Regulation of amyloid beta generation and its involvement in synaptic function: studies in human iPSC-derived cortical neurons

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Gothenburg 2024

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ISBN: 978-91-8069-617-3 (PRINT)
ISBN: 978-91-8069-618-0 (PDF)

Printed in Borås, Sweden 2024
Printed by Stema Specialtryck AB

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease that affects millions of individuals worldwide and exerts a profound societal and economic impact. Clinically characterized by a gradual loss of memory, cognitive and functional abilities, AD begins decades before the onset of symptoms with the accumulation of an endogenously produced peptide, amyloid beta (Aβ). Aβ is produced through the enzymatic cleavage of amyloid precursor protein (APP) by β- and γ-secretases and its functions include regulating synaptic plasticity and activity, although excessive accumulation can disrupt neuronal function. Inhibition of Aβ generation could enable early disease prevention, however greater insights into the mechanisms of Aβ production and its functions at the synapse are needed to avoid side-effects. Furthermore, a deeper understanding of Aβ's toxic effects on synapses would improve our ability to detect Aβ-induced synaptic dysfunction and degeneration in patients, allowing to better monitor effective treatments. Therefore, this thesis aims to deepen our understanding of Aβ generation and its pathophysiological effects on synapses in human neurons.

In **paper I**, using a cellular model of human iPSC-derived neurons we found that increased Aβ secretion correlated with increased APP/β-secretase

colocalization in early endosomes, and a possible inhibitory function of APP-CTFβ, the intermediate product of β-cleavage, on β-secretase. In **paper II**, we investigated the secretion of ten potential biomarkers of synaptic dysfunction in AD, from human iPSC-derived neurons. We found that synapse formation, neuronal activity and exposure to exogenous toxic oligomeric Aβ affected secretion of the synaptic proteins differently. In **paper III**, we explored the consequences of high-dose β-secretase inhibition on synaptic function in human iPSC-derived neurons. We found that acute synaptic dysfunction following β-secretase inhibition seems to involve mechanisms other than reduction of Aβ secretion or APP accumulation at synapses. Finally, in **paper IV**, we developed a protocol to differentiate human stem cells into mature, synaptically active neurons without the need for glial support. Collectively, our insights into the intricate mechanisms of APP trafficking and cleavage, Aβ generation and its impact on synaptic function and dysfunction will advance the field of AD research and will hopefully provide directions to enhance the success rate of clinical trials targeting AD.

Keywords: Alzheimer's disease, Aβ, APP, human iPSC, cortical neurons, BACE1, synaptic formation, synaptic transmission, multi electrode array, synaptic dysfunction

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SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom är den vanligaste formen av demens, en sjukdom som leder till minnesförlust och påverkar miljontals människor runt om i världen. Vid Alzheimers sjukdom ses ansamlingar av proteinet Amyloid beta (Aβ) som plack i hjärnan. Aβ bildas genom att ett större protein, amyloid precursor protein (APP), klyvs av specifika enzymer (β- och γ-sekretaser). Aβ bildas normalt i den friska hjärnan och spelar en roll i den normala funktionen när nervceller kommunicerar. Vid Alzheimers sjukdom leder däremot en ökad mängd Aβ till att den normala funktionen av nervceller och deras synapser (där information överförs från en nervcell till en annan) störs. Att stoppa uppbyggnaden av Aβ i plack kan bidra till att förhindra sjukdomen. Vi behöver dock lära oss mer om hur Aβ tillverkas och fungerar för att undvika biverkningar av läkemedel. Dessutom kan förståelsen för hur Aβ skadar synapser hjälpa oss att enklare diagnosticera sjukdomen. Denna avhandling syftar till att få en bättre förståelse för hur Aβ bildas och hur det påverkar synapser både i den friska hjärnan och vid Alzheimers sjukdom. Som forskningsmodell använde vi cellkulturer av mänskliga nervceller som skapats från stamceller.

I **det första delarbetet** fann vi att Aβ bildas när APP och β-sekretas är nära varandra i en del av celler som kallas endosomer, de delar av cellen som ansvarar för sortering av material från cellens yta. Vi fann också att CTFβ, en mellanprodukt vid APP-klyvning, kan fungera som en hämmare av β-sekreta och förhindra bildningen av Aβ. Synaptisk dysfunktion kan studeras i patienter genom att mäta mängden av synaptiska proteiner i ryggmärgsvätska.

Det är fortfarande inte känt hur dessa synaptiska proteiner frisätts från nervcellerna till ryggmärgsvätskan vid Alzheimers sjukdom. I **det andra delarbetet** fann vi att nervceller frisätter flera synaptiska proteiner när synapser bildas, och både synaptisk aktivitet och skadliga former av Aβ kan påverka denna frisättning. Att stoppa β-sekretas, som en behandling för Alzheimers sjukdom, verkade lovande resultat i kliniska prövningar, men orsakade också kognitiva bieffekter.

I **det tredje delarbetet** testade vi hypotesen att en ansamling av APP vid synapser orsakar skador på synapserna. Det visade sig dock att ansamling av APP inte var inblandad den synapsskada man sett när β-sekretas stoppas. Detta inträffade i stället bara vid mycket högre doser än de som behövs för att hämma Aβ-produktionen till en fördelaktig nivå.

Slutligen, i **det fjärde delarbetet** utvecklade vi en metod för att göra en försöksmodell med mänskliga nervceller utan stödjeceller, en annan viktig celltyp i hjärnan som tidigare tänktes vara avgörande för synapsernas utveckling och funktion.

Våra upptäckter om hur Aβ bildas och dess effekter på synapser bidrar till att öka vår förståelse för hur Alzheimers sjukdom kan uppkomma och hur man kan behandla och diagnosticera den. Vi hoppas att denna kunskap kommer att leda till bättre kliniska prövningar och förbättra chanserna att hitta effektiva behandlingar.

RIASSUNTO IN ITALIANO

La malattia di Alzheimer (o piú comunemente Alzheimer) è una complessa malattia neurodegenerativa che porta alla perdita di memoria e al declino cognitivo in milioni di persone in tutto il mondo. Alla base dell' Alzheimer c'è un piccolo frammento proteico chiamato amiloide beta (Aβ). La produzione di Aβ inizia con una proteina più grande chiamata proteina precursore dell'amiloide (APP), che, quando tagliata da specifici enzimi (β- e γ-secretasi), rilascia Aβ nello spazio tra i neuroni. Aβ è normalmente prodotto nel cervello sano ed é coinvolto nella normale funzione delle sinapsi, le comunicazioni tra i neuroni. Tuttavia, nell'Alzheimer, un accumulo eccessivo di Aβ disturba il normale funzionamento dei neuroni e delle sinapsi, con meccanismi che sono ancora sotto indagine. Fermare questo accumulo in una fase precoce potrebbe aiutare a prevenire la malattia, ma dobbiamo imparare di più su come l'Aβ è prodotto e quali sono le sue funzioni per evitare eventuali effetti collaterali dei trattamenti. Inoltre, comprendere come l'Aβ danneggia le sinapsi potrebbe aiutarci ad identificare e monitorare meglio la malattia nei pazienti, rendendo più facile verificare l'efficacia dei trattamenti che modificano la malattia. Questa tesi mira ad una migliore comprensione di come l'Aβ è prodotto e come influisce sulle sinapsi sia in condizioni fisiologiche che patologiche. Come modello di ricerca abbiamo utilizzato la coltura cellulare di neuroni umani derivati dalla differenziazione forzata delle cellule staminali pluripotenti.

Nell'**articolo I**, abbiamo scoperto che l'Aβ è prodotto quando l'APP e la β-secretasi si trovano in stretta prossimità negli endosomi precoci, organelli intracellulari atti allo smistamento di materiale dalla superficie cellulare. Abbiamo anche scoperto che il CTFβ, un prodotto intermedio del taglio dell'APP, potrebbe funzionare come un inibitore della β-secretasi, prevenendo la secrezione di Aβ.

La disfunzione sinaptica può essere monitorata nei pazienti misurando la concentrazione di certe proteine sinaptiche nel liquido cerebrospinale. Tuttavia, i meccanismi del rilascio di queste proteine dai neuroni durante la malattia sono sconosciuti. Nell'**articolo II**, abbiamo scoperto che i neuroni secernono molteplici proteine sinaptiche durante il processo di formazione delle sinapsi e attività sinaptica e che forme tossiche di Aβ possono influenzare diversamente questo rilascio.

L’inibizione della β-secretasi ha mostrato risultati promettenti nei trial clinici, ma ha causato anche effetti collaterali cognitivi. Nell’**articolo III**, abbiamo ipotizzato che l’accumulo di APP alle sinapsi causi disfunzione sinaptica. Tuttavia, abbiamo scoperto che l’accumulo di APP non era coinvolto nella disfunzione sinaptica, che invece si verificava solo a dosi molto più elevate di quelle necessarie per inibire la produzione di Aβ ad un livello benefico.

Infine, nell’**articolo IV**, abbiamo sviluppato un protocollo per ottenere una coltura cellulare di neuroni umani senza il supporto degli astrociti, altro importante tipo di cellula nel cervello che in precedenza si pensava fosse cruciale per il corretto sviluppo e funzione delle sinapsi.

Le nostre scoperte su come viene prodotto Aβ i suoi effetti sulle sinapsi hanno avanzato la nostra comprensione dell’Alzheimer. Confidiamo che questa conoscenza porterà a migliori trial clinici e aiuterá nello sviluppo di nuovi trattamenti efficaci.

LIST OF PAPERS

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

I. **Sandra Roselli**#, Tugce Munise Satir#, Rafael Camacho, Stefanie Fruhwürth, Petra Bergström, Henrik Zetterberg, Lotta Agholme "APP-BACE1 Interaction and Intracellular Localization Regulate Aβ Production in iPSC-Derived Cortical Neurons" Cell Mol Neurobiol. 2023;43(7):3653-3668. doi:10.1007/s10571-023-01374-0

first co-authorship

II. **Sandra Roselli**, Johanna Nilsson, Parasto Shahrouki, Kaj Blennow, Henrik Zetterberg, Ann Brinkmalm Westman, Lotta Agholme "Regulation of synaptic degeneration biomarkers secretion in iPSC-derived cortical neurons" (submitted)

III. **Sandra Roselli**, Parasto Shahrouki, Linnéa Mundin, Johanna Nilsson, Berta Marcó De La Cruz, Stefanie Fruhwürth, Fredrik Sterky, Lotta Agholme, Henrik Zetterberg "BACE inhibition-mediated synaptic dysfunction is independent on APP accumulation at synapses" (manuscript)

IV. **Sandra Roselli**, Berta Marcó De La Cruz, Alexander Back, Lydia Moll, Christina Nodin, Johan Pihl, Fredrik Sterky, Henrik Zetterberg, Lotta Agholme "Generation of synchronously active cortical neurons through Ngn2 induction of iPSCs without glia" (manuscript)

CONTENT

ABBREVIATIONS

ADAM9	A disintegrin and metalloproteinase 9
ADAM10	A disintegrin and metalloproteinase 10
ADAM17	A disintegrin and metalloproteinase 17
AICD	App intracellular domain
AMPAR	A-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AP	Action potential
AP2B1	Adaptor protein complex 2 - subunit β
APLP1	Amyloid precursor-like protein 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARC	Activity-regulated cytoskeleton-associated protein
ARIA	Amyloid-related imaging abnormalities
Aβ	Amyloid β
BACE1	β-site app cleaving enzyme 1
BACE2	β-site app cleaving enzyme 2
BDNF	Brain-derived neurotrophic factor
BMP4	Bone morphogenetic protein 4
BN-PAGE	Blue native-polyacrylamide gel electrophoresis
BPM	BrainPhys medium
BSA	Bovine serum albumine
CAMKIIB	Calcium/calmodulin-dependent protein kinase II β
CCD	Charge-coupled device
cDNA	Copy DNA
CHL1	Close homolog of L1

CNS	Central nervous system
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTFα	C-terminal fragment α
CTFβ	C-terminal fragment β
DAPI	4′, 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
Dpi	Days post induction
EAAT	Excitatory amino acid transporter
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
fAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
FDG	Fluorodeoxyglucose
Fgf2	Fibroblast growth factor 2
flAPP	Full-length APP
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI1	Rab GDP dissociation inhibitor α
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Gln	Glycine
GWAS	Genome-wide association study
HFIP	Hexafluoro isopropanol

HKG	House-keeping gene
HKP	House-keeping protein
HMW	High molecular weight
HPLC	High-performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyl transferase 1
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
ICM	Inner cell mass
iGluts	Induced glutamatergic neurons
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilization
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LMW	Low molecular weight
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2
MCI	Mild cognitive impairment
MEA	Multi electrode array
mGluR	Metabotropic glutamate receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSD	Meso scale discovery
NB	Network burst
NeuN	Neuronal nucleus
NFT	Neurofibrillary tangles
NGN2	Neurogenin 2

NIA-AA	National institute on aging-Alzheimer's association
NMDAR	N-methyl-D-aspartate receptor
NMM	Neuronal maintenance medium
NPC	Neuronal progenitor cells
NPTX2	Neuronal pentraxin 2
NPTXR	Neuronal pentraxin receptor
NSC	Neuronal stem cell
oAβ_{42}	Oligomerized Aβ_{42}
PAP	Peri-synaptic astrocyte process
PAX6	Paired box 6
PET	Positron emission tomography
PLA	Proximity ligation assay
PM	Plasma membrane
PSC	Pluripotent stem cells
PSD	Post-synaptic density
PSD95	Post-synaptic density protein 95
PSEN1	Presenilin 1
PSEN2	Presenilin 2
pTau	Phosphorylated tau
qPCR	Quantitative polymerase chain reaction
RPL27	Ribosomal protein L27
sAD	Sporadic Alzheimer's disease
sAPPα	Soluble APP α
sAPPβ	Soluble APP β
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEZ6	Seizure protein 6 homolog
SEZ6L	Seizure 6-like protein
SNAP25	Synaptosomal-associated protein, 25kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SPE	Solid phase extraction
STX7	Syntaxin 7
SV	Synaptic vesicle
SV2A	Synaptic vesicle protein 2A
SYT1	Synaptotagmin 1
TBR1	T-box brain protein 1
TBS	Tris-buffered saline
TGFβ	Transforming growth factor β
TGN	Trans-Golgi network
TMB	Tetramethylbenzidine
TREM2	Triggering receptor expressed on myeloid cells 2
vGlut	Vesicular glutamate transporter
α7nAChR	α7 nicotinic acetylcholine receptor

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 Epidemiology and clinical presentation

Alzheimer's disease (AD) is a neurodegenerative disease and the most common cause of dementia, defined as a chronic and progressive loss of cognitive functions. To date, AD affects more than 35 million people worldwide, and the prevalence is expected to double every 20 years (1). Clinical presentation of AD starts with short-term memory loss connected to neuronal degeneration in the entorhinal cortex and hippocampus. As the pathology progresses to the cerebral cortex, other areas of cognition, such as language, decision making, and executive functions are gradually impaired (2). Eventually neurological symptoms appear and the disease is fatal, usually due to dysphagia and aspiration pneumonia (3).

1.1.2 Etiology and pathological hallmarks

Rare autosomal dominant inherited forms of AD, termed familial AD (fAD), are caused by genetic mutations in either the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or *PSEN2* genes (4). These forms typically have an early onset (<65 years of age) and account only for 1% of all forms of AD. Ninety-nine percent of cases are, instead, defined as sporadic (sAD) typically with late-onset (>65 years of age). Hereditary factors are estimated to contribute to about 80% of the sAD risk (2, 5). The greatest risk factors for sAD are age, family history in a first degree relative, and *APOE ε4* allele (4). Several other genetic risk loci have been identified through genome-wide association studies (GWAS) and their functional relation to AD pathogenesis is currently under investigation (6). Pathological hallmarks of AD are extracellular deposition of the peptide amyloid beta (Aβ) into the form of "senile plaques", intraneuronal aggregation of hyperphosphorylated microtubule-associated protein tau into neurofibrillary tangles (NFTs), neurodegeneration, synaptic degeneration, microgliosis and astrogliosis (2).

