain lifestyle changes, including restricted diet and a physical exercise training programme, have beneficial effects on cognition in relation to AD (Ngandu *et al.* 2015).

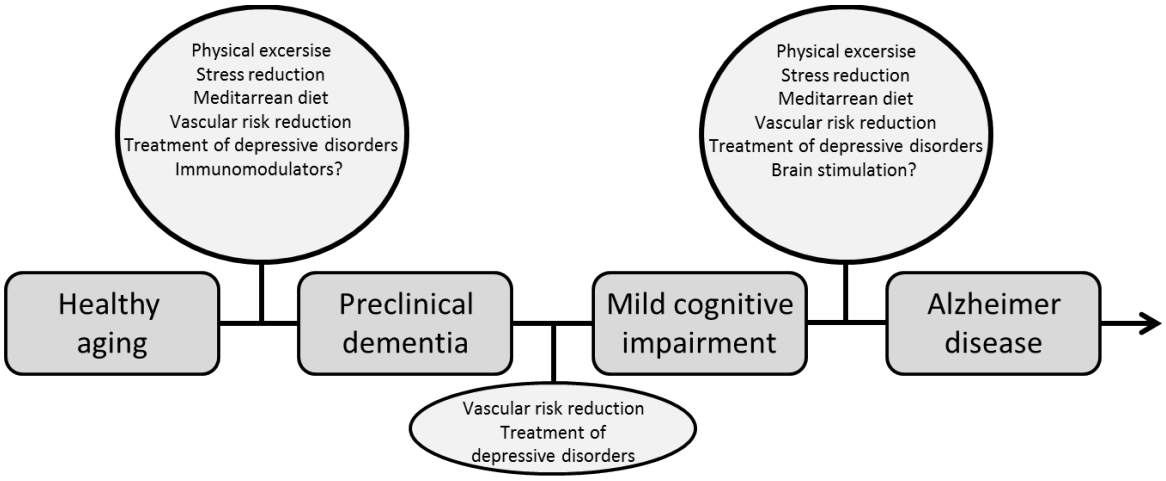

Figure 3. Graphic summary of possible promising preventive strategies at different stages of the development of Alzheimer disease. Adapted from Rakesh *et al.*, 2017.

Genetic risk factors that increase the risk of developing AD have also been identified. *APOE* is the most common risk gene associated with sporadic AD and the gene product, apolipoprotein E, is a lipid-binding protein involved in cholesterol metabolism. The ε4 allele of *APOE* greatly increases the risk of AD while the ε2 allele is associated with decreased risk (Raber *et al.* 2004). Genome-wide association studies have identified several other genes linked to an increased risk of AD, including complement receptor 1 (*CR1*), sortilin-related receptor 1 (*SORL1*), phosphatidyl-inositol binding clathrin assembly protein (*PICALM*) and clusterin (*CLU*) (Tosto and Reitz 2013). These risk genes can be clustered into four major pathways; 1) amyloid processing 2) inflammation, 3) lipid transport and

metabolism and 4) synaptic function/endocytosis (Tosto and Reitz 2013) emphasising the importance for these pathways in the development and progression of AD.

1.1.9 Treatment

Despite the fact that more than 100 years have passed since AD was first described, there still is no cure or treatment that effectively would stop the progression of the disease. Two classes of drugs are available but they only give symptomatic relief: choline esterase inhibitors and the N-methyl-D-aspartic acid (NMDA) receptor antagonist memantine (Silvestrelli *et al.* 2006).

The cholinergic system is severely impaired in AD, contributing to memory decline and cognitive impairment. Choline esterase inhibitors supress the degradation of the neurotransmitter acetylcholine at the synaptic cleft, thus enabling more acetylcholine to activate the postsynaptic cell and maintain neurotransmission (Wenk 2006). Treatment with choline esterase inhibitors can increase the synaptic levels of acetylcholine but is unable to stop the progression of AD.

Increased basal Ca^{2+} levels, in particular in glutamatergic neurons, are common in AD. The mechanisms are not clear but possibly involve dysfunctional mitochondria at synapses as well as increased Ca^{2+} entry through NMDA receptors (Wenk 2006). Memantine maintains normal neurotransmission by blocking extrasynaptic NMDA receptors which reduces excitotoxicity and excessive glutamate signalling. Memantine is a low-moderate affinity NMDA receptor antagonist with fast on/off kinetics which is crucial for its ability to inhibit excessive NMDA receptor stimulation while allowing normal, physiological neuro-transmission (Silvestrelli *et al.* 2006; Wenk 2006).

Reducing the levels of Aβ in the brain is a major treatment strategy where there currently is much focus. γ- and β-secretase inhibitors and GSMs which cause a reduction in Aβ production, have been in clinical trials, so far without success (Mikulca *et al.* 2014; Bachurin *et al.* 2017; Kandalepas and Vassar 2014). A phase I clinical trial of the BACE inhibitor verubecestat or MK-8931 markedly reduced the levels Aβ in the CSF (Winblad *et al.* 2016). Results from a phase III clinical trial in prodromal AD and MCI are expected in 2019. However, another phase III clinical trial with the same drug on mild to moderate AD patients had to be prematurely withdrawn due to lack of effect (Alzforum). As mentioned in section 1.1.4, no success has been achieved with GSIs or GSMs. Drugs targeting γ-secretase are rather associated with toxicity and adverse effects, probably due to

accumulation of other, toxic APP-derived fragments, off-target effects and dosage (Bachurin *et al.* 2017; Winblad *et al.* 2016).

Specifically reducing Aβ toxicity by destabilising or neutralizing oligomeric Aβ is a promising approach, however no major break-through has been made so far. Active and passive immunisation against Aβ has proven efficacious in animal models although not yet in humans (Silvestrelli *et al.* 2006; Wang *et al.* 2017; St-Amour *et al.* 2016). A phase II clinical trial of the AN1792 vaccine against Aβ42 had to be terminated due to the development of aseptic meningoencephalitis in 6 % of the participants. Yet immune-responding patients also showed reduced levels of Aβ in the brain and, importantly, a slight decline in the rate of cognitive impairment, emphasising the potential of the approach (Gilman *et al.* 2005; Masliah *et al.* 2005). Immunisation using modified antigens, such as truncated Aβ, which cause less immunological responses, is currently in clinical trials (St-Amour *et al.* 2016). BIIB37, or aducanumab, is a monoclonal antibody against a conformational epitope of Aβ. Positive, dose-dependent effects with improved cognition have been reported from a large phase I clinical trial and aducanumab will now proceed directly into phase III (Biogen web page; Sevigny *et al.*, 2016). Anti-tau vaccines have also been developed and some are in clinical trials (St-Amour *et al.* 2016). Stimulating regeneration to increase neurogenesis is a rather new and promising approach (Felsenstein *et al.* 2014).

