Increasing the interpretability of Alzheimer-related biomarkers:

cell- and cerebrospinal fluid-based studies with focus on neurogranin

Faisal Hayat Nazir

Department of Psychiatry and Neurochemistry

Institute of Neuroscience and Physiology

Sahlgrenska Academy at the University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Faisal.nazir@neuro.gu.se

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To my parents

Everything finishes when utilised except knowledge

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Gothenburg, Sweden

ABSTRACT

Biomarkers for Alzheimer's disease (AD) is a growing field of research. A particularly vibrant field during recent years has been biomarkers for synaptic dysfunction. Sensitive assays for a synaptic protein called neurogranin (NRGN) have produced very interesting results when applied on cerebrospinal fluid (CSF) from AD patients and there are several other biomarker candidates that are thought to reflect different aspects of AD pathophysiology. The aim of this thesis was to investigate the expression and secretion of selected Alzheimer-associated biomarkers in a newly developed model of stem cell-derived cortical neurons that recapitulate the *in vivo* time frames of cortical development. For NRGN, we further investigated the processing and detection of its various molecular forms in CSF.

First, human induced pluripotent stem cell (hiPSC)-derived cortical neurons were used to determine the expression and processing of one of the core AD biomarkers, amyloid precursor protein (APP)-derived amyloid beta (Aβ). Our findings suggested that APP was expressed throughout the differentiation, but its processing shifted during neuronal stages. The AD-associated amyloidogenic pathway was activated in mature cortical neurons. Although amyloid and tau pathology are the defining neuropathological lesions, synaptic dysfunction and degeneration are

thought to be the earliest events in AD. Thus, secreted synaptic proteins in CSF during neurodegeneration could serve as potential AD biomarkers; a notion that has been supported by several studies on changes in concentration of NRGN in CSF in AD during recent years. To learn more about this biomarker, its expression and secretion were investigated in hiPSC-derived cortical neurons. We also examined three additional markers, namely synaptotagmin-1, SNAP-25 and GAP-43. NRGN, synaptotagmin-1 and SNAP-25 expression peaked in mature neurons, while GAP-43 expression was highest in immature cortical neurons and its secretion peaked in mature cortical neurons. The increased expression of synaptic proteins coincided with neurite network formation, which suggests that secretion of these proteins to the extracellular space reflects synapse maturity.

For one of the synaptic proteins, NRGN, C-terminal peptides have been detected at increased levels in CSF from AD patients. Nonetheless, the enzyme(s) that generate these peptides were not known. Here, we identified calpain 1 (CALP1) and prolyl endopeptidase (PREP) as enzymes that cleave NRGN and its fragments. The fragments generated through cleavage by human CALP1 and PREP may suggest an increase in the activation and/or expression of these enzymes in AD. Further, CSF analysis revealed the presence of several molecular forms of NRGN that may represent NRGN fragments, monomers and oligomeric forms, or complexes of NRGN with yet unidentified binding partners. Furthermore, we determined that the ratio of C-terminal fragments to total-NRGN was about 50% in a CSF pool.

Taken together, the results of this thesis show that a human-derived neuronal model can teach us a great deal on biomarker processing and secretion into biofluids, which may increase the interpretability of the biomarker results and tell us more about the underlying disease processes, which they may reflect.

Keywords: Alzheimer's disease, biomarker, neurogranin, APP, human iPSCs, CSF

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Sammanfattning på svenska

Forskningsfältet gällande biomarkörer för Alzheimers sjukdom (AD) är i en stark tillväxtfas och de senaste åren har biomarkörer för synapsskada fått speciellt mycket uppmärksamhet. Nyligen utvecklades nya och mycket känsliga metoder för att mäta synapsproteinet neurogranin (NRGN) i bland annat cerebrospinalvätska (CSF). Detta har möjliggjort analyser av NRGN i CSF från Alzheimerpatienter och kontroller, vilket har gett intressanta resultat. Ytterligare synapsbiomarkörer tros återge olika aspekter av patofysiologin vid AD. Målet med den här avhandlingen var att undersöka uttrycket och utsöndringen av utvalda Alzheimerassocierade biomarkörer i en nyligen utvecklad nervcellsmodell. Denna modell består av humana kortikala nervceller av stamcellsursprung, vars differentiering kan jämföras tidsmässigt med utvecklingen av nervceller *in vivo*. Vidare undersöktes neurogranin mer detaljerat, genom att mekanismerna för dess klyvning analyserades. Vidare karaktäriserades förekomsten av olika molekylära former av NRGN i CSF.