1.1.3 Diagnosis

Historically, AD has been diagnosed at late stages of the disease when cognitive symptoms appeared, with a definitive diagnosis only made through a post-mortem neuropathological evaluation (7, 8). In 2011, the National

Institute on Aging and Alzheimer's Association (NIA-AA) provided guidelines for how to clinically diagnose AD and defined two distinct clinical phases of AD: mild cognitive impairment (MCI) and dementia, with dementia further divided into mild, moderate, and severe. The difference between the MCI and dementia rests in the "significant interference in the ability to function at work or in usual daily activities" (9-11). Furthermore, AD pathophysiological processes start decades before onset of symptoms and thanks to imaging and fluid biomarkers, this pre-clinical phase of AD has also been defined (9, 12). However, it is important to note that 10-30% of cognitive unimpaired individuals present AD neuropathological changes at autopsy (9), which means that having AD does not automatically translate into developing dementia in the lifetime. This has created confusion with what AD diagnosis actually means.

1.1.4 *In vivo* biomarkers

More recently, in the updated NIA-AA work group in 2018, AD has been redefined as an "aggregate of neuropathological changes" rather than a clinical diagnosis, which needs to be defined by *in vivo* biomarkers, classified in the "AT(N) system" (9, 13). This system is designed to aid especially in the research and clinical trial setting, where individuals can be assigned a biomarker phenotype, maximizing the benefits for targeted treatments.

- "A" stands for amyloid pathology, it is measured through amyloid positron emission tomography (PET) or concentration of soluble Aβ in cerebrospinal fluid (CSF) and it defines if an individual is in the Alzheimer's continuum.
- "T" is for tau pathology, it is measured through tau PET or concentration of phosphorylated tau (pTau) in CSF or plasma and it determines if an individual in the AD continuum has AD. In this updated diagnostic criteria A+T+ cognitively unimpaired individuals would be labelled as having preclinical AD. If they lived long enough (maybe around 10 years), most will develop AD-like symptoms.
- Finally, "N" stands for neurodegeneration or neuronal injury, it is measured by concentration of CSF total tau, magnetic resonance imaging (MRI) or fluorodeoxyglucose (FDG) PET and it determines the severity of the disease.

The category (N) is still evolving with the potential to include biomarkers related to synaptic pathology, a key early aspect in the pathophysiology of AD.

These biomarkers are clinically significant due to the direct and strong correlation between synaptic degeneration and cognitive decline (14). Therefore, synaptic biomarkers are crucial for aiding in diagnosis, disease staging, but also monitoring the outcomes of clinical trials aimed at stopping or minimizing synaptic damage (15). The most promising synaptic biomarkers are the pre-synaptic proteins synaptosomal-associated protein 25 (SNAP25) and the post-synaptic protein neurogranin, the concentration of which have been found to be particularly elevated in CSF of AD patients and can predict future cognitive decline (16, 17). These levels start to rise during the pre-symptomatic phase of AD, concurrent with the emergence of Aβ-containing plaques, indicating early synaptic dysfunction and degeneration in AD (18, 19). Synaptic dysfunction in the brain can also be visualized *in vivo* using imaging technologies. Recently, reduced density of synaptic vesicle protein 2A (SV2A) measured by PET was reported in AD patients (20). To date, a significant number of synaptic proteins which may serve as potential markers for synaptic dysfunction remain uninvestigated.

1.1.5 Pathogenesis

1.1.5.1 The amyloid cascade hypothesis

Based on genetic evidence and temporal emergence of pathological hallmarks, in 1992, Hardy and Higgins postulated the "amyloid cascade hypothesis" (21). According to this now widely accepted hypothesis, Aβ deposition is the earliest and causative event of AD pathology that induces a cascade of modifications in the brain eventually leading to neuronal and synaptic loss and dementia. Aβ peptides are physiologically produced in different lengths and continuously secreted by neurons, astrocytes and oligodendrocytes in the heathy brain (22). The most abundant form of Aβ detected in the CSF of adult healthy brain is 40 amino acids long ($A\beta_{40}$), while the longer form $A\beta_{42}$, is 10 times less abundant and highly prone to aggregation. When the balance between these two Aβ forms is shifted towards more production (mainly in fAD) or less clearance (mainly in sAD) of $A\beta_{42}$ over $A\beta_{40}$, pathological processes begin in the brain (2).

The biochemical phase

The sequence of biochemical events for Aβ aggregation from monomers to fibrils, the most abundant Aβ form in plaques, have been extensively studied

(23). In their soluble state, Aβ polypeptides natively assume a random-coil disorganized structure. However, based on parameters such as pH, temperature, protein concentration and interaction with other molecules they convert into a β-sheet structure, which aggregates into larger oligomers in a phase called primary nucleation. Oligomers are only intermediates that elongate into protofibrils and mature fibrils, which are relatively stable and inert. However, shear forces can break fibrils into oligomers (fragmentation) or monomers can aggregate through interaction with fibrils (secondary nucleation). Soluble, diffusible Aβ oligomers are the most neurotoxic Aβ species (24). The process of Aβ fibrillation is described in **Figure 1**.

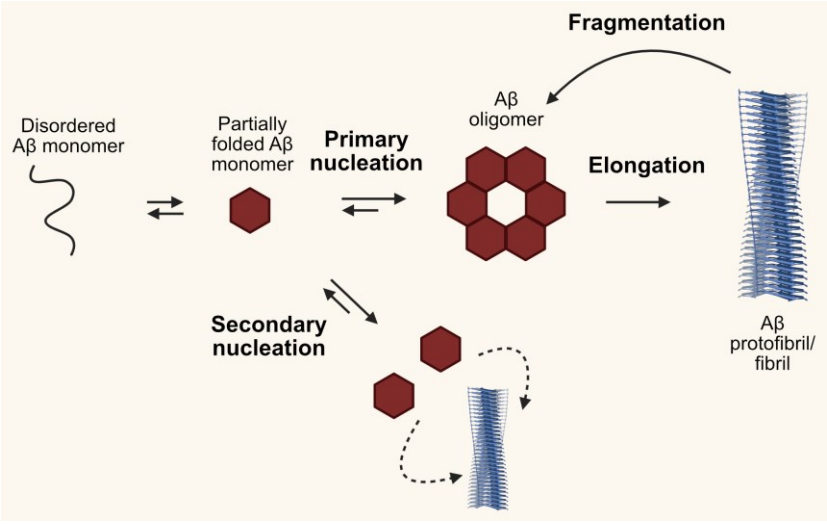


Figure 1. The process of Aβ fibrillation. Created with Biorender.com

The cellular phase

Aβ aggregates cause hyperphosphorylation of tau, which also aggregates into oligomers and then tangles. Both Aβ and tau aggregates spread in a prion-like fashion in a temporal sequence which is undefined (25, 26). The misfolded proteins interfere intracellularly with normal cellular processes and organelles, such as endoplasmic reticulum (ER), endosomes, lysosomes, autophagosomes (27-29). Since lysosome and autophagosomes are needed for protein degradation, dysfunction in these pathways exacerbate the aggregation of Aβ (30). Mitochondrial and oxidative stress have also been linked to Aβ-mediated neuronal death (31). Although direct neurotoxicity mechanisms of Aβ and tau aggregates have been studied for decades in animal models and cell culture

(for reviews see 32, 33), it is clear how all of these negative effects are not immediately dangerous for the survival and well-being of the cell, since individuals with abnormal AD biomarkers usually live for decades without any symptoms. Even in more severe cases of autosomal dominant fAD, patients will have increased levels of Aβ - therefore increased toxic Aβ oligomers - already early in life but will only develop dementia in mid-life. This resilience is likely due to homeostatic cellular mechanisms that counteract the toxic ones. When these mechanisms become insufficient, a second phase, termed the "cellular phase" begins (reviewed in 34).

Beyond neurons, astrocytes and microglia are involved in both protective and later deleterious mechanisms. For example, reactive astrogliosis found in AD brains, which involves hypertrophy and hyperproliferation of astrocytes, is actually a protective mechanism which promotes post-injury neuronal function recovery (35). Indeed, in mouse models with decreased astroglial activation, plaque load and dystrophic neurites were subsequently increased (36). However, reactive astrocytes downregulate the glymphatic flow, reducing Aβ clearance efficiency (37) and release pro-inflammatory mediators, leading to a cascade of events culminating with neuronal injury. Furthermore, chronic neuroinflammation brings to increased reactive oxidative stress and exacerbate Aβ and tau pathology (38). Atrophic astrocytes have also been observed in AD mouse models, in association with synaptic dysfunction and excitotoxicity (39).

Microglia, immune cell of the central nervous system (CNS), is another cell type that lately gained a lot of attention in AD pathogenesis. Rare mutations in the microglial triggering receptor expressed on myeloid cells 2 (*TREM2*) gene elevate the risk of sAD by approximately 3-5-fold (40, 41). Furthermore, GWA studies have uncovered more than 20 genetic loci strongly linked to the risk of sAD, a significant number of which are predominantly or solely found in microglial cells (42). These findings indicate that dysfunction in microglia is an important event in disease pathogenesis. Normal functions of microglia cover elimination of synapses and excessive neurons during development via phagocytosis or induced apoptosis (43). However, in AD activated microglia phagocytises more synapses, exacerbating synaptic dysfunction (44). Interestingly, a study demonstrated that microglia actively makes plaques, especially dense-core ones, by phagocyting Aβ protofibrils and oligomers, compacting them in their lysosomes and then resending them into the extracellular space to form plaques (45). In the absence of phagocyting microglia amyloid accumulated more around the blood vessels, causing

cerebral amyloid angiopathy (45). Thus, microglia help pack amyloid into tight aggregates in isolated parenchymal regions, which may inhibit toxicity. Emerging roles of these cells in disease-development and possible therapeutic intervention are currently being explored.

1.1.5.2 Alternative hypotheses

Although alternative hypotheses exist, none of them can completely replace the main Aβ aggregation hypothesis. As AD pathogenesis is complex and multifactorial, it is likely that all mechanisms cited below are involved in the disease development and exacerbation in an Aβ-related manner.

The cholinergic hypothesis

The cholinergic hypothesis is the oldest hypothesis based on cholinergic dysfunction and current available drugs are based on this hypothesis (46). Studies found abnormal levels of enzymes connected to the synthesis, transport, release and degradation of the neurotransmitter acetylcholine in AD brain (47). This led to the hypothesis that a decline in cholinergic neurotransmission in the cerebral cortex and other regions played a major role in the deterioration of cognitive abilities in AD. Cholinesterase inhibitors were developed, but they revealed to be only symptomatic treatment, while failing to prevent or delay AD progression in MCI patients (46, 48).

The tau hypothesis

Hyperphosphorylation and aggregation of tau disrupts its normal function, resulting in a pathological alteration of its structural and regulatory roles of the cytoskeleton. This impacts neuronal morphology, axonal transport, and causes synaptic dysfunction and neurodegeneration (49). The tau hypothesis suggests that the formation of tau tangles occurs before the development of Aβ plaques. The primary argument for this hypothesis is that several diseases exist in which tau aggregation is the primary neurotoxic feature (primary tauopathies), in the absence of Aβ pathology (50). However, therapeutics targeting tau phosphorylation or aggregation for the prevention of AD have also failed in showing clinical efficacy (51). Perhaps the biggest evidence that tau pathology is, instead, downstream to Aβ pathology is provided by the decrease in tau biomarkers in patients treated with anti-Aβ antibodies-based therapeutics (52-54).

The oxidative stress hypothesis

Animal and human research has consistently suggested that oxidative stress is a prevalent aspect of AD brain pathology, highlighting a potential pathogenic role in the disease's development (55, 56). Aβ itself is cause of oxidative stress, through mitochondrial dysfunction (57). However, randomized clinical trials using antioxidants have not delivered the expected outcomes suggested by these studies.

1.1.6 Aβ generation

Aβ is produced by cleavage of the amyloid precursor protein (APP) (58). APP is a type I single pass transmembrane protein, composed of a long N-terminal ectodomain protruding on the extracellular or luminal side of certain intracellular vesicles, a transmembrane part, including the Aβ sequence, and a short C-terminal part on the cytosolic side (**Figure 2**, **left panel**). APP undergoes sequential cleavage that produces several soluble and membrane-bound fragments, including Aβ, through two main pathways (**Figure 2**, **right panels**).

The amyloidogenic pathway begins with cleavage of the aspartyl protease β-secretase at the β-site (59, 60). The most active neuronal isoform of β-secretase is BACE1 (61). This cleavage releases a long soluble N-terminal fragment called sAPPβ and leaves a short membrane-bound C-terminal fragment (CTFβ). CTFβ is then the substrate for the aspartyl protease γ-secretase that cleaves at the γ-site, which releases amyloidogenic Aβ extracellularly (or in the vesicles) and APP intracellular domain (AICD) in the cytosol. γ-Secretase is a multi-protein complex, in which the active site is contained in presenilin 1 (PSEN1) or PSEN2 (62). The level of processivity of γ-secretase defines the final length of the secreted Aβ (37 to 43 amino acids long), of which forms $Aβ_{42}$ is highly amyloidogenic (63).

In the non-amyloidogenic pathway, a third protease, called α-secretase cleaves 16 aa downstream from the β-site, within the Aβ region, therefore preventing Aβ production. α-cleavage produces a longer soluble fragment (sAPPα) and a shorter CTFα. Subsequent cleavage of CTFα by γ-secretase produces a shorter and non-amyloidogenic peptide (p3) and AICD (64). Alternatively, C-terminally truncated forms of Aβ (up to 16 amino acids long) have been described to be produced by combined activity of α- and β-secretase (65).

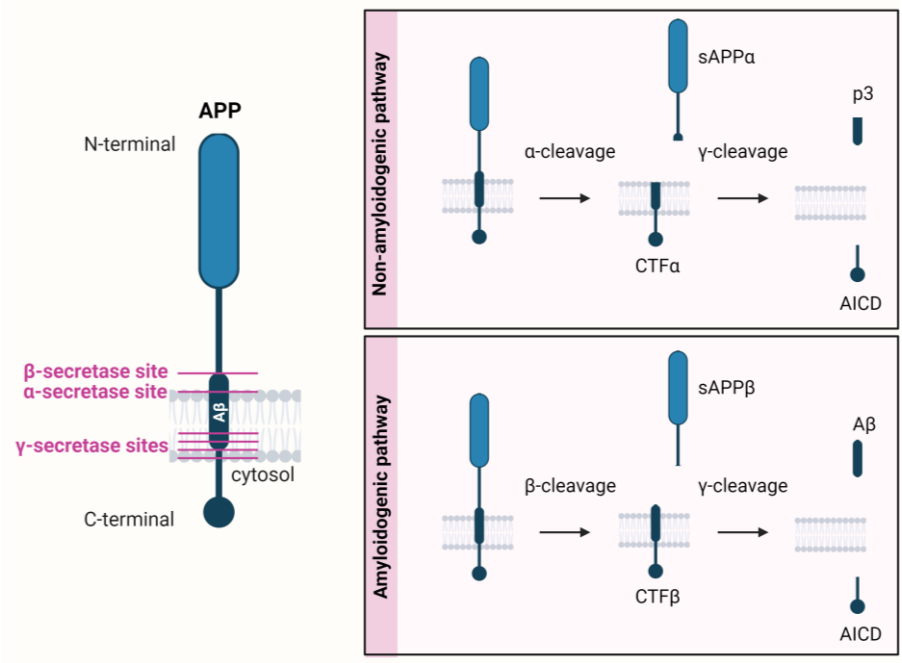


Figure 2. Schematic representation of APP with cleaving sites for α-, β- and γ-secretase (left) and the two canonical pathways of APP proteolytic processing (right).

