The physiological processing of Alzheimer-associated amyloid beta precursor protein in human and animal-derived neuronal models

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To my very dear sister, mother and father
Anneme…

-Enough is as good as a feast
Aβ42

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ABSTRACT

Alzheimer's disease (AD) is characterized by cognitive impairment due to the loss of structure and/or function of neurons, and amyloid plaques composed of aggregated-amyloid beta (Aβ) peptides, primarily species ending at the amino acid 42 (Aβ42), are one of the major neuropathological hallmarks of AD. Aβ peptides of different lengths are produced by sequential cleavage of amyloid beta precursor protein (APP) by α-, β- and γ- secretases. Aβ peptides are often considered "toxic", but they are also involved in many biological processes such as neuronal differentiation and synaptic activity. Therefore, this thesis aims to increase the understanding of APP and Aβ regulations by investigating when, where and how APP is processed in cortical neurons and how this is linked to neuronal maturation and synaptic activity.

In Project I, we measured secreted Aβ peptides during cortical differentiation of human induced pluripotent stem cells (iPSCs) and showed that APP processing changes during differentiation. In neuroprogenitor cells (NPCs), APP is predominantly processed via the non-amyloidogenic pathway (α-/β-secretase) producing short Aβ peptides, whereas with the formation of a neuronal phenotype and increased synaptic function, the processing of APP shifts towards the amyloidogenic pathway (β-/γ-secretase) producing longer Aβ peptides. Next, we hypothesized that secretion of the longer,

potentially amyloidogenic Aβ peptides requires a neuronal phenotype-dependent co-localization of APP and APP-cleaving enzymes. **Project II** thus aimed at investigating if co-localization of APP with APP-cleaving enzymes could explain the changes in Aβ secretion. We showed that APP co-localization with PSEN1 (γ-secretase) correlated with secretion of the longer Aβ peptides, supporting our initial hypothesis. **In Project III**, we differentiated the NPCs in a culture medium designed to increase synaptic activity, to investigate the effects of accelerated neuronal and synaptic maturity on APP processing, and showed that increased neuronal maturity and activity increased the secretion of Aβ peptides along with sAPPα/β. We also showed that the secretion of Aβ peptides in our model was regulated in part, but not entirely, by synaptic activity. **In Project IV**, we investigated if reducing Aβ secretion by inhibiting APP-cleaving enzymes would affect synaptic transmission and showed that reduction in Aβ42 exceeding 50% decreased synaptic transmission, suggesting that Aβ42 (or altered APP processing) may have a regulatory effect on the synaptic activity in a concentration-dependent manner.

In conclusion, we found that APP is differentially processed depending on neuronal and synaptic maturation and presented a platform for future studies targeting APP/Aβ function and dysfunction.

Keywords: Alzheimer's disease, APP, Aβ, human iPSCs, cortical neurons, BACE1, PSEN1, neuronal activity, neuronal differentiation

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SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom karaktäriseras av nedsatt kognitiv förmåga på grund av att nervceller i de delar av hjärnan där minnen bildas bryts ner. Ett tydligt kännetecken för Alzheimers sjukdom är att det bildas aggregat av peptiden amyloid-beta (Aβ) i hjärnan. Aβ-peptider av olika längd produceras då proteinet amyloid beta precursor protein (APP) klyvs av olika kombinationer av enzymer, så kallade sekretaser. Aβ-peptiderna anses ofta vara skadliga på grund av deras inverkan på Alzheimer sjukdom, men de är också inblandade i ett flertal biologiska processer. Dessa innefattar bland annat processer då nervceller utvecklas och bildar nätverk, samt då nervcellerna kommunicerar med varandra. Många läkemedel mot Alzheimers sjukdom har varit inriktade på att minska produktionen av Aβ-peptider i hjärnan, men hittills har de flesta kliniska studier med sådana läkemedel fått avbrytas på grund av allvarliga bieffekter. En anledning till detta skulle kunna vara att de naturliga funktionerna hos peptiderna också påverkats negativt av behandlingarna. Det övergripande målet med försöken presenterade i den här avhandlingen är att öka förståelsen för hur APP och dess klyvningsprocesser regleras och vad de har för funktion då nervceller utvecklas och kommunicerar med varandra.

I **Projekt I** undersökte vi hur utsöndringen av olika långa Aβ-peptider förändrades under utvecklingen av nervceller. För att göra detta använde vi oss av en metod där celler framtagna från hudbiopsier från donatorer i laboratoriet tillbakabildas till stamceller, så kallade inducerade pluripotenta stamceller (iPSC), med förmåga att bilda vilken annan celltyp som helst. Stamcellerna kan i sin tur utvecklas vidare till nervceller och användas som modell för hur nervceller beter sig i hjärnan. Under utvecklingen från stamcell till nervcell mätte vi utsöndringen av Aβ-peptider och såg att korta Aβ-peptider utsöndrades under den tidiga nervcellsutvecklingen, medan de längre Aβ-peptider som ses i aggregat i hjärnan hos patienter med Alzheimers sjukdom började utsöndras först då nervcellerna mognat och bildat fungerande synapsnätverk. Klyvningen av APP förändrades alltså under

nervcellernas utveckling och **Projekt II** syftade till att ta reda på om den här förändringen kunde förklaras av var i nervcellerna som APP träffar på de sekretaser som samverkar för att bilda Aβ-peptiderna. Genom att använda oss av en teknik som kan mäta samlokalisation av APP med de olika sekretaserna kunde vi se att samlokalisation av APP med γ-sekretas, det enzym som står för bildandet av de långa Aβ-peptiderna, ökade då utsöndringen av de långa peptiderna ökade. I **Projekt III** undersökte vi hur utsöndringen av de långa Aβ-peptiderna hängde ihop med hur aktiva synapserna var. För att göra detta, odlade vi nervcellerna i ett speciellt cellodlingsmedium framtaget för att öka synapsaktivitet. Vi såg att odling i detta medium både ökade hur aktiva nervcellernas synapser var och dessutom skyndade på nervcellsutvecklingen. Vi såg också att utsöndringen av de långa Aβ-peptiderna ökade och att detta till viss del var beroende av synaptisk aktivitet. I **Projekt IV** undersökte vi hur molekyler som använts i kliniska studier för att minska aktiviteten hos ett av de sekretaser som står för produktionen av Aβ skulle påverka nervcellernas kommunikation med varandra. Vi såg att en sänkning av Aβ med mer än 50% minskade nervcellskommunikationen, men att en modest sänkning inte hade någon negativ påverkan.

Sammanfattningsvis visar vi att klyvningen av APP förändras under nervcellsutvecklingen och att utsöndringen av långa Aβ-peptider beror av nervcellernas mognad och till viss del av synaptisk aktivitet. Vi presenterar också en modell för framtida studier av funktion- och dysfunktion hos APP och Aβ.

TÜRKÇE ÖZET

Alzheimer hastalığı ("AH"), bunama hastalıklarının en sık rastlanan çeşitlerinden biri olup zihinsel bozukluk ve hafıza kaybına yol açar. AH'na sahip bireylerin beyinlerindeki sinir hücreleri ölmüş, işlevlerini ve yapısal özelliklerini kaybetmiştir. AH'nin en önemli patolojik bulgusu beyindeki amiliod plaklardır. Amiliod plaklar, amiloliod-beta (Aβ) adı verilen peptidlerden, özellikle de Aβ42'den, oluşmaktadır. Aβ peptitleri çeşitli boyutlarda olabilir ve amiloid-beta-öncülü proteinin (APP) farklı enzimler (α-, β- and γ- sekretaz) tarafından kesilmesi sonucu meydana gelir. Aβ peptitleri genellikle toksik olarak değerlendirilseler de birçok biyolojik işlevin gerçekleştirilmesinde; örneğin sinir hücrelerinin farklılaşmasına ve sinir hücrelerinin aktivitelerini gerçekleştirmesine katkı sağlar. Bu yüzden, bu tez çalışmasında APP ve Aβ`nin hücreler tarafından nasıl düzenlendiği araştırılmakla birlikte özellikle, APP´nin ne zaman, nerede ve nasıl işlem gördüğünün ve bu işlemlerin sinir hücrelerinin gelişimi ve aktiviteleri ile nasıl bir ilişkide olduğunun ortaya çıkarılması amaçlanmıştır.

Proje I´de, hiPSCs (indüklenmiş pluripotent insan kök hücresi) ´ni kortekste yer alan sinir hücrelerine dönüştürülmüş, bu süreç boyunca Aβ peptitlerinin nasıl salgılandığı incelenmiş ve Aβ salgılanma profilinin nasıl değiştiği gösterilmiştir. Kısaca; sinir öncül (NP) hücrelerinde APP´ nin daha çok anti-amiloid yollar ile (α-/β-sekretaz aracılığı ile) kesildiği, kısa Aβ peptitlerinin meydana geldiği ve öncül hücrelerin sinir hücreleri formunu almasıyla APP´nin daha çok amiloid yollar ile (β-/γ- sekretaz) kesildiği ve uzun-agregasyona meyilli Aβ peptitlerinin meydana geldiği ortaya konulmuştur. Buradan yola çıkarak uzun-agregasyona meyilli Aβ peptitlerinin meydana gelmesi için sinir hücresi fenotipinin ve APP ile APP kesici enzimlerin (sekretazlar) kolokalizasyonunun gerekli olduğu öne sürülmüştür. Bu sebeple **Proje II**´de, APP ile APP kesici enzimlerin (sekretazlar) kolokalizasyonunun, Aβ salınımı ile olan ilişkisi incelenmiştir. Bu projede uzun-agregasyona meyilli Aβ peptitlerinin salınımının artan APP-PSEN1 (γ-sekretaz) ile ilişkili olduğu gösterilmiştir.

Proje III´de, artan sinir hücresi olgunluğunun ve aktivitesinin, uzun-agregasyona meyilli Aβ peptitlerinin salınımına olan etkisinin incelemenmesi

amacı ile NP hücreleri, sinir hücrelerinin aktivitesini arttırdığı bilinen bir besi yerinde sinir hücrelerine dönüştürülmüştür. Sonuçlarda, artan olgunluk ve aktivitenin Aβ peptitlerinin salınımını ve β-site kesimini artırdığı gözlemlenmiştir. Aynı zamanda bu projede Aβ peptitlerinin salınımının tamamen olmasa da kısmen sinir hücrelerinin aktivitesi ile düzenlendiği ortaya konulmuştur.

Proje IV´de, β-sekretaz aktivitesi farklı BACE (β-sekretaz) inhibitorleri ile inhibe edilerek azaltılmış Aβ salınımının, sinir hücrelerinin aktivitesi üzerindeki etkileri incelenmiştir. Bu projede % 50´den daha fazla azaltılmış Aβ42 salınımının sinir hücrelerinin iletişimini olumsuz etkilediği bulgulanmıştır. Bu sonuçlar, Aβ42 peptidinin ya da değişmiş APP kesiliminin sinir hücrelerinin iletişimini düzenleyici görevini ve bu görevin doza bağlı olduğunu önermektedir.

Özetle bu tezde; APP´nin sinir hücrelerinin olgunluk ve aktiviteye bağlı olarak kesime uğradığı gösterilmiş ve APP/Aβ biyolojik fonksiyonlarının ve işlev bozukluklarının çalışılabileceği bir sinir hücresi-kültür sistemi sunulmuştur.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Bergström P, Agholme L, Nazir FH, **Satir TM**, Toombs J, Wellington H, Strandberg J, Bontell TO, Kvartsberg H, Holmström M, Boreström C, Simonsson S, Kunath T, Lindahl A, Blennow K, Hanse E, Portelius E, Wray S, Zetterberg H**.** Amyloid precursor protein expression and processing are differentially regulated during cortical neuron differentiation.
Scientific Reports 2016 Jul 7; 6:29200

II. **Satir TM**, Roselli S, Camacho R, Agholme L, Zetterberg H. Bergström P. Relationship between neuronal Aβ secretion and co-localization of APP with APP-cleaving secretases.
Manuscript

III. **Satir TM**, Nazir FH, Vizlin-Hodzic D, Hardselius E, Blennow K, Wray S, Zetterberg H, Agholme L, Bergström P. Accelerated neuronal and synaptic maturation by BrainPhys medium increases Aβ secretion and alters Aβ peptide ratios from iPSC-derived cortical neurons.
Scientific Reports 2020 Jan 17; 10: s41598-020-57516-7

IV. **Satir TM**, Agholme L, Karlsson A, Karlsson M, Karila P, Illes S, Bergström P, Zetterberg H. Partial reduction of amyloid β production by β-secretase inhibitors does not decrease synaptic transmission.
Submitted

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ABBREVIATIONS

AD	Alzheimer´s disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
APLP	APP like protein
ApoE	Apolipoprotein E
APP	Amyloid beta precursor protein
APs	Action potentials
Aβ	Amyloid beta
BACE1	Beta site APP-cleaving enzyme 1
BMPs	Bone morphogenetic proteins
CNS	Central nervous system
CR	Cajal–Retzius
CSF	Cerebrospinal fluid
CT	Cycle threshold
CTF	C-terminal fragment
DAPI	4’,6-Diamidino-2-phenylindole
DIV	Days in vitro
ECL	Electrogenerated-chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
FAD	Familial Alzheimer’s disease
GABA	Gamma-Aminobutyric acid
GFAP	Glial fibrillary acidic protein
hiPSCs	Human induced pluripotent stem cells
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IP	Immunoprecipitation
iPSCs	Induced pluripotent stem cells
LTP	Long term potentiation

MAPT	Microtubule-associated protein tau
MEA	Multielectrode arrays
MS	Mass Spectrometry
NfL	Neurofilament light
NMM	Neuronal maintenance media
NPCs	Neuroprogenitor cells
NSCs	Neural stem cells
PAX6	Paired Box 6
PCR	Polymerase chain reaction
Pen-2	Presenilin enhancer-2
PFA	Paraformaldehyde
PLA	Proximity ligation assay
PSEN	Presenilin
RA	Retinoic acid
RG	Radial glia
SAD	Sporadic Alzheimer's disease
sAPPα	Soluble amyloid beta precursor protein alpha
sAPPβ	Soluble amyloid beta precursor protein beta
SVZ	Subventricular zone
TGFβ	Transforming growth factor beta
TGN	Trans-Golgi network
TM	Transmembrane
TMD	Transmembrane domain
TREM2	Triggering Receptor Expressed On Myeloid Cells 2
TTX	Tetrodotoxin
VZ	Ventricular zone

1 INTRODUCTION

"Pray, do not mock me.
I am a very foolish fond old man,
Fourscore and upward, not an hour more nor less.
And to deal plainly
I fear I am not in my perfect mind.
Methinks I should know you, and know this man.
Yet I am doubtful, for I am mainly ignorant
What place this is, and all the skill I have
Remembers not these garments. Nor I know not
Where I did lodge last night. Do not laugh at me,
For as I am a man, I think this lady
To be my child Cordelia."

King Lear by William Shakespeare - 1606

1.1 ALZHEIMER´S DISEASE

1.1.1 History

Dementia (from the Latin *demens*, without mind) is a term used to describe the decline in mental abilities, such as impaired capacity to remember, reduction in thinking abilities that affects daily life, emotional problems and difficulties with language and eating, which progress with age [1]. Mental deficiency and cognitive impairment among elderly people have long been a recognized condition. Memory decline was already acknowledged in ancient Egypt, showing that dementia has been present in human history long before it was named [2]. In the Early Ages, dementia was described as an irreversible, chronic disorder. However, in the Middle Ages, due to the domination of theocracy, dementia was referred to as punishment of man's sins; thus, research into dementia was prevented [3].

As medical knowledge and research progressed, dementia was reported as a form of "vascular dementia" for the first time in an academic treatise in the 17th century [4], and was accepted as a medical diagnostic term in the 18th century [3]. In the mid-18th and early 19th centuries, it became possible to distinguish between age-related dementia and other mental disorders, and "senile (old age) dementia" became a defined term for the condition. Although impossible to explain, loss of brain weight was considered the cause of senile dementia in the early 19th century. Increasing numbers of dementia cases, due to alcoholism and central nervous system (CNS) syphilitic infection, then allowed medical doctors to perform more autopsies on demented brains. They found that the cerebral atrophy observed in dementia was actually the result of cell death that was often associated with cerebrovascular changes, causing a decrease in the blood supply to the cells. With time, arteriosclerotic (thickening, hardening and loss of elasticity) brain atrophy and atheromatous (abnormal accumulation of lipids) degeneration of blood vessels were accepted as the major causes of senile dementia [5].

In the late 19th century, improved staining techniques made it possible to clearly visualize the cellular components of neurons and helped to identify neurofibrils among the neurons in demented brains [6]. At a meeting in Tübingen in November, 1906, using these new staining techniques, Dr. Alois Alzheimer described new histopathological findings from a brain that belonged to Auguste Deter, who had died from clinically unusual dementia at the age of 55. A year later, Dr. Alzheimer published his case report, describing the pathology as "excessive neurodegeneration accompanied with neurofibrils and widespread presence of plaque pathology" (for English translation [6]). This was the first time that neurofibrillary tangles were described in relation to dementia. However, Dr. Oscar Fischer had already described the cerebral plaques in relation to neuronal alterations in senile dementia [7]. In his paper, Dr. Alzheimer reported Auguste Deter's case as an undefined disease, due to her "young age, rapid course of the disease progression and severity of the neuropathology" [6]. In 1910, one of the most famous psychiatrists at the time, Emil Kraepelin, called this undefined disease, "Alzheimer's disease", a special type of dementia: "pre-senile dementia".

Today, we acknowledge Alzheimer's disease (AD) as the most common form of dementia, causing progressive memory loss due to the impaired structure and function of the synapses in the cerebral cortex, irrespective of age, and characterized by neurofibrillary tangles and deposition of amyloid plaques [8].