In the Dominantly Inherited Alzheimer Network (DIAN) individuals with FAD are registered and studied. Pathological changes in the brain of these individuals often arise long before clinical symptoms (Winblad *et al.* 2016). The DIAN Trials Unit (TU) is a preventive trial with drugs targeting Aβ (immunisation and BACE inhibitor) to evaluate drug safety, tolerability and effectiveness. The main aim is to delay, prevent or even reverse the pathological changes and clinical symptoms of AD (clinicaltrials.gov).

Discovering reliable biomarkers for early diagnosis is urgently needed for the treatment of AD. Identifying preclinical stages of sporadic AD would enable design of clinical trials aiming at preventing the onset of dementia by reversing synaptic and neuronal dysfunction. For effective disease modifying treatment early initiation is necessary.

1.2 SYNAPTIC FUNCTION

Synapses are highly specialised structures at which neurons communicate. Remodelling of synaptic connections in the brain is a prerequisite for memory formation and enables us to

interact with the world around us. Synaptic dysfunction and loss of synapses, on the other hand, may impair brain network activity.

Simultaneous activity in nearby synapses leads to LTP which persistently strengthens and sensitizes the synapses for further stimulation. In a similar fashion does asynchronous stimulation decrease synaptic strength by causing long-term depression (LTD) (Kandel et al 2014). NMDA receptors play a pivotal role in synaptic plasticity. Depending on the amount of Ca^{2+} influx through these receptors and the subsequent signalling cascades activated at the dendrites, either LTP or LTD is induced (Kullmann and Lamsa 2007). LTP induces increased insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to the postsynaptic membrane and growth of dendritic spines whereas synapse loss and spine shrinkage is associated with LTD (Kullmann and Lamsa 2007). Impaired synaptic plasticity is often detrimental and can lead to cognitive decline and memory dysfunction, as is the case in AD (Spires-Jones and Hyman 2014).

1.2.1 Synaptic alterations in Alzheimer disease

The number of synapses in the brain decrease during normal aging, not only as a consequence of neurodegeneration. However, the synapse-to-neuron ratio is significantly lower in the brains of AD patients compared to elderly persons without AD, especially at brain areas particularly affected by AD. For example, at the hippocampus of AD patients the synapse-to-neuron ratio is up to 50 % lower than the ratio in the same area of non-demented elderly and adults (Bertoni-Freddari *et al.* 1990). At the cortex of AD patients there are 25-30 % less synapses than at the cortex of persons without AD and 15-35 % fewer synapses per cortical neuron (DeKosky and Scheff 1990; DeKosky *et al.* 1996; Davies *et al.* 1987). In line with this, the levels of both pre- and postsynaptic proteins are lower in AD patients compared to age-matched control subjects (Reddy et al., 2005). Accordingly, synaptic loss correlates better with cognitive decline than amyloid plaques, NFTs or neuronal loss (Terry *et al.* 1991; DeKosky and Scheff 1990).

1.2.2 Aβ at the synapse

Soluble forms of intracellular Aβ interfere with synaptic function and contribute to the loss of synapses and synaptic proteins (Selkoe 2008; Bayer and Wirths 2010; Musardo and Marcello 2017). Aβ co-localise with postsynaptic density-95 (PSD-95) protein in AD brain (Lacor *et al.*, 2004) and the levels of PSD-95 are decreased in the brains of AD patients in an Aβ dependent manner, correlating with the severity of dementia (Gylys *et al.* 2004).

Spine loss and reduced levels of AMPA receptors at the synaptic membrane is observed in parallel to the decreased PSD-95 levels (Gylys *et al.* 2004).

Aβ oligomers are present both pre- and postsynaptically, particularly in synapses surrounding amyloid plaques (Koffie *et al.* 2012; Pickett *et al.* 2016; Koffie *et al.* 2009) and impair synaptic structure and function both *in vitro* and *in vivo* (Hsia *et al.* 1999; Kamenetz *et al.* 2003; Mucke *et al.* 2000; Shankar *et al.* 2008; Walsh *et al.* 2002; Koffie *et al.* 2009). Aβ oligomers may activate caspases and calcineurin and thereby cause Ca^{2+} dys-homeostasis (Abdul *et al.* 2009; Kuchibhotla *et al.* 2008). Moreover, Aβ disturbs excitatory synaptic transmission, particularly by affecting AMPA and NMDA and receptor endo-cytosis and thus the availability of these receptors at the synapse (Musardo and Marcello 2017; Shankar *et al.* 2008; Hsieh *et al.* 2006; Li *et al.* 2009). Through these actions, oligomeric Aβ inhibits LTP and facilitates the induction of LTD (Koffie *et al.* 2011; Musardo and Marcello 2017).

Oligomeric forms of Aβ may also affect neuronal glutamate reuptake (Li *et al.* 2009) and cause an increase in glutamate release from astrocytes (Talantova *et al.* 2013). The excess of glutamate around the synapses may then activate extrasynaptic NMDA receptors which may cause excitotoxicity (Talantova *et al.* 2013).

Learning is disrupted in rats after injection of naturally secreted human soluble oligomeric Aβ (Cleary *et al.* 2005) and soluble oligomeric Aβ impairs synaptic plasticity also in neuronal cultures (Townsend *et al.* 2006). Oligomeric Aβ may induce cognitive deficits before amyloid plaque formation in APP transgenic mice (Westerman *et al.* 2002; Koistinaho *et al.* 2001).

Neurons of the AD affected brain appear to be presynaptically hyperactive and neuronal activity is impaired in AD, both at the level of network circuits and at individual synapses (Palop and Mucke 2010; Palop *et al.* 2007). Increased synaptic activity cause an increase in extracellular and decrease in intracellular Aβ levels (Cirrito *et al.* 2005; Kamenetz *et al.* 2003; Tampellini *et al.* 2009). Aβ may enter cells through membrane permeabilisation (Kayed *et al.* 2009) and endocytosis (Nath *et al.* 2012) but the mechanism by which Aβ is secreted is still unknown.

To prevent further synapse loss, inhibit excitotoxicity or even facilitate synaptogenesis, removal of oligomeric Aβ from synapses is an attractive approach. However, no anti-

amyloid immunotherapeutic studies aiming at clearing Aβ from the brain has yet been successful (Montoliu-Gaya and Villegas 2016).

2 AIMS

The main aim of this thesis was to investigate the secretion mechanisms of Aβ as well as the subcellular localisation of its precursor and the enzymes involved in its production at the synapse.