Aβ production requires proximity of APP and the secretases in the same subcellular compartment (66). As a type I transmembrane protein, APP traffics through the secretory pathway. After synthesis and ER and Golgi post-translational processing, APP is directed to the plasma membrane (PM), where part of it is processed in the non-amyloidogenic pathway, while a bigger fraction is re-internalized into endocytic compartments. From here, most of APP is directed to late endosomes and lysosomes for degradation, while a smaller fraction is recycled to the PM via recycling endosomes or retrograded to the trans-Golgi network (TGN). At the steady state, most of APP resides in the Golgi and TGN. Amyloidogenic cleavage by β- and γ-secretase happens mainly in endosomes and lysosomes (67-69), where the low pH enhances their proteolytic activity. β- and γ-secretase cleavage at the lipid rafts has also been reported (70, 71). However, site and mechanisms of Aβ production are still widely under investigation.

1.1.7 Treatment

1.1.7.1 Approved therapeutics

Until 2021, only symptomatic treatments were available for AD, comprising acetylcholinesterase inhibitors (e.g., donepezil and galantamine) and the partial antagonist of N-methyl-D-aspartic acid receptor (NMDAR), memantine, to counteract the disbalance in cholinergic and glutamatergic signaling, respectively (72). Although these drugs help easing cognitive and functional deficits and behavioral symptoms, they do so only partially and temporarily.

The last three years, two disease-modifying drugs have been approved in the US: aducanumab and lecanemab (53, 73). Both are monoclonal antibodies directed towards aggregated forms of Aβ, that have shown to reduce plaque loads and NFT deposition in the brain (measured by PET) and normalized both Aβ and pTau biomarkers in the CSF. Lecanemab, specifically, showed a reduction in cognitive decline by 27% in 18 months compared to placebo in patients with early AD (MCI or mild AD)(54). A similar effect was also seen with another monoclonal antibody in phase III, donanemab (52). A significant side-effect that comes with all anti-amyloid treatments is the amyloid-related imaging abnormalities (ARIA), which involve brain edema, microbleeds, and in some cases, significant brain hemorrhages, which have been linked to fatalities in certain clinical trials (74).

Although it is debated whether the observed slowing in clinical progression is clinically significant enough to justify the risk of side effects, and the expense of treatment, these revolutionizing data have for the first time confirmed in human the validity of the amyloid cascade hypothesis as causation of the disease. In the meantime, more than 100 pharmacological compounds are currently being tested in clinical trials as disease-modifying treatments for AD, mainly targeting Aβ, tau, inflammation and synaptic plasticity/neuroprotection (75).

1.1.7.2 Therapeutics in the pipeline: the case of BACE inhibitors

Another class of disease-modifying drugs that showed promising results in reducing Aβ biomarkers in clinical trials is the BACE inhibitors (76, 77). BACE1 is the enzyme that initiates APP cleavage to produce Aβ, therefore its inhibition theoretically reduces Aβ secretion, thereby its accumulation. Since BACE inhibitors are small molecules that can be administered orally, the

advantage over the monoclonal antibodies would be the cost, ease of administration and potentially less side-effects. Unfortunately, this was not the case, as all clinical trials with BACE inhibitors, although successfully reducing Aβ biomarkers in brain and CSF, have been halted so far due to lack of efficacy in slowing cognitive decline or severe adverse cognitive side effects (77).

However, the scientific community thinks it is still too early to abandon this class of drugs, while new data from past clinical trials have shown where we did wrong (78). Due to apparent normal phenotype in BACE1 null mice, inhibition of BACE1 seemed to be safe and was targeted to as high as 90% in the first clinical trials (79). As a result, patients in the treatment group experienced faster cognitive decline, weight loss and brain atrophy compared to the placebo group and all trials were consequentially halted (77). Future investigations showed that these side-effects were partially reversible once the treatment stopped (77). In the meantime, research in animal models and cell culture had found that the side-effects may be related to loss-of-function of several products of BACE1 proteolysis, such as seizure protein 6 homolog (SEZ6), seizure 6-like protein (SEZ6L), amyloid precursor-like protein 1 (APLP1) and close homolog of L1 (CHL1) (80-82), while low-dose BACE1 inhibition may spare the deleterious effects (77, 83).

1.2 THE SYNAPSE

According to the Campbell's Psychiatric Dictionary "cognition is a […] high level of processing of specific information including thinking, memory, perception, motivation, skilled movements and language". In the brain, this information processing is done by billions of excitable neurons through trillions of fundamental units, named synapses (84).

Neurons are polarized cells with an input-end (soma and dendrites) and an output-end (axon terminals). Synapses are the asymmetrical intercellular junctions that mediate rapid information transfer between the output and input neurons, which are therefore called pre- and post-synaptic neuron, respectively. The most common synapses are axo-dendritic and axo-somatic, although axo-axonic connections are also seen (85). In the most common type, the chemical synapse, neurotransmitters released by the pre-synaptic neuron into the synaptic cleft (a space around 15-20 nm wide) bind receptors on the post-synaptic membrane to transduce the input signal. Most synapses are formed *un-passant* by axons, which create numerous synapses, typically organized "like pearls on a string" (86). The most common neurotransmitter in

the brain is glutamate and glutamatergic synapses account for 80% of all synapses in the brain, particularly in areas related to memory and cognition (86, 87).

1.2.1 Structure

The structure and activity of a typical glutamatergic synapse is schematically summarized in **Figure 3**. In the pre-synaptic end, glutamate is packed into synaptic vesicles (SVs) through a vesicular transporter (vGlut) and then released by the fusion of the SV with the membrane in response to increased concentration of Ca^{2+}. The standard pre-synaptic release machinery comprises of: 1) SNARE proteins, involved in SV fusion, 2) syntaxins and complexins that sense increased Ca^{2+} levels and initiate SV fusion and 3) Rab3-interacting molecules, key components of the active zone that tether SVs and attract Ca^{2+} channels to sites where neurotransmitters are released (86).

At the post-synaptic membrane, glutamate binds specific receptors, which can be either ionotropic, such as the NMDAR and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) or metabotropic (mGluRs). Both NMDAR and AMPAR are transmembrane ion channels that can depolarize the membrane by allowing the influx of cations, while the mGluRs operate through second-messengers. The post-synaptic density (PSD) functions to maintain receptors packed near the sites where glutamate is released (86).

1.2.2 Fast synaptic transmission and action potential

The main functional difference between NMDA and AMPA receptors lays in the speed and mode of signal transmission. AMPAR is exclusively ligand-gated, which means that it is the first one to open, allowing sodium influx and depolarizing the post-synaptic membrane. When the depolarization reaches a certain threshold at the axon hillock, the post-synaptic neuron fires an action potential (AP), an all-or-nothing strong membrane depolarization input, which travels unidirectionally along the axon and reaches the synaptic terminals. Depolarization of the membrane at the axonal terminal opens voltage-gated Ca^{2+} channels and inflow of Ca^{2+} activates SV fusion (88).

1.2.3 Slow synaptic transmission and synaptic plasticity

On the contrary, NMDAR needs both pre- and post-synaptic stimuli, such as binding to glutamate (and the co-activator D-serine) and partial depolarization of the post-synaptic membrane. However, NMDAR is also much more permeable to calcium than AMPAR and therefore it initiates a cascade of second-messenger signal transduction, such as long-term synaptic plasticity. Synaptic plasticity refers to the activity-dependent modification of synaptic transmission. This plasticity can either enhance (long-term potentiation, LTP) or reduce (long-term depression, LTD) the strength and efficiency of synaptic connections. In the case of NMDAR-mediated LTP, when high-frequency transmission activates NMDAR, calcium ions activate calcium/calmodulin-dependent protein kinase II (CAMKII). This kinase phosphorylates AMPAR, leading to the increased expression and stabilization of AMPARs at the PSD, increasing future fast synaptic transmission, thus synaptic strength (89). Dendritic spines subject to LTP also grow in size. Conversely, low levels of transmission can induce LTD, characterized by dephosphorylation of AMPAR, leading to receptor internalization through endocytosis and pruning and shrinking of spines (90).

1.2.4 The tripartite synapse

Synapses not only consist of pre- and post-synaptic neurons but often also include contact with a peri-synaptic astrocyte process (PAP). Astrocytes participate to glutamatergic synaptic transmission in several ways. First, glutamate is removed from the synaptic space predominantly by excitatory amino acid transporters (EAATs) located on nearby astrocytes and, only to a smaller degree, on neurons. Glutamate in the astrocytes is converted into glutamine, which is then re-transported into the pre-synaptic bouton (91). Re-uptake of glutamate from astrocytes is the major path to stop glutamate signaling and impairment in this route can cause cell death through excitotoxicity (92). In addition, PAPs express mGluRs which get activated by the nearby neuronal activity and respond by increasing the intracellular Ca^{2+} concentration, which exerts second messenger-mediated effects in the astrocyte (93, 94).

Astrocytes also play a role in synaptogenesis by secreting synaptogenic factors, such as aforementioned D-serine, brain-derived neurotrophic factor (BDNF) and transforming growth factor β (TGFβ) (95).

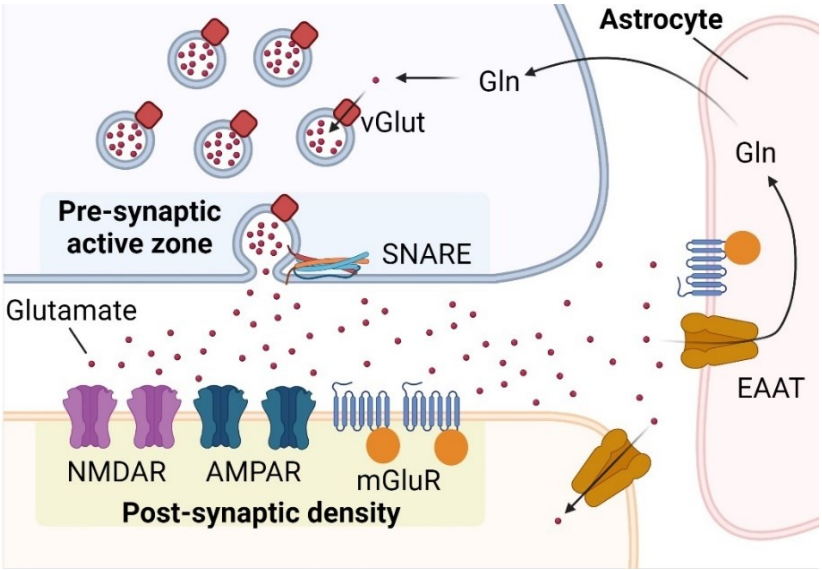


Figure 3. Schematic representation of an active glutamatergic synapse. Glutamate is pumped into the synaptic vesicles (SVs) through vGlut. Tethering and fusion of the SVs at the pre-synaptic active zone, involving interaction of the SNARE proteins, induces secretion of glutamate into the synaptic cleft. The post-synaptic density (PSD) gathers ionotropic (NMDAR and AMPAR) and metabotropic (mGluR) receptors opposite to the active zone for an efficient synaptic transmission. Re-uptake of glutamate is made through excitatory amino acid transporters (EAATs) on post-synaptic neurons or the peri-synaptic astrocyte process (PAP). The astrocytes participate to the glutamate metabolism, by converting it into glutamine (Gln) and resending it to the pre-synaptic neuron, where it will convert back into glutamate. mGluRs on the PAP allow the astrocyte to sense and modulate synaptic transmission. Created with biorender.com.

1.2.5 $A\beta_{42}$ in synaptic function

Despite its implication in the etiology of AD, $A\beta_{42}$ is endogenously produced and secreted in the healthy brain throughout life, suggesting that it exerts physiological functions in neurons (96). This dual nature of $A\beta_{42}$ seems to be mostly dependent on dose, exposure time or aggregation state. Indeed, picomolar concentrations of $A\beta_{42}$ were shown to positively modulate synaptic plasticity and memory (97-100) and recent research demonstrated even a synaptogenic effect of $A\beta_{42}$ (101). Furthermore, synaptic activity and $A\beta_{42}$ generation seem to be inter-dependent. Indeed, increased synaptic activity enhances $A\beta_{42}$ generation and extracellular secretion while opposite effect is seen with decreased synaptic activity (102-104).

1.2.6 $A\beta_{42}$ in synaptic dysfunction

Given the documented role of $A\beta_{42}$ in synapse function and plasticity, it has been speculated that synaptotoxicity of oligomeric Aβ species is nothing more than exacerbated physiological effects of Aβ, consequence of impaired homeostasis of its metabolism. High nanomolar concentrations or prolonged exposure of oligomerized $A\beta_{42}$ ($oA\beta_{42}$) has been shown to damage synaptic function through over 20 candidate receptors (reviewed in 105, 106).

For example, nanomolar concentration of $oA\beta_{42}$ was shown to depress excitatory synaptic transmission and impair synaptic plasticity and memory in AD animal models via α7nAChR activation (107, 108). On the other hand, hyperactivated neurons have also been found in the hippocampus of early AD animal models (109), healthy hippocampi injected with exogenous $oA\beta_{42}$ (110) and in regions surrounding the plaques in mouse models (111). $oA\beta_{42}$-mediated neuronal hyperactivation has been explained either by impairment in inhibitory transmission or increased synaptic glutamate concentration due to decreased re-uptake and/or increased secretion (112). Prolonged neuronal hyperactivation causes cell death through NMDAR-mediated excitotoxicity (92).

Synaptic dysfunction can be monitored *in vivo* by PET imaging or CSF concentration of synaptic biomarkers, as explained in **paragraph 1.1.4**. Evidence shows that Aβ influences these biomarkers change. For example, a significant reduction in synaptic density is seen in pre-clinical AD, before formation of plaques and tangles, therefore probably as a direct consequence of increased Aβ oligomers (18). However, the direct relationship between Aβ pathology and synaptic biomarker release remains elusive.

1.3 THE USE OF HUMAN PLURIPOTENT STEM CELLS FOR AD RESEARCH

Small mammals like mice and rats are frequently used in disease modeling. However, since rodents do not spontaneously develop amyloid plaques and NFTs, these conditions must be artificially created. Several transgenic rodent models have been developed for AD pre-clinical research, mainly expressing mutated forms of human *APP* or *PSEN1*, and have helped understanding many processes of AD pathogenesis (113, 114). However, cerebral amyloidosis in these models mirrors familial rather than sporadic forms of AD and tauopathy is essentially absent in these animals. Therefore, the ability to translate findings

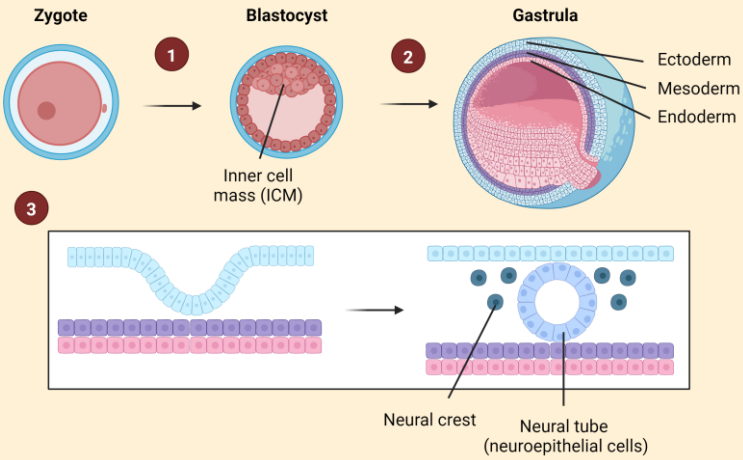
to human AD has been questioned. The generation of human models of the disease is now possible thanks to the ability to culture pluripotent stem cells (PSCs) and to differentiate them into virtually any cell type of the CNS (115).