Identification of AD was a cumulative effort. Thus, besides Dr. Alzheimer, we should also acknowledge Dr. Max Bielschowsky and Dr. Franz Nissl for their contribution to biochemical staining techniques, Dr. Oscar Fischer for his significant contribution to the identification of

plaque pathology, as well as many other scientists who worked hard to document their observations on demented brains and Auguste Deter for her collaboration [2].

1.1.2 Neuropathology

AD brains display atrophy, especially at the medial temporal lobes due to degeneration and death of neurons [9]. As a result of cortical thinning due to the loss of brain tissue, the lateral ventricles are enlarged in the AD brain [9].

At the microscopic level, AD is characterized as the presence of β-folded protein sheets: i) intracellular neurofibrillary tangles, ii) extracellularly accumulated amyloid-containing plaques, and iii) cerebrovascular amyloid fibril deposition. A low molecular weight protein component, 4-kDa, in the plaques, amyloid beta (Aβ), has been shown to be the major component of the amyloid plaques in AD [10]. The complete amino acid sequence of the Aβ led to the identification of its precursor protein, the amyloid beta precursor protein (APP) [11].

Amyloid plaques are considered to be a neuropathological hallmark of AD and consist of Aβ peptides, which are the cleavage products of APP (described in more detail in **paragraph 1.3**) [8]. Amyloid plaques are commonly classified as diffuse and core plaques according to their morphology and their staining abilities with thioflavin-S [12], known to bind beta sheet-rich structures [13]. Dense core plaques are mostly made up of accumulated fibrillar amyloid peptides and the core of the plaque stains positive with thioflavin-S. Dense core plaques are associated with cognitive impairment and surrounded by reactive astrocytes and active microglia and thus linked to neuronal loss and synaptic toxicity [12], whereas thioflavin-S-negative diffuse plaques do not cause glial responses or neurotoxicity and can be found in the brains of cognitively healthy, elderly individuals [12, 14]. Therefore, diffuse plaques are not considered to be a pathologic marker of AD.

In addition to amyloid plaques, neurofibrillary tangles (NFTs) are another pathological hallmark of AD, which were first described by Dr. Alzheimer [6]. NFTs consist of hyperphosphorylated tau proteins [8]. Tau proteins are microtubule-binding proteins contributing to axonal stability and neurite extension [15]. Like many other proteins, tau is also a post-translationally modified protein. However, pathologically, hyperphosphorylated tau inhibits its own microtubule assembly activity, triggers self-aggregation and segregates other microtubule organizing proteins from each other, and, in turn, disturbs microtubule assembly [16-18]. Therefore, intra-neuronal accumulation of tau is considered to be the major reason for the axonal degeneration observed in AD [19].

1.1.3 Amyloid cascade hypothesis

The exact cause of AD is still a matter of debate; however, among a number of hypotheses, the "Amyloid Cascade Hypothesis", proposed in 1991, is the best accepted and most studied hypothesis [20, 21]. According to the amyloid cascade hypothesis, imbalance between the production and clearance of Aβ is the leading disease factor [20]. Gradually, increased accumulation and oligomerization of Aβ42 cause the formation of plaques in the brain parenchyma, along with an inflammatory response and increased tangle formation, and eventually cause neurotoxicity and synaptic loss leading to cognitive decline (explained in **Figure 1**) [22].

Down syndrome patients display AD pathology at an early age, due to trisomy in the region of chromosome 21 that contains the *APP* gene [23]. Therefore, in the amyloid cascade hypothesis, the aggregation and deposition of Aβ peptides have been suggested to be the initiating events for the AD pathology [20]. Since the amyloid cascade hypothesis was formulated, literature supporting this hypothesis has accumulated. For example, mutations in the *APP* gene, which result in increased Aβ peptide production, were discovered and shown to cause dominantly inherited familial AD (FAD) [24, 25]. More mutations resulting in increased Aβ production were later discovered in the presenilin 1 and 2 genes (*PSEN1*, *PSEN2*) that encode the catalytic subunits of γ-secretase, which is one of the main APP-cleaving enzymes (described further in **paragraph 1.3.3**). The mutations in *PSEN1* account for most of the dominantly inherited FAD, while the mutations in *APP* and *PSEN2* are seen less often [26-28].

APP is sequentially cleaved by β- and γ-secretases and gives rise to Aβ peptides of different lengths (discussed more in **paragraph 1.3.4**). However, the 40 and 42 amino acid-long peptides, Aβ40 and Aβ42, are more abundantly produced [29], and the increased ratio of Aβ42 to Aβ40 in cell culture media from FAD patient cells suggested that Aβ42 is the pathogenic Aβ peptide [30]. Furthermore, supporting this claim, cerebrospinal fluid (CSF) concentrations of Aβ42 were found to be decreased along with the decreased Aβ42/Aβ40 in AD patients and this ratio was reversely correlated with the amyloid load in the AD brain [31]. This signifies that the Aβ42 is the peptide that accumulates to become the amyloid plaques in the AD brain [31].

Even though some cases of AD are the familial from, which is dominantly inherited and also related to early onset of the disease (typically between age 30 and 60) [32], almost 95% of the AD cases are the sporadic form, which begins later in life (above age 65, late onset AD) and is thus called sporadic AD (SAD).

Even though there is no defined cause of SAD, some important risk factors are suggested, including both genetic and environmental factors [32]. One of the most important genetic risk factors for SAD is the apolipoprotein E (*APOE*) gene, which plays an

important role in lipid metabolism [33]. *APOE* has three common alleles (ε2, ε3 and ε4), with the ε4 allele being a risk factor, while the ε2 allele is considered to protect against AD [34]. ApoE was found to be co-deposited with Aβ in amyloid plaques of AD brains, indicating a relationship between Aβ and apoE in the pathogenesis of AD [35]. Aβ peptides are predominantly produced in neurons and are cleared by i) proteolytic degradation [36], ii) cellular clearance by neurons [37], astrocytes and microglia cells [38], iii) interstitial fluid drainage [39], or iv) are transported out of the brain via the blood-brain barrier [40]. Problems occurring in one of these pathways induce Aβ accumulation into amyloid plaques extracellularly and even sometimes inside neurons [41]. ApoE is mainly produced by glial cells and assists Aβ clearance by activating enzymatic degradation and phagocytic pathways in the microglia [42]. Cells carrying the *APOE* ε4 allele have reduced capacity to induce these pathways [42]. Thus, *APOE* ε4 is a risk factor for SAD in relation to Aβ clearance and thus also supports the amyloid hypothesis. Another risk factor for SAD is mutations in *TREM2* (triggering receptor expressed on myeloid cells 2). The microglia regulate plaque dynamics [43] and TREM2 has been shown to assist microglia cell proliferation around the plaques [44]. Mutations in *TREM2* that negatively affect the activity of TREM2 cause increased amyloid plaque diffusion and, hence, enhance amyloid-related neuronal damage [44].

Despite a large number of studies regarding Aβ as the target for treatment, most of the clinical trials have failed and, thus, the amyloid cascade hypothesis was targeted as concern of relevance [45]. The reason may be that the treatments were started too late in the disease progression or/and AD may also be caused by Aβ-independent factors [46]. Indeed, there are new studies showing that APP and its cleavage products may cause the neuronal cell death observed in AD, regardless of Aβ [47]. On the other hand, another reason for the failures in clinical trials may be imperfect knowledge of the physiological roles of APP and Aβ. For this reason, our aim in this thesis is to contribute with some insights regarding the physiology of APP and Aβ.

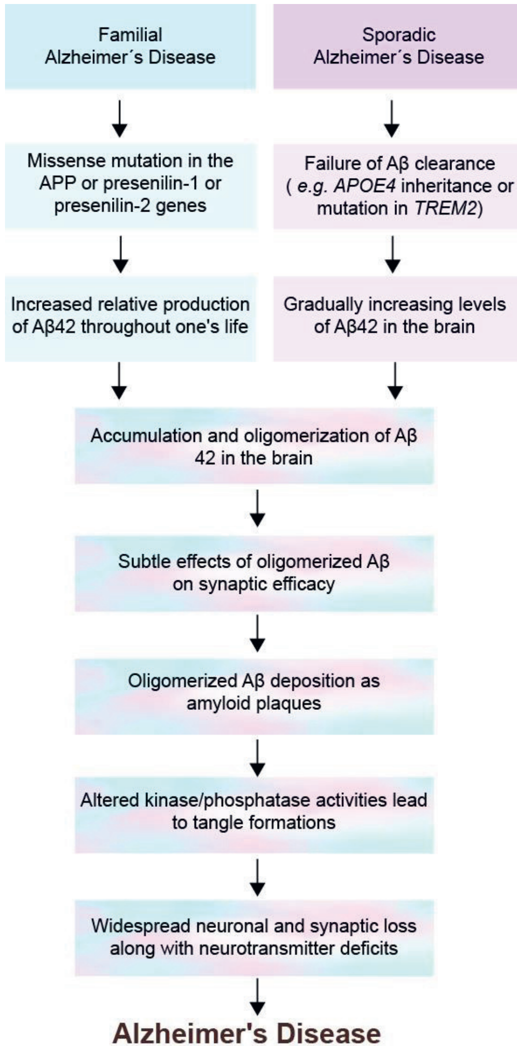


Figure 1. ***The amyloid cascade hypothesis***
The amyloid cascade hypothesis proposes that progressive Aβ42 accumulation is the main cause of both familial and sporadic forms of AD. Due to increased levels, Aβ42 accumulates and eventually forms oligomers. These oligomers sequentially cause plaques, tangle formation, toxicity and inflammation. This in turn causes synaptic loss, cellular death and eventually AD [22].

1.2 The amyloid precursor protein

Human APP is a member of a larger family of APP proteins, including APP-like proteins (APLP1 and APLP2) [48]. APP, APLP1 and APLP2 are single-pass transmembrane proteins with large extracellular glycosylated N-termini and shorter cytoplasmic C-terminal domains

[49]. The large ectodomain of APP shares common individual domains with both APLP1 and APLP2 (**Figure 2**) [50]. APLP1 and APLP2 are both processed in a similar manner as APP, but they do not contain the Aβ sequence [51].

The human *APP* gene is located on the distal arm of chromosome 21q21 and contains at least 18 exons [49]. It undergoes several alternative splicing events, ranging in size from 639 to 770 amino acids (**Figure 2**). The major splicing isoform in the brain is APP_{695} and this variant has been extensively investigated with regard to AD [49].

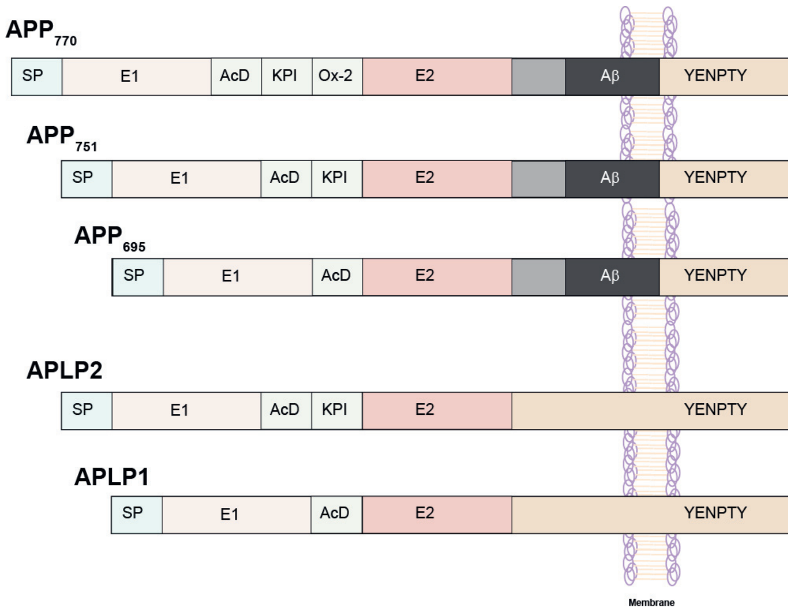


Figure 2. ***Domain structure of the isoforms of APP and APP-family proteins.***
All the isoforms and the family proteins of APP share a conserved large extracellular domain and a short cytoplasmic tail with the YENPTY motif. However, only APP has the Aβ sequence and it is unique to APP [50]. They all carry a signal peptide domain (SP) at the beginning of their N-terminal domain [51]. They are all multi-domain proteins with E1, E2 and a transmembrane binding domain [52]. The E1 domain contains the growth factor-like domain (GFLD), which holds the first heparin-binding domain (HBD) inside and the copper-binding domain (CuBD). The E2 domain contains a second HBD, which plays a role in cellular iron transfer [53], and the REMS motif, which may have roles in the trophic function of APP-family proteins [54]. The E1 and E2 domains are shared domains among the family members as well as in the APP isoforms [51]. Linked to the E1 domain, there is an acid-rich domain (AcD), which is rich in glutamic acid and aspartic acid [51]. The AcD is another shared domain among the proteins. In APP_{770}, APP_{751} and APLP2 following the AcD, there is the Kunitz-like serine protease inhibitory domain (KPI), which is shown to be involved in iron homeostasis [55]. However, APP_{695} and APLP1 do not contain this domain. Following the KPI domain, there is the Ox-2 antigen domain, which only exists in the APP_{770} isoform [51].

1.3 Ectodomain shedding of APP by the main APP-cleaving secretases

Like other transmembrane proteins, APP undergoes ectodomain shedding and proteolytic processing, mainly through two alternative pathways: i) the "amyloidogenic pathway", which generates long and aggregation-prone Aβ peptides, and ii) the "non-amyloidogenic pathway", which prevents the production of the aggregation-prone peptides.

"Ectodomain shedding" is the term used for the first proteolytic cleavage of transmembrane proteins, which may then be followed by additional cleavages at the remaining transmembrane (TM) domain of the shedded protein [56]. The "shedding" refers to the liberation of a protein's ectodomain from the membrane to an extracellular space, and the enzymes performing these activities are consequently called "sheddases" or "secretases" [56].

Shedding at the TM domain of the proteins does not only occur at the plasma membrane, but also at the membranes of subcellular organelles that are involved in secretory and recycling pathways [57]. Depending on the subcellular site of the shedding, the ectodomain part of the protein is liberated and secreted either into the extracellular or into the lumen of the organelle.

Sheddases are divided into two categories: i) sheddases cleaving their substrates at the juxtamembrane domain, in close proximity to the membrane, are called "canonical sheddases/secretases" ii) the ones cleaving their substrates within the TM domain or at the membrane anchored part are called "non-canonical sheddases/secretases". α- and β-secretases belong to the "canonical sheddases/secretases" family while γ-secretase belongs to the "non-canonical sheddases/secretases" family [56].

APP has three identified main cleavage sites. While two of them are located in close proximity to the TM domain, the third is located within the TM domain. Starting from the amino terminus side of APP, the cleavage sites are termed β-, α- and γ-cleavage sites, respectively [58], and the three main proteases that perform cleavage at these sites are called β-, α- , and γ-secretases [52].

Briefly, APP is initially cleaved by either α- or β-secretase, generating membrane-bound α- or β-C-terminal fragments (CTFα/CTFβ), and soluble α- or β-fragments (sAPPα/sAPPβ) that are released extracellularly [52]. When CTFs are cleaved by γ-secretase, AICD (an intracellular domain of APP) is produced along with Aβ peptides of different lengths [59].

1.3.1 α-Secretase

Ectodomain shedding of APP by α-secretase is a key regulatory event preventing the generation of long and aggregation-prone forms of Aβ peptides, as it cleaves APP within the Aβ domain (**Figure 3**). When APP is initially cleaved by α-secretase, secreted sAPPα and a membrane-bound C- terminal fragment-α (CTFα or C83) are generated. This fragment can be further processed by γ-secretase generating Aβ17-40/42 (also known as p3) [60]. Thus, the initial cleavage of APP by α-secretase precludes the formation of full length Aβ [60] and is therefore called the non-amyloidogenic pathway (**Figure 3**).

In 1994, α-secretase was shown to have the characteristics of metalloproteases. Different metalloproteases have been shown to cleave APP and overexpression of these enzymes increase APP cleavage at the α-site [61]. The most studied α-secretases are the members of a disintegrin and metalloprotease (ADAM) family, such as ADAM9, ADAM10, ADAM17 and ADAM19 [61]. ADAM10 is the most studied and best characterized canonical α–secretase [62] with regard to APP processing and AD, due to its harmonized expression with APP in the human brain that is seen less often for other ADAM family members [63].

1.3.2 β-Secretase

β-secretase cleavage of APP is the initial step for generation of long and aggregation-prone Aβ peptides, and this cleavage pathway is therefore called the “amyloidogenic pathway”. β-secretase cleaves APP at the N-terminus of the Aβ domain and generates extracellular sAPPβ and membrane-anchored CTFβ (C99), which contains the entire Aβ domain (**Figure 3**). CTFβ can then be further cleaved by γ-secretase, giving rise to Aβ peptides of different lengths. These peptides can start at the first amino acid of the Aβ sequence and end at the amino acid 37-43 [64].

Like the other sheddases, β-secretase only cleaves membrane-bound substrates [65]. The β-secretase activity is detected throughout the body [66], but is most active in the brain, especially in neurons [67, 68]. Interestingly, it has also been shown to be active in astrocytes [69], although less than in neurons. Still, due to the high content of astrocytes in the brain, even a slight increase in β-secretase activity in the astrocytes may contribute greatly to the production of Aβ [70].

BACE1 (beta-site APP-cleaving enzyme 1) is the major enzyme performing β-secretase activity in neuronal tissue and is essential for the generation of Aβ peptides. BACE1 is well known due to its involvement in AD pathology. For example, there is both increased protein expression and increased activity of BACE1 in the AD brain [71-73]. Further, a mutation in *APP* (called the Swedish mutation), which results in elevated BACE1 cleavage and thus increased Aβ generation, is linked to FAD [74], and another mutation in *APP*

(called the Icelandic mutation), which alters the BACE1 cleavage site of APP and reduces Aβ production, instead protects against AD [75]. Consequently, BACE1 has been investigated with regard to AD and has been a target for AD treatment.