The specific aims of the individual papers were:

PAPER I: Activity-independent release of the amyloid-β peptide from rat brain nerve terminals

To test the hypothesis "Aβ is produced and stored in synaptic vesicles and released together with neurotransmitters during synaptic stimulation".

PAPER II: ADAM10 and BACE1 are localized to synaptic vesicles

To investigate the synaptic localisation of the APP processing enzymes.

PAPER III: Similar pre- and postsynaptic distribution of ADAM10 and BACE1 in rat and human adult brain

To investigate the *in situ* synaptic localisation of APP and the APP processing enzymes in rat and human adult brain.

PAPER IV: A 25 kilodalton amyloid precursor protein C-terminal fragment is abundant in human but not in mouse or rat brain

To investigate the levels of 25 kDa CTF as well as other APP-derived fragments in human AD and control brain and in the brains of other animals.

3 METHODOLOGICAL CONSIDERATIONS

3.1 ETHICAL CONSIDERATIONS

All studies in this thesis involving humans and other animals were performed in accordance with the declaration of Helsinki, the current European Law (Directive 2010/63/EU) as well as the local ethical review board and the guidelines at Karolinska Institutet or the guidelines at the university or facility where the animals were kept or the experiments conducted. The human *post mortem* brain tissue used in paper III was obtained from the Harvard Brain Tissue Resource Center at the NIH Brain Bank, US, while the human brain material used in paper IV was obtained from the Brains for Dementia Research, London, UK, and the Brain Bank at Karolinska Institutet, Stockholm, Sweden.

3.2 PREPARATION OF SYNAPTOSOMES

In order to study synaptic mechanisms without involvement of cell body events, synaptosomes were isolated from rat brain. Synaptosomes are pinched off and resealed nerve endings which, in case they are prepared from fresh brain tissue, are functional and may be stimulated to release neurotransmitters (Fig 4). Therefore synaptosomes have been extensively used to study the regulation of neurotransmitter release as well as other synaptic events under different conditions.

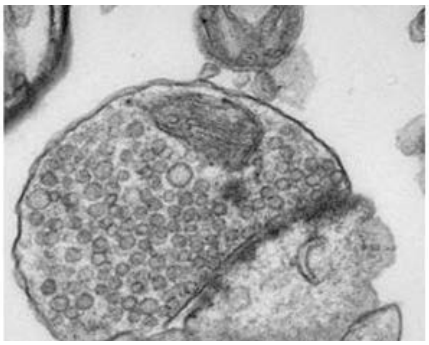

Figure 4. A synaptosome with a synaptic vesicle-filled presynpastic terminal, synaptic cleft and dense postsynaptic density. The presynaptic terminal also contain mitochondria.

Synaptosomes were prepared essentially as described before by Nicholls (1978). In brief, cerebellum and cortex of five weeks old Wister rats were homogenised in sucrose buffer using a pestle homogeniser. Nuclei and cell debris were first spun down. The subsequent supernatant was further centrifuged to collect synaptosomes, mitochondria and other membrane organelles in the pellet which was resuspended in sucrose buffer, layered on a discontinuous ficoll gradient (6-9-13 %) and centrifuged at 62 500 x g for 35 min at 4 ºC. The fraction containing synaptosomes was collected from the 9-13 % ficoll interface and centrifuged at 9 500 x g for 12 min at 4 ºC. The synaptosomes were then resuspended in sucrose buffer.

3.3 SYNAPTOSOMAL STIMULATION AND GLUTAMATE RELEASE ASSAY

KCl and 4-aminopyridine (4-AP) are widely used to experimentally induce action potentials and synaptic activity. 4-AP blocks voltage-gated potassium channels, thereby inhibiting potassium efflux which normally stabilises the membrane potential after the initial depolarisation caused by sodium influx. When potassium cannot leave the depolarised axon, the synapses remain active. Addition of KCl to a synaptosomal solution would increase the extrasynaptosomal potassium levels thus decreasing the concentration gradient of potassium between the (normally high potassium) cytosol and the (normally low potassium) extracellular environment. Potassium would not leave the synaptosomes against its chemical gradient, thus leaving the synapses continually active.

KCl- and 4-AP-induced glutamate release was measured by the conversion of NADP to NADPH by glutamate dehydrogenase. In the presence of water and NADP, glutamate dehydrogenase converts glutamate into α-ketoglutarate while NH_4^+ and NADPH are produced as by-products (Fig 5). Since NADPH is fluorescent and its levels directly correspond to the levels of glutamate in the solution, increased fluorescence in response to KCl or 4-AP directly correlates to glutamate release.

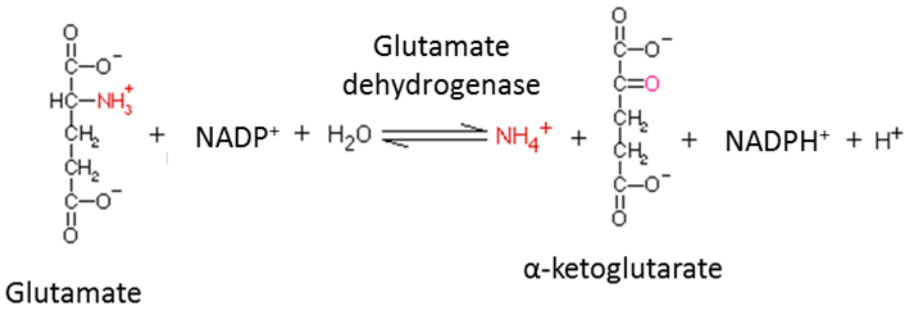

Figure 5. The reversible enzymatic reaction catalysed by glutamate dehydrogenase.

Synaptosomes were spun down and resuspended in sodium buffer containing protease inhibitors and subsequently placed in a fluorometer at 37 ºC. The synaptosomal solution was allowed to reach the correct temperature by incubation for 3 min. $CaCl_2$ or EDTA (a calcium chelator used as negative control) and NADP was subsequently added. After 1.5 min incubation glutamate dehydrogenase was added and the fluorescence measurement started. After another 5 min the fluorescence level had stabilised since the enzymatic reaction had had time to reach equilibrium, and KCl or 4-AP was added to a final concentration of 50 mM and 1 mM, respectively. The measurement continued for another 6 min.

In figure 6, glutamate release in response to KCl (a) and 4-AP (b) is presented as an increase in fluorescence when NADP is converted into NADPH in the presence of KCl or 4-AP. As EDTA is a calcium chelator, and calcium is necessary for SV exocytosis and thus neurotransmitter release, less glutamate is released in the presence of EDTA than $CaCl_2$. However, to our surprise, 4-AP was much less effective in inducing synaptic glutamate release (and thus synaptic activity) than KCl (Fig 6).