1.3.1 The origin of pluripotent stem cells

PSCs have the ability to differentiate into any cell type. Since 1981 from mouse surgically explanted blastocysts (116) and 1998 from human blastocysts produced with *in vitro* fertilization (117), PSCs can be isolated from the inner cell mass (ICM) and maintained undifferentiated long-term *in vitro.* See **Box 1** for a detailed explanation of early embryogenesis and neurogenesis and of the potency of the cell types involved. Cells isolated from the ICM, termed embryonic stem cells (ESCs) have three characteristics: a) the capacity for extensive proliferation (self-renewal), b) the origin from a single cell (clonality), and c) the competence to develop into various types of cells (potency) (118). However, since the creation of ESCs involves the destruction of embryos, research with hESC suffers of both availability and ethical limitations.

In 2006 Shinya Yamanaka's lab discovered that overexpression of four factors (Oct3/4, Sox2, Klf4, and c-Myc) in terminally differentiated somatic cells, such as skin fibroblasts, is sufficient for the cells to regain the pluripotency state, similar to the one in ESCs (119). This discovery was awarded the Nobel Prize in Medicine or Physiology in 2012 and the reprogrammed cells were defined induced pluripotent stem cells (iPSCs). Since then, protocols to differentiate iPSCs into several cell types, including neurons, astrocytes, oligodendrocytes and microglia have arisen (115).

Box 1 | ***Potency during differentiation of the nervous system***



The cell of the zygote is defined **totipotent** because it can differentiate in all the known cell types included in both embryonic and extraembryonic tissue, forming the embryo and the placenta. After a phase of cleavage, the zygote proceeds into the blastulation phase (**1**), and cells differentiate into two distinct layers that define the blastocyst: the outer trophoblast which will form extraembryonic structures, and the inner cell mass (ICM). Cells in the ICM, although losing the ability to form the placenta, conserve a **pluripotent** nature, which means that they can generate any embryonic tissue in the body. During the subsequent gastrulation phase (**2**), ICM cells differentiate into three distinct germ layers – ectoderm, mesoderm and endoderm – each giving rise to different linings and organs in the fetus. During the following phase of neurulation (**3**), part of the ectoderm folds and fuses into the neural tube, which will become the adult brain and spinal cord. The neural tube comprises **multipotent** neuroepithelial cells, which are able to generate neurons, astrocytes and oligodendrocytes. Some cells of the neuroectoderm (neural crest) during this process migrate out of the neural tube to give rise to the peripheral nervous system. The first cell types in the developing CNS to have a restricted cell fate are the radial glial cells, generated from asymmetrical division of neuroepithelial cells. Radial glial cells are therefore **unipotent** neuronal progenitor cells (NPCs) which can self-renew or terminally differentiate into a restricted cell type. Most neurons in the CNS differentiate from radial glial cells (120). This illustration was created using biorender.com.

1.3.2 Cortical differentiation of iPSCs

Two main strategies exist to differentiate iPSCs into cortical neurons: 1) the morphogen-based neuronal induction, in which iPSCs are pushed towards neuroectodermal differentiation similar to the *in vivo* embryogenesis and 2) the transcription factors-based differentiation, in which overexpression of cell type-specific transcription factors induce rapid differentiation of iPSCs into terminally differentiated cortical neurons (115).

1.3.2.1 Morphogen-based cortical differentiation

During early embryonic development *in vivo*, pluripotent ICM cells differentiate into three germ layers following morphogenic cues (**Box 1**). Thereafter, part of the ectodermal germ layer differentiates into the neuroectoderm and subsequently the neural tube, a radially-organized structure comprising multipotent neuroepithelial cells, or neuronal stem cells (NSCs). This process, called neurulation, is facilitated *in vitro* by inhibition of TGFβ and bone morphogenic factor 4 (BMP4). Since both morphogens activate SMAD-type transcription factors, this protocol is defined dual SMAD inhibition. Dual SMAD inhibition allows neuroectodermal differentiation of iPSCs into radial structures called "neural rosettes", which resemble the neural tube and function as a source of NSCs in as soon as 10 days (121). NSCs can be expanded in the presence of fibroblast growth factor 2 (fgf2) and will spontaneously differentiate into neurons in non-self-renewal conditions (without fgf2). This will give rise to a heterogenous culture of neurons and astroglia, which normally becomes spontaneously active in as long as 60 days (121, 122).

This approach can be adapted by incorporating certain growth factors or small molecules to accelerate the differentiation process towards neurons. The most commonly used factors are BDNF (induces differentiation of NPCs into neurons), glial cell line-derived neurotrophic factor (GDNF – improves neuron survival) and cyclic adenosine monophosphate (cAMP – activates CREB-mediated gene transcription, influencing dendritic length and the maturity of young neurons) (115).

1.3.2.2 Transcription factor-based cortical differentiation

Transient overexpression of transcription factors can induce rapid differentiation of iPSCs into neuronal types. The first successful generation of so called glutamatergic induced neurons (iGluts) from iPSCs was achieved

through the forced expression of the transcription factors *NGN2, BRN2, ASCL1*, and *MYT1L* (123). Later it was shown that expression of *NGN2* was sufficient to induce morphologically mature neurons in two weeks. Development of synaptically mature neurons required co-culture with astrocytes (124, 125).

These cultures have been used as human models for examining the mechanisms of human neural development *in vitro*, for modeling human CNS-related diseases, and for drug discovery and testing. As of 2022, for AD solely, over 60 studies had been conducted using iPSC-derived neuronal models for *in vitro* research on the disease (115).

2 AIM

2.1 GENERAL AIM

The aim of this thesis is to increase the understanding of mechanisms of Aβ generation in neurons as well as Aβ-related pathophysiological processes at the synapse, using an entirely human model system.

2.2 SPECIFIC AIMS

I. To investigate how Aβ generation and secretion is regulated by APP/secretases interaction in a manipulation-free human neuronal model

II. To establish how oligomeric Aβ affects the release of biomarkers of synaptic dysfunction from neurons

III. To evaluate if synaptic dysfunction caused by BACE1 inhibition-mediated reduction in Aβ secretion is caused by accumulation of APP at the synapses

IV. To develop a protocol of human Ngn2-induced cortical neurons that form synchronous spontaneous synaptic network in the absence of glia

3 METHODOLOGICAL CONSIDERATIONS

3.1 HUMAN PSC-DERIVED CORTICAL NEURONS

AD is a disease of the cerebral cortex; therefore, cortical neurons were chosen as neuronal model to study Aβ pathophysiology. Three hiPSC lines were used: Ctrl1 (126), ChiPSC22 (Takara Bio Europe) and WTSIi015-A (EBiSC/Sigma Aldrich), all originated from reprogrammed fibroblasts (**Table 1**). In **paper IV**, the hESC line WAe009-A (WiCell) was additionally used.

Table 1. Human pluripotent stem cell lines used in the thesis.

	Type	Origin	Age of donor (years)	Sex chromosomes
Ctrl-1	hiPSC	skin fibroblasts	78	XY
ChiPSC22	hiPSC	skin fibroblasts	32	XY
WTSIi015-A	hiPSC	skin fibroblasts	65-69	XX
WAe009-A	hESC	blastocyst/embryo	n.a.	XX

n.a.= not applicable

Cortical neuronal differentiation of hPSCs was initiated using two methods.

3.1.1 Cortical neuronal differentiation using dual SMAD inhibition

In **paper I, II and III**, iPSCs were induced using **dual SMAD inhibition** as described before (122). Briefly, a confluent monolayer of hPSCs on Matrigel-coated well was incubated with neuronal maintenance medium (NMM) supplemented with TGFβ1-inhibitor SB431542 and the BMP4 antagonist Noggin or Dorsomorphin for 10 days. Afterwards, cells were enzymatically detached by incubation with dispase, a protease formulation that more efficiently dissociates mesenchymal cells, precursors to connective tissue, into single cells, while leaving neuroepithelial cell populations intact. Cells were maintained on laminin in NMM supplemented with fgf2 for 4 days to promote self-renewal of NSCs. At this stage, the differentiating culture often carries some contamination from mesenchymal cell, which can be removed by dispase passaging, as described above. Appearance of neuronal rosettes, signaled the differentiation into NPCs. Around 20-25 days post induction (dpi), radial glial cells displaying longer neurites appear at the edges of the NPC populations. At

this stage, pure NPC cultures are dissociated into single cells using gentle dissociation enzymes, and plated onto laminin. NPCs were frozen between 20 and 30 dpi or expanded until 30-40 dpi, plated onto poly-L-ornithine and laminin and then maintained in NMM with medium exchange every second day. For studies involving synaptically active neurons, NMM was switched to BrainPhys medium (BPM), of which composition and ion concentrations more closely resemble human CSF (127), supplemented with pro-neuronal differentiation factors, including BDNF, GDNF and cAMP (see **paragraph 1.3.2.1**). Recipes of each neuronal medium are found in the method section of the papers.

3.1.2 Cortical neuronal differentiation using Ngn2 overexpression

In **paper III** and **IV**, **Ngn2 overexpression** was used as a second method to generate cortical neurons from hPSCs, as described before (128). Briefly, hiPSC and hESCs were transduced with two plasmids (**Figure 4**):

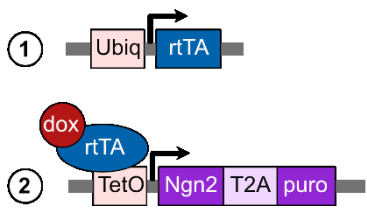


Figure 4. Plasmids used in Ngn2 neuronal induction.

1. **FU-M2rtTA**, integrating lentiviral vector containing the gene for M2-rtTA, a reverse tetracycline trans activator particularly sensitive to doxycycline activation;
2. **Tet-O-Ngn2-puromycin**, containing human *NGN2* transcribed under control of the TetOn system, together with the gene of resistance to puromycin for the selection of transduced clones.

Ngn2/puromycin expression was induced on dpi 0 by addition of doxycycline in N2-medium containing pro-neuronal factors BDNF and NT3 for 3 days, while puromycin addition caused cell death in non-infected cells. Ngn2 expression rapidly induce the transformation of hPSCs into cortical neurons. On dpi 3, cells were re-plated onto mouse primary glia and maintained in Neurobasal-A-based medium containing pro-neuronal survival factor B27. Cytosine arabinoside (araC) was added to stop glia proliferation. From dpi 10,

FBS was added to support glial function. In **paper III** we differentiated hiPSCs using this protocol for functional analysis of spontaneous neuronal activity. A variation of this protocol was used in **paper IV**, where cells at dpi 3 were plated on poly-D-lysin and human laminin without glia and maintained in BPM for several days.

3.1.3 Comparison of the two neuronal models

Both neuronal induction protocols generate cortical glutamatergic neurons. As explained in **paragraphs 1.3.2.1 and 1.3.2.2**, the main difference between the two cell models is that dual SMAD inhibition produces NPCs which differentiate to neurons similarly to how they do *in utero*, while Ngn2-overexpression forces PSCs into neurons, skipping the NPC stage. This creates some regional and functional differences (summarized in **Table 2**).

Table 2. Comparison between two methods of cortical differentiation of PSCs.

	Dual SMAD inhibition	**Ngn2 overexpression**
Neurotransmitter type	Glutamate	Glutamate
Cortical layers	Deep IV-VI (day 40) Upper III (day 70-80) Upper II (day 90)	Upper II, III
Receptors	NMDAR+AMPAR	AMPAR
Spontaneous astrocyte differentiation	Yes	No
Functional synapses	45 days	7 days
Functional network	60-90 days	20 days (in co-culture with astrocytes)

First, physiological differentiation of NPCs generates neuronal types found in both deep and upper layers of the cerebral cortex (121). Instead, Ngn2 forced expression generates a more homogenous culture of layer II and III neurons (124). Ionotropic NMDAR is usually expressed first during glutamatergic synaptogenesis, therefore SMAD inhibition-induced neurons with functional synapses express both NMDAR and AMPAR, while in Ngn2-induced neurons NMDA currents are hardly detected until very late stages (145 days) (129, 130). Functional synapses appear at 45 days in SMAD inhibition-induced neurons, although for a functional network 60-90 days are required (121). With Ngn2-induction a functional network develops in 20 days, though only when co-cultured with astrocytes (124).

To choose the right method of differentiation it is crucial to carefully consider the research question being posed. For example, SMAD inhibition may be more useful in answering developmental questions. However, the model suffers from high variability of cell types that result in each differentiation, even within the same iPSC line (131). Furthermore, it is time consuming and expensive to obtain neurons with electrical and synaptic functionality, making this protocol not feasible for high-throughput studies with electrophysiological output analysis. On the other hand, Ngn2-induction generates reproducible homogenous populations of glutamatergic neurons in considerably less time (**Figure 5**). Therefore, for studies with pure and terminally differentiated neuronal population, Ngn2-induction offers a better choice. However, since astrocytes are absent in the culture, culturing with astroglia feeder layer of conditioned medium is required to reach functional network.

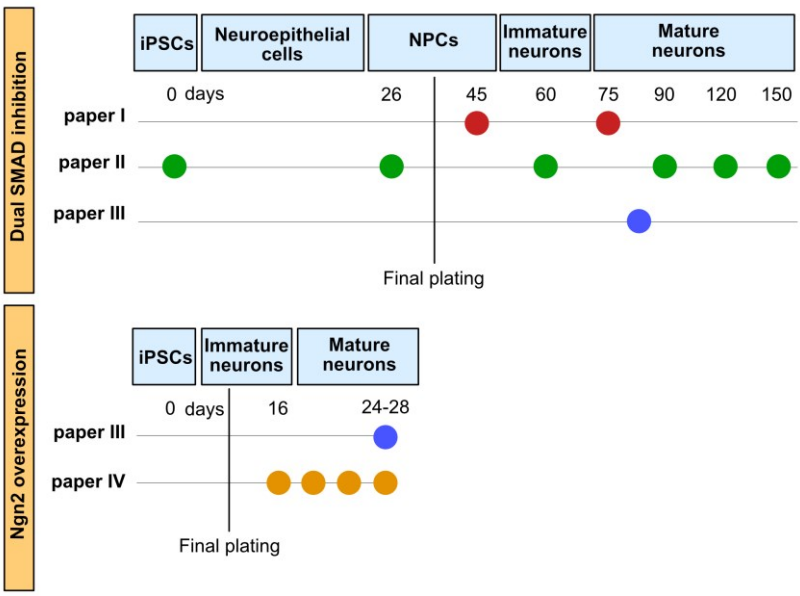


Figure 5. Cortical differentiation protocols and experiment timeline used in this thesis. Experiments (circles) were done at different time points of neuronal differentiation based on the research question. Dual SMAD inhibition (upper panel) induced iPSCs through all physiological stages of embryonic neuronal development, allowing comparison between cell types of different differentiation stages. It takes around 70-90 days to obtain mature neurons. In **paper I** and **II** we compared different time points of differentiation from iPSCs to mature neurons. In **paper III**, we did experiments with mature neurons only. Ngn2 overexpression (lower panel) induces rapid differentiation of iPSCs into neurons, synaptically active in less than 20 days, therefore it is ideal for analysis of terminally differentiated neurons, without

developmental questions. In **paper III** and **IV**, electrophysiological experiments were performed in Ngn2-induced neurons.

3.1.4 Ethical considerations

The derivation and use of each iPSC and ESC line were approved for research purposes (**Table 3**). All donors had signed an informed consent.