I den första delen av avhandlingen använde vi oss av kortikala nervceller, differentierade från humana inducerade pluripotenta stamceller (hiPSC), för att bestämma produktionen och klyvningen av en av huvud-biomarkörerna för AD, amyloidprekursorprotein (APP)-deriverat amyloid beta (Aβ), under nervcellsutvecklingen. Vi fann att APP uttrycktes under hela differentieringen, men att klyvningsmönstret var olika i de olika utvecklingsstadierna, då det Alzheimerrelaterade klyvningsmönstret bara var aktiverat i mogna nervceller. Även om Aβ och tau är de huvudsakliga biomarkörerna för AD i nuläget, så tros dysfunktion och nedbrytning av synapser vara en av de tidigaste händelserna vid AD. Det gör synapsproteiner som utsöndras till CSF till potentiella biomarkörer för AD. Detta understryks av flera studier de senaste åren, som visar att koncentrationerna av NRGN sjunker i AD CSF jämfört med kontroller. För att få mer kunskap om denna biomarkör så undersökte vi produktionen och utsöndringen i kortikala nervceller med ursprung i hiPSC. Vi undersökte även ytterligare tre synaptiska proteiner med potential att vara AD-biomarkörer; synaptotagmin-1, SNAP-25 och GAP-43. Uttrycket av NRGN, synaptogtagmin och SNAP-25 var som högst i mogna nervceller,

medan uttrycket av GAP-43 var som högst i omogna kortikala nervceller. Utsöndringen av GAP-43 var dock högst i mogna kortikala nervceller. Den ökade produktionen av synaptiska proteiner sammanföll med bildningen av neuronala nätverk, vilket kan betyda att utsöndringen av dessa proteiner till extracellulärvätskan reflekterar synaptisk mognad.

För ett av de synaptiska proteinerna, NRGN, har vi kunnat se C-terminala peptider i CSF, men vilket eller vilka enzymer som producerar dessa peptider har varit okänt. Vi identifierade calpain 1 och propyl endopeptidas (PREP) som NRGN-klyvande enzymer. Dessa klyvningar resulterade i flera C-terminala peptider som var förhöjda i CSF från AD-patienter jämfört med kontroller, vilket kan betyda att enzymerna calpain 1 och PREP har en förhöjd aktivitet vid AD. Vidare så resulterade analysen av CSF i att flera olika molekylära former av NRGN kunde detekteras, både fragment, monomerer, och oligomerer, alternativt komplex mellan NRGN och andra, ännu okända, proteiner. Vi kunde även bestämma förhållandet mellan C-terminala fragment och fullängds-NRGN till ungefär 50% i CSF.

Sammantaget så visar den här avhandlingen att en human nervcellsmodell kan ge oss ovärderlig kunskap om potentiella biomarkörers klyvning och utsöndring från nervceller, vilket kan ge oss en ökad förståelse för biomarkörprofilen och ge oss ytterligare kunskap om de sjukdomsmekanismer som biomarkörerna kan tänkas återspegla.

List of papers

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

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

II. **Nazir FH,** Becker B, Brinkmalm A, Höglund K, Sandelius Å, Bergström P, Satir TM, Öhrfelt A, Blennow K, Agholme L, Zetterberg H. *Expression and secretion of synaptic proteins during stem cell differentiation to cortical neurons.* Neurochem Int. 2018 Dec;121:38-49.

III. Becker B, **Nazir FH,** Brinkmalm G, Camporesi E, Kvartsberg H, Portelius E, Boström M, Kalm M, Höglund K, Olsson M, Zetterberg H, Blennow K. *Alzheimer-associated cerebrospinal fluid fragments of neurogranin are generated by calpain-1 and prolyl endopeptidase.* Mol Neurodegener. 2018 Aug 29;13(1):47.