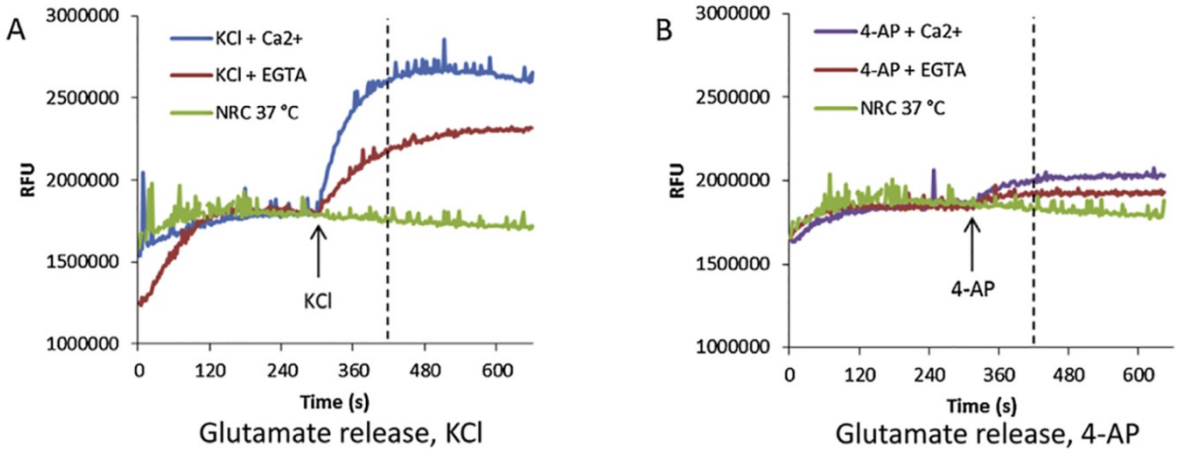

Figure 6. Glutamate release from synaptosomes in response to stimulation by KCl or 4-AP. Representative figures of glutamate release as assessed by conversion of NADP to fluorescent NADPH by glutamate dehydrogenase. 50 mM of KCl (a) or 1 mM 4-AP (b) was added at the indicated time-point. Fluorescence was measured in the presence (blue line for KCl and purple line for 4-AP) or absence (red line) of Ca^{2+}. NRC (non-reaction control, green line) denotes non-stimulated synaptosomes. Numbers denote relative fluorescence units (RFU). (From Lundgren *et al.*, 2014)

3.4 ENZYMATIC ACTIVITY ASSAYS

To determine ADAM10 enzymatic activity in brain homogenates and SVs, we used the commercial SensoLyte® 520 ADAM10 Activity Assay Kit which is based on the FRET substrate 5-FAM/QXLTM 520 with excitation/emission of 490/520 nm. The required reagents were provided in the kit and the manufacturer's protocol was followed. Homogenate or SVs were resuspended in reaction buffer in the presence or absence of the matrix metalloproteinase inhibitor GM-6001 and placed in a black 96 well plate. The enzymatic reaction was started by addition of substrate and fluorescence was measured every 5 min for 60 min at 37 °C at an excitation/emission of 485/520 nm.

3.5 PROXIMITY LIGATION ASSAY

Proximity ligation assay (PLA) is a sensitive method for *in situ* detection of proteins which are in close proximity to each other. It is superior to most other co-localisation methods as it only requires a distance of maximum 40 nm between the two labelled proteins in order to

give rise to a signal. Two proteins are labelled, first with primary antibodies and then with secondary oligonucleotide probes directed toward the primary antibodies. If the two proteins are in sufficiently close proximity (≤40 nm), the secondary oligonucleotide probes can be ligated, amplified and subsequently detected as a PLA-signal visualised by either a fluorescent or light microscope, depending on the PLA kit used (Fig 7) (Söderberg *et al.* 2008). As negative controls we either used only one of the primary antibodies or omitted primary antibodies altogether.

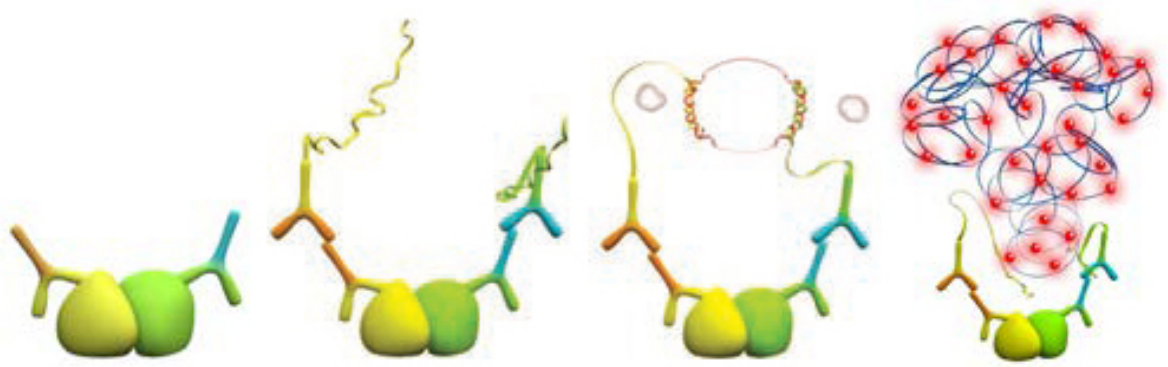

Figure 7. The proximity ligation assay method. The proteins of interest are labelled with primary antibodies which are recognised by secondary, oligonucleotide-labelled probes. If these probes are in less than 40 nm proximity, the oligonucleotides may be ligated and amplified by a rolling circle amplification mechanism and fused to fluorescently-labelled complementary nucleotides. Where the two proteins are in close proximity, fluorescent dots are detected by confocal microscopy. For brightfield PLA, detection and substrate solutions are also needed for production of signals visible by light microscope. (Amplified Detection, Duolink® Proximity Ligation Assay (PLA), Merck, Sigma)

In the present thesis fluorescent PLA was used in paper II to investigate the proximity of ADAM10, BACE1 and a probe for active γ-secretase with the SV protein synaptophysin in mouse primary hippocampal neurons. In paper III, we used PSD-95 in addition to synaptophysin, in order to also detect postsynaptic localisation of the secretases. In that paper we applied brightfield PLA since human brain often is auto-fluorescent which may interfere with the detection of fluorescent PLA signals.