1.3.3 γ-Secretase

γ-Secretase plays an important role in both the amyloidogenic and the non-amyloidogenic pathways. While the amyloidogenic pathway involves the sequential cleavages of APP by β- and γ-secretases, the non-amyloidogenic pathway involves the sequential cleavages of α-secretase and γ-secretase. Cleavage of CFTs by γ-secretase liberates different lengths of Aβ peptides and AICD [60].

γ-Secretase is a multi-protease complex consisting of four subunits: presenilin 1 (PSEN1) or presenilin 2 (PSEN2), nicastrin, anterior pharynx defective-1 (Aph-1), and (presenilin enhancer-2 (Pen-2) [76, 77]. While all these four subunits are required for the γ-secretase complex to be active, PSEN1 and PSEN2 contain the aspartyl residues constituting the critical catalytic domain of γ-secretase activity [78, 79]. Nicastrin acts as a gatekeeper for substrate entry into the active part of the γ-secretase complex [80], although it is not actively involved in cleavage. If the ectodomain part of the substrate is long, nicastrin would not allow the substrate to reach the active domain. Hence, the substrate first needs to undergo membrane shedding for γ-secretase to perform the cleavage [81]. For that reason, APP needs to first be cleaved by either α- or β-secretases for γ-secretase to be able to bind to the membrane-anchored part of APP (CTFs) at the TMD and generate Aβ peptides. This is the reason why γ-secretase belongs to the non-canonical secretases [82].

PSEN has two forms, which are encoded by two individual genes called *PSEN1* and *PSEN2* [83]. The γ-secretase complex exerts diverse activities and various sub-cellular localizations and that depends on whether PSEN1 or PSEN2 are present in the γ-secretase complex [84]. *PSEN1* knockout mice show abolished γ-site cleavage of APP, such as decreased turnover of CTFs and decreased production of Aβ [85]. Therefore, PSEN1 is widely studied with regard to APP cleavage, as well as in this thesis.

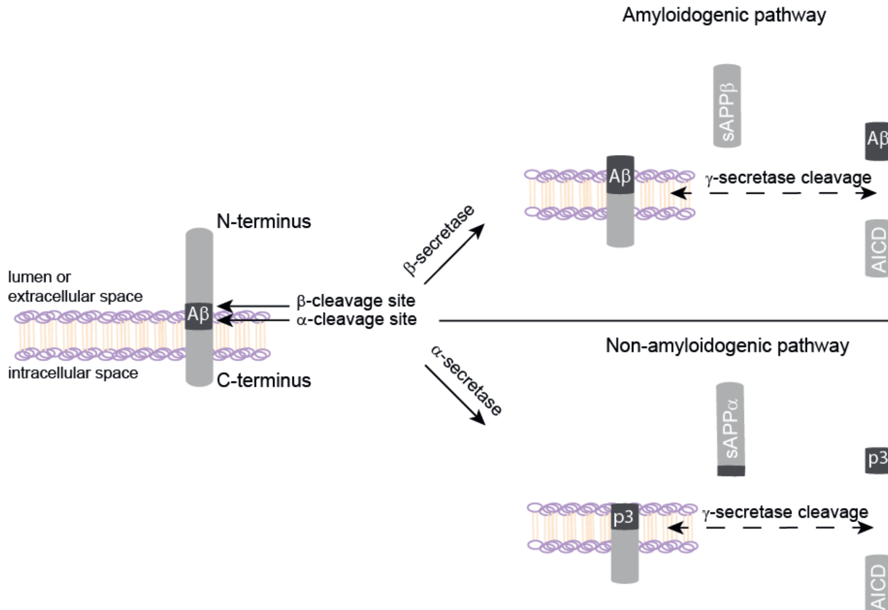


Figure 3. ***Schematic illustration of the proteolytic procession of APP.***
APP can be cleaved via two alternative pathways: The amyloidogenic pathway (upper picture), where APP is cleaved sequentially by β- and γ-secretases, producing amyloidogenic Aβ peptides, or the non-amyloidogenic pathway (lower picture), where APP is sequentially cleaved by α- and γ-secretases, producing p3 peptides. In both pathways, AICD is also produced on the inside of the membrane.

1.3.4 Production of Aβ peptides

The sequential cleavage of APP by α-, β-, and γ- secretases, respectively, gives rise to at least 15 different Aβ peptides, ranging in length from 14 to 43 amino acids [86]. (**Figure 4**). The length of the produced Aβ is determined by the enzyme that cleaves APP first (β- or α-secretase).

-***The non-amyloidogenic pathway***

α-Secretase cleaves APP within the Aβ domain at amino acid 16/17. If α- and β-secretase acts on the same APP molecule, several shorter Aβ fragments such as Aβ1-15 and Aβ1-16 are produced that may act like endogenous neuromodulators [87]. In addition, β-site cleavage can be followed by other β-secretase cleavages, giving rise to short Aβ peptides, such as Aβ1-19 and Aβ1-20 (**Figure 4**, upper panel).

-***The amyloidogenic pathway***

When APP is initially cleaved by β-secretase, membrane-anchored CTFβ is produced and binds to the active site of γ-secretase. Their proteolysis starts with ε (epsilon) cleavage, at

amino acid 49/48. This cleavage is then followed by ζ (zeta) cleavage at amino acid 46/45, and finalized by γ-site cleavage at amino acids 37, 38, 39, 40 and 42. Aβ1-42 is the peptide considered to be the most aggregation-prone form of Aβ and is produced when CTFβ is initially cleaved at amino acid 48 by ε-cleavage, followed by γ-site cleavage [88]. (Figure 4, lower panel)

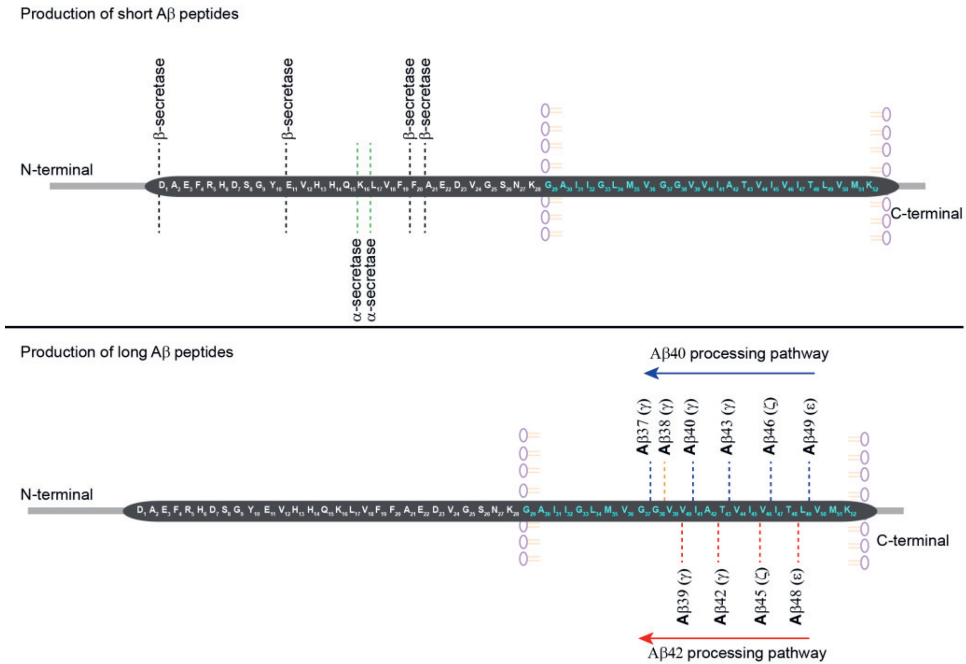


Figure 4. Sequential processing of the Aβ domain by α-, β- and γ-secretase.

Production of short Aβ peptides: α- and β-secretases can cleave the Aβ domain at different amino acids and give rise to short Aβ peptides such as Aβ1-15, Aβ1-16, Aβ1-19 and Aβ1-20 [86]. Production of long Aβ peptides [88]: γ-Secretase cleaves the Aβ domain at multiple sites within the TM domain. The stepwise cleavage of γ-secretase at the Aβ domain begins within the ε-site, just after amino acid 49 or 48. This cleavage is then followed by ζ-site cleavage at amino acid 46 and 45 and terminates with γ-site cleavage, predominantly at amino acid 42 or 40 (but also at amino acids 43, 39, 38 and 37). Aβ42 is produced when ε-site cleavage is initiated at amino acid 48 and followed by cleavage after amino acid 45 and terminated at amino acid 42. Aβ40 is produced when ε-site cleavage takes place at amino acid 49 and is followed by cleavages at amino acids 46 and 43.

1.4 Trafficking of APP and APP-cleaving enzymes in neurons

The subcellular localization of proteins is a determining factor for their interaction with other proteins. Thus, in order for proteins to function correctly, they need to be transported to the correct subcellular compartments. As other membrane proteins, APP is constantly being trafficked through the cells during its maturation. After translation, nascent APP undergoes several posttranslational modifications during its intracellular trafficking in secretory, endosomal/lysosomal and recycling pathways [89]. In the secretory pathway, APP is trafficked from the endoplasmic reticulum (ER) via the Golgi to the plasma membrane and can be cleaved by secretases during its way to the plasma membrane. Once APP reaches the plasma membrane, it is either cleaved there or reinternalized into early endosomes to go through the endosomal/lysosomal and recycling pathways. During its subsequent trafficking through endocytic and recycling organelles to the trans-Golgi network (TGN) or back to the cell surface, APP can interact with different combinations of α/β, and γ-secretases, producing different APP cleavage products [89]. Thus, the complex proteolytic processing of APP is regulated to a large extent by cellular distribution of APP and APP-cleaving enzymes [90].

β-Secretase has its maximum activity in acidic compartments like endosomes [91], but is also active in the Golgi and TGN. This indicates that the active site of β-secretase is located in the lumen of these subcellular compartments [92, 93]. However, β-site cleavage of APP occurs within endosomal compartments as β-secretase needs an acidic environment to function [94-96]. ADAM10 is mostly active in the TGN, at secretory pathway compartments and at the plasma membrane. Therefore, the α-secretase-mediated APP cleavage predominantly occurs on the cell surface, as well as in the TGN [97]. Therefore, plasma membrane retention of APP favours the non-amyloidogenic processing of APP whereas the retention in acidic organelles favours the amyloidogenic processing [98].

Proteolytically active PSEN1 was found to be localized in the plasma membrane and the endosomal/lysosomal system including phagosomes and autophagosomes [99]. At which site of the Aβ sequence where γ-secretase would cleave APP has been shown to depend on the subcellular localization of the enzyme, as well as the membrane properties of the compartment where it is localized [100]. Therefore, the subcellular compartment where APP interacts with the active γ-secretase has a big impact on the length of the Aβ produced.

Neurons have a complex transportation system compared with other cells, due to their polarized morphology [101]. Once APP leaves the TGN, it is transported to the axons and dendrites via post-Golgi transport vesicles [102]. While α-secretase cleavage mainly occurs on the cell surface, it has recently been shown that active ADAM10 co-localizes with BACE1

in axons and dendrites [103], as well as in synaptic vesicles [104]. The similar distribution of ADAM10 and BACE1 in synaptic vesicles, along with enriched CTFs, suggests that initial cleavage of APP may also take place in synaptic vesicles [104]. However, neither Aβ nor active γ-secretase was found to be enriched in synaptic vesicles, implying that the γ-secretase cleavage of CTFs for Aβ production may mostly take place elsewhere [104, 105]. Moreover, γ-secretase is present in synapses and distal axons [106], and both BACE1 and APP have also been shown to be co-transported along the axons and localized in dendritic vesicles [107]. However, given the complex morphology of neurons and the variable properties of γ-secretase, it is still not clear where γ-secretase processes APP. Yet, the amyloidogenic processing of APP is believed to occur mainly in the endocytic organelles [88] (**Figure 5**). For this reason, in this thesis we focused on the localization of APP, PSEN1 and BACE in the endosomal/lysosomal system.

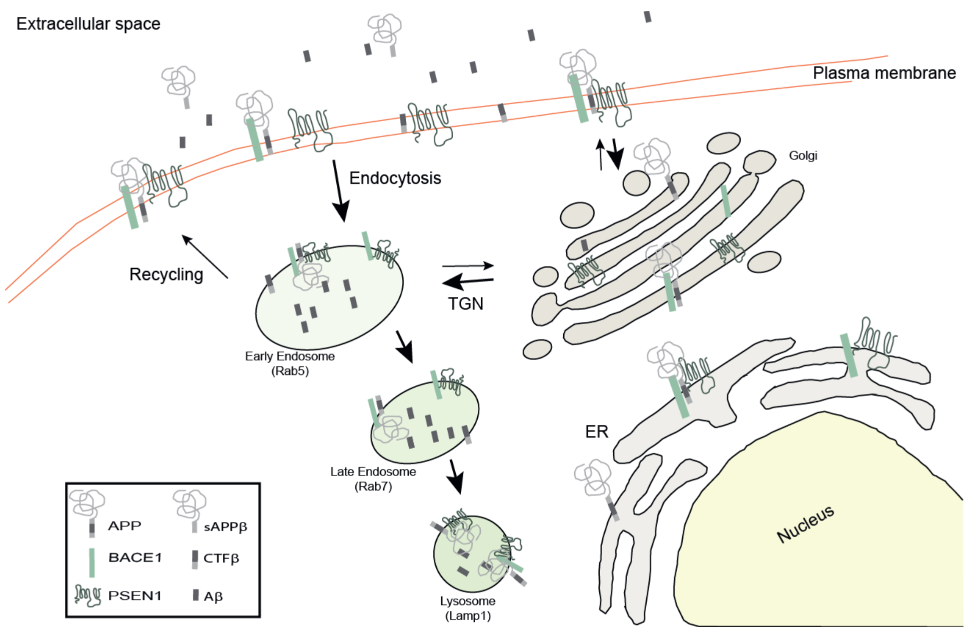


Figure 5. Endosomal/lysosomal trafficking of APP, PSEN1 and BACE1.
Once translated, nascent APP, BACE1 and PSEN1 are trafficked from the endoplasmic reticulum (ER), to the Golgi and the trans-Golgi network (TGN) to the cell membrane. During this trafficking, they interact with each other and APP can be cleaved. Intact APP or CTFs can be endocytosed from the plasma membrane to early endosomes and can then be trafficked to late endosomes and lysosomes. Through this trafficking,intact APP or CTFs can be cleaved by PSEN1 or BACE [88].

1.5 Physiological function of APP and APP cleavage products

The APP family of proteins is evolutionarily preserved across a variety of species, including vertebrates and invertebrates [48]. However, prokaryotes, plants and yeasts do not have APP family proteins, and the presence of APP family proteins appears to have emerged with the evolution of a nervous system with functional synapses [48]. Therefore, APP and its cleavage products cannot be solely associated with AD, as they have also been shown to be involved in many biological functions [108], such as neuronal development and neurogenesis [109, 110]. In order for neuronal networks to be built and reach functional maturity, neurons go through complex stages. In the early stages of neuronal development, newborn neurons migrate from the subventricular zone (SVZ) to the cortical plate, which is a strictly controlled event where APP works together with cell adhesion molecules [111, 112]. Cell-cell adhesion provides mechanical and chemical connections between adjacent cells [113], so it is an important event for synaptic plasticity and nervous system development [114]. The extracellular domain of APP family proteins has cell adhesion properties and can promote cell-cell interaction, thereby possibly promoting the generation of functional synapses [115]. Indeed, APP-overexpressing HEK cells that were co-cultured with primary neurons were shown to increase the synaptogenesis in contacting axons [116]. Moreover, APP interacts with several extracellular matrix (ECM) molecules [117]. For example, APP binds to reelin and, together with integrin, promotes neurite outgrowth and dendritic arborization [118]. For that reason, the ancestral APP may be the transmembrane protein that is responsible for the interactions at the synaptic junctions in the early CNS evolution as well as in neuronal development.

APP is expressed throughout the early development, and its expression increases and reaches its highest level coincidentally with the formation of neuronal structures, particularly in the neural tube and developing motor neurons [119]. In addition, APP is highly expressed in radial glial cells, suggesting its possible contributions to neurogenesis, neuronal proliferation and differentiation [120, 121]. Moreover, APP has been shown to be involved in synaptogenesis, memory formation, and increased neural stem cell proliferation by acting as a growth factor [122, 123].

Besides the biological roles of full-length APP, its cleavage products are also involved in physiological cellular processes [124]. Both sAPPα and sAPPβ are involved in neuronal differentiation [125]. sAPPα and sAPPβ share domains N-terminally, which promotes neurite and axonal outgrowth [126]. On the other hand, their C-terminals are slightly different from each other [127]. This difference gives sAPPα a neuroprotective effect [51, 127] and a contributing role for cognitive functions, such as long-term potentiation (LTP) [128].

AICD can be generated through both the amyloidogenic and the non-amyloidogenic pathways. Detection of AICD is a challenge due to its highly unstable nature [129]. However, AICD has been shown to regulate transcription [130] and interact with many adaptor proteins to regulate cell signalling [51]. In addition, overexpression of membrane-anchored AICD was shown to induce neurite outgrowth [131].