IV. **Nazir FH,** Camporesi E, Brinkmalm G, Zetterberg H, Blennow K, Becker B. *Molecular forms of neurogranin in cerebrospinal fluid.* Manuscript.

Content

Abbreviations

Aa	Amino acids
AD	Alzheimer's disease
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
AICD	An intracellular cytoplasmic domain
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
APLP	Amyloid precursor-like proteins
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
BACE	Beta-site amyloid precursor protein-cleaving enzyme
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
CALP1	Calpain 1
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deribonucleic acid
CNS	Central nervous system
CREB	cAMP response element binding protein

CSF	Cerebrospinal fluid
CT	Computed tomography
CTs	Cycle thresholds
CTF	C-terminal fragment
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ES cells	Embryonic stem cells
fAD	Familial AD
FDG	Fluoro-deoxy-D-glucose
FGF-2	Fibroblast growth factor-2
FRET	Fluorescence Resonance Energy Transfer
GAP-43	Growth-associated protein-43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hiPSCs	Human induced pluripotent stem cells
HPRT-1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IP	Immunoprecipitation
IP-MS	Immunoprecipitation mass spectrometry
iPSCs	Induced pluripotent stem cells
KO	Knock-out

LC-MS	Liquid chromatography–mass spectrometry
LDH	Lactate dehydrogenase
LTD	Long-term depression
LTP	Long-term potentiation
MALDI	Matrix-assisted laser desorption/ionization
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
MSD	Meso Scale Discovery
MW	Molecular weight
MWCO	Molecular weight cut-off
NFTs	Neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
NMM	Neural maintenance media
NPC	Neuro-progenitor cell
NRGN	Neurogranin
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFA	Paraformaldehyde
PHFs	Paired helical filaments

PKC	Protein kinase C
PREP	Prolyl endopeptidase
PSD-95	Post-synaptic density protein-95
PSEN	Presenilin
P-tau	Phospho-tau
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RNA	Ribonucleic acid
RPL-27	60S ribosomal protein L27
RPL-30	60S ribosomal protein L30
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
sAD	Sporadic AD
sAPP	Soluble APP
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SMAD	*Caenorhabditis elegans* Sma genes and the Drosophila Mad, Mothers against decapentaplegic
SNAP-25	Synaptosomal associated protein-25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SPR	Surface plasmon resonance
SV2	Synaptic vesicle protein 2
SVZ	Sub-ventricular zone

SYT	Synaptotagmin
TGF	Transforming growth factor
TOF	Time of flight
T-tau	Total-tau

1. Introduction

1.1 Alzheimer's disease

1.1.1 History

Age-related mental illnesses in the elderly have been described for hundreds of years. However, in the early 1900s, a German physician, Dr. Alois Alzheimer diagnosed a form of dementia that eventually was to become named after him, Alzheimer's disease (AD). Dr. Alois Alzheimer described his patient Auguste Deter, showing certain symptoms, such as memory and language impairment, delusions, sleep disturbances and aggressiveness. These symptoms matched dementia (a definition known at that time), but the patient was unusually young (she started showing symptoms in her 40s and was diagnosed when she was 51 years of age). Therefore, she was diagnosed with "pre-senile dementia" (Alzheimer, 1907). Auguste Deter was hospitalised and her disease progression was closely monitored and documented by Dr. Alzheimer. She died in 1906 at the age of 55 and Alzheimer performed an autopsy. He reported his findings in 1906 at a German psychiatry conference, where he described a "particular malady of the cerebral cortex" of his patient. The following year, he published a paper where he described his findings at the *post-mortem* examination. He observed the cortical atrophy, including extracellular "miliary bodies" (neuritic plaques) in the neuropil, and "bundles of fibrils" (neurofibrillary tangles, NFTs) in the nerve cells (Alzheimer, 1907;Alzheimer et al., 1995;Graeber and Mehraein, 1999). Alzheimer's mentor, Dr. Emil Kraepelin named the condition "Alzheimer's disease" in the 8th edition of his book *Psychiatrie*, published in 1910 (Kraepelin, 1910). Alzheimer published numerous figures and drawings including histopathology of his first case together with his second case report in 1911 (Alzheimer, 1911;Graeber et al., 1997). For decades, AD was considered an unusual form of dementia, mainly affecting relatively young people. However, during the 1960s and 1970s, reports accumulated suggesting that many elderly who had died with dementia showed the typical AD pathology that Auguste Deter had displayed, which led to the diagnostic term senile dementia of Alzheimer-type. From the 1980s, dementia with plaque and tangle pathology is classified as AD, irrespective of the age at onset (Blennow et al., 2006).