An SV is about 40 nm in diameter and there are approximately 32 copies of synaptophysin per vesicle (Takamori *et al.* 2006). Thus, since all labelled proteins within 40 nm distance give rise to PLA signals, all proteins in an individual SV should give rise to PLA signals when labelled together with synaptophysin. This makes PLA a very sensitive method for co-localisation studies. However, we have found that it is challenging to quantify the results since PLA is not always very reproducible and large inter-experimental differences are

common. Careful sample preparation and handling seem to be extremely important and it is necessary to ensure the whole tissue section is covered during all incubation steps, especially during incubations at 37 ºC when the solutions may evaporate. Consequently, small variances might affect the results as PLA is such a sensitive method.

3.6 ACCELL siRNA

Cortical neurons isolated from 16 days old mouse embryos were seeded on poly-D-lysine coated 24 well plates. At 4 days *in vitro* (DIV) half of the Neurobasal media was exchanged to BrainPhys media and 1.5 mM Accell SMARTPool siRNA (APP or scrambled, non-coding control siRNA) was added. Half of the media was replaced by new BrainPhys media at 8 DIV. At 14 DIV, Alamar Blue cell viability assay was performed to ensure that the neurons were healthy and metabolically active. Thereafter the neurons were washed and lysed in Benzonase buffer.

Using this technique we have managed to very efficiently abolish the expression of some genes in our primary neuronal cultures. However, we have experienced large difficulties in silencing the expression of some other genes. Consequently, we are currently trying to better manage this technique. Yet, we suspect that our unsuccessful experiments are mainly results of varying efficiency of the different siRNAs.

4 RESULTS AND DISCUSSION

4.1 GLUTAMATE AND Aβ ARE RELEASED FROM SYNAPSES THROUGH DIFFERENT MECHANISMS

Aβ is continuously secreted from neurons (Moghekar *et al.* 2011) and increased synaptic activity cause an increase in Aβ release both *in vitro* and *in vivo* (Lazarov *et al.* 2002; Kamenetz *et al.* 2003; Cirrito *et al.* 2005; Cirrito *et al.* 2008). In addition, Wei *et al.* (2010) have demonstrated that Aβ released from both pre- and postsynaptic compartments can have local neurotoxic effects on dendritic spines and synaptic function. Our group and others have shown that Aβ can be produced at the synapse and that the SVs contain all components needed for Aβ production (Frykman *et al.* 2010; Groemer *et al.* 2011). In paper I we show that Aβ can be produced in pure SVs and hypothesised that it would be released from synapses through normal SV exocytosis during synaptic activity. We used KCl and 4-AP to induce synaptic activity in synaptosomes and subsequently measured the levels of glutamate and Aβ released into the extracellular solution. Contrary to what we expected, we found that Aβ secretion was not affected by synaptic stimulation, but that there was considerable activity-independent release of Aβ from synaptosomes kept at 37 ºC. Glutamate release, on the other hand, was highly dependent on synaptic activity (Fig 6).

In conclusion, we demonstrated that small amounts of Aβ can be produced in SVs but that Aβ is not released from nerve terminals through normal SV exocytosis. Consequently, the release mechanisms of Aβ and glutamate are different.

Contrary to our findings, Kim *et al.* (2010) observed a time-dependent increase in Aβ secretion from KCl-stimulated synaptosomes at 25 ºC. However, they had not included a negative control of unstimulated synaptosomes, thus it is not possible to know if the increase in Aβ release actually was activity-dependent.

By using synaptosomes, we were able to study the effect of synaptic activity on Aβ release without involvement of cell body events such as translation and trafficking of proteins. Dolev *et al.* (2013) demonstrated that presynaptic events (electrically induced spike bursts) affect the conformation of presenilin at the postsynaptic membrane, consequently increasing Aβ40 secretion and the extracellular Aβ40/42 ratio. Single spikes caused release of equal amounts of Aβ40 and Aβ42. However, in our system the secretion of both Aβ40 and Aβ42 was continuous and activity-independent, indicating that the effects observed by Dolev *et al.* (2013) were dependent on intact cells and possibly even on larger neuronal

circuits. Yet, we use other techniques than Dolev *et al.* (2013) to induce synaptic activity and this could possibly affect the results.

However, it is still also possible that the activity-dependent release of Aβ is dependent on intact postsynaptic compartments and/or entire dendritic spines. Delivering pre- or postsynaptically targeted APP to rodent primary hippocampal neurons by lentiviral transduction, DeBoer *et al.* (2014) found that more Aβ is released into the conditioned media from dendrites than from axons. Moreover, by two-photon microscopy of organotypic rat hippocampal slice cultures, Wei *et al.* (2010) demonstrated that spine density is decreased in the vicinity of both dendrites and axons of APP overexpressing neurons. This effect was absent in the presence of the γ-secretase inhibitor L685,458 indicating that it was Aβ dependent. Similarly, Wei *et al.* (2010) further demonstrated that Aβ released from both dendrites and axons also caused impairments of other synaptic functions. These findings support the notion that the activity-dependent release of Aβ might, at least in part, be of postsynaptic origin. Yet, more studies are needed to also clarify the role of protein trafficking and other cell body dependent mechanisms in the activity-dependent release of Aβ.

4.2 SYNAPTIC DISTRIBUTION OF ADAM10 AND BACE1

Aβ levels are affected by protein trafficking and the subcellular localisation of different proteins, especially the enzymes involved in its production (Haass *et al.* 2012). Genome-wide association studies put emphasis on the importance of protein trafficking in the pathology of AD. Many AD patients carry genetic variances in genes coding for several proteins involved in trafficking and endocytosis (Tosto and Reitz 2013). Because of the synaptotoxicity of Aβ and the known synaptic dysfunction and degeneration in AD, targeting trafficking of APP producing proteins could be an attractive therapeutic approach. This, however, requires careful understanding of how the trafficking is regulated, as well as knowledge of the precise cellular location of the APP processing enzymes.