Aβ peptides are extensively studied regarding their neurotoxic effects in AD, but they are also produced under non-pathological conditions and shown to be involved in biological processes [132-134]. Production of Aβ peptides occurs during brain embryogenesis [135] and they are important for the viability of the CNS [133]. Although aggregated forms of Aβ peptides cause neurotoxicity, monomeric Aβ40 and Aβ42 have been shown to accelerate NPCs proliferation and neurogenesis [136]. While Aβ40 was shown to stimulate the neurogenesis of NPCs, Aβ42 was shown to induce astrocyte lineage differentiation *in vitro* [136]. The short Aβ fragments, derived from α- and β-secretase cleavage of APP, are shown to act as synaptic regulators and do not contribute to the pathogenic activity of Aβ [137]. As those short fragments do not contain a hydrophobic domain, they do not form aggregates [137, 138]. The inhibitory effect of high concentrations of full-length Aβ (Aβ1-42) in LTP is a well-documented phenomenon [139]. However, exogenous addition of Aβ1-15 can rescue the impaired LTP that was initially caused by the full-length Aβ [138]. Besides, exogenously added oligomeric Aβ42 peptides in picomolar concentrations have been shown to increase the number of neurons and the expression of neuron stem cell-specific markers *in vitro* [140]. Altogether, Aβ peptides are involved in synaptic activity, neuronal development and differentiation, as well as proliferation and neurogenesis, through different pathways in a concentration-dependent manner.

1.6 Modulating secretase activity as a treatment of AD

Reducing the level of amyloidogenic Aβ peptides has been the major strategy of AD treatment. Therefore, the focus has been on modulating or inhibiting the activity of γ- and β-secretases [141]. However, the clinical trials have, so far, been unsuccessful [142].

γ-Secretase activity is necessary for amyloidogenic peptides to be produced. Inhibitors of γ-secretase activity were thus the first targets in clinical trials but, to date, none of the γ-secretase inhibitors/modulators have been successful, due to lack of efficacy and/or side effects [143]. The adverse effects of some γ-secretase inhibitors were shown to be due to alterations in Notch signaling [144]. The increased accumulation of APP-CTFβ after inhibition of γ-secretase may also contribute to these side effects, causing endosomal abnormalities and neurotoxicity [145].

Inhibiting β-secretase activity successfully reduced Aβ secretion, both in animals and humans [146, 147], but led to a number of physiological and behavioral deficiencies, including increased astrogenesis, impaired axonal structure, disturbed synaptic functions, impaired neuronal maturation and migration, deficiency in motor neurons due to altered myelination, impairments in LTP and LDP, as well as cognitive and emotional memory deficiencies [147-149]. Although several small-molecule BACE inhibitors are currently being tested, two of the recent trials have stopped (verubecestat, ClinicalTrials.gov identifier NCT01739348, and LY2886721, Eli Lilly), due to lack of efficacy and/or to adverse side effects (including cognitive decline). Although the exact reason behind these failures is unclear, they may be related to the late administration of the treatment, when the amyloid cascade has already taken off in the disease process, or to BACE1 inhibitors having off-target effects on the physiologically important BACE substrates [147]. Aβ peptides are shown to be involved in neuronal activity [138, 150, 151], and another possible explanation may be that altered Aβ peptide production causes impaired neuronal activity. Conversely, partially reduced β-secretase activity by heterozygous gene deletion in 5XFAD mice [152], knock-down of hippocampal BACE1 in $APP751_{Swe·Lon}$ mice [153], and immune therapy in APP_{Swe} Tg2576 mice with the BACE1 ectodomain [154] reduced Aβ40 and Aβ42 secretion by 35-45% and improved the cognitive deficiencies caused by excess Aβ in transgenic mice.

In transgenic animals, partially reduced BACE activity is amendatory to impaired cognition, which was due to an increased Aβ burden. This could actually be due, at least to some extent, to enhanced efficacy of BACE1 reduction with increased substrate availability of APP in the transgenic mice. Therefore, it is important to evaluate the effects of partially reduced BACE1 levels by inhibitors in wild-type animal or *in vitro* models. This may pave the way for improving the quality of life and health of AD patients/high-risk individuals through delaying the AD progression in existing cases and as prophylactic treatment in predicted cases [155].

As α-secretase cleavage of APP prevents production of long Aβ peptides, increasing the α-secretase cleavage is another potential treatment for AD. Therefore, several molecules, such as indirect α-secretase activators, have been tested in clinical trials. RX-03140, a 5-HT4 agonist known to stimulate α-secretase, showed to improve cognition in AD patients, even though no further results have been announced yet [156]. Etazolate (EHT 0202), a GABA receptor modulator [157], was shown to increase sAPPα production and protect against Aβ-induced toxicity *in vitro*, and was also shown to relieve symptoms and to modulate the disease progression [158]. Etazolate was tested in a small group of patients with mild to moderate AD and found to be well tolerated. However, future studies will reveal the efficacy of the treatment on AD [159]. Bryostatin 1, a protein kinase C (PKC) modulator, is also known to increase the secretion of sAPPα and reduced both Aβ40 and Aβ42 [160] *in*

vitro. Bryostatin 1 has been clinically tested and shown to be tolerable, recommending further trials with more focus on cognition [161].

“There is no genuine motor activity without a previous thought, and there is no genuine thought if is not duly referred to a motor activity and enhanced by its relation with it”

El Hombre y la Gente
by José Ortega y Gasset-1957

1.7 The cerebral cortex

The ability to solve problems is the essential cognitive skill for animals to survive. With the environmental challenges that animals faced, the need for more sophisticated cognitive skills increased [162]. Thus, the evolution of the cerebral cortex has played a key role in the process of humans becoming “human”.

The cerebral hemispheres are the largest part of the brain, where the cerebral cortex is also located. The cerebral cortex is located at the outer layer of the cerebral hemisphere and is responsible for the cognitive actions of everyday life. The cortex has a highly convoluted shape allowing it to accommodate a large number of neurons [163]. In addition, the phylogenetically most elaborate cerebral cortex is the human cerebral cortex [164].

The largest part of the human cerebral cortex is called the “neocortex”, which is organized in six functional layers. These layers are numbered from the pia mater to the white matter [163].

- → *Layer I* is a cell-sparse layer. It is the area where dendrites and axons of pyramidal neurons that are located deeper in the cortex make connection [163]. In addition, unique Cajal-Retzius (CR) cells are also located in layer I.
- → *Layer II* mainly contains granule cells and is therefore also called the “*external granule cell layer*”.
- → *Layer III* contains a variety of cell types, but mainly pyramidal neurons. For this reason, it is also called the “*external pyramidal cell layer*”.
- → *Layer IV*, like layer II, mainly contains granule cells and is thus also called the “*internal granule cell layer*”.
- → *Layer V* mostly contains pyramidal neurons, which are larger than those in layer III, and is called the “*internal pyramidal cell layer*”.
- → *Layer VI* is a heterogeneous layer, containing a variety of cortical neurons, and is thus called the “*multiform layer*”.

Although the layers are defined by the presence or absence of neuronal cell bodies and the most common cell type they accommodate, each layer also contains axons and dendrites deriving from neurons located in other layers [163].

The cerebral cortex mainly consists of two major types of neurons: projection neurons and interneurons [165].

Projection neurons constitute 75-80% of the neurons in the cortex [166]. They have a pyramidal shape and are mostly located in layers III, V and VI. The projection neurons stretch their axons to make connection in distinct cortical areas and subcortical regions, as well as in the contralateral hemisphere [166, 167]. They use the excitatory amino acid glutamate as a neurotransmitter [167].

Interneurons constitute 20-25% of the neurons in the cortex; Interneurons have several forms, the most common forms are the inhibitory neurons, which use γ-amino-butyric acid (GABA) as a neurotransmitter [167]. However, some proportion of cortical-interneurons has excitatory features and utilizes glutamate as a neurotransmitter. Interneurons act locally, and are located in all the cortical layers [163].

1.7.1 Corticogenesis

Early in the development, the telencephalon is composed of undifferentiated neuroepithelial cells as progenitor cells. As these cells proliferate, some differentiate into radial glial (RG) cells and establish the ventricular zone (VZ) [168]. As the RG cells proliferate to self-renew, they also give rise to other progenitor cells, such as the outer RG cells and the intermediate progenitor cells, as well as neurons [169, 170]. During the development, the outer RG cells and the intermediate progenitor cells form the subventricular zone (SVZ) and increase the neuron production [171, 172].

The RG cells regulate the thickness of the cortex and are used as a scaffold by newborn neurons during their migration through the cortical layers. As the neurogenesis progresses, the earliest-born neurons migrate away from to the VZ and the SVZ, separate from the progenitor cells and eventually form the preplate. The later-born neurons then migrate from the VZ and the SVZ, respectively, into the preplate. There, they establish the cortical plate and eventually the marginal zone and the subplate. During the rest of the corticogenesis, newborn neurons migrate into the cortical plate and establish the cortical layers in an 'inside-out' manner [166]. While the early-born neurons settle in the deeper cortical layers and establish layer VI and then layer V, the late-born neurons populate the upper cortical layers and first establish layer IV, then layer-III/-II [166].

The establishment of layer I, the most superficial cortical layer, is different from the other cortical layers. Layer I is the first cortical layer that is produced during the corticogenesis [173] and contains Cajal-Retzius (CR) cells. CR cells are GABAergic neurons [174] and have horizontal axons, which can form synaptic connections with pyramidal neurons [175]. CR cells are most likely generated in the VZ [175] and they migrate to the outer edge of the cortex [173]. Then, along with the RG cells, CR cells form the marginal zone and, eventually, cortical layer I. CR cells control the radial glial migration of projection neurons; hence, they are important for the human neocortex development and patterning of later-born neurons [175, 176]. The corticogenesis is summarized in **Figure 6**.

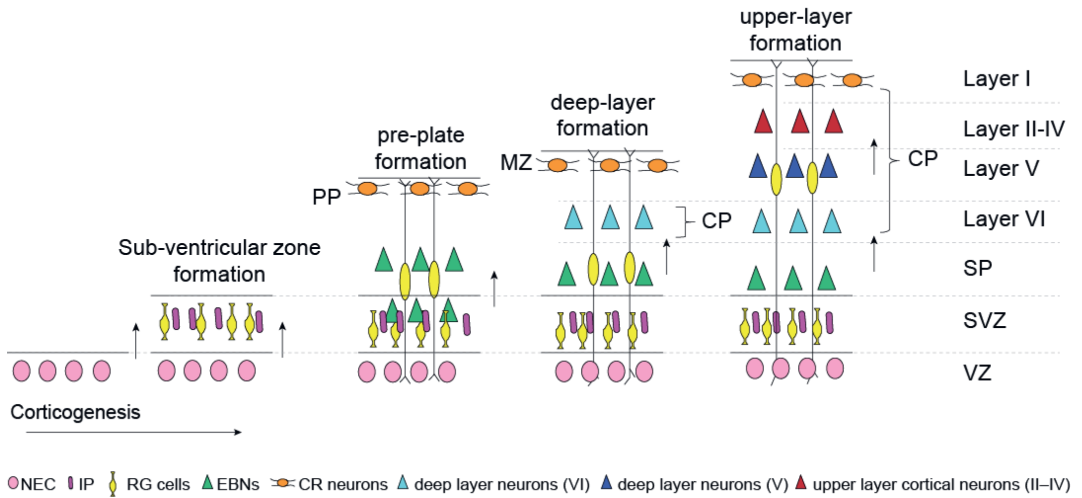


Figure 6. Schematic illustration of the corticogenesis.

During the early neurogenesis, neuroepithelial cells (NEC) form the ventricular zone (VZ) and they differentiate into radial glial (RG) cells. While the RG cells proliferate to self-renew, they also give rise to other progenitor cells, such as the outer RG cells and the intermediate progenitor (IP) cells. ***The*** *outer RG and IP cells eventually form the subventricular zone (SVZ). RG cells that are located in the SVZ differentiate into early-born neurons (EBNs). EBNs migrate towards the SVZ and form the preplate (PP). The later-born neurons then migrate towards the preplate (PP) and eventually the subplate (SP) and the marginal zone (MZ). After the establishment of the SP and the MZ, deep-layer (V and VI) neurons are generated. Deep-layer neurons establish the cortical plate (CP). During the rest of the corticogenesis, newly-born neurons migrate into the CP and establish the cortical layers in an 'inside-out' manner. During the corticogenesis, Cajal-Retzius (CR) neurons control the migration of projection neurons to the radial glial cells and hence assist the patterning of later-born neurons.*

1.8 Induced pluripotent stem cells — iPSCs

In 2006, Takahashi and Yamanaka published their milestone strategy to reprogram adult somatic mammalian cells back to a pluripotent state by retroviral integration of four transcription factors: OCT4 (also known as POU5F1), SOX2, KLF4 and MYC (also known as c-MYC) [177, 178]. The cells obtained from reprogramming are known as induced pluripotent stem cells (iPSCs).

These iPSCs are functionally and molecularly similar to embryonic stem cells (ESCs) [179, 180]. Like ESCs, iPSCs have the ability to differentiate into cells from all germ layers; i.e., the endoderm, ectoderm and mesoderm (**Figure 7**). Thus, iPSCs technology leads the way for patient-specific donor cell therapy and tissue repair, as well as for understanding of molecular disease pathophysiology in general [181-183] and brain disease in particular.

The iPSC technology is one of the most exciting discoveries of our century, giving us an opportunity to work with human brain cells in “a dish”.

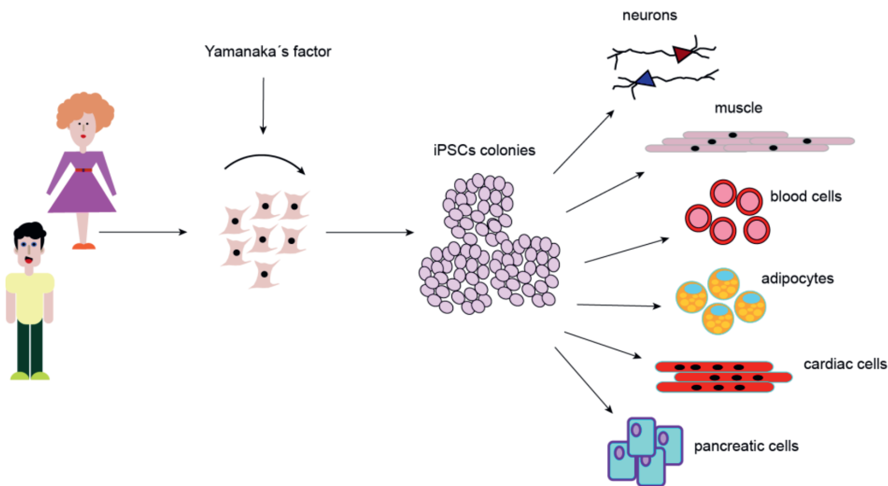


Figure 7. Induced pluripotent stem cells (iPSCs).
iPSCs are generated directly from somatic cells by somatic cell reprogramming. Briefly, administration of four transcription factors, OCT4 SOX2, KLF4 and MYC (also known as Yamanaka´s factors), which are highly expressed in embryonic stem cells, converts the somatic cells into a pluripotent state. iPSCs have the potential of differentiating into all three germ layers: the mesoderm, the endoderm and the ectoderm, from which neurons are derived. iPSC-derived cells provide material for studying personalized medicine, drug screening, regenerative and developmental medicine and especially neurobiology and related disorders.

1.8.1 Cortical differentiation of iPSCs

The human cerebral cortex is different from the rodent cerebral cortex in many ways [184], including relative size, variety of progenitors and diversity of neurons in the cortical layers [185]. Thus, it is important to study human diseases of the cerebral cortex, such as AD, in human cortical neurons. With the iPSCs technology, human NPCs can now be generated by mimicking the *in vivo* development [186] and can be used to generate human neurons.

The goal of differentiating iPSCs into cortical neurons is to obtain a functional *in vitro* cortical neuronal network, resembling *in vivo* networks [187]. As briefly described in **paragraph 1.7.1.**, neuroepithelial cells found in the VZ and the SVZ are the neuroprogenitors that increase the neuronal diversity in the cortical layers [171, 172]. Thus, in this project we initially differentiated iPSCs into cortical progenitor cells, which mimic the neuroepithelial cells that are seen throughout *in vivo* human embryonic development. Further differentiation and maturation of these progenitor cells gave rise to cortical projection neurons from all the cortical layers in 90-100 days [187-189]. The differentiation of iPSCs into cortical neurons is summarized in the Materials and Method section, **Figure 8**.

The protocol used here for cortical differentiation of iPSCs that is based on a dual-SMAD inhibition approach [190]. SMAD proteins are downstream transducers of TGFβ signaling [191], and SMAD inhibition has been shown to induce neuroectodermal lineage commitment [192]. SMAD proteins maintain pluripotency and induce ectoderm and mesoderm specifications [193]. Nodal and BMPs (Bone morphogenetic proteins) signaling controls the endoderm and mesoderm germ layer specification and their inhibition induces neuroectoderm formation [194, 195]. Therefore, at the initial phase of the differentiation, in order to induce *in vitro* neuroectoderm formation, we employed two molecules to inhibit the downstream activities of the SMAD protein: i) a small molecule "SB431542", which is a Nodal receptor antagonist [196], to inhibit Nodal/Activin/TGFβ signaling, and ii) Noggin, which is a BMPs receptor antagonist [197].