1.1.2 Epidemiology

Dementia is defined as a decline in mental ability in one or several aspects of cognitive performance that interferes with daily life (Kocsis, 2013). AD is the most common form of dementia, accounting for 60-80% of all cases worldwide (Association, 2018). As of reports from 2017, AD is the second most common cause of death world-wide with an increase of 13.5% from 2007 to 2017 (IHME, 2017a), while it is the third major cause of premature death in Sweden (IHME, 2017b). Reports from the Center for Disease Control indicate that the number of people aged over 65 years will increase from 7% to 12% by 2030 worldwide (CDC, 2003;Qiu et al., 2009). Aging is the biggest risk factor for the development of AD. The incidence rate is approximately 1% for people aged 65 to 70 years and 6-10% between 80 to 89 years (Jorm and Jolley, 1998;Fratiglioni et al., 2000). As of 2018, there are 50 million dementia patients worldwide and this is expected to increase to 82 million in 2030 and 152 million in 2050. Further, there will be one new case of dementia every three seconds (Patterson, 2018). AD has a profound effect on patients, families, careers, as well as on the economy and society (Association, 2018). The increase in prevalence and the cost of healthcare expenditure, which amounts to trillions of dollars, make dementia a global healthcare problem that needs immediate attention.

1.1.3 Neuropathology

Gross neuropathological examination of an AD brain reveals cortical atrophy and enlargement of sulci and ventricles (Selkoe and Podlisny, 2002). The end-stage AD brain typically weighs 8 to 10% less than a normal aged-matched brain (Terry et al., 1981). The medial temporal and occipital lobes, along with the primary sensory and visual cortices, are the most vulnerable brain regions. The atrophy is initially seen in the hippocampus and entorhinal cortex, mainly because of degenerating neurons (Terry et al., 1981;Serrano-Pozo et al., 2011), and the symmetrical dilations of the lateral ventricles are due to brain tissue loss (Perl, 2010). Microscopically, AD is characterised by extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) in the medial temporal lobes and cortical areas of the brain (Braak and Braak, 1996;Serrano-Pozo et al., 2011).

Amyloid plaques are formed by the deposition of amyloid beta (Aβ), mainly its 42 amino acid-long form Aβ42, produced after sequential enzymatic processing of amyloid precursor protein (APP) (O'Brien and Wong, 2011). The APP

processing is described in detail in section 1.2.2. During amyloidosis, soluble Aβ monomers are polymerised to intermediate structures, including oligomers and proto-fibrils. This is followed by formation of insoluble fibrils that eventually form plaques (Serpell, 2000;Yang et al., 2017). Thal *et al.* described five phases of amyloidosis in the brain. Phase 1 starts with deposition of Aβ in neocortical regions including frontal and temporal lobes. The second phase involves allo-cortical brain regions, including hippocampus, amygdala and entorhinal cortex, followed by phase 3, where Aβ is deposited in subcortical nuclei, including diencephalic nuclei and the striatum. In phase 4, Aβ is deposited in distinct brain stem nuclei and finally, in phase 5, Aβ is deposited in the cerebellum (Thal et al., 2002). The plaques can be grouped according to morphology into diffuse plaques found in cognitively normal elderly individuals (Masliah et al., 1990) and dense-core plaques associated with AD, which disrupt synapses (Knowles et al., 1999). The dense-core plaques often have activated astrocytes and microglia in their vicinity and are associated with neuronal and synaptic loss (Pike et al., 1995;Vehmas et al., 2003).