Performing more Western blotting of the SVs from my first project, we found that both ADAM10 and BACE1, but only presenilin of the components of the γ-secretase complex, were highly enriched in SVs compared to total rat brain homogenate (Fig 8). Likewise, CTF-α and -β, the products of ADAM10 and BACE1 cleavage of APP, were also highly enriched (Fig 8). BACE1 has long been known to localise to the presynaptic compartment and Del Prete *et al.* (2014) have found this enzyme also in SVs. However, we use a different protocol for SV purification and found more enrichment of BACE1 in our highly pure SVs

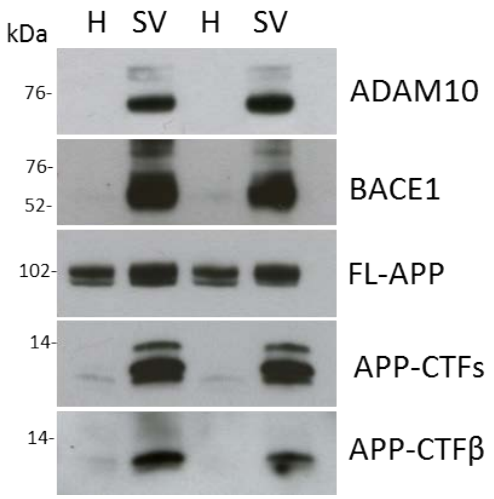

Figure 8. Western blot of rat brain homogenate and synaptic vesicles. ADAM10, BACE1 and the C-terminal fragments (APP-CTFs) are highly enriched in synaptic vesicle fractions (SV) compared to total brain homogenate (H).

compared to total brain homogenate than Del Prete *et al*. Moreover, in contrast to Del Prete *et al*., we also detected enrichment of the CTFs in SVs, indicating that ADAM10 and BACE1 are also active in the SVs. Indeed, we were able to show ADAM10 activity in SVs using an *in vitro* enzymatic activity assay although we did not succeed in detecting BACE1 activity. Yet, the enrichment of both the enzyme and cleavage product suggests that BACE1 is also active in SVs. The assays available are probably not optimal for biological samples since most BACE1 assays are based on recombinant protein as well as on synthetic substrates and were originally designed for compound screening. In spite of this, other groups have previously been able to measure BACE1 activity in human brain homogenate (Fukumoto *et al.* 2002; Li *et al.* 2004). Another option, though unlikely, is that the CTFs that are enriched in the SVs have been transported there from elsewhere.

Using PLA, we further demonstrated that a large amount of both ADAM10 and BACE1 was in close proximity to the SV protein synaptophysin in mouse embryonic primary hippocampal neurons. This was a new and interesting finding, since to our knowledge ADAM10 had not previously been reported to localise to the presynapse. In addition, since PLA of active γ-secretase and synaptophysin only gave rise to a few PLA signals, these results indicate that the secretases are present at different presynaptic locations, which might be a limiting factor for synaptic Aβ production. The CTFs produced in SVs might thus be trafficked to other synaptic compartment(s) for γ-secretase cleavage and Aβ production. Recycling endosomes, autophagic vesicles and exosomes have been reported to be involved in Aβ secretion (Rajendran *et al.* 2006; Nilsson *et al.* 2013; Udayar *et al.* 2013) and are therefore likely γ-secretase containing compartments where Aβ production could take place.

By using confocal microscopy and PLA of mouse primary neurons, Nigam *et al.* (2015) observed that much less C-terminally labelled APP co-localised with presynaptic proteins than did N-terminally labelled APP. However, in neurons from BACE1 knock-out mice, C-terminally labelled APP was increased presynaptically due to more full length (FL)-APP

(N-terminally labelled APP) being present (and probably processed) there. This could be considered contradictory to our findings of enrichment of CTFs in SVs. However, also AICD, generated by γ-secretase cleavage of CTFs, would be labelled with the APP C-terminal antibodies used by Nigam *et al.* The AICD functions as a transcription factor and is thus translocated to the nucleus directly after it is produced (Multhaup *et al.* 2015). Therefore, it could be assumed that the synaptic levels of AICD would be low. Consequently, APP C-terminal antibodies would strongly label perinuclear AICD during confocal imaging which could have a neutralising effect on the enrichment of CTFs we observe in SVs by Western blotting. We also show that the levels of FL-APP are somewhat enriched in SVs compared to total brain homogenate, although not to the same extent as the CTFs. However, this does not necessarily mean that there are more copies of CTFs than APP in SVs as the total amount of cellular APP probably exceeds the total cellular amount of CTFs. Regardless, it would be interesting to analyse the levels of the different APP-derived fragments in SVs isolated from BACE1 knock out animals as well as to perform the same set of PLA experiments on primary neurons or brain slices from these animals.

The findings of Nigam *et al.* (2015) are supported by those of DeBoer *et al.* (2014) who used lentiviral transduction of rodent primary neuronal cultures to demonstrate that surface FL-APP is primarily localised to axons while intracellular FL-APP as well as C-terminally labelled APP is more equally distributed between axons and dendrites. Thus, using confocal microscopy as performed by Nigam *et al.* (2015), it is quite evident that FL-APP would appear to co-localise more with presynaptic proteins than would C-terminally labelled APP. Yet, considering the great enrichment of CTFs in SVs, it reasonable to assume that the majority of presynaptic, axonal CTFs could reside in SVs, supporting our results.

Following up on these findings, in paper III we further investigated the synaptic distribution of ADAM10 and BACE1 as well as their substrate APP. Again PLA was used, but this time in sections of rat and human adult brain. In addition to synaptophysin, we also performed PLA of the secretases together with the postsynaptic protein PSD-95. We confirmed that both ADAM10 and BACE1 were present presynaptically and could demonstrate that they were both also found postsynaptically (Fig 9). Likewise, APP was also in close proximity to both synaptophysin and PSD-95, as well as to both ADAM10 and BACE1. We also confirmed the pre- and postsynaptic localisation of both ADAM10 and BACE1 by Western blotting of SV and PSD fractions from rat brain.