The differentiation of iPSCs into synaptically active cortical neurons *in vitro* follows the *in vivo* human embryonic corticogenesis and consists of four chronologically distinct steps:

1. Neuroepithelial-neuroprogenitor cell formation:
   In this initial step, stimulated retinoid signaling and dual-SMAD inhibition force the iPSCs to commit to primitive ectoderm lineage with forebrain identity [187]. This stage resembles the neuroepithelial neuroprogenitor cells (NPCs) forming the neural plate *in vivo*.
2. Rosette-type neuroprogenitor cell formation:
   After initial induction of neuroepithelial formation, the cells are further differentiated in the absence of dual-SMAD inhibition, but in the presence of stimulated retinoid signaling to promote corticogenesis [198]. As the differentiation continues, neuro-epithelial NPCs start to form neural "rosette" structures [187]. Those neural rosettes are progenitor cells with the capacity to differentiate into various region-specific neuronal and glial cell types [199]. Fascinatingly, their differentiation capacity is similar to the NPCs that emerge after closing of the neural tube *in vivo* [200]. Dr. Tabar and her colleagues transplanted human embryonic stem cell-derived neural rosettes in young adult rats and showed that they integrated into adult SVZ and contributed to the neurogenesis [201], supporting the theory that the rosettes in this step are the progenitor cells that will eventually become cortical projection neurons. At this stage, following the temporal order (inside-out manner) cortical layer VI (deep layer) neurons also start to appear.
3. Radial glia (-like) neuroprogenitor cell formation:
   Further differentiation of neural rosettes gives rise to radial glia-like NPCs, which will then differentiate into postmitotic neurons [202]. The cortical layer V and IV neurons also start to appear at this stage.
4. Neurogenesis and astrogenesis
   At this stage, further differentiation and maturation of radial glia (-like) NPCs give rise to upper layer cortical neurons. Before astrogenesis, the cortical neurons start to form functional, glutamatergic synapses. In the later phase of this stage, a relatively small number of astrocytes start to appear in the cultures.

2 AIM

2.1 The general aim

The overall aim of this thesis is to increase the understanding of APP processing and secretion of Aβ peptides in cortical neurons.

2.2 Specific aims

Paper I

To investigate how APP is regulated and processed during cortical differentiation of human iPSCs.

Paper II

To investigate how Aβ secretion correlates with co-localization of APP and APP-cleaving secretases, as well as where this co-localization takes place in hiPSC-derived cortical neurons.

Paper III

To investigate the effects of increased neuronal maturation and activity on the processing of APP in hiPSC-derived cortical neurons.

Paper IV

To investigate the effects of reduced Aβ secretion by BACE inhibitors on neuronal activity in primary rat cortical neurons.

3 METHODS

In this thesis, various methods were used to obtain the results. The methods have been described thoroughly in each paper, but key methods used are described briefly in this section.

3.1 Ethical permit

The use of human iPSCs in **Papers I-III** was approved by the Regional Ethics Review Board in Gothenburg (ethical permit 2014-731). Experiments performed in Paper IV were in agreement with the rules of the ethical committee in Gothenburg (ethical permit 5.8.18-11305/2018) and followed the guidelines of the Swedish National Board for Laboratory Animals.

3.2 Cell models

3.2.1 Human iPSCs-derived cortical neurons

Papers I-III are based on iPSC-derived cortical neurons differentiated from five different human iPSC lines: femoral condyle chondrocyte-derived iPSCs (A2B) [203], in-house reprogrammed fibroblast-derived iPSCs (BJ1a), and the fibroblast-derived iPSCs Con1/Ctrl1 [204], ChiPSC22 (Takara Bio Europe) and WTSIi015-A (EBiSC/Sigma Aldrich).

In order to differentiate iPSCs into cortical neurons we used a protocol that gives rise to functional cortical neurons from deep- and upper cortical layers within 90 days [205], with small modifications [189]. Briefly, neuronal induction was initiated on confluent monolayer iPSCs with neural maintenance medium (NMM) supplemented with SMAD protein inhibitors (media formulations are described in the **Papers I-III**) for 10 days (Figure 8). When a neuroepithelial layer had formed, the cells were detached with Dispase (a gentle enzyme, which does not disassociate the cells into single cells) and re-plated in NMM supplemented with FGF2 to promote self-renewal and to expand neuronal-rosettes (Figure 8). In order to clean the cultures from single cells and keep the neuronal rosettes expanding, the cell colonies were further passaged two to three times with Dispase before day 25. Around day 25, when neurogenesis started to occur, the cells were dissociated with Accutase an enzyme, which dissociates the cell-colonies into single cells. The cells were further passaged with Accutase for expansion before the final plating around day 35. After the final plating, the cells were kept for an extended time for further differentiation and maturation, with media changes every second day. The cells were cultured for up to 90

days after the final plating in **Paper I**, and for 40 days in **Paper II**. In **Paper III**, the NMM was replaced with BrainPhys Neuronal Medium (media composition is described in detail in the **Paper III**) the day after the final plating, and the cells were further matured for up to 55 days.

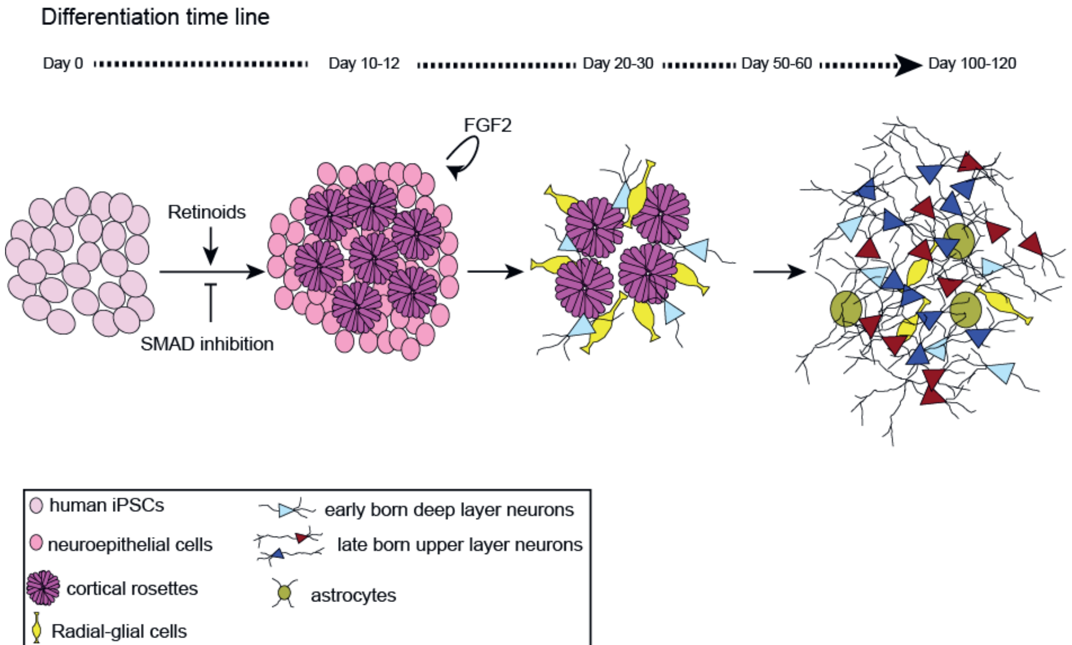


Figure 8. ***Schematic illustration of differentiation of human iPSCs into cortical neurons.***
Cortical differentiation of iPSCs started with neuroepithelial differentiation and was followed, chronologically, by cortical rosette formation, neurogenesis and terminal neuronal differentiation.

3.2.2 Primary rat cortical neurons

Primary cells are isolated directly from the tissue of interest, in our case from the rat cortex. The primary neuronal cultures are prepared by dissociation of the relevant brain region through enzymatic or mechanical procedures and maintained in a suitable growth medium. In **Paper IV**, primary cortical neurons from E18 Sprague Dawley rats were used. Briefly, the cortex was separated from the brain, and the cortical tissue was dissociated mechanically by gentle titration in the medium using a sterile, silanized glass Pasteur pipette. The solution with the cortical tissue was left for a couple of minutes for the non-dissociated parts to settle, before the supernatant was centrifuged down and the pellet carefully re-suspended in growth medium. The cell suspension was then strained on a 40-

µm diameter cell strainer to clean the suspension from the aggregated tissue before the cells were plated.

3.2.3 *SH-SY5Y* cell culture

In **Paper II**, *SH-SY5Y* cells were differentiated into neuron-like cells with BrainPhys media to observe potential changes in Aβ secretion with an increased neuronal phenotype. *SH-SY5Y* cells constitute a subline of the originally parental line of *SK-N-SH* cells (ATCC® HTB-11™), which were derived from a metastatic bone marrow biopsy from a girl with neuroblastoma. *SH-SY5Y* cells are easy to culture and maintain and the fact that they can easily be differentiated into neuron-like cells with RA treatment and serum deprivation make them widely used in the field of neurobiology.

3.2.4 Comparison of the cell models

The human cortex is different from the cortex of other mammalians, and the possibility to model the human cortex with hiPSC-derived neurons has been invaluable in research aiming at understanding mechanisms underlying brain diseases and the physiological roles of proteins in the diseases. However, iPSC-derived cortical neurons may represent embryonic neurons rather than the mature neurons found in the adult brain. Hence, mimicking the neurodegeneration observed in diseases such as AD using hiPSCs is challenging.

Culturing iPSC-derived neurons are time consuming and to obtain synaptically functional, communicating human cortical neurons takes about 75-90 days. Although the results obtained from primary rodent neurons may not be directly translated to humans, these neurons are active already after 14 *DIV*, making primary cortical cultures a good alternative for investigating the effects of new/current therapeutic targets in regards to neuronal activity.

Cell lines are immortalized and can be expanded on a large scale within a short period, facilitating mechanistic studies *in vitro*. *SH-SY5Y* cells can easily be differentiated into neuron-like cells, with high reproducibility and low cost. However, this line is derived from cancerous cells, which can alter the native functions of the proteins of interest and responses of the cells to stimuli/treatments. The results obtained from cell lines should thus optimally be confirmed in either primary or iPSC-derived cells.

3.3 Protein analysis

3.3.1 Western blot

In **Papers I-III**, the western blot technique was used to determine changes in the protein levels of APP, APP-cleaving enzymes and synaptic proteins. Western blot includes two main steps. Firstly, the proteins are separated according to size in a gel and secondly, they are blotted onto a membrane for detection with protein-specific antibodies [206].

Prior to western blot, the cells were lysed and a protein extract were prepared. To load the same amount of protein from each sample, protein concentrations were measured (detailed protocol can be found in **Papers I-III**). The proteins were then denatured and separated by size using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred (blotted) onto a membrane. The membranes were then blocked to avoid unspecific bindings, and incubated with antibodies directed to the protein of interest. To remove un-bound primary antibodies, the membranes were washed and horseradish peroxidase (HRP)-conjugated secondary antibody was used to detect the primary antibody. After the secondary antibody incubation, the membranes were washed and developed with a developing agent containing luminol, which is oxidized in the presence of HRP and peroxidase buffer and forms an excited state (chemiluminescence) that emits the light at 425 nm (luminescence). The luminescence was then detected by a light-sensitive camera and the band intensities were calculated using Image Lab software (BioRad laboratories). The intensity of the protein of interest, which was calculated with densitometric analysis, was normalized to the intensity of a housekeeping-protein, in our case GAPDH, to adjust for loading variability.

3.3.2 Immunochemical quantification of tau and NfL

In **Paper III**, enzyme-linked immunosorbent assay (ELISA) was used to detect media concentrations of secreted tau and NfL. ELISA is a technique that is used to detect a molecule (most often a protein) in liquid samples with an antibody directed to the molecule of interest [207, 208]. Briefly, the sandwich ELISA employs two or more antibodies against the same antigen; i) the capture antibody, which is attached to the surface of the well and captures the antigen in the samples and ii) the detection antibody, which is usually conjugated with biotin and detected by streptavidin-conjugated HRP and chromogenic solution (**Figure 9**). The color change is proportional to the amount of the captured antigen and can be measured, *e.g.*, by using a spectrophotometer. In **Paper III**, two different commercially available sandwich ELISAs were used for tau and NfL (details can be found in **Paper III**).

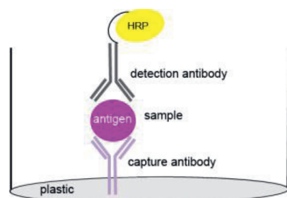


Figure 9. Schematic illustration of a sandwich ELISA.

3.3.3 Immunochemical quantification of sAPPα, sAPPβ and Aβ peptides

In **Papers I-IV**, electrogenerated-chemiluminescence (ECL) immunosorbent assay was used to detect media concentrations of secreted sAPPα, sAPPβ and Aβ peptides. ECL assays, are based on the same principle as the traditional sandwich ELISA, but the detection method differs. In ECL assays, the wells in microplates are covered with carbon-ink electrodes that are coated with the capture antibodies. The detection antibodies are conjugated with a ruthenium complex [$Ru(bpy)_3^{2+}$-(4-methyl sulfonate)] (**Figure 10**). When the detection antibody binds to the antigen, ruthenium ions on the detection antibody will be in close proximity to the electrodes and voltage in the electrodes will trigger an oxidation reaction in the ruthenium [209]. This reaction will eventually produce light and, as long as voltage is applied, the emitted light will be detected and measured by a charge-coupled device camera. In this study, commercially available ECL assays from Meso Scale Discovery (MSD) were used. Assay details can be found in all four papers in the thesis.

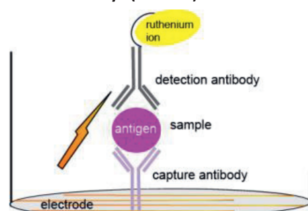


Figure 10. Schematic illustration of a sandwich ECL assay.

3.3.4 Immunoprecipitation followed by mass spectrometry for detection of Aβ peptides

In **Paper I**, immunoprecipitation (IP) followed by mass spectrometry (MS) was used to identify Aβ peptides of different lengths that are secreted into cell-conditioned media. IP is an antibody-based technique where magnetic beads are used to pull down a protein of interest from a sample. Antibodies that identify a specific antigen (peptides in this case) are conjugated onto the magnetic beads, followed by sample incubation and final elution of the protein/peptides of interest. The eluate is then analyzed by MALDI TOF/TOF mass spectrometry.

3.3.5 Immunocytochemistry

Immunocytochemistry (ICC) is an antibody-based method that allows for the detection of expression and localization of proteins in cells using an optical microscope.

In **Papers I-III**, ICC with fluorescence detection was used to investigate neuronal transcription factors, markers for cortical neurons, synaptic proteins and glial cells, as well as to visualize dendrites and axons. Firstly, the cells were fixed to protect the cell morphology and to retain the antigenicity of the target proteins. In this thesis, paraformaldehyde (PFA) was used as the fixative agent. The cells were then permeabilized with Triton-X to allow antibodies to penetrate through the cell membranes, before the cells were blocked with donkey serum to avoid unspecific antibody binding. After preparing the specimens, they were incubated with primary antibodies against the target proteins, followed by fluorescently conjugated secondary antibody incubation. In order to stain the nuclei, 4′, 6-diamidino-2-phenylindole (DAPI, a fluorescent dye that binds to adenine–thymine rich regions in DNA) was used and the slides were mounted with appropriate mounting media.

3.3.6 Proximity ligation assay (DuoLink)

DuoLink is a proximity ligation assay (PLA), which was designed to detect proximity between two target proteins [210]. In **Paper II**, we used the method to investigate co-localization of APP–BACE1, APP–PSEN1 and PSEN1–BACE1 in low- and high Aβ-secreting cells.

Briefly, selected primary antibodies (in our case, antibodies against APP, BACE1, and PSEN1) were used and detected with respective PLA probes conjugated with oligonucleotides. If two PLA probes are in close proximity (less than 40nm) [210, 211], oligonucleotides on the PLA probes can be joined into a circular DNA with the help of a ligase. The circled DNA is then amplified by a rolling-circle amplification mechanism and hybridized with fluorescently labelled complementary nucleotides (**Figure 11**). The signal obtained from the PLA assay was considered as co-localization of APP and APP-cleaving enzymes and was detected using a confocal microscope.

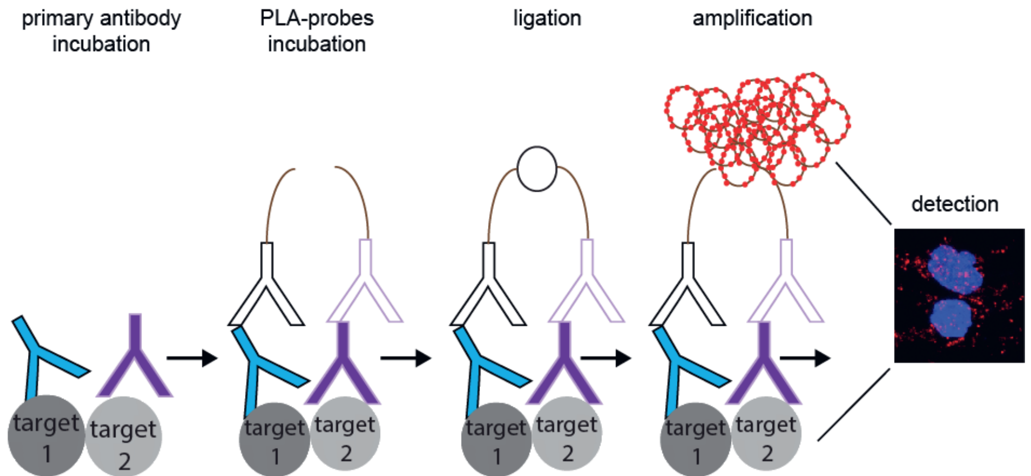


Figure 11. Schematic illustration of the DuoLink method.
When primary antibodies bind to the target, they are detected by the related PLA probes. Then oligonucleotides on the PLA probes are ligated and amplified. The amplification step includes the fluorescently labelled oligonucleotides that can be detected by an optical fluorescence microscope when two target proteins are within 0-40 nm of each other.

3.3.7 Comparison of the protein analysis methods

All the protein analyses used in this thesis were based on antibodies and thus rely on the specificity of the antibodies.

Western blot is a semi-quantitative, sensitive method that can detect both full-length proteins and truncated fragments of the proteins. Western blot can detect several proteins of different sizes, which will reduce the amount of sample and reagents needed. As for other antibody-based methods, unspecific interactions between antibodies and proteins may occur and give false positive bands. Therefore, the antibody for the target protein has to be chosen wisely, and if possible, negative and positive controls should be used during the initial optimizations.