Table 3. List of the ethical permits for all PSC lines used in the thesis

	Ethical committee	Ethical approval number	Purchased from/in-house	Obtained consent for research use
WTSIi015-A	NRES Committee Yorkshire & The Humber - Leeds West	15/YH/0391	EBiSC-Sigma Aldrich	yes
ChiPSC22	EudraCT and the French Ethics Committee		Takara Bio Europe	yes
WAe009-A	University of Wisconsin IRB	95-623-239	WiCell	yes
Ctrl-1	National Hospital for Neurology and Neurosurgery and the Institute of Neurology Joint Research Ethics Committee	09/H0716/64	In-house	yes

In general, iPSCs were derived from a donated skin biopsy which are free of health and safety risks and might only cause some temporary discomfort. The ESC line was derived from an IVF embryo with consent from both parents. By differentiating PSCs into neurons, neuronal cell preparations from animals were mostly replaced.

Methods of isolation of primary glia from mouse brain was revised via an animal ethics committee, in accordance with the Swedish Animal Protection Act. Experiments were conducted provided that no other system could replace the use of animals for this research and the benefits that this research will have for humans.

3.2 QUANTITATIVE POLYMERASE CHAIN REACTION

The first step of protein synthesis is the DNA transcription into messenger RNA (mRNA). Levels of mRNA can be quantified using quantitative polymerase chain reaction (qPCR).

Total RNA is first isolated from the cell lysate using solid phase extraction (SPE) and eluted in RNase-free water and then retro-transcribed into a copy DNA (cDNA). With the use of specific primers, the target gene is copied 40 times thanks to the thermostable Taq polymerase, giving rise to an amplified number of cDNA molecules (n^{40}). Thanks to the insertion of fluorescent probes (TaqMan probes), the reaction can be followed real-time and the cycle threshold (Ct), which is the cycle in which the fluorescent signal surpasses the noise, can be identified. The original amount of gene cDNA can easily be calculated according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt refers to the difference with Cts of target gene minus house-keeping gene (HKG) and target sample minus calibrator sample. In **paper I**, the calibrator was NPCs sample, while in **paper III** it was the control sample.

The choice of the HKG is very important, as the gene should not be affected by the treatment (difference of more than one cycle between conditions was excluded). Especially in **paper I**, where we compared two different stages of neuronal differentiation, it was important that the HKGs were stable throughout change in potency and cell-cycle. In the whole thesis, the enzyme for nucleotide synthesis hypoxanthine phosphoribosyltransferase 1 (HPRT1) and the ribosomal protein L27 (RPL27) were used as HKG.

3.3 INTRACELLULAR AND SECRETED PROTEIN QUANTIFICATION

3.3.1 Western blot

Transcription level does not always reflect protein level. Therefore, we evaluated intracellular protein levels of targeted proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot.

Briefly, cells were lysed in strong non-ionic detergent mixture containing Triton-X-100. However, since western blot of whole cell lysate does not

distinguish between subcellular localization, when intracellular location of the antigen is of interest, subcellular organelles can be isolated prior to blotting. For example, in **paper III**, we wanted to investigate APP accumulation at synapses. Therefore, we isolated synaptosomes from the neuronal culture using a specific buffer composition and steps of high-speed centrifugation (132).

Extracted proteins in the sample are separated in a gradient gel by application of an electric field and then transferred to a nitrocellulose membrane. The membrane is blocked with a solution high in protein (e.g. milk) and then incubated with antigen-specific primary antibody and species-specific horseradish peroxidase (HRP)-linked secondary antibody, with thorough washes with non-ionic detergent solution in between to remove unbound antibodies. Finally, the membrane is incubated with the developing solution, where luminol oxidizes and produces light in the presence of HRP. This output light signal, which has the shape of a band, can be detected by a charge-coupled device (CCD) camera and has a linear relationship with the amount of antigen. The intensity of the bands can be measured and compared to a loading control (house-keeping protein, HKP), to account for gel loading variability.

HKP choice depended on the type of sample. For whole cell lysate the cytosolic protein and common HKP GAPDH was used. For synaptosomes in **paper III,** cytoskeletal protein β-actin was more indicated, since GAPDH is mainly localized in the cytoplasm, while in **paper III** β-III-tubulin (neuron-specific) allowed for normalization on total neuronal protein extract in co-culture with astrocytes.

Protein quantification in western blot is only relative, however it allows for recognition of differently sized isoforms/fragments of the protein of interest and possible exclusion of off-targets. Furthermore, multiplexing is possible as long as the target proteins have different molecular weight. However, specificity of the antibody has to be validated on different types of tissues. Furthermore, western blot requires relatively high technical skills and large sample amount, which makes it hardly adaptable to higher throughput analysis.

3.3.2 Sandwich immunoassays

Sandwich immunoassays are another type of antigen quantification technique, with several advantages over western blot: they are fast and highly sensitive techniques that require very little sample preparation and allow for absolute quantification with the help of a standard curve. However, usually only one analyte can be quantified in each sample, limiting the multiplexing.

In this type of assay, the soluble analyte is immobilized by a capture antibody pre-conjugated in the bottom of a plate and then recognized by of a second antibody against a different epitope, forming a "sandwich" structure. The antibody-analyte complex is detected by an enzymatic reaction (e.g. colorimetric, luminescence, etc.), of which intensity of the signal depends on the amount of complex.

For example, the quantification of intracellular CTFβ in **paper I** and secreted sAPPα and β in cell culture media in **paper III** was performed using commercially available ELISA kits with colorimetric type of detection.

In a typical colorimetric ELISA, the antibody-linked enzyme HRP oxidizes the chromogenic substrate Tetra Methyl Benzidine (TMB) that produces a blue color. The optical density of this solution can be measured with a regular spectrophotometer at the wavelength of 450 nm (**Figure 6A**). Finally, the signal released from the unknown sample is quantified by interpolation in a signal/concentration curve generated using dilutions of a standard sample in each experiment. This method of detection requires instruments that are inexpensive and commonly found in biology labs, but it is not the most sensitive.

The quantification of secreted Aβ40, Aβ42 in the cell culture supernatant in both papers and secreted sAPPβ and intracellular Aβ40 in **paper I** was done using the Meso Scale Discovery (MSD) system, a sandwich immunoassay with electrochemiluminescence (ECL) detection. In this method, the detection antibody is conjugated to a ruthenium complex that emits light when oxidized. In this case, the oxidation will start by application of a high voltage from the electrode at the bottom of the well (**Figure 6B**). Like in colorimetric detection, the signal, here luminescent, is detected by a camera and can be correlated to the concentration of the analyte, obtained by interpolation in a standard curve. ECL has several advantages over colorimetric detection, such as low background noise (absence of background optical signal), easy and homogenous reaction start time (by applying the potential) and ultra-high sensitivity (133). In addition, thanks to the high sensitivity, MSD plates can detect more than one analyte in each well (e.g. Aβ38, Aβ40 and Aβ42), adding all the advantages of multiplexing to the assay, such as low sample volume required and low intra-sample variability. Assay details can be found in **paper I and III**.

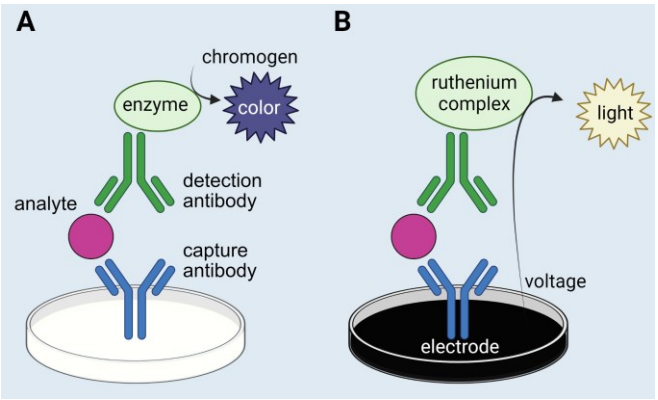


Figure 6. Illustration of two methods of antigen detection in sandwich immunoassays: (**A**) chromogenic ELISA and (**B**) electrochemiluminescence (ECL). Created with biorender.com.

3.3.3 Liquid chromatography - tandem mass spectrometry

In **paper II**, we investigated how the secretion of synaptic dysfunction biomarkers was regulated in neurons. Nine-teen proteins, including SNARE proteins, syntaxins, VAMP-2, AP2B1, complexin-2, PEBP-1, synucleins, GDI-1, neuronal pentraxins, and 14-3-3 proteins were measured using well-characterized liquid chromatography - tandem mass spectrometry (LC-MS/MS)-based assays (17, 134, 135).

Reverse-phase high-performance liquid chromatography (HPLC) allows for separation of the proteins in the sample based on the hydrophobicity. As the separated sample exits the chromatography column, the target proteins are nebulized, ionized and let run into a magnetic field where their mass/charge ratio is quantified. The particles are then fragmented and the mass/charge ratio of the product ions is quantified again. This transition between precursor and product ion is very specific to the structure of the initial protein, allowing a highly sensitive and selective identification (136).

With MS, absolute quantification is possible, but relative quantification to an internal standard makes the assay more reproducible. For the detection of synaptic proteins in this thesis, stable isotope-labeled peptides of all targeted peptides were added as internal standards to the sample, to adjust for pipetting error and any variation that happened to the sample. The sample was digested with trypsin and then purified from contaminants such as salts and detergents, based on hydrophobicity, using SPE.

For low-abundance proteins, such as SNAP25 and SYT1, further enrichment was done using immunoprecipitation, where capture antibodies conjugated to pre-coated paramagnetic beads can be immobilized during washing steps and then dissociated by addition of formic acid, enriching the sample of the target proteins.

MS does not rely on antibody detection, thus avoiding the possible non-specific recognition and batch-to-batch variations. Due to high sensitivity and possibility of multiplexing, the sample volume can be drastically reduced compared to other protein detection methods. On the other hand, MS is relatively low throughput, instruments are costly and the sample preparation is complex compared to other protein quantification methods, such as ELISA.

3.4 *IN SITU* PROTEIN QUANTIFICATION

3.4.1 Immunocytochemistry

Proteins can also be visualized and quantified *in situ*, maintaining the spatial information that is otherwise lost in the lysis step before western blot or ELISA. Immunocytochemistry (ICC) is an antibody-based technique used to visualize specific molecules in their cellular localization.

Briefly, cells are first fixed with a cross-linking agent to preserve cell morphology, prevent degradation and immobilize antigens, then permeabilized to allow entry of the antibodies. Incubation with a serum-containing solution is used to block the unspecific antibody bindings, thus removing background signal. The species of origin of the serum should be the same as the one of the secondary antibodies. Primary antibody-antigen recognition and subsequent fluorophore-labelled secondary antibody introduces a fluorescent label to the antigen, which can be visualized with a fluorescent microscope.

Multiple distinct antigens can be visualized in the same sample, as long as the primary antibodies are produced in different species and the excitation and emission spectrum of the different fluorophores do not excessively overlap. To visualize nuclei, cells are typically counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) or Hoechst33342.

3.4.2 Confocal microscopy and image analysis

Fluorescent molecules absorb light in a specific wavelength and emit it at a longer one. In a fluorescent microscope, the specimen is illuminated and a filter

is used to separate this emitted fluorescence from the brighter illumination light. Fluorescent microscopes can be wide-field, where the whole sample volume is illuminated, or confocal, where thanks to a pinhole, a beam of light is used to illuminate the sample in one focal point and another one to filter out any out-of-focus light before it reaches the detector, thus decreasing blur. The wide-field microscope suffers from low z-axis resolution and high background, especially in auto-fluorescent samples. Therefore, we chose confocal microscopy to image the iPSC-derived neurons.

Usually, objective lenses of light microscopes are adjusted for diffraction index of glass coverslips 0.17 mm thick and a refractive index of 1.52, which allow for the brightest and crispest imaging (137). However, dual SMAD inhibition-induced neurons are not able to grow long-term on glass, even when pre-coated. Plastic (polystyrene) coverslips exist but they are usually autofluorescent, block UV light and scatter the light, making them a bad substitute for glass coverslips. Ibidi has designed a polymer that has the same thickness and diffraction index of glass, can replace glass in microscopy. We therefore cultured cells on µ-ibidi slides for confocal microscopy (138).

For dual SMAD inhibition-induced neurons in **project I and II**, we used a Nikon A1 Eclipse Ti-E inverted confocal microscope, while in **project IV** we imaged Ngn2-induced neurons with the Operetta High Content Imaging System (Revvity) in confocal mode. The latter microscope was preferred, since it allowed fully automatic, high-throughput and high-content imaging with cost- and time-effectiveness. However, the use of the image analysis with the connected Harmony Image Analysis Software was more restricted to automatic in-built algorithms and allowed less customization compared to the open-source ImageJ.

In this thesis, ICC and confocal microscopy were used for both qualitative and quantitative assays. A summary of the cell markers used is shown in **Table 4**.

Table 4. List of cellular, neuritic and synaptic markers investigated using ICC

Antigen	Cell marker	Intracellular location	Quantified
Pax6	NPC (cortical)	Nucleus	/
Nestin	Neuronal stem/progenitor cell	Cytoskeleton	/
Tau	Neuron	Axon	/
Tbr1	Deep layer neuron	Nucleus	/
β-III-tubulin	Neuron	Neurite	/
NeuN	Neuron	Nucleus	/
GFAP	Astrocyte	Cytoskeleton	/
MAP2	Neuron	Dendrite	Segment length
Synapsin I	Neuron	Synapse	Puncta number, size, intensity

3.4.3 Proximity ligation assay

In **paper I**, we investigated proximity of APP with the secretases in iPSC-derived NPCs and neurons through proximity ligation assay (PLA), a technique that relies on antibody detection of two target proteins combined with amplification of circular DNA (139). Briefly, cells are fixed, permeabilized, blocked and incubated with primary antibody as for regular ICC. The secondary antibodies, instead of fluorophores, are conjugated to single-stranded oligonucleotides. The cells are then incubated with a ligase and afterwards with a DNA polymerase in the presence of with fluorescent oligonucleotides. If the two target proteins are as close as 40 nm or less to each other, the two DNA strands will be ligated into a circular DNA, which will be amplified and hybridized with fluorescently-labelled nucleotides. Proximity of the two proteins will then result in a fluorescent "dots" under the confocal microscope (**Figure 7**).

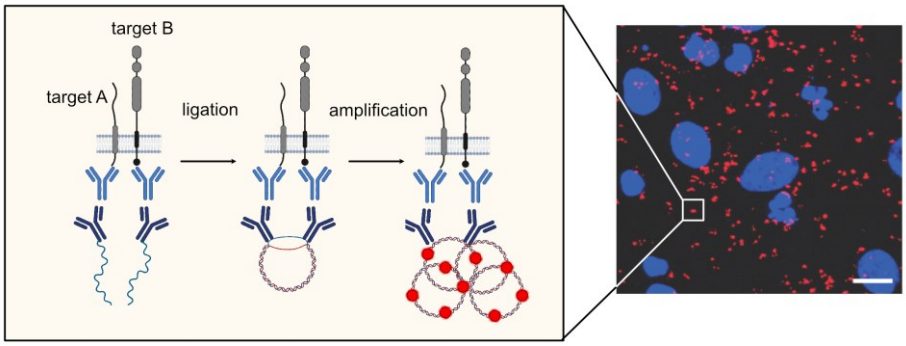


Figure 7. Schematic illustration of PLA for the colocalization of target A with target B. The secondary antibodies are conjugated to single strand-DNA sequence, which are ligated, amplified and hybridized with fluorescent DNA probes (left panel). Each proximity event will result in a red dot, visible under the fluorescent microscope (right panel). Blue= DAPI. Scale bar= 10 µm. Created with biorender.com.