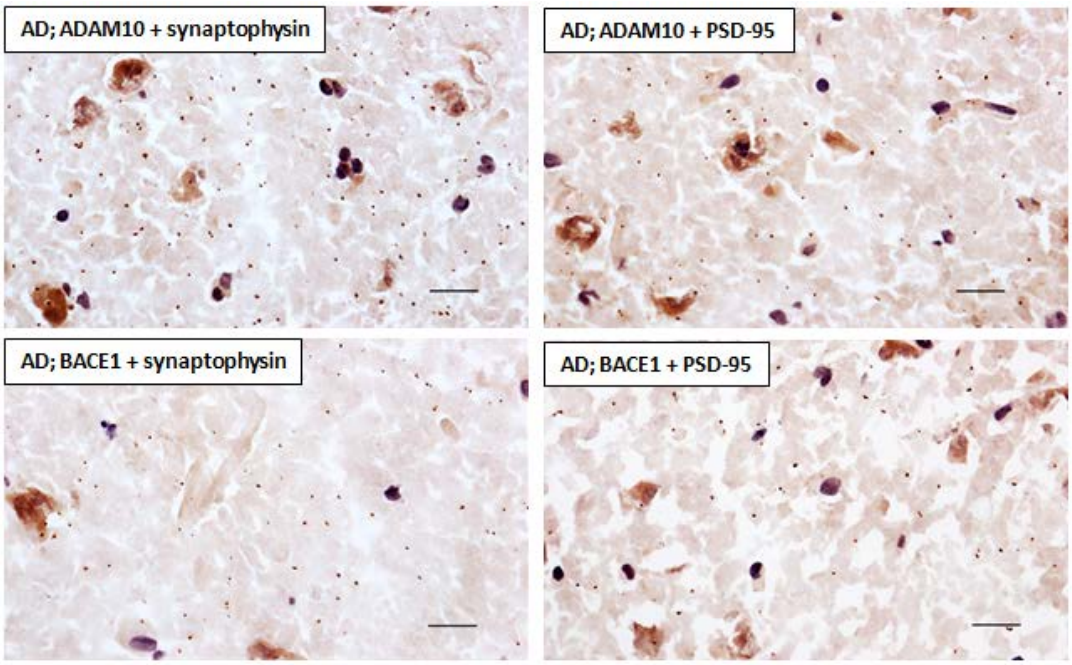

Figure 9. Proximity ligation assay of human AD brain. Human AD brain sections were subjected to PLA using antibodies toward ADAM10 or BACE1 and the presynaptic marker synaptophysin or the postsynaptic marker PSD-95. Both ADAM10 and BACE1 are localised both pre- and postsynaptically. Scale bar 20 µm.

PLA gives rise to signals if the two labelled proteins are within 40 nm distance from each other. Consequently, one drawback of using PLA when studying synaptic proteins is that the synaptic cleft is only approximately 20 nm. Thus, it is possible that labelled presynaptic proteins could give rise to PLA signals together with PSD-95, and that labelled post-synaptic proteins likewise could give rise to PLA signals together with synaptophysin. However, given the amount of signals generated in our experiments, we find it highly unlikely that such false positive signals would have an impact on our conclusions. In addition, we also confirmed our PLA results using subcellular fractionations both in Paper II and III.

Our results are further supported by the fact that both pre- and postsynaptically residing proteins have been identified as substrates for both ADAM10 and BACE1 (Munro *et al.* 2016; Zhu *et al.* 2016; Kuhn *et al.* 2016). Accordingly, our co-author Marcello *et al.* (2007) have previously shown enrichment of not only ADAM10, but also BACE1 in PSD fractions purified from mouse brain. Thus, we consider that the localisation of ADAM10 and BACE1 both pre- and postsynaptically has physiological relevance. Nevertheless, we were unfortunately unable to quantify potential differences between control and AD cases due to inter-experimental variations.

4.3 A 25 kDa C-TERMINAL FRAGMENT IS THE MOST ABUNDANT APP-CTF IN HUMAN BRAIN

When working with the first papers of this thesis, we used the Y188 APP antibody for Western blotting and repeatedly found a 25 kDa CTF to be the most abundant APP-derived fragment in human brain. This CTF was hardly detectable in rat and mouse brain. It has been shown that not only Aβ, but also APP-CTFs and AICD can exert neurotoxic effects (Passer *et al.*, 2000; Kim *et al.* 2003; Saura *et al.*, 2004; Bittner *et al.*, 2009; Jiang *et al.*, 2010; Lauritzen *et al.*, 2012), emphasising the importance of careful elucidation of APP processing and the different fragments produced thereof. In addition, both Wang *et al.* (2015) and Willem *et al.* (2015) had fairly recently discovered an APP-CTF, CTF-η, that migrates around 25 kDa on an SDS-PAGE gel. Hence, we considered it important to further study the pattern of APP-derived fragments in the brains of humans and other animals.

Using five different APP antibodies, we consistently found this 25 kDa CTF to be abundant in human brain homogenate, but present at much lower levels in rat brain homogenate (Fig 10). Yet, the ratio between this and other CTFs varied in human brain depending on which antibody we used for detection. Differences in epitope exposure and/or antibody affinity are probable reasons for this discrepancy. Other groups have previously shown that APP-derived fragments migrating between 20 and 30 kDa on SDS-PAGE gels are present in human brain (Haass *et al.* 1992; Tamaoka *et al.* 1992; Estus *et al.* 1992) although they have not thoroughly characterised or identified these fragments. However, we have found that different molecular markers do not migrate consistently on SDS-PAGE gels and different gel systems might also give rise to somewhat different protein patterns during Western blotting. Therefore it is likely that at least some of these CTFs are in fact identical to CTF-η and/or the 25 kDa CTF detected by us. Furthermore, when using 16 % tricine gels, which efficiently separate proteins of low molecular weight, we found that the 25 kDa CTF could actually be distinguished as two bands on the

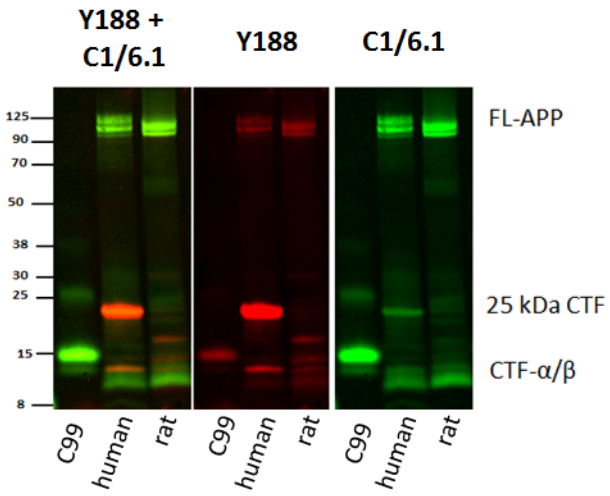

Figure 10. Pattern of APP-derived fragments in human and rat brain homogenate. Western blot of C99-flag (synthetic CTF-β) and human and rat brain homogenates. The Y188 and C1/6.1 antibodies recognising the C-terminal part of APP were used for detection. The molecular marker does not migrate correctly as C99 has a molecular weight of 12 kDa. The 25 kDa CTF is abundant in human brain but not in rat brain.

blots. This is in agreement with Wang *et al.* (2015) who observed that CTF-η is detected as "a cluster of two or more fragments close in size".

Even though we did not manage to immunoprecipitate the 25 kDa CTF from human brain homogenate, a strong indication of it being a true APP-derived fragment is the fact that it is absent in the lysates of APP siRNA treated mouse embryonic primary neurons. The 25 kDa CTF was hardly detectable in mouse brain homogenate. However, when loading human brain homogenate and mouse embryonic primary neurons on the same gel, we detected a 25 kDa CTF in untreated and scrambled treated mouse embryonic neurons migrating at the same position as the human 25 kDa CTF. Yet, the murine 25 kDa CTF was present at much lower levels. Accordingly, Willem *et al.* (2015) detected CTF-η in both adult and postnatal day 10 wild type mouse brain.