ECL and ELISA assays are quantitative methods. ECL is more sensitive than the ELISA and can employ several capture antibodies that allow multiple antigen detection in one single well. IP-MS provides a highly specific cleaved-peptide profile of a distinct protein. However, it gives a relative quantification of a single peptide among the peptide pool of the sample that may increase the variation among the samples.

Both ICC and PLA employ a fixation step, preserving the cellular structures and allowing for observation of morphology along with expression patterns of proteins simultaneously. The choice of antibody, the fixation method and the blocking steps are critical steps for both techniques, as the antibody specificity is essential in order not to get unspecific

signals. When it comes to co-localization analyses, PLA (DuoLink) is more sensitive than traditional ICC since a signal will be detected only when the two target proteins are in close proximity.

3.4 Quantitative PCR

In **Papers I-III**, quantitative polymerase chain reaction (qPCR) was used to determine changes in gene expression by measuring messenger-RNA (mRNA) levels. In order to measure mRNA levels of the gene of interest, total RNA is isolated from the cells and the mRNA is converted to complementary-DNA (cDNA). In this thesis, reverse transcriptase, which uses the single-stranded RNA as a template and oligo (Dt) as primers to produce single-stranded cDNAs, were employed to convert RNA into cDNA. The single-stranded cDNA was then converted to double-stranded cDNA by DNA polymerase and ligase (**Figure 12**) and was used as a template for qPCR.

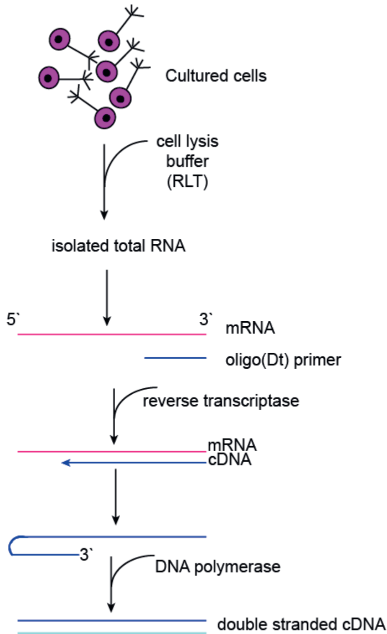


Figure 12. Schematic illustration of the workflow of double-stranded cDNA production from the lysed cells.

The qPCR technique is based on traditional PCR that is combined with DNA-binding fluorescent dyes. Hence, the amount of the product can be visualized in real time, after each amplicon has been produced. In this thesis, TaqMan probes with a FAM (fluorescein

amidite) reporter were used. TaqMan probes have a fluorophore molecule at the 5` end and a quencher at the 3` end, and as long as the quencher is in close proximity of the fluorophore, it quenches the light emitted by the fluorophore. As the Taq polymerase synthesizes the PCR products using the primer, it comes across to the 5´ end of the TaqMan probe, where the fluorophore is located, and due to the exonuclease activity of the Taq polymerase, it cuts the probe and releases the fluorophore. When the fluorophore is free from the quencher, it emits light that can be detected by the qPCR thermal cycler. The signal increases with each amplification cycle. (**Figure 13**) [212].

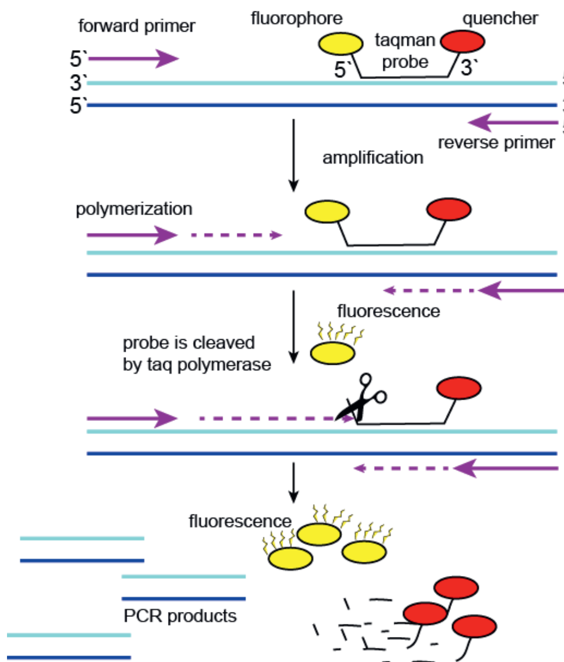


Figure 13. Schematic illustration of the workflow of qPCR with TaqMan probe.

The signal received from the fluorophore by the qPCR thermal cycler can be analyzed to give both absolute and relative quantification. In this thesis, the relative quantification method was used to determine relative changes in mRNA levels between the samples. To this end, the ΔΔCT method [213] was used. Briefly, the numbers of cycles to achieve a cycle threshold (CT) are measured. The less number of cycles to reach the CT, the more substrate the sample contain from the beginning, why this number has an inverse relationship with the amount of amplicons present in the sample. The principle behind the ΔΔCT method is based on the differences between CT values of reference genes and the gene of interest. The CT values of the reference genes, measured in each experiment, are subtracted from

the CT values of the target genes, giving a "relative quantity" value for each sample. This value is then compared between samples and correlated to either a control sample (**Paper III**) or to the sample with the highest expression (Paper I and II).

3.5 Fluorescent Microscopy

3.5.1 Confocal microscopy

ICC and DuoLink samples in **Papers I-III** were imaged using a Nikon A1 Eclipse Ti-E inverted confocal microscope with a 60x objective, or a Zeiss LSM 800 confocal microscope with a 63x objective.

Confocal microscopes have fluorescence optics, which can focus the laser light at a single spot with a defined depth on the sample. When the laser hits the fluorescence-conjugated secondary-antibody- or DuoLink probes with a photon of excitation light, the fluorescent molecule absorbs the light, which turns it into an excited state. The fluorescent molecule then emits light that is detected by the microscope [214]. Confocal microscopes have better resolution then regular epi-fluorescent microscopes, because of the emission pinhole, which prevents out of focus emission lights from entering the light detector.

iPSC-derived cortical neurons do not grow in mono-layers, but rather form semi-3D structures. In order to not lose information and create three-dimensional images, the samples were scanned with at least 20 optical planes (Z-stack) and acquired at 0.5 µm distances between the Z-stacks. The images were analyzed using ImageJ with the same settings for each figure [215]. Image analyses by ImageJ were explained in detail in Papers II-III.

3.5.2 High resolution confocal microscopy "AiryScan"

In **Paper II**, the amplified oligonucleotides in the DuoLink assay create complex helical structures, which require a higher resolution than the traditional confocal microscope. Therefore, AiryScan high-resolution confocal microscopy was used to investigate co-localization of APP with APP–cleaving enzymes inside organelles. As mentioned above, emission pinholes in traditional confocal microscopes allow the emission light from only one point to reach the detector. This reduces the noise, but at the expense of losing some of the signal. In order to improve the image quality, ZEISS designed a 32-channel area-detector, which collects the focused light that comes from the lenses. Each 32 channels in the Airy-detector behaves as a single pinhole and hence increases the resolution compared with traditional confocal microscopes [216].

3.6 Measuring neuronal activity

3.6.1 Patch-clamp

In **Paper I**, in order to characterize the electrophysiological maturity of the iPSCs-derived cortical neurons, whole-cell patch-clamp with current-clamp mode was used. In this technique a hollow glass patch pipette is attached to the cell membrane, creating a suction that allows for recording of currents through the membrane of the entire cell. The current-clamp mode was used to record the membrane potential, by applying series of hyperpolarizing and depolarizing current injections to the cells. [217].

3.6.2 Multi-electrode array

In **Paper III**, we used the multi-electrode array (MEA) technology to measure the changes in spontaneous neuronal activity over time. The MEA technology has been developed to characterize *in vitro* neuronal networks [218]. The neurons are cultured in culture dishes covered with electrodes capable of sensing changes in currents caused by neuronal activity without interfering with the membrane integrity (**Figure 14**). This technology records from multiple cells that grow on the electrodes.

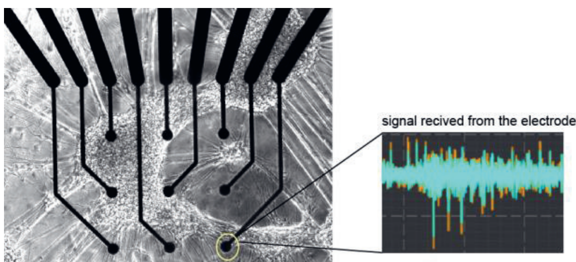


Figure 14. A representative image of neurons cultured in a MEA dish and an example of a signal from spontaneous neuronal activity.

The MEA dishes used in this thesis have nine individual electrodes per well.

3.6.3 Electrical field stimulation

In **Paper IV**, electrical field stimulation (EFS) by Cellaxess Elektra was used to measure changes in synaptic transmission after BACE-inhibitor treatments, to investigate the effects of decreased Aβ secretion on synaptic transmission. The Cellaxess Elektra platform employs a capillary electroporation system, which can introduce different types of molecules, as well as electrical stimulation, to adherent cells [219]. In this thesis, rat primary neurons were incubated with calcium indicators prior to electrical stimulation and were simultaneously imaged. Changes in Ca^{2+} fluorescence of unstimulated cells in another part of the well were quantified as synaptic transmission (**Figure 15**).

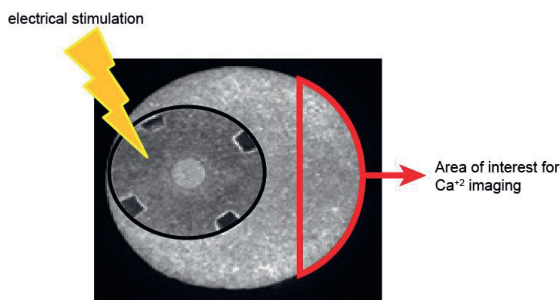


Figure 15. Schematic illustration of measuring synaptic transmission with the Cellaxess Elektra platform.

3.6.4 Comparisons of the assays used for neuronal activity

The patch-clamp technique allows for identification of the effects of a certain stimuli or a pharmacologic manipulation on specific neuronal functions and channels. The whole-cell patch-clamp technique can only be performed on a single cell at a time, which makes population-based estimations practically difficult and time consuming, especially if the cell population is heterogeneous with different neuronal maturation stages.

The MEA technique measures the activity of a population of cells, providing a measurement of the average activity of smaller sub-populations in the neuronal culture. Recording of the action potentials is highly dependent on the number of neurons that grow on top of the electrodes. The Cellaxess Elektra system provides a high-throughput analysis of the whole neuron population in the cell culture wells and is thus suitable for testing multiple drug candidates at different concentrations simultaneously.

3.7 Statistical analysis

GraphPad was used to perform statistical analyses and to create graphs (Prism version 7.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical significance was defined as $p < 0.05$.

In **Paper I**, ordinary one-way ANOVA (analysis of variance), followed by Tukey's *post-hoc* correction, was used to compare mRNA, protein and secretion levels between the differentiation time points. In **Paper II**, the unpaired two-tailed t-test was used to compare mRNA, protein, secretion and co-localization analyses between low- and high Aβ-secreting cells. In **Paper III**, the unpaired two-tailed t-test was used to compare mRNA, protein and secretion levels between NMM- and BrainPhys-cultured neurons. The statistical analysis of the secretion of Aβ peptides after TTX treatment was performed using the one-tailed t-test, given that decreased secretion was expected on the basis of earlier results [220]. Two-way

ANOVA followed by Bonferroni's multiple comparison test was used to analyze changes in neuronal activity over time. In **Paper IV**, the unpaired two-tailed t-test was used for analysis of secretion and synaptic transmission.

4 RESULTS

4.1 Paper I

In this first paper, we characterized the human neuronal cell model and performed a detailed analysis of the secretion of APP cleavage products during cortical differentiation of human iPSCs (hiPSCs). The secretion of sAPP and Aβ peptides differed during the cortical differentiation of hiPSCs, even though APP and the APP-cleaving enzymes ADAM10 (α-secretase), BACE1 (β-secretase) and PSEN1 (γ-secretase) were expressed throughout the differentiation.

The hiPSCs were differentiated into cortical neurons for up to 150 days, and we found that the differentiation could be divided into four different stages according to their Aβ secretion profiles:

I. The undifferentiated stem cell stage — no Aβ secretion

The undifferentiated hiPSCs did not secrete any of the APP cleavage products.

II. The neuroepithelial stage — secretion of shorter Aβ peptides

This stage was initiated with the differentiation of iPSCs into neuroepithelial cells through dual SMAD inhibition.

The cells gained neuroepithelial morphology after about ten days and started to secrete short Aβ peptides, the non-amyloidogenic peptides, and sAPPα into the cell-conditioned medium. This indicates that APP is processed via the non-amyloidogenic pathway, which is driven by α/β-secretase, during the neuroepithelial cell stage.

III. The radial glia (-like) neuroprogenitor cells stage — low Aβ peptide secretion

Further differentiation of neuroepithelial cells gave rise to radial glia (-like) NPCs (days 35-45). At this stage, the cultures continued to secrete sAPPα, and sAPPβ started to be detected in the cell-conditioned media. However, the overall secretion of Aβ peptides was much lower compared with neuroepithelial cells.

IV. The neuronal stage — secretion of longer Aβ peptides

Further differentiation of the radial glia (-like) NPCs gave rise to cortical projection neurons. At this stage, as the neuronal differentiation and maturation continued, cells started to develop an extensive neuronal network, express synaptic proteins and gained electrophysiological properties. As the neurons matured, the processing of APP shifted towards the β-/γ-secretase-dependent amyloidogenic pathway, which produced longer Aβ peptides, such as Aβ1-34, Aβ1-38, Aβ1-40, and Aβ1-42. The shift in APP processing coincided with the formation of a neuronal phenotype and increased synaptic maturity.

In summary, in this project we described a human cortical model that enabled us to study regulation of APP processing and Aβ secretion *in vitro*. The main findings in **Paper I** are summarized in **Figure 16**.

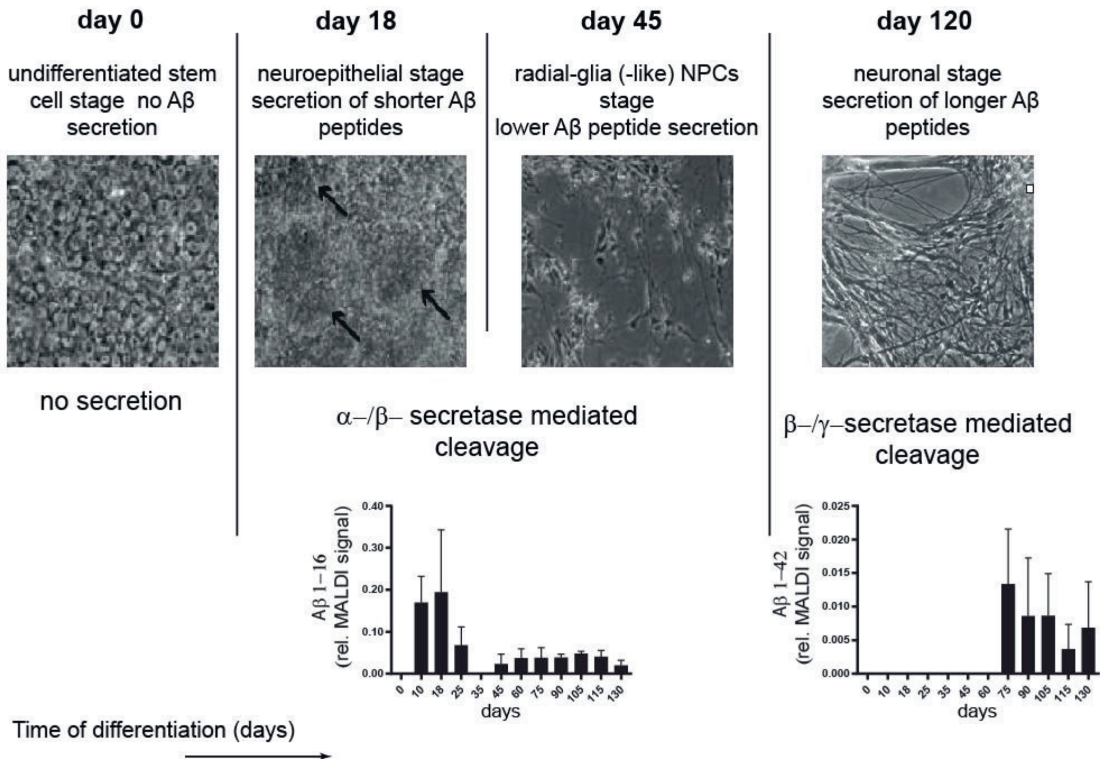


Figure 16. Schematic illustration of the main findings in Paper I.
As the cortical differentiation of hiPSCs progresses, the profile of secreted Aβ peptides changes. In the undifferentiated stage (day 0), no secretion is observed, but as the cells differentiate into neuroepithelial and neuronal rosette cells (from day 10 to 25), they start to secrete short Aβ peptides. Once the cells become radial glia (-like) NPCs (from day 35 to 45), Aβ secretion into the cell media decreases. Still, APP is mainly processed via the α-/β-secretase-dependent, non-amyloidogenic pathway, in both neuroepithelial and radial glia (-like) NPCs. As the cells mature and gain a neuronal morphology, they start to secret longer Aβ peptides. This suggests that APP is processed via the β-/γ-secretase-dependent, amyloidogenic pathway in the neuronal stage.