As for any antibody-based assay, primary antibody specificity has to be confirmed and antibody dilution optimized to avoid unspecific binding and background. In this thesis, the concentration needed for a good signal-to-noise ratio in PLA was double the concentration used for regular ICC for the same antibody. Specificity of the assay with C-terminal APP and BACE1 antibodies was evaluated using APP knock-down SH-SY5Y cell lines.

3.4.4 Super-resolution microscopy and image analysis

PLA dot-like structures actually formed complex helical shapes, which spanned in all three dimensions of the specimen and required higher resolution than the regular confocal microscope to be accurately detected. Therefore, to image the PLA dots, we utilized the Airy Scan microscope (Zeiss) which is a confocal microscope equipped with a special array detector that surpasses the diffraction limit of light. Normally when light from a point source crosses the lens of an objective, it creates diffraction, which sets the resolution limits of a light microscope to 250 nm. Reducing the pin-hole size increases this resolution, but also reduces the signal, decreasing sensitivity and increasing signal-to-noise ratio. In the Airy Scan microscope, each of the 32 hexagonal detector elements operates like a small, individual pinhole, increasing the resolution but collectively maintaining the light-collection efficiency (140).

PLA dots count and their colocalization with organelle markers were analyzed using a custom-made macro code on ImageJ. The detailed image analysis procedure is described in **paper I**.

3.5 OLIGOMERIZED $A\beta_{42}$ PREPARATION AND CHARACTERIZATION

In **paper II**, we exposed iPSC-derived neurons to exogenous oligomerized $A\beta_{42}$ to determine its effect on synaptic proteins secretion. For experiments, several sources of Aβ exist: brain-derived (extracted from autoptic brain), synthetic, cell-derived (mammalian) and recombinant (E. Coli). We used synthetic Aβ since it showed similar pathogenic effects in *in vitro* experiments than the most physiological, though hard to obtain brain-derived Aβ (141). $A\beta_{42}$ is highly aggregation-prone and every step of its handling is crucial for a reproducible oligomerization, rather than fibrillation. Specifically, it should be kept on ice at all times, avoid unnecessary shaking or pipetting. Furthermore, to avoid losing peptide, the use of low-bind plastics and pre-coated plastic with 1% BSA is advised.

$A\beta_{42}$ is stored as a dry solid film and the first step is to remove any pre-existing aggregates by dissolving it in a strong alcohol hexafluoro isopropanol (HFIP). HFIP-$A\beta_{42}$ solution was used to make working aliquots, dried under vacuum and frozen at -80 until use. Before experiments, $A\beta_{42}$ was resuspended to a concentration of 1 mM in dimethyl sulfoxide (DMSO), sonicated and then dissolved in phenol-red free Neurobasal cell culture medium to a final concentration of 100 μM, mixed only by vortexing for 30 seconds and incubated overnight in the fridge. The next day, oligomerized $A\beta_{42}$ ($oA\beta_{42}$) is diluted in normal culturing media and added to the cell at a final concentration of 0.5 or 1 μM. Scrambled $A\beta_{42}$ preparations were performed identically and diluted to 1 μM as a control.

As explained in **paragraph 1.1.3.1**, $A\beta_{42}$ aggregates in several different species of different molecular weight, of which the most biologically active are the smaller oligomers. We therefore characterized the aggregate composition of $oA\beta_{42}$ by both SDS-PAGE, non-reducing conditions) and blue native-PAGE (BN-PAGE, non-denaturating conditions). SDS confers an homogenous negative charge to the protein aggregates, allowing them to migrate based on their change only (142). However, SDS has been shown to both enhance the formation of high molecular weight (HMW) protofibrils, which partially dissociate during electrophoresis, thereby creating artifacts. Therefore, we

additionally performed BN-PAGE where the Coomassie confers the negative charge to the protein aggregates without denaturating them. After gel electrophoresis, the proteins were visualized either by Coomassie staining, using a commercial kit, or by western blot and detection by the widely used anti-$A\beta_{42}$ antibody 4G8 (143). Antigen-retrieval by boiling the membrane in TBS before blocking was fundamental to detect any signal afterwards.

3.6 SYNAPTIC MEASURES

For most projects of this thesis, we needed to evaluate the synaptic phenotype either of mature pre-formed synapses in response to drugs or stressors (**paper II** and **III**), or in developing synapses in different culture conditions (**paper IV**).

3.6.1 Synaptic formation

Morphological signs of synapse formation can be observed through ICC, such as pre-synaptic vesicle proteins which appear as small dots located near MAP2-positive dendrites. The number, size and intensity of these so-called pre-synaptic "puncta" frequently show a correlation with functional synaptic parameters, such as electrophysiology (144). Therefore, in **project IV**, we evaluated synaptic formation using ICC and confocal microscopy as described in **paragraphs 3.4.1** and **3.4.2**.

3.6.2 Synaptic transmission

Electrophysiology is the gold-standard technique to measure synaptic activity. The Multi Electrode Array (MEA) is an extracellular electrophysiological technique. The MEA platform consists of an array of electrodes attached to the bottom of a cell culture plate on which neurons are grown (**Figure 8A**). The electrodes detect the change in voltage in the vicinity of the neuron that fires an action potential and measures it as a "spike".

Since the electrodes are only in the vicinity of the cell, rather than intracellularly, such as in the gold-standard electrophysiological technique patch clamp, MEA is less sensitive, it suffers of higher noise and the amplitude of the spikes is less informative, since it is strictly dependent on the distance between the firing neuron and the electrode. However, the biggest advantage of MEA over patch clamp is the non-invasiveness which allows non-destructive recording of neuronal activity over a long time. Furthermore, because the array of electrodes spans throughout the well, spikes from different

locations of the same neuronal culture can be measured at the same time, providing information about neuronal network activity, thereby synaptic transmission (145).

Neurons in culture usually fire action potentials in two patterns: 1) tonic firing, seen as a typical background activity, consisting in single spikes with a constant frequency and 2) burst firing or bursts, which is a train of spikes with higher frequency, followed by a longer period of quiescence (**Figure 8B**). Bursts have shown to be more effective in depolarizing the post-synaptic neurons, compared to tonic firing. Furthermore, research *in vitro* indicated that bursts initiate network formation via increased synaptic efficacy (146), while *in vivo* it helps connect distant areas of the brain, for example during attention (147). When bursts are synchronously detected in multiple electrodes in the same well, it is defined as a network burst (NB), which is a sign of functional maturation of the neuronal network (145).

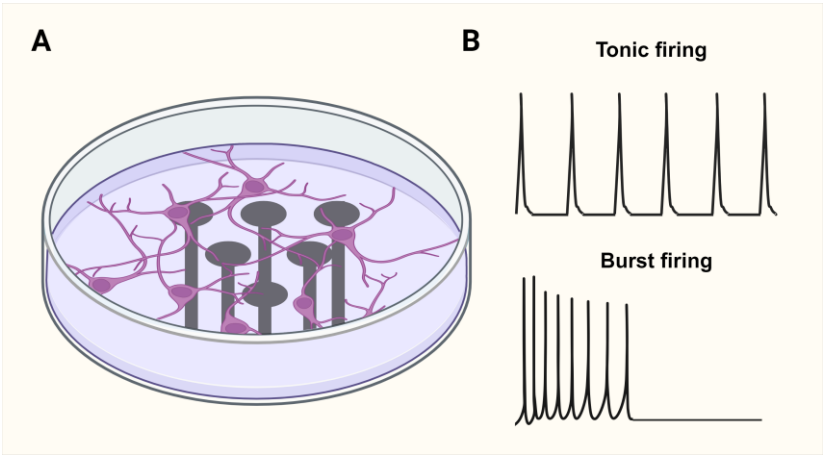


Figure 8. Illustration of multi-electrode array (MEA). (**A**) Schematic of a neuronal network (purple) growing in a MEA plate (electrodes in black), (**B**) two types of firing pattern in cortical neurons: tonic firing and burst firing. Created with Biorender.com.

In this thesis two different MEA systems were used: the MEA2100-System from Multi Channels Systems MCS GmbH (**paper II**) and the Maestro Edge multi-well MEA and Impedance system from Axion Biosystems (**paper III and IV**). The biggest difference between the two systems was in cell attachment to the compatible labware and the ease-to-use of the system and annex software for data acquisition and analysis. Details of recording and data analysis are described in each paper.

3.6.3 Synaptic plasticity

Long-term synaptic plasticity is dependent on the activation of genes that transduce the signal and strengthen synapses in response to high frequency pulses. Therefore, changes mRNA expression of these genes, such as CAMKIIB and activity-regulated cytoskeleton-associated protein (ARC) used in this thesis, indicate changes in neuronal activity-dependent synaptic plasticity (104). We measured these changes using qPCR, as described in **paragraph 3.3.1**.

3.6.4 Synaptic loss

The first indication of a bulk loss of synapses can be evaluated by measuring protein levels of proteins ubiquitously found in functional synapses, such as synaptic vesicular proteins or structural proteins. In this thesis the SNARE protein SNAP25 and the post-synaptic density protein 95 (PSD95) were measured in cell lysates to detect signs of pre- or post-synaptic degeneration, respectively, using western blot, as described in **paragraph 3.3.2**.

4 RESULTS & DISCUSSION

4.1 PAPER I

Improving the clearance or blocking the production of Aβ in the brain are two of the approaches to prevent or treat AD. However, unselective targeting of β- or γ-secretase may result in severe side effects, due to their ubiquitous nature and multiple substrates in the brain and other tissues (148, 149). Alternatively, the molecular interaction of APP with the secretases could be selectively blocked, avoiding off-target effects. Therefore, in-depth understanding of the interaction between APP and the cleaving enzymes upon physiological and pathological production of Aβ is needed for the development of successful treatment strategies.

Previous studies of APP-secretase interaction have mainly used overexpression of fluorescent-tagged proteins. However, recent work has demonstrated that co-expression of APP with BACE1 leads to disrupted APP trafficking and maturation, affecting Aβ production (150).

Therefore, we took advantage of a non-overexpressing cellular model based on two stages of human iPSC-derived neurons: young NPCs secreting low levels of Aβ, and mature neurons having increased Aβ secretion. In this model, we investigated the colocalization of APP with β- and γ-secretase in endo-lysosomal organelles using a proximity ligation assay (PLA). This technique allows to monitor the interaction of protein and enzyme without the use of florescent labels, that might disturb the protein structure, function, localization and stability.

Secretion of $A\beta_{40}$, $A\beta_{42}$ and sAPPβ was increased by at least 10-fold in neurons compared with NPCs, while intracellular levels of the intermediate product CTFβ remained stable, indicating a rapid cleavage of CTFβ by γ-secretase. Unchanged mRNA and protein expression of APP and BACE1 (the neuronal β-secretase isoform), and even decreased protein levels of the active subunits of γ-secretase PSEN1 suggested that protein expression was not a mechanism for the regulation of increased amyloidogenic processing of APP.

Investigating APP/secretase interaction using PLA revealed interesting and partially unexpected results.

Colocalization of full-length APP (flAPP) with BACE1 was increased in neurons (**Figure 9A**) in endosomes, while it was decreased in late endosomes and lysosomes, suggesting early endosome as one intracellular site of β-cleavage. This was in line with previous reports in the literature (151-153).

Colocalization of APP-CTF with PSEN1 was generally increased in neurons (**Figure 9B**) but it was decreased in all endo-lysosomal organelles, suggesting a different site for cleavage-dependent interaction. Lipid rafts and Golgi apparatus are suggested locations (154, 155).

Interestingly, colocalization of APP-CTFβ (product of β-cleavage) with BACE1 was at least 6.7-fold higher than flAPP/BACE1 colocalization in NPCs (**Figure 9C**), suggesting a possible inhibitory role for this intermediate product of APP processing over Aβ production.

In conclusion, our study presents a suitable manipulation-free human neuronal model for the study of APP and secretases trafficking, APP processing and endogenous Aβ production.

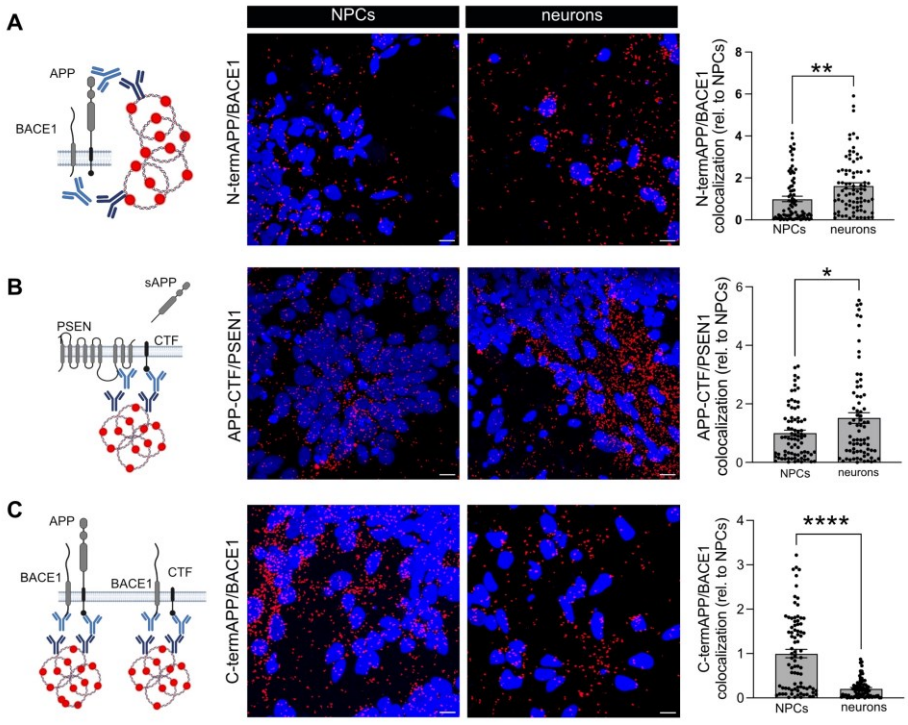


Figure 9. Colocalization of APP/secretases in low-Aβ secreting NPCs and high-Aβ secreting neurons. Colocalization and APP with BACE1 and PSEN1 was measured using proximity ligation assay (PLA) using antibody pairs directed to N-terminal APP, recognizing only full-length APP (flAPP) and BACE1 (**A**), C-terminal APP and PSEN1 (**B**), C-terminal APP, recognizing both flAPP and APP-CTF and BACE1 (**C**). For each antibody pair, a schematic representation of the PLA complex (left panel), example images of the PLA dots (red) and DAPI (blue) under confocal microscope (middle panel) and calculation of the PLA/DAPI area using ImageJ (right panel) are shown. Scale bar= 10 µm. Bars represent mean PLA/DAPI area +/- SEM. All means were compared using Student's t-test. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. Edited with permission from (156).

4.2 PAPER II

Synapses are the functional unit of information transfer inside the brain. Therefore, it is not surprising that synaptic dysfunction and loss is the best pathophysiological correlate of cognitive decline in AD (14). Since the first study in the 1990s, synaptic proteins can be detected in the CSF and have been studied as potential synaptic degeneration biomarkers (15, 157).

Interestingly, many synaptic markers are also increased in Aβ+ cognitive unimpaired individuals compared to Aβ-, showing that synaptic dysfunction is one of the earliest events following Aβ pathology, appearing earlier than symptoms (19). On the other hand, a group of 3 proteins, the neuronal pentraxins (NPTXs), is consistently shown to be decreased in the CSF of individuals with AD and other neurodegenerative diseases compared to control, but only in the later symptomatic stages of the disease (19, 134). Despite all the knowledge on these synaptic proteins as biomarkers, very little is known about the mechanisms of their release from neurons. For some of the synaptic proteins, their CSF levels rise, while their immunoreactivity in the brain is reduced (158). Therefore, a "leakage" from damaged synapses has been speculated.