One of Dr. Alzheimer's major findings was "bundles of fibrils" commonly referred to as neurofibrillary tangles (Alzheimer, 1907;Alzheimer et al., 1995), which is the other variant of amyloidosis in the AD brain. Tau is a microtubule-associated protein and it forms the major component of NFTs. Physiologically, tau provides stability to microtubules by binding to tubulin multimers; however, in AD, tau is truncated and phosphorylated, which triggers misfolding and aggregation of the protein into tangles (Grundke-Iqbal et al., 1986a;Grundke-Iqbal et al., 1986b;Iqbal et al., 2010). NFTs are detected by silver staining (Gallyas technique), or Congo Red staining applying polarized light (Stokes and Trickey, 1973). These techniques stain both plaques and tangles. Tangles may be specifically detected using anti-tau antibodies. NFT pathology progression has been characterised by Braak and Braak in a scheme that is commonly referred to as Braak staging (Braak et al., 2006). According to this scheme, NFT pathology starts in the trans-entorhinal cortex and then progresses to entorhinal cortex, hippocampus, temporal neocortex, insular cortex, superior temporal gyrus, occipital lobes and, finally, to occipital neocortex. The progression of NFT pathology correlates well with the cognitive decline (Braak and Braak, 1991;1996;Braak et al., 2006). Intracellular NFTs are strongly associated with axonal and dendritic degeneration (Serrano-Pozo et al., 2011). Figure 1 shows schematic representations of neurons, as well as the AD-defining NFT and amyloid plaque pathologies.

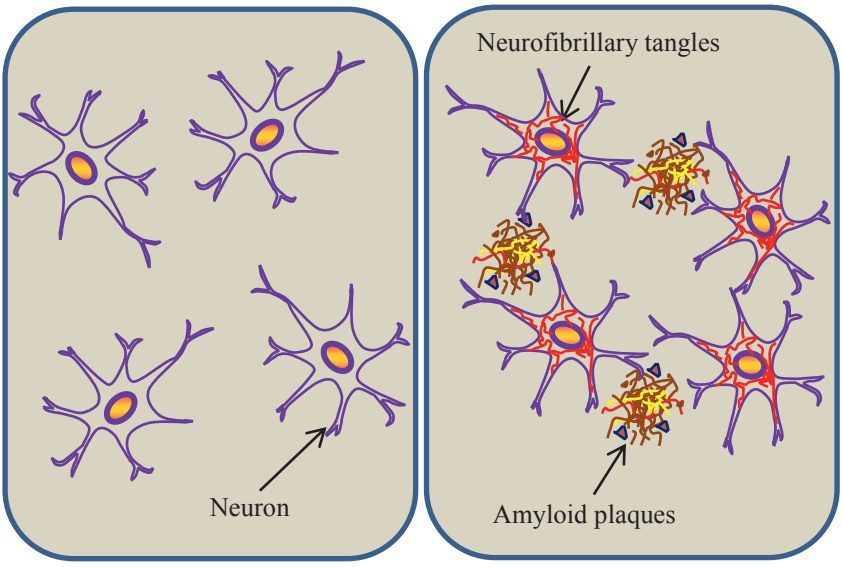

Figure 1 *A cartoon of neurons in healthy brain is shown on the left and Alzheimer's disease neurons are shown on the right depicting amyloid plaques and neurofibrillary tangles.*

1.1.4 The amyloid cascade hypothesis

Aberrant processing of APP with aggregation of Aβ into plaques was suggested as the initiating pathophysiological event in AD pathogenesis in 1987 (Kang et al., 1987). The amyloid cascade hypothesis, which in detail discusses the interplay between Aβ and downstream events, was first postulated in 1991. This hypothesis posits that the deposition of the Aβ peptide in the brain parenchyma is the crucial triggering event in AD (Hardy and Allsop, 1991;Selkoe, 1991;Hardy and Higgins, 1992;Selkoe and Hardy, 2016). The aggregation of Aβ could be due to over-production or dysfunctional