4.2 Paper II

In **Paper II**, we investigated whether the secretion of long Aβ peptides, produced via the amyloidogenic pathway, is regulated by co-localization and subcellular localization of APP and the secretases. To this end, taking advantage of the model we described in **Paper I**, we chose two different differentiation stages: radial glia (-like) NPCs (referred to as NPCs), where almost no long Aβ peptides are secreted, and the neuronal stage, where the long Aβ peptides are highly secreted.

To define the differences between the NPCs and the neurons in terms of secretion and protein levels, we first measured the secretion profiles of the APP cleavage products and the protein levels of APP and the secretases. We confirmed that the NPCs secreted lower levels of Aβ peptides compared with the neurons. We also found that the protein levels of PSEN1 were higher in neurons than in NPCs, whereas the protein levels of BACE1 did not change. These findings are summarized in **Figure 17**.

In order to investigate if the secretion of long Aβ peptides could be correlated with co-localization of APP and the respective secretases involved in Aβ production, we examined the co-localizations of APP–BACE1 and APP–PSEN1 in NPCs and neurons, using a PLA method called DuoLink [210]. We found that APP co-localization with PSEN1 was higher in neurons secreting more Aβ peptides, whereas APP co-localized to a higher degree with BACE1 in the low Aβ-secreting NPCs.

APP is a membrane-bound protein and, therefore, it is trafficked through the intracellular membranes [90]. During this trafficking, APP converges with the secretases, enabling cleavage [221]. Hence, to investigate the possible production site of the long Aβ peptides, we next investigated the distribution of co-localizations of APP and the secretases in endosomes and lysosomes. In the high Aβ-secreting neurons, APP–PSEN1 localized to a higher degree in early endosomes than in late endosomes and lysosomes, while in the low Aβ-secreting NPCs, the distribution was the opposite. Regardless of the Aβ secretion levels, APP–BACE1 localized primarily in late endosomes and lysosomes, rather than in early endosomes.

The PSEN1–BACE1 interaction is suggested to be important for the long Aβ peptides to be generated [222]. Therefore, we also investigated the co-localization of PSEN1–BACE1 in both NPCs and neurons and found that PSEN1–BACE1 co-localization was higher in NPCs than in neurons, although not reaching statistical significance. As both PSEN1 and BACE1 are transmembrane enzymes, their activities are regulated by the membrane lipid compositions [223, 224] and the acidification of the compartment [225], in which they are located. Therefore, we also investigated the subcellular site of PSEN1–BACE1 co-localization and found it to be higher in early- and late endosomes than in lysosomes in low Aβ-secreting NPCs, but increased in the lysosomes in high Aβ-secreting neurons. These findings are summarized in **Figure 18**.

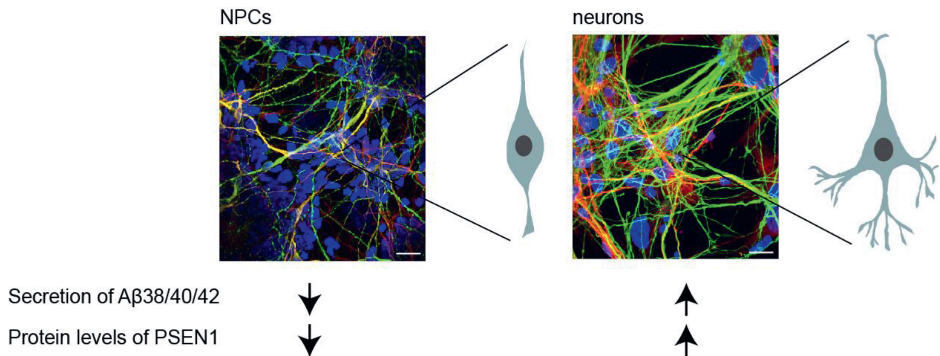


Figure 17. Morphology and secretion profiles of NPCs and neurons.
The neurons have an extended complex neuronal network as compared with the NPCs. Neurons secrete more Aβ38, Aβ40, and Aβ42 than the radial-glia (-like) NPCs. In addition, the protein levels of PSEN1 are higher in the neurons than in the radial-glia (-like) NPCs. (The arrow facing up indicates "high" while the arrow facing down indicates "low.")

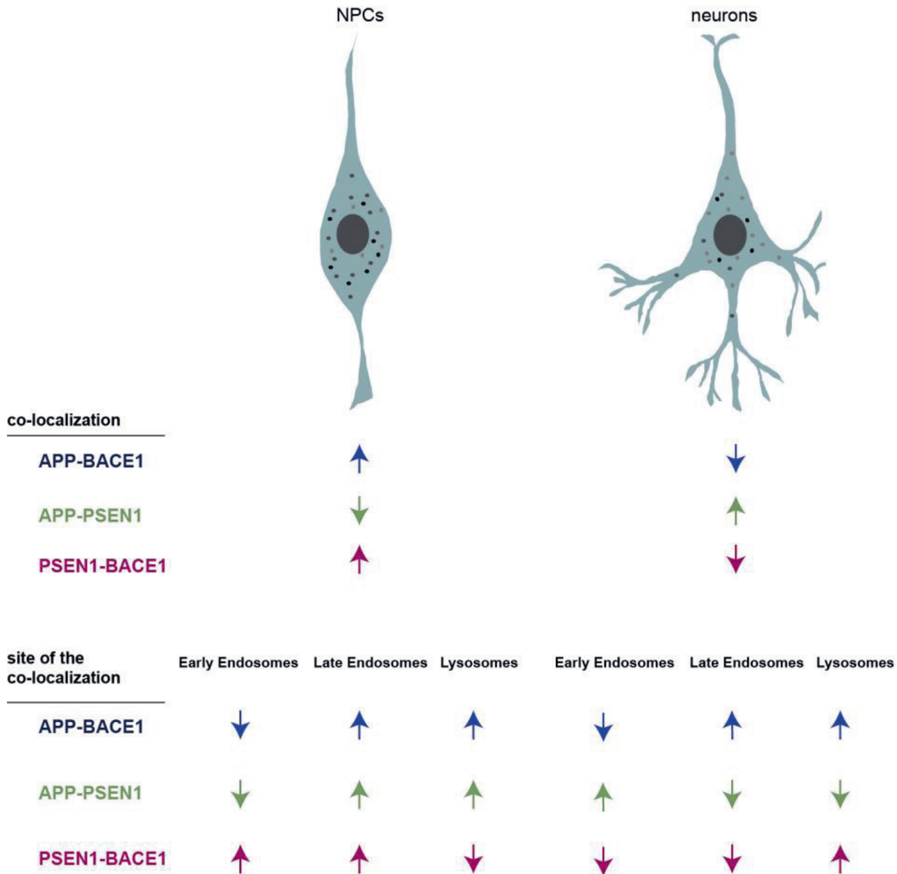


Figure 18. Schematic illustration of the main findings in Paper II.
Co-localizations of APP-BACE1 and PSEN1-BACE1 are higher in the low Aβ-secreting NPCs compared with the high Aβ-secreting neurons, whereas co-localization of APP-PSEN1 is higher in the neurons. Regardless of cell type and Aβ secretion, APP-BACE1 primarily localizes in late endosomes and lysosomes, rather than in early endosomes. APP-PSEN1 localizes to a higher degree in early endosomes than in late endosomes and lysosomes in high Aβ-secreting neurons, while in the low Aβ-secreting NPCs, the distribution is the opposite. In the NPCs, PSEN1-BACE1 co-localization is higher in early- and late endosomes than in lysosomes, while it is higher in lysosomes in the neurons. (The arrow facing up indicates "high" while the arrow facing down indicates "low". Each color represents the respective co-localization).

4.3 Paper III

In **Paper I**, we showed that the production of the amyloidogenic Aβ peptides increased as the neurons and synapses matured. To investigate in greater detail how neuronal maturation and increased neuronal activity is linked to APP processing and Aβ secretion, we differentiated the radial-glia (-like) NPCs for 25-40 days in BrainPhys medium, which was formulated to enhance the electrophysiological and synaptic properties of the neurons [226], and compared it with neurons differentiated in the traditional neuronal medium (NMM).

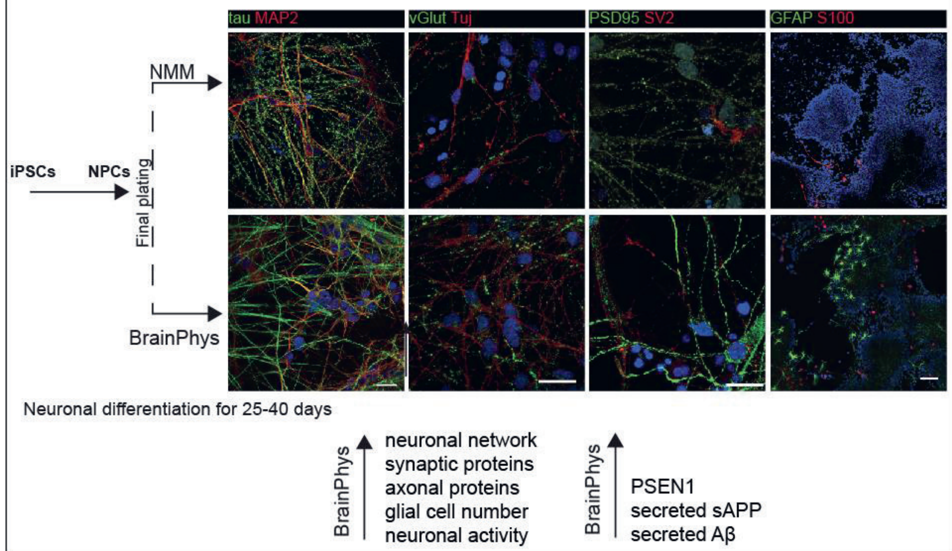
In **Paper III**, we performed a comparative study and showed that culturing the radial-glia (-like) NPCs cells in BrainPhys accelerated neuronal maturation and increased neurite branching, axonal proteins, expression of synaptic markers and spontaneous neuronal activity compared with NMM. In addition, BrainPhys culturing also increased the number of glial cells in the cultures. This means that the BrainPhys medium accelerated the differentiation of NPCs into mature and more active neurons.

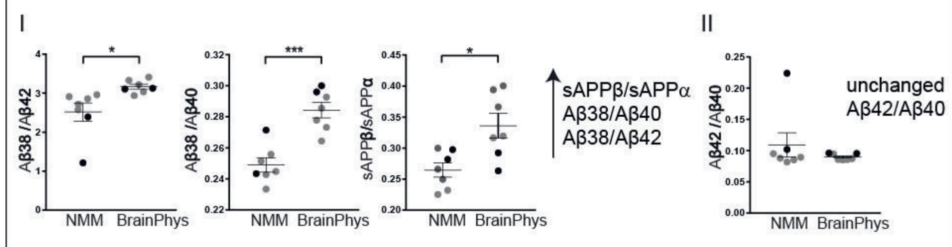
BrainPhys also significantly increased the overall secretion of APP cleavage products with altered ratios compared with NMM. Culturing the radial-glial NPCs in BrainPhys increased the ratios of Aβ38/Aβ42, Aβ38/Aβ40 and sAPPβ/sAPPα, while the Aβ42/Aβ40 ratio was sustained. This indicates that BrainPhys increased overall APP processing and specifically increased the β-site cleavage of APP, as well as the cleavage at amino acid 38 of the Aβ domain. In the first paper we found that in order for the long Aβ peptides to be produced, a neuronal phenotype is necessary. In line with this, BrainPhys also enhanced the neuronal phenotype in SH-SY5Y cells along with increased Aβ secretion.

To investigate how much of this increased secretion was due to increased neuronal activity, we blocked the synaptic activity with TTX (tetrodotoxin) in BrainPhys cultures. As expected, TTX fully blocked the neuronal activity even after 24 hours of treatment and decreased the secreted Aβ peptides by approximately 20%. This suggests that the increased Aβ secretion in BrainPhys cultures was at least, in part, due to increased neuronal activity.

Next, we analysed if increased expression of APP and APP-cleaving enzymes to were responsible for the increased Aβ secretion. We found that

the protein levels of APP and BACE1 remained stable, whereas PSEN1 increased significantly in the BrainPhys cultures compared with in NMM. This indicates that the protein level of PSEN1 is an important regulator of amyloidogenic Aβ peptide production, in line with the findings in **Paper II**. The main findings in **Paper III** are summarized in **Figure 19**.


Box 1. BrainPhys accelerates neuronal maturation and increases Aβ secretion
tau MAP2
vGlut Tuj
PSD95 SV2
GFAP S100
NMM
iPSCs
NPCs
Final plating
BrainPhys
Neuronal differentiation for 25-40 days
BrainPhys
neuronal network
synaptic proteins
axonal proteins
glial cell number
neuronal activity
BrainPhys
PSEN1
secreted sAPP
secreted Aβ


Box 2. BrainPhys alters sAPPβ/sAPPα, Aβ38/Aβ40 and Aβ38/Aβ42 ratios but does not change Aβ42/Aβ40
I
Aβ38 /Aβ42
Aβ38 /Aβ40
sAPPβ/sAPPα
NMM
BrainPhys
sAPPβ/sAPPα
Aβ38/Aβ40
Aβ38/Aβ42
II
Aβ42 /Aβ40
unchanged
Aβ42/Aβ40

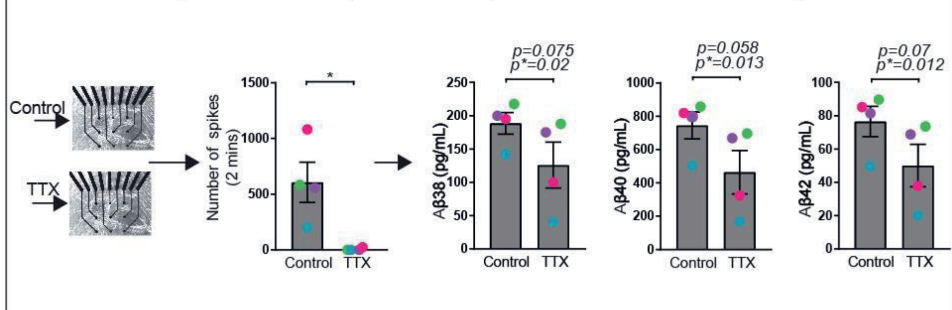

Box 3. Inhibiting neuronal activity decreases Aβ secretion but does not change the ratios
Control
TTX
Number of spikes (2 mins)
Aβ38 (pg/mL)
Aβ40 (pg/mL)
Aβ42 (pg/mL)
p=0.075
p*=0.02
p=0.058
p*=0.013
p=0.07
p*=0.012

Figure 19. Schematic illustration of the main findings in Paper III.
Box 1. *The BrainPhys medium accelerates NPCs differentiation into mature neurons, leading to increased neuronal phenotypes and networks, expression of synaptic markers, the number of glial cells, neuronal activity, secretion and expression of axonal proteins, PSEN1 expression and APP processing.* ***Box 2.*** *The BrainPhys medium alters the secreted Aβ and sAPP ratios. BrainPhys increases the ratios of Aβ38/Aβ42, Aβ38/Aβ40 and sAPPβ/sAPPα, compared with NMM, while the Aβ42/Aβ40 ratio remains stable.* ***Box 3.*** *Inhibiting synaptic activity in BrainPhys-cultured cells by TTX decreases the secretion of Aβ38, Aβ40 and Aβ42, but the ratios remain stable.*

4.4 Paper IV

BACE is widely known for its involvement in AD [71-73]. Since it is the enzyme performing the initial step of aggregation-prone Aβ-peptide generation, it has long been targeted in the treatment of AD. While inhibiting BACE in clinical studies reduced the production of Aβ as expected [146, 147], the treatment caused side effects, including cognitive decline [147]. The exact reason for the side effects is unknown, but could in theory be related to alterations in APP or Aβ processing linked to neuronal functions [109, 136, 227, 228].

In **Paper IV**, we investigated if decreasing Aβ peptides by inhibiting BACE would affect synaptic transmission. To this end, we treated rat primary cortical neurons with three different BACE inhibitors at 0.04, 0.3 and 3 µM for four days and screened for effects on Aβ secretion and synaptic transmission.

Lanabecestat has been shown to reduce both CSF-Aβ and the amyloid load in the brain in clinical trials [229], but has also been shown to cause cognitive problems [230]. Here, we showed that lanabecestat decreased both Aβ40 and Aβ42 by approximately 70% at all the concentrations tested. Consequently, all the concentrations decreased synaptic transmission.

LY2886721 has been shown to reduce both brain amyloid deposition and CSF-Aβ in clinical trials [231], but also to cause physiological side effects [232]. Here, we showed that LY2886721 decreased Aβ secretion in a concentration-dependent manner, but that synaptic transmission was significantly decreased only when Aβ42 decreased with more than 50%.

BACE inhibitor IV has been shown to decrease Aβ *in vitro* [233], but has not been tested in clinical trials. Here, we showed that BACE inhibitor IV also decreased Aβ secretion in a concentration-dependent manner and that only the highest concentration decreased synaptic transmission. Interestingly, the lowest concentration, which significantly decreased both Aβ40 and Aβ42 by approximately 40%, significantly increased synaptic transmission, showing that BACE inhibition on neuronal activity is not only dependent on Aβ levels, but that other factors may also contribute.

The data presented here suggest that β-secretase-mediated APP processing should be modestly inhibited to avoid impaired synaptic transmission.

The main findings in Paper IV are summarized in **Figure 20**.