In **paper II**, we wanted to investigate the regulation of synaptic protein release from neurons in physiological conditions and in the presence of toxic oligomeric Aβ. To do so, we measured multiple synaptic proteins in the cell culture supernatant of hiPSC-derived cortical neurons using mass-spectrometry-based protocols well-established for CSF analysis (19, 135, 159). Out of the 19 proteins analyzed in the assay (AP2 complex - subunit β, complexin 2, β-synuclein, γ-synuclein, 14-3-3η, ε, ζ/δ, neurogranin, rab GDP dissociation inhibitor α, syntaxin 1B, syntaxin 7, phosphatidylethanolamine-binding protein 1, and neuronal pentraxin 1, 2 and receptor, SNAP25 and synaptotagmin 1), only 10 proteins were readily detected (**Table 5**).

First, we measured the secreted concentration of 8 synaptic proteins at several time points during iPSC neuronal differentiation (from 0 to 150 dpi). Secretion of SNAP25 and synaptotagmin 1 throughout differentiation had already been established in a previous study (160). We therefore established three main patterns of secretion of synaptic proteins (**Figure 10, Table 5**).

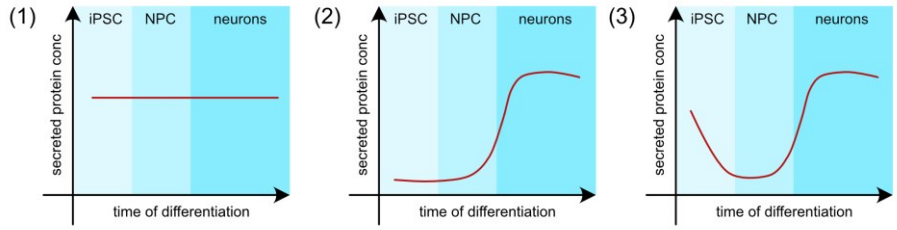


Figure 10. Patterns of secretion of synaptic proteins from iPSC-induced neurons throughout neuronal differentiation. 1) No changes between iPSCs and mature neurons; 2) Low concentration throughout pluripotent and NPC stage, sharp increase at the neuronal stage, plateau in more mature neurons; 3) Higher levels in pluripotent stage, decrease at the NPC stage, sharp increase at the neuronal stage, plateau in more mature neurons.

Table 5. Summary of the results of paper II. Changes in secretion of synaptic proteins from hiPSC-derived neurons (this work) and comparison with CSF levels of the same proteins in AD compared to control (previous work).

Synaptic protein	iPSC-derived neurons				AD CSF (17, 19, 135)
	Secretion pattern through neur. diff. (Fig. 10)	Blockage of action potential (TTX)	oAβ42		
			0.5 µM	1 µM	
NPTX2	(1)	=	↓	↓	↓
NPTXR	(2)	=	=	=	↓
GDI	(2)	↓↓	=	=	↑
STX7	(2)	↓↓	=	=	↑
SYT1	(3)*	↓↓	=	=	↑
SNAP25	(2)*	↓↓	↓↓	↓↓	↑
AP2B1	(3)	↓↓	=	↓	↑
14-3-3 η	(3)	↓↓	=	↓↓	↑
14-3-3 ζ/δ	(3)	↓↓	=	↓↓	↑
14-3-3 ε	(3)	↓↓	=	↓↓	↑

Note: NPTX2= Neuronal pentraxin 2, NPTXR= neuronal pentraxin receptor, GDI= rab GDP dissociation inhibitor α, STX7= syntaxin 7, SYT1= synaptotagmin 1, AP2B1= AP2 complex - subunit β; * from previously published work (160); = no change; ↑ increase; ↓ less than 25% decrease; ↓↓ more than 25% decrease

Only one protein, NPTX2, followed pattern 1 of secretion, with steady secretion throughout differentiation. Secretion of NPTXR, GDI1, STX7 and SNAP25 followed pattern 2, with a sharp increase from NPCs to neurons, stabilizing at the mature neuronal phase. SYT1, AP2B1 and 14-3-3 proteins followed pattern of secretion 3, which resembled pattern 2 from NPCs to neurons, but with higher secretion levels in pluripotent stage, suggesting a

possible function of these proteins in maintenance of pluripotency and self-renewal. Overall, except for NPTX2, all other proteins' secretion quickly increases with the development of synapses (between 60 and 90 dpi), suggesting a connection of the protein' secretion with synapse formation and/or function. However, no further increase was seen beyond 90 dpi, despite an increase in synapse number and activity, which usually occurs as neurons mature in culture (122), indicating perhaps a major role in synaptic formation.

Thereafter, to establish if the synaptic protein release was affected by neuronal activity, we blocked the propagation of action potentials in neurons using TTX. TTX exposure decrease secretion of almost all proteins by more than 25%, whereas NPTX2 and NPTXR secretion was unaffected.

Finally, we exposed the neurons to two toxic concentrations (0.5 and 1 μM) of oligomerized $A\beta_{42}$. SNAP25 and NPTX2 secretion was decreased by both concentrations of $oA\beta_{42}$. Secretion of AP2B1, 14-3-3 η, 14-3-3 ζ/δ, 14-3-3ε was decreased by only the highest concentration and the secretion of 4 proteins (NPTXR, GDI, STX7 and SYT1) was not affected by any concentration of $oA\beta_{42}$. The results of all experiments are summarized in **Table 5**.

Most of these proteins mainly function at the pre-synapse in synaptic transmission or recycling of synaptic vesicles (**Figure 4 in paper II**). NPTX2 is specifically secreted during glutamatergic synaptic transmission and when bound to NPTXR on the post-synaptic membrane, it induces LTD, indicating a possible protective mechanism against excitotoxicity (161, 162). However, NPTXs were the only synaptic proteins here investigated of which secretion did not depend on the firing of action potential, while NPTX2 decreased in the presence of exogenous $oA\beta_{42}$ with signs of pre-synaptic damage. This is intriguing, since changes in CSF concentration of NPTX2 were only found in later symptomatic stages of the disease, unlike other synaptic proteins (19, 134). Therefore, it may be speculated that while other biomarkers, such as SNAP25 and 14-3-3 proteins, changed due to synaptic dysfunction, NPTXs changed only due to a clear synaptic damage or loss. The response of all other proteins' secretion to $oA\beta_{42}$ was a bit more puzzling. While their CSF levels increase with Aβ pathology, their secretion from neurons decreases for 5 proteins and did not change for the other 3, without highlighting a clear pattern related to their molecular pathways and functions. SNAP25 is one of the most studied biomarkers and out of all proteins the best predictor of cognitive decline (19). Here, it was the most sensitive synaptic protein to both changes in neuronal function and $oA\beta_{42}$-dependent synaptic damage.

In conclusion, our research shows that 10 synaptic biomarkers are released by human cortical neurons during neuronal development and activity and that this release is affected by exposure to oAβ_{42}, which led to a decreased release of most, but not all proteins. This study demonstrates that iPSC-derived cortical neurons are a suitable model for examining the differential secretion of synaptic biomarkers, which are key in diagnosing and tracking the progression of neurodegenerative diseases.

4.3 PAPER III

One promising preventive strategy for AD is the inhibition of APP β-cleavage, the rate-limiting step of amyloid generation (58). Unfortunately, several BACE inhibitors have failed in clinical trials due lack of efficacy and appearance of cognitive side-effects (76). At the time these clinical trials took place, the fact that BACE1 KO mice had showed no adverse phenotype provided a false evidence that inhibition of BACE1 activity was safe (163). It has later been shown, both *in vitro* and *in vivo* that BACE1 inhibition impairs synaptic plasticity and cognitive functions (164). Yet, the exact processes behind these effects are still being explored. Understanding how BACE inhibitor-induced synaptic dysfunction occurs could help avoiding adverse effects and reevaluating this therapeutic approach.

In **paper III**, we aimed at understanding the underlying mechanisms of synaptic dysfunction mediated by BACE1 inhibition. Specifically, we hypothesized that excessive inhibition of APP β-cleavage induces pathological accumulation of APP at the synaptic terminal, and that this could impar synaptic function.

iPSC-derived cortical neurons were treated with LY2886721, a potent allosteric BACE inhibitor, or DMSO (≤0.1%) as vehicle control and changes in APP processing and turnover were evaluated after 1 to 3 days exposure to 0.33 μM LY2886721. While $A\beta_{40}$, $A\beta_{42}$ and sAPPβ in cell supernatant were reduced by up to 80% following BACE inhibitor exposure, sAPPα was unchanged at all time points, suggesting that uncleaved APP was not rerouted to the α-secretase pathway. This was in contrast with earlier data in pre-clinical and clinical trials with multiple BACE inhibitors (165, 166), in which CSF reduction in Aβ levels was accompanied by increased sAPPα.

LY2886721 exposure of cortical neurons also caused intracellular accumulation of APP after 2 days, which returned to normal levels after 3 days. However, APP accumulation was also found in isolated synaptic terminals, and did persist even 3 days post treatment. The reason for this selectively located increase is intriguing. It was previously shown that decreased BACE1 activity increases APP anterograde axonal transport (167). Thus, the observed accumulation of APP at the synapse might be attributed to a disruption in the normal anterograde axonal transport of APP. Nevertheless, these changes in APP turnover did not seem to cause any synaptic loss (intracellular protein level of SNAP25 and PSD95) nor synaptic plasticity dysfunction (mRNA

expression of *CAMKIIB* and its downstream gene *ARC*). Moreover, secretion of synaptic proteins, investigated in **paper II**, was not changed.

Finally, changes in synaptic transmission were investigated using the extracellular electrophysiological technique MEA. Since long-term culturing of SMAD inhibition-induced neurons required to reach mature neuronal activity resulted in cell detachment from the MEA plates, a different method for cortical differentiation of iPSCs was used to produced stable and highly synchronous network in as soon as 20 dpi (see **paragraph 3.1.2**): a co-culture of Ngn2-induced glutamatergic neurons with mouse primary glia. We treated neuron/astrocyte co-cultures with the same concentration of LY2886721 (0.33 µM) and two higher concentrations (3.3 and 10 µM). Neuronal activity was impaired only with 3.3 and 10 µM, while APP intracellular accumulation was progressively smaller. APP protein level was also unchanged in synaptosomes of SMAD-inhibition induced neurons exposed to 3.3 and 10 µM LY2886721, excluding APP intracellular and synaptic accumulation as a cause for the harmful synaptic phenotype. None of these concentrations affected cell viability. The results of this paper are schematically described **Figure 11**.

In conclusion, we demonstrated that APP accumulation in neurons following BACE inhibition is not involved in the synaptic phenotype, therefore a different mechanism may be involved in the cognitive side-effects experienced during the clinical trials. Furthermore, BACE1 inhibition-mediated Aβ reduction up to 60-70% was not per se harmful to the synapses and neuronal activity in human cortical neurons, suggesting that human neurons may be more resilient than previously reported to short-term Aβ lowering.

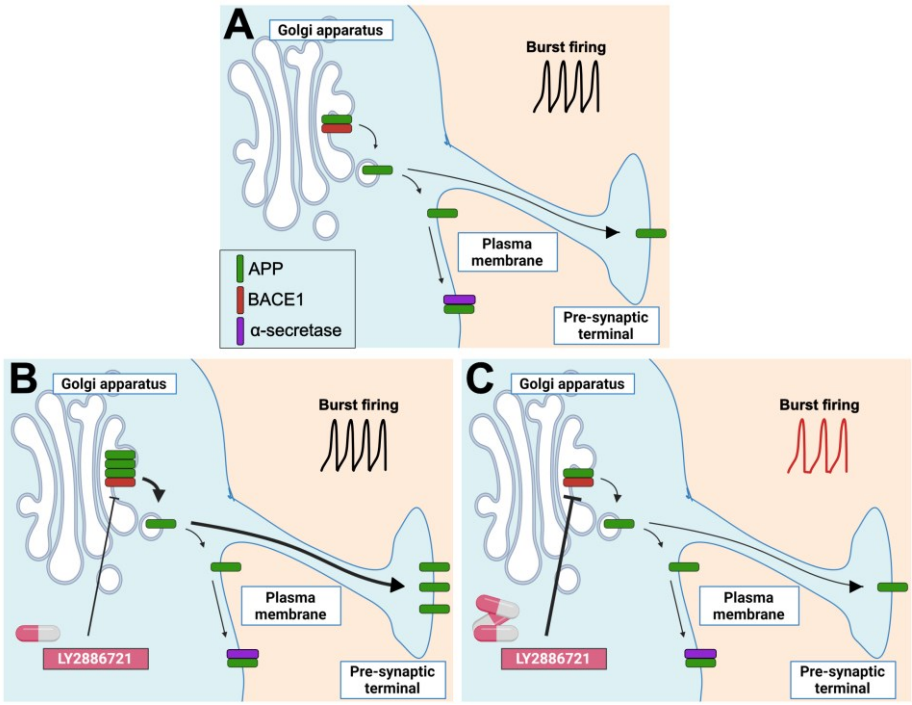


Figure 11. BACE inhibition-induced impaired neuronal activity is independent of APP accumulation at synapses. Schematic model of APP trafficking and neuronal activity in hiPSC-derived cortical neurons upon exposure to BACE inhibitor LY2886721. **A**) In untreated neurons, APP traffics from the Golgi apparatus either to the plasma membrane, where is mainly processed by α-secretase, or to the synaptic terminal through anterograde axonal transport. This latter process is influenced by BACE1 activity. Neuronal activity mostly comprises of burst firing activity. **B**) In neurons exposed to 0.33 µM LY2886721, BACE1 activity is reduced and APP trafficking to the synapse terminal is thereby increased, resulting in APP accumulation at the synaptic terminal. α-secretase-mediated processing does not change. Neuronal activity is unimpaired. **C**) Thirty times higher LY2886721 concentration (10 µM) does not induce any APP accumulation at the synaptic terminal. However, burst firing is reduced in terms of spike frequency and burst duration. Created with biorender.com.

4.4 PAPER IV

As numerous protocols to differentiate several types of neuronal cells from iPSC have been developed, a clear goal has been to produce mature, synaptically active neurons. Evidence from *in vivo* corticogenesis and *in vitro* culture suggests a strong dependency of neurons on astrocytes for the development of fully functional synapses (91, 168). While dual SMAD inhibition yields functional, synaptically connected neurons along with naturally occurring astrocytes, Ngn2 overexpression produces pure astrocyte-free cultures of glutamatergic neurons, thus not naturally synaptically active (125). Culturing these neurons on rodent or human glial preparations has shown some success, but this requires extensive resources (124, 169).

In **paper IV**, we produced a novel protocol that differentiates human iPSCs into mature, synaptically active neurons using Ngn2 overexpression, without the need for glial support. These cells, cultured on poly-D-lysine coating, were kept in regular neurobasal-based medium until DIV 17 and then switched to complete BPM, a culture medium for enhanced neuronal activity (127, 170) as described in **paragraph 3.1.1**.

Neuronal activity was measured using MEA and synaptically active neurons were defined by their ability to generate NB, as explained in **paragraph 3.6.2**.

The results demonstrate that neurons differentiated with Ngn2 in co-culture with mouse primary glia showed increasing neuronal activity over time, with the appearance of NBs. In contrast, neurons cultured without glia initially showed lower activity. However, when switched to BPM these neurons rapidly developed synchronous network activity. Furthermore, ICC studies revealed that neurons without glia initially had shorter dendrites and less intense pre-synaptic puncta, but these aspects improved significantly after switching to BPM. Schematic results are shown in **Figure 12**.