To further characterise the 25 kDa CTF, we examined its expression pattern in different species and found that it was present in human, guinea pig and macaque brain. At the same time, using the C1/6.1 antibody, a fragment migrating just above the 25 kDa CTF was detected in rat, mouse and, to some extent in guinea pig brain but not in human or macaque brain. Whether this band is the same CTF with other and/or more post-translational modifications or unspecific binding of the antibody to the membrane was unclear. Alternatively, the exact cleavage site could be different in different species or this could be yet another APP-derived fragment. In any case, the 25 kDa CTF was not present in the brains of transgenic mice expressing human APP with the Swedish/London mutation. This implies that it is not the human sequence of APP that determines the production of this fragment, but rather the environment in the human brain. However, production of the 25 kDa CTF in the brains of these transgenic mice might be competed out by the increased β-cleavage caused by the Swedish mutation of APP. However, although more β-cleavage of APP takes place in the brains of these transgenic mice, they also greatly overexpress APP (Westerman *et al.* 2002; Kawarabayashi *et al.* 2001) and therefore one could assume that there would be some production the 25 kDa CTF also in these mice if they had all component needed for the process.

Investigating the 25 kDa CTF in human CSF, García-Ayllón *et al.* (2017) observed increased levels of this fragment in both familial and sporadic AD patients as well as in aged Down syndrome individuals as compared to control CSF. However, when quantifying the 25 kDa CTF from Western blots of ten sporadic AD and ten control brain homogenates we were not able to detect any significant differences. Neither were there any apparent differences in the levels of this fragment between the familial AD and control brains we

analysed. Yet, we observed large inter-individual differences. Since CTFs are membrane-bound proteins, a general degradation of membranes in AD brain would therefore presumably release more CTFs into the CSF, whereas the CTF levels in the brain would more closely correlate to the total brain protein content. This could possibly explain why we, contrary to García-Ayllón *et al.* (2017), could not detect any differences in the brain levels of the 25 kDa CTF between AD and control subjects.

Altogether, these data reveal that a 25 kDa CTF is among the most abundant APP-derived fragments in human brain but not in rat and mouse brain. It is necessary to take the species difference into consideration when designing clinical trials based on previous animal experiments. Moreover, these differences may be a possible explanation as to why humans, but not rats and mice, naturally develop amyloid plaques and AD. Previous translational problems in AD drug discovery might partly be explained by this.

5 CONCLUSION AND FUTURE CONSIDERATIONS

Synaptic dysfunction is emerging as one of the earliest and most severe pathological hallmarks of AD (Selkoe 2002). This thesis contributes with knowledge about the synaptic localisation of particularly ADAM10 and BACE1, but also of γ-secretase. Moreover, we demonstrated that Aβ is continuously secreted from synapses in an activity-independent manner and that a 25 kDa CTF is the most abundant APP-derived fragment in human brain as opposed to rat and mouse brain where this fragment is barely detectable.

Although several studies have shown Aβ release to be activity-dependent (Cirrito *et al.* 2005; Tampellini *et al.* 2009; Kamenetz *et al.* 2003; Dolev *et al.* 2013) the mechanism of Aβ release is still elusive. We demonstrated that the mechanism of Aβ release is distinct from that of normal neurotransmission and concluded that intact cells are necessary for Aβ to be secreted from neurons. Nilsson *et al.* (2013) have demonstrated that conditional knockout of autophagy-related gene 7 (Atg7) resulted in reduced Aβ secretion and extracellular plaque load, implying a role of autophagy in Aβ secretion. Aβ release also appears to be associated with exosomes (Rajendran *et al.* 2006). We are currently investigating the role of protein trafficking and autophagy in the release of Aβ.

Understanding the mechanism of Aβ secretion is facilitated by understanding its production. To that end this thesis has contributed substantially. We showed that both the α-secretase ADAM10 and the β-secretase BACE1 are located both pre- and postsynaptically and that both enzymes are highly enriched in SVs. However, the activities of these two enzymes have contrasting effects since ADAM10 cleavage of APP precludes Aβ formation while BACE1 cleavage promotes it. Since nature seem to primarily promote the most energy-effective processes, it is conceivable that cleavage of APP in SVs is not random. Consequently, still unknown regulatory mechanisms probably direct the processing of APP in SVs towards either amyloidogenic or non-amyloidogenic processing. Alternatively, ADAM10 and BACE1 might be present in different SV pools. However, this is fairly unlikely since the highly pure SVs in which we detected enrichment of ADAM10 and BACE1 were isolated based both on density and size and were thus very homogeneous. Furthermore, since active γ-secretase is not enriched in SVs, the CTF-β need to be transported from the SVs to yet another cellular location for Aβ to be produced. Consequently, more research is still required in order to fully appreciate the regulation and location of APP processing.

Our finding of considerable species differences in the levels of the 25 kDa CTF is rather remarkable. Willem *et al.* (2015) has pointed out the possibility that the synaptotoxicity of Aβ

dimers might actually be attributable to Aη-α, which is produced after ADAM10 processing of CTF-η and is of the same size as Aβ dimers. Since the levels of the 25 kDa CTF in rat and mouse brain are very low, it is assumable that these animals do not have substantial amounts of Aη-α in their brains either. Consequently, they would be expected not to suffer from Aη-α and CTF-η induced toxicity. This is yet another reason as to why rats and mice are not to be considered optimal models for AD. Much more research should be done in order to enable replacement, or at least considerable reduction, of the use of animals in research. A lot is already being done in this regard, yet the use of transgenic animals is standardised in this field of research which renders it more difficult to develop alternative models.

It is fascinating that a novel APP processing pathway was identified as recently as 2015 (Wang *et al.* 2015; Willem *et al.* 2015), although the product of this processing had probably been observed and reported before (Haass *et al.* 1992; Tamaoka *et al.* 1992; Estus *et al.* 1992). Aβ was identified in the 1980s and its pivotal role in AD pathology has long been recognised (Selkoe 2011). However, there is still no Aβ modifying AD treatment and it is not known where Aβ production takes place, nor how the production is regulated or how Aβ gains toxicity and exerts its toxic effects. Furthermore, the mechanistic link between Aβ and synaptic dysfunction remains elusive. This sheds light on the extremely important role of basic research for the advancement of AD research and future development of AD therapies. If we do not understand APP processing and the physiological and pathophysiological roles of Aβ, we will not cure AD. Yet, however we may be able to alter Aβ levels in the brain, we will not cure AD if we do not simultaneously ameliorate the cognitive dysfunction and memory loss.

In summary, development of effective treatment strategies for AD is urgently needed and dependent on the findings from basic research. Therefore this thesis facilitates future development of AD therapies and is thus of outmost importance for the AD field.

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