Name of the BACE inhibitor	Concentration	Reduction in Aβ40 secretion (%)	Reduction in Aβ42 secretion (%)	Reduction in Synaptic Transmission
Lanabecestat	0.04 µM	~70%*	~70%*	~14-18%*
	0.3 µM	~70%*	~70%*	~14-18%*
	3 µM	~70%*	~70%*	~14-18%*
LY2886721	0.04 µM	39%	37%	-
	0.3 µM	53%*	43%	-
	3 µM	57%*	57%*	39%*
BACE inhibitor IV	0.04 µM	39%*	38%*	13%* increased
	0.3 µM	62%*	58%*	-
	3 µM	65%*	61%*	16%*

Figure 20. Summary of the main findings in Paper IV.
Synaptic transmission is only affected when the decrease in Aβ exceeds 50%, which is marked in blue. (Indicates statistically significant differences).*

5 DISCUSSION

5.1 APP processing during cortical neuronal maturation

Here, we analyzed cell-conditioned media from cortical neurons for secreted APP cleavage forms and linked it to intracellular processes and synaptic activity. Using a human iPSC-derived neuronal cell model, we defined three stages of neuronal differentiation where Aβ secretion differed significantly, providing an opportunity to study APP processing in relation to intracellular events as well as in a developmental time manner. As the cells differentiate into a neuronal phenotype, the membranes and cytoskeletons are reorganized and membrane trafficking and cytoskeleton assembly are key events for cells to generate neurites, and eventually axons and dendrites. As APP is a membrane-bound protein and suggested to be involved in neuronal differentiation along with its cleavage products (see under **paragraph 1.5**), these developmental processes would affect the processing of APP during the neuronal differentiation.

5.2 APP processing in neuroepithelial progenitors

The first stage that we defined during the cortical differentiation of hiPSCs consisted primarily of neuroepithelial NPCs, where APP was processed along the β-/α-secretase-dependent pathway producing short Aβ peptides. NPCs, especially PAX6-positive NPCs, assist cortical lamination [167], where APP may act as an adhesion molecule on the cell surface along with other cell adhesion molecules [111, 112]. During this process, interaction of APP with the other cell adhesion molecules may increase the surface retention of APP [118], and this, in turn, increases α-site cleavage [97]. The lack of longer Aβ peptides, but presence of both APP and BACE1/PSEN1 mRNA at this stage, may be explained by the γ-secretase complex instead cleaving the membrane-anchored CTFα and producing AICD, which is then translocated to the nucleus to regulate the proliferation and differentiation of NPCs [234].

5.3 APP processing in radial-glia (-like) progenitors

The next stage that we defined was a differentiation stage where the overall secretion of Aβ was low and the cultures consisted mostly of radial-glia (-like) NPCs, along with some early-born deep-layer neurons. The radial-glia (-like) NPCs are known to guide neurons to find their correct positions during the corticogenesis. High levels of APP expression has been shown in these cells [235], suggesting that APP may promote the adhesion of neurons to the radial-glia (-like) NPCs. This also means that APP should retain and interact with its cleavage enzymes, especially α-secretase, at the cell surface. However, at this stage, sAPPβ also started to appear in the cell-conditioned medium, suggesting that the initial cleavage of APP by β-secretase increased [52]. The β-site cleavage of APP usually occurs in acidic subcellular compartments [91], but BACE1 has also been shown to interact with APP at the cell membrane [236], and it is possible that APP is initially cleaved by either α- or β-secretase at the cell surface at this stage, secreting sAPPs and producing membrane-bound CTFs. The CTFs are then internalized via endocytosis, where most of the internalized CTFs go into degradation pathways [237] and only some are further processed by γ-secretase. Another explanation for the low levels of secreted Aβ peptides observed in radial-glia (-like) NPCs, compared with neuroepithelial NPCs and neurons, could be that the necessary co-localizations of APP and APP-cleaving enzymes are lacking.

A relatively recent study proposes that APP is differently trafficked and processed in the nervous system to meet particular requirements of different neuronal subtypes [238]. Indeed, sAPPβ has been shown to induce neuronal differentiation and neurite outgrowth [125, 126] that could be related to the onset of a neuronal phenotype at this stage. The amount of APP located at the cell surface of newborn deep-layer neurons may be essential for the adhesion to radial-glial cells during the cortical plate formation [234].

We initially hypothesized that secretion of Aβ would correlate with the intracellular co-localization of APP and the respective APP-cleaving enzymes. In support of this, we found that APP–PSEN1 co-localization was significantly lower in these cells than in the high Aβ-secreting neurons. In line with the

literature, we also found that APP–BACE1 localization was higher in late endosomes and lysosomes than in the less acidic early endosomes [91, 225, 239], regardless of the Aβ secretion levels. This could be due to APP–BACE1 localizing in a similar manner in endosomal compartments in both low Aβ-secreting NPCs and high Aβ-secreting neurons, but can be regulated differently in recycling endosomes or in secretory pathways [107]. Future studies of the co-localization of APP–BACE1 at the plasma membrane and in other subcellular compartments, such as synaptic vesicles, as well as investigation of the intercellular APP cleavage products, would help to understand the regulation of the β-site cleavage of APP and Aβ secretion.

PSEN1 and BACE1 have been shown to be transported together [240] and, here, we found higher co-localization of the two secretases PSEN1 and BACE1 in the low Aβ-secreting NPCs. This could be due to the possible regulatory action of PSEN1 in the maturation of BACE1 through regulation of the subcellular trafficking of BACE1 [241].

5.4 APP processing in neurons

Next, we showed that as the radial-glia (-like) NPCs differentiated into mature neurons, the proportion of Aβ peptides derived from the amyloidogenic pathway increased. Notch protein and APP-CTFs are competing substrates for γ-secretase [242], and Notch signaling is known to be inactivated during the neurogenesis [243]. Therefore, the increased proportion of amyloidogenic Aβ peptides may be the result of increased γ-secretase cleavage of APP-CTFs by inactivated Notch signaling, as the proportion of postmitotic neurons increased in the culture. Altogether, this proposes that cleavage of APP may be neuronal maturation-dependent.

Previously, it has been shown by us and others that APP is favorably cleaved via the amyloidogenic pathway in neurons [189, 244, 245]. We thus hypothesized that a neuronal phenotype is necessary for APP to be cleaved via the amyloidogenic pathway. We further supported this hypothesis by our finding that an increased neuronal phenotype in BrainPhys-differentiated SH-SY5Y cells and NPCs increased the secretion of amyloidogenic Aβ peptides.

We also showed that as the neurons matured, the expression and secretion of axonal proteins (tau and NfL) increased along with the increased secretion of amyloidogenic Aβ peptides. This suggests a relationship between Aβ and axonal proteins. Indeed, exogenous treatment of tau has been shown to increase Aβ40 and Aβ42 production by altering the initial cleavage of APP through increased β-secretase cleavage over α-secretase cleavage [246]. It is possible that secreted Aβ induces tau secretion, and that tau secretion induces Aβ secretion, suggesting a tau–Aβ-secretion-perpetuating loop; although the exact relationship between tau and Aβ is not completely clear [246]. This possible tau–Aβ loop may be one of the reasons for the increased Aβ secretion with maturation in our cell model.

As the neurons mature, they create complex neuronal networks of axons and dendrites and establish functional synapses with pre- and post-synaptic vesicles that are all known to be important sites for Aβ production and secretion [247, 248]. Accordingly, another reason for the increased Aβ secretion in mature neurons could be that increased maturity enhances the trafficking and internalization of APP into synaptic vesicles. There, it can be cleaved by BACE1, increasing the production of CTFβ. The CTFβ, in turn, can converge with γ-secretase to be cleaved into Aβ peptides, thus increasing Aβ secretion.

As the cells differentiate into neurons, they polarize into axons, soma and dendrites, and cargo vesicles with distinct lipid and protein compositions are produced [88]. This provides APP with opportunities to be transported through membranes and converge with different secretases that would ultimately increase the amyloidogenic cleavage of APP. Indeed, in line with the previous finding that PSEN1–APP proximity has a regulatory effect on Aβ secretion [249], we found an overall increase in PSEN1–APP co-localization, along with increased PSEN1 protein levels, in the neurons compared with the NPCs. This indicates that the protein levels of PSEN1 and APP–PSEN1 co-localization have direct regulatory roles in the Aβ secretion. We also found an increased APP–PSEN1 co-localization in early endosomes in high Aβ-secreting neurons. Together with earlier findings [145, 250], this suggests that Aβ peptides may also be produced in early endosomes. We further showed that PSEN1–BACE1 co-localization, which is shown to be necessary

for amyloid Aβ peptide production [222, 240], localized primarily in lysosomes in high Aβ-secreting neurons, whereas it localized mainly in early- and late endosomes in low Aβ-secreting NPCs. Even though further investigation is needed, these results could imply a regulatory role for the subcellular site of PSEN1–BACE1 interaction with regard to Aβ production.

5.5 Aβ secretion and neuronal activity

The secretion of amyloidogenic peptides has been shown to correlate positively with increased synaptic activity [227, 251, 252]. In support of this, we observed that increased neuronal activity in our cultures also increased the secretion of both sAPPβ and the longer forms of Aβ, suggesting both increased β- and γ-cleavage of APP. It has been shown that with more synaptic activity, more synaptic vesicles fuse with the plasma membrane and increase the overall endocytosis, which, in turn, increases the recycling of APP from the plasma membrane to the endosomal vesicles [252]. This will eventually increase the interaction of APP and β-secretase in the endosomes, enabling increased β-site cleavage [227]. Induced neuronal activity has also been shown to increase APP–PSEN1 proximity in mouse primary neuron cultures [249]. In our human neuronal cell model, APP–PSEN1 co-localization increased as the cells matured, which could indicate that increased accessibility of γ-secretase for APP may be one reason for the observed increased secretion of Aβ with neuronal activity. Furthermore, changes in synaptic activity cause conformational changes in γ-secretase at the pre- and post-synaptic membranes that may change its tendency to cleave the Aβ domain at different amino acids [253, 254]. This could possibly explain the increased Aβ38 over Aβ40 and Aβ42 that we observed with increased synaptic activity.

By inhibiting neuronal activity using TTX, we saw around 20% reduction in Aβ secretion. This is consistent with earlier findings, where Aβ secretion was reduced, but not completely stopped, by blocking the synaptic activity with TTX in animal models [227, 252]. This indicates that the secretion of Aβ is only partially regulated by neuronal activity, which is also in line with our findings that neurons start secreting Aβ before any measurable neuronal activity is observed [189].

Inhibiting neuronal activity did not have significant effects on the ratios of secreted Aβ38 over Aβ40 and Aβ42, indicating that increased cleavage at the amino acid 38 over 40 and 42 may be explained by other factors than neuronal activity. An increased number of astrocytes was observed in the BrainPhys cultures. This most likely promoted neuronal activity, as the astrocytes are important for neurons to form healthy and functional synapses [255, 256]. While neurons are the main Aβ-secreting cell type, glial cells also contribute to Aβ secretion [257], although which Aβ peptides that are produced in glial cells have not yet been described in detail. The increased Aβ38 secretion could thus, in theory, be the result of increased numbers of glial cells in the cultures. Another explanation could be that BrainPhys culturing promoted a relative increase in deep-layer neurons, and that these neurons are responsible for the increased Aβ38 secretion.

To evaluate the effects of Aβ secretion levels on neuronal activity one step further, cortical rat neurons were treated with different BACE inhibitors. We showed that while all the BACE inhibitors reduced Aβ secretion as expected, BACE inhibition only resulted in reduced neuronal activity when the inhibitor concentrations were high enough to decrease Aβ42 secretion by at least 50%. This suggests a threshold effect, rather than direct dose dependency, of the Aβ42 levels on synaptic transmission and is in line with the fact that the Icelandic mutation, which decreases Aβ production by around 30% without impairment in synaptic function, is protective against AD [258]. The reduced synaptic transmission we observed here, however, could also be due to overall altered APP processing. Future studies will reveal whether the reduction in synaptic transmission was solely due to decreased Aβ42, or if other factors were involved.

The relationship between Aβ secretion and neuronal activity is still a matter of debate and different correlations have been suggested: increased neuronal activity inhibits amyloidogenic Aβ production [259]; or increased neuronal activity increases amyloidogenic Aβ production [227, 251, 252]; or increased amyloidogenic Aβ peptide production inhibits neuronal activity [227]; or amyloidogenic Aβ peptides induce neuronal activity [150, 151]. Moreover, a negative feed-back mechanism has been proposed between Aβ and synaptic transmission. According to this notion, β-site cleavage of APP is regulated by neuronal activity, meaning that increased neuronal activity

enhances Aβ secretion and the increased Aβ secretion, in turn, depresses synaptic transmission and, consequently the production of Aβ [227, 249]. Our results partially support this concept and propose that a neuronal activity feed-back mechanism operated by Aβ peptides may not only represent negative feed-back, but it may rather represent a concentration-dependent feed-back loop, where the amount of Aβ that is present in the synapses determines whether it will cause synaptic depression or synaptic transmission.

6 CONCLUSION

APP and its cleavage products have been shown to be involved in many biological processes [108], such as neuronal development and synaptogenesis, neurite outgrowth and dendritic arborization [109, 110, 115, 116, 118]. However, the conclusive understanding of APP functions that is necessary in order to identify efficient treatments for AD and to avoid severe side effects is still lacking. This thesis contributes with the following knowledge to the field:

i) APP processing in human cortical neurons changes from α-/β-mediated cleavage to β-/γ-secretase mediated cleavage as the neurons mature.
ii) Increased secretion of amyloidogenic Aβ peptides correlates with both increased PSEN–APP co-localization and increased APP and PSEN1 protein levels.
iii) The secretion of amyloidogenic Aβ peptides correlates with increased APP–PSEN1 co-localization in early endosomes.
iv) The secretion of amyloidogenic peptides is dependent on a neuronal phenotype and is partially regulated by synaptic activity.
v) Amyloidogenic APP processing and/or the secreted amount of Aβ42 may act as a regulator of synaptic transmission in a concentration-dependent manner with a threshold effect.

7 FUTURE PERSPECTIVES

The amyloid cascade hypothesis proposes that the accumulation of Aβ peptides in the AD brain is due to imbalance between Aβ42 production and clearance [22, 260]. Hence, treatment strategies for AD have focused on reducing the amounts of Aβ, either by modulating APP-cleaving enzymes or by reducing the amyloid load in the AD brain by immunotherapies [261]. Despite intense research, most of the clinical studies have failed. Reasons behind these failures may be either that the treatments are initiated too late in the disease process, when irreversible brain damage has already occurred, or that the drugs are reducing APP processing too much, and thus affect the physiological functions of the APP cleavage products. For example, it is known that endogenously produced Aβ42 is important for maintaining synaptic connections, as well as for normal LTP and memory function [151, 228]. In line with this, we showed in **Paper I** that the longer Aβ peptides, including Aβ42, increase naturally as the neurons mature and form functional networks. In addition, in **Paper IV,** we saw that excessive reduction of the endogenous Aβ42 production using BACE inhibitors affected synaptic activity negatively. Future studies should investigate in depth which APP cleavage products that are responsible for these effects. For example, in **Paper III,** we showed a relative increase in Aβ38 with increased synaptic activity. Whether this peptide has a direct role for synaptic function or simply reflects an increased efficacy of γ-secretase at amino acid 38 upon synaptic activity remains to be elucidated.

Aducanumab is a monoclonal antibody that binds aggregated forms of Aβ. Aducanumab was recently tested on patients with mild AD and was shown to slow down cognitive decline [262]. This is very promising news, but it also implicates that the stage of the disease may play an important role for the treatment. Although several BACE inhibitors are currently being tested, most of the clinical studies involving modulation or inhibition of β-secretase activity have so far been halted due to inefficacy or side effects [263]. The reason for these side effects have not yet been defined, but it has been shown in APP-transgenic mouse models that partial inhibition of β-secretase activity that reduces Aβ42 with 30-50% does not cause known side

effects in animal [152-154]. In **Paper IV**, we showed that partially decreased BACE activity does not interfere with synaptic activity in wild-type rat primary cultures. Although we did not explore other possible side effects, our data indicates that a partial reduction in BACE activity can be tolerable. Possible future treatment strategies against AD could therefore include the combination of immunotherapy to decrease the Aβ burden in the AD brain, along with low-dose β-secretase inhibition to prevent growth of new and existing plaques. Indeed, the combination of BACE inhibitors and anti-Aβ antibodies has been tested in a mouse model. The results showed that the overall anti-amyloid effect was enhanced by this combination [264], supporting the potential of combination therapies for future clinical studies.

Inhibiting BACE has also been shown to increase α-cleavage resulting in increased sAPPα [265]. As mentioned previously, sAPPα has positive effects on neuronal plasticity and memory [260]. In **Paper IV**, we showed that the lowest dose of BACE inhibitor IV even increased synaptic transmission slightly. Although the reason behind this increase must be further investigated, moderate BACE inhibition could potentially increase α-cleavage and thereby indirectly have a beneficial effects on synaptic dysfunction observed in AD [260].

BACE1 has many other substrates apart from APP (reviewed in [70]) and the affinity of BACE1 to its substrates changes with the sub-cellular localization [266]. For example, while BACE1 seems to cleave many of its substrates in non-endosomal compartments, BACE1 cleavage of APP takes place to a large extent in acidic endosomal compartments [266], something that was supported by the data presented here in **Paper II**, showing that APP and BACE1 co-localized to a large degree in endosomal compartments in human neurons. BACE1 is also known to be localized in post- and pre-synaptic vesicles [104, 267], and Aβ to be secreted from synaptic vesicles [268]. Further studies using the DuoLink method presented in **Paper II**, in combination with synaptic vesicle markers, has potential to increase the understanding of the secretase activities in the synapses. This increased knowledge could in turn enable design of secretase inhibitors specific to particular subcellular compartments that would not affect the processing of other substrates. Indeed, such a molecule was shown to inhibit the endosomal BACE1 activity in human iPSC-derived neurons [269], and to

reduce Aβ production, without affecting the cleavage of other BACE substrates [266].

In summary, targeting BACE activity in the organelles where β-secretase primarily cleaves APP, with low doses of inhibitors may be a future approach to prevent or treat AD.

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