Since the limiting factor in the neuron-only culture was the physical contact between glial cells and synapses, we speculate that some factors in BPM replaced glial secreted factors, allowing synaptogenesis and synaptic function. Candidate factors are BDNF, GDNF and cAMP, well-known glia-derived factors that enhance synaptogenesis (171, 172) and synaptic plasticity (173). Furthermore, basal BPM has an osmolarity and ion concentration closer to the CSF, thus more physiological compared to Neurobasal formulation, while B27 - absent in BPM complete medium - has been found to increase neuronal

survival but inhibit neuronal activity (127). It would be intriguing to explore whether a single factor, a combination of some, or all these factors together are necessary to achieve the observed outcome.

Interestingly, the morphology of the NBs was different between the two culture conditions. In co-cultures, NBs appeared to be divided into a sequence of smaller bursts, each separated by increasing interburst intervals. Conversely, in mono-cultures, NBs were observed as one single, large burst. The subdivision in smaller bursts in the co-culture is likely dependent on the physical contact of astrocytes, for example through the re-uptake of glutamate through EAATs (91). A schematic summary of the electrophysiological results of this paper are shown in **Figure 12**.

In summary, we have successfully developed a human exclusively neuronal culture model that is suitable for conducting synaptic transmission assays. Furthermore, some of these findings add cues to the unique role and molecular pathways of astrocytes in the formation of glutamatergic synapses and their involvement in synaptic transmission.

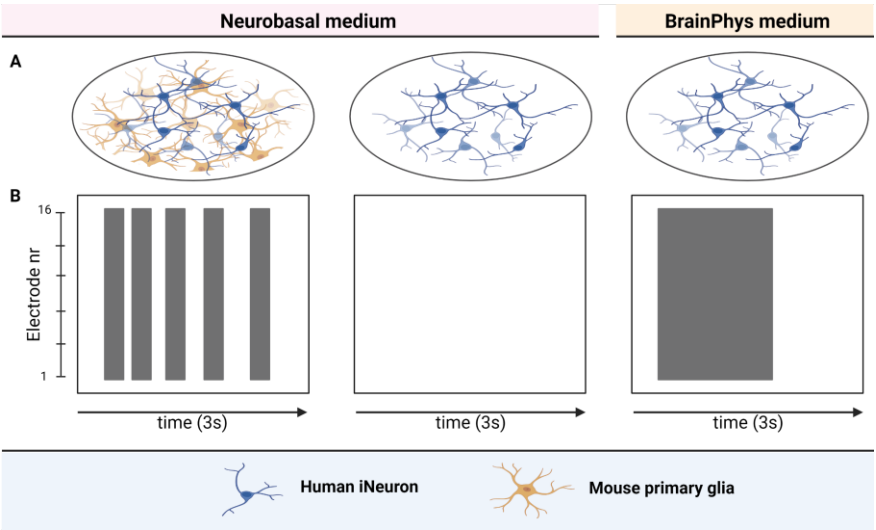


Figure 12. Spontaneous neuronal activity of human Ngn2-induced neurons cultured with or without glia. A) Human Ngn2-induced neurons were cultured in three conditions: 1) in co-culture with mouse primary glia, 2) without primary glia in Neurobasal-based medium, 3) without primary glia in BrainPhys-based medium. The neuronal activity was measured using multi-electrode array (MEA). **B**) Boxes show schematic network burst (NB)-type neuronal activity of 16 electrodes for 3 seconds recording. NBs (shown in grey) have a different morphology between co-culture and neuronal-culture in BrainPhys, while they are absent in neuronal-culture in Neurobasal.

5 CONCLUDING REMARKS & FUTURE PERSPECTIVES

5.1 Aβ GENERATION

The process of Aβ generation and secretion remains a complex and not fully understood phenomenon. It involves the intricate trafficking of APP and three secretases. APP internalization through clathrin-mediated endocytosis tends to result in amyloidogenic cleavage by β- and γ-secretase in endosomes and trans-Golgi network (TGN)(174). Alternative non-amyloidogenic α-cleavage seemed to happen mainly at the plasma membrane, even though recent report found the TGN as a predominant α-cleavage site (175). Every aspect concerning the localization of APP and its proximity to the secretases unveils new possible avenues for therapeutic interventions in the treatment of AD.

Notably, much of the research on APP trafficking and cleavage has been conducted in non-polarized non-neuronal cells (174). Our research in human neurons (**paper I**) revealed that increased endogenous Aβ production and secretion are associated with increased colocalization of APP and BACE1 in early endosomes. Colocalization of PSEN1 and APP was also overall increased, though not in the endo-lysosomal system. Future research shall aim at exploring other cellular locations, like PM, TGN and synaptic vesicles, for their potential roles in this process. Two additional pathways warrant investigation in light of our observations of increased Aβ in this model. Firstly, exploring the role of PSEN2, another active subunit of γ-secretase primarily found in late endosomes and lysosomes, involved in creating an intracellular pool of Aβ (176). Secondly, examining the colocalization of APP with α-secretases ADAM9, 10, and 17, as this pathway seems to offer protection against amyloidogenesis (177).

The intermediate product of APP amyloidogenic processing, CTFβ is also of interest, as it has been shown to be neurotoxic by causing lysosomal, autophagosomal and mitochondrial dysfunction (178, 179). Therefore, future research shall investigate the possible inhibitory interaction between CTFβ and BACE1, which could prevent both Aβ secretion and toxic effects of CTFβ.

APP exerts many of its functions at the synapse and trafficking to the axon is dependent on BACE1 activity (167, 180). In line with this, in **paper III** we found that inhibition of BACE1 by LY2886721 increased APP especially in

isolated synaptic terminals. However, α- and β-processing did not seem to be interdependent, as there was no change in sAPPα secretion, despite a significant reduction (around 80%) in β-processing. Furthermore, no accumulation of APP at higher concentrations of LY2886721 argued against neurotoxic effects of uncleaved APP, and the reasons behind this phenotype are intriguing and merit further investigation.

5.2 $A\beta_{42}$ IN SYNAPTIC FUNCTION

$A\beta_{42}$ is known to be involved in synaptic formation and plasticity (101, 181), yet in our model, synaptic transmission impairment only occurred with over 80% reduction of $A\beta_{42}$ secretion. The exact molecular mechanisms underlying this synaptic dysfunction remain unclear. Future experiments shall investigate whether introducing exogenous $A\beta_{42}$ can reverse these effects.

5.3 $A\beta_{42}$ IN SYNAPTIC DYSFUNCTION

Synaptic dysfunction and loss are increasingly recognized as key predictors of cognitive decline as well as early responders to Aβ pathology. Yet, the impact of Aβ on the secretion of these biomarkers in neurons has not been explored.

Paper II in this thesis provided key evidence that several synaptic biomarkers are secreted alongside synaptogenesis and synaptic activity, but also that application of exogenous $oA\beta_{42}$, with evidence of pre-synaptic damage, caused a decrease in most of the biomarkers. SNAP25, most promising synaptic dysfunction biomarker was the most affected with 40% decrease both as secretion and intracellular level. On the other hand, NPTX2 changes seemed to mirror synaptic damage, rather than function, although this hypothesis needs to be explored deeper.

A central question emerges from this study: how does reduced secretion of synaptic proteins relate to their increased protein concentration in CSF? First, it may be related to neuronal activity, which we saw influencing biomarker secretion in this study. Indeed, since acute exposure to $oA\beta_{42}$ has been observed to induce both hyperexcitability and LTD in neurons (110, 182), increased CSF biomarkers may be caused by increased protein secretion due to neuronal hyperactivation. Secondly, it may be a homeostatic mechanism of the fewer functional synapses to keep the level of synaptic transmission in the neuronal circuits. Alternatively, other cell types may contribute to the CSF synaptic protein increase. Astrocytes and microglia, well recognized for their

increased activity in engulfing synapses in AD than in healthy brain (44) may be also secreting synaptic proteins. Future studies of neuron-astrocyte-microglia co- or tri-culture will elucidate their role. Our study paves the way for extensive research into the secretion of synaptic proteins and their mechanisms.

Lastly, in **paper IV**, we showed that neurons are capable of forming a functional synaptic network independently of glial cells, offering a novel platform for exploring Aβ's role in both synaptic function and dysfunction.

ACKNOWLEDGEMENTS

This thesis wouldn't have been possible without the help and support of many people who've been with me through my journey of learning, facing challenges, and seeking answers. I want to sincerely thank everyone who contributed, offering their guidance, support, and encouragement along the way.

The first thanks go to my main supervisor, **Lotta Agholme**. Throughout five years you have taught me everything: from how to culture cells and design experiments to the crucial soft skills of planning and writing in a concise way, essentially you made a scientist out of me. You always knew how to motivate me when I was low or you reminded me to take a break when I was stuck, and always started a meeting with the sincerest "how are you?". Your love for science combined with your electric energy and your immense kindness have deeply inspired me to accomplish what I have today. There are no words to describe how grateful and proud I am to have been your student.

Next, I thank my co-supervisors **Henrik Zetterberg**, **Petra Bergström** and **Stefanie Fruwürth** for offering me supervision but also being an inspiration for what it means to be a scientist.

Henrik, the boss and supervisor that anyone can ask for, always positive, enthusiastic, motivating and promoting new ideas. When I started working in your lab, I have hoped to learn at least a fraction of your immense knowledge in Alzheimer's and neurology and I believe I have succeeded, thanks to your guidance and input. Thank you for believing in me.

Petra, you showed me that our job is as much about heart as it is about mind, because when you love what you are doing (and the beautiful rosettes and neurons) it is less hard to handle the disappointments when they come. I was inspired by your patience and learned from you that if you stare at the data long enough, it will eventually start to make sense. Thank you for being there.

Stefanie, your planning and multitasking ability is impressive. You taught me structure and dedication and to dig deeper instead of stopping at the surface of a question. Thank you for never stopping to seek an answer to every smaller or bigger problem I faced during my PhD until you found it, I could always count on your advice.

Besides my supervisors, I want to express my gratitude to a few mentor figures that have influenced my journey substantially. To **Tugce Munise Satir**, you

have been my introduction to the PhD world and I have learned many valuable lessons in the lab from you, especially with cell culture and microscopy. Thank you for finding time in what I can imagine was the busiest year of your PhD to teach me and always made sure both of us learned from the time spent together in the lab. I feel grateful and proud to have had the opportunity to work with your most beloved project. To **Dzeneta Vizlin Hodzic**, my former co-supervisor in my master's thesis work, the time when you thoroughly taught me my first steps with ICC, microscopy and research. You are the first person ever (perhaps even before myself) who has believed that academia was the right place for me, you introduced me to the group and advised me to take this path and never stopped believing that I would succeed. Thank you because I would have not been here if it wasn't for you. To **Debora Kaminski**, six years ago you kindly accepted to teach me, a random mater's student in desperate need for help, how to dissociate brains and culture neurons, showing what big heart you have for people and science. I have learned so much from you during that period and ever since. I was lucky enough to share five more years in the same lab learning from your extremely joyful approach to science and friendship.

Thanks to my collaborators from the Neurochemistry lab **Johanna Nilsson** and **Ann Brinkmalm**, for introducing me closer to the biomarker world, it was the greatest pleasure and fun to blend clinical and pre-clinical with you and explore together the translational value of our research. To **Berta Marcó De La Cruz** and **Fredrik Sterky** for teaching me Ngn2 induction, which added an extremely valuable model to this thesis, and for your great input to the manuscripts. **Berta**, you have not only been a great lab-mate and collaborator, always sending me papers and being genuinely happy to discuss all the newest techniques available in neuroscience, but also a great friend, listening and sharing and supporting me. I'm very happy we are reaching this goal together! Big thanks to **Johan Pihl**, **Alexander Back**, **Lydia Moll** and **Christina Nodin** for a valuable collaboration and for allowing me to discover the immense joys of the "high-throughput" (let the robot do it for you), and to the whole **Cellectricon crew** for welcoming me from the very first moment as part of the family. To **Rafael Camacho** for being the best microscopy expert, course leader and support person at the Center for cellular imaging, always open to explain and explore new quests with a big excitement and a friendly smile. To **Eric Hanse**, **Julia Izsak**, **Sebastian Illes** and **Tim Lyckenvik**, for helping me set-up the MEA platform and always being available for suggestions and discussions on the best way to culture on MEA or general neuroscience facts.

To my students **Linnéa Mundin**, well-organized and structured, you have achieved in a few months a great deal for the BACE inhibitor project, and with the most fun and singing attitude and **Parasto**, I enjoyed the time we worked together in the lab, how quickly you became independent, worked hard and delivered results and showed me that even PCR can be fun after all. I know you will achieve great things!

To everyone in the "cell and zebrafish" group: **Ajay Pradhan**, I appreciated very much your input in every scientific discussion, and all the pleasurable talks, especially in cell culture at strange times of the day and evening, your dedication to science is outstanding; **Alexandra Abramsson**, who loves science discussions and always has the trickiest and most thought-inspiring questions; **Maryam Rahmati** I could always count on you for help in the lab, exchange of knowledge or liberating talks; **Katarina Türner Stenström** always so keen to help me and everyone else; **Olena Tkachova** for bringing some more playfulness to our group and forcefully getting everyone out of their shell; **Fatemeh Razaghi** for always working hard in every challenge that was given to you; **Maria Olsson** for refreshing and pleasurable conversations. To all the former and visiting group members which had great input and brought original point of views to my research: **Sofia Darin**, **Marcelina Sakr**, **Dèsirée Axén**, **Jasmine Chebli**, **Rakesh Kumar Banote** and a great scientist and friend **Maria De Los Angeles Cortes Gomez.** To the whole **Neurochemistry lab** in Mölndal for the great Friday seminars' discussions and the fun lagdagen and after-works. Special thanks to **Celia Hök Fröhlander** for all the help with the administrative part and for organizing the get-togethers.

To all of the people who have brightened my days in the lab and office. My office and lab mates, new and old, **Christina Heiss**, **Carl Öberg**, **Mahnaz Nikpour**, **Aditi Chaudhari, Kristin Oskardotter, Mona Engström** and my dear friend **Linnéa Sjölin** for sharing lunches, fika breaks and long long talks. The "fun office" with **Angela Molinaro** and **Sanhita Mitra**, the new-comers **Joana Rodrigues**, **Debora Dreher Nabinger**, **Myra Nett**, **Melis Çelik**, **Niklas Bengtsson**, **Bingquing He** and everyone else at the **Clinical Chemistry lab** for creating a friendly and enjoyable environment for labwork and science discussions.

To the PhD day crew **Licínia Santos**, **Linnea Sjölin** and **Finn Huynh**, it has been super fun and a great satisfaction to organize the PhD day 2022 with you. Now that I am close to the end I can actually say it with confidence: "It was worth it!"

To all current and past members of the **Neuroscience and Physiology committee** and **DoR**, thank you for discussing together important matters and organizing events for PhD students and make our life and education a little bit better during these years. It has been a great part of my formative PhD years.

To **Justin Schneiderman**, for helpful coaching sessions which have helped me set reachable goals and get out of a no-period. One big thanks to **Maria Björkevik**, the undisputed "angel" of the PhD students at the Neuroscience institute, for always replying within minutes to every question and solving it right away, for being so keen to organize events for PhD students and for being so refreshingly positive all the time.

Thanks to **all of my friends**, near and far, who have helped me get through these - at times - challenging years, with a special thanks to **Licínia Santos** and **Lydia Moll**, for countless ways you have supported me and accompanied me in this crazy journey. I am the luckiest person to have you as friends!

To my partner and friend **Francesco Grieco** for teaching me how to relax, forcing me out after days of reclusion writing and for the constant support and love you have given me throughout. I owe my preserved sanity to you!

Alla mia famiglia, **mamma**, **papà**, **Raffa**, **Lianna** e **Sabino** per avermi dato l'opportunità, l'ispirazione e la motivazione per studiare, viaggiare, trovare il mio posto ed inseguire grandi obiettivi. Questa tesi è dedicata a voi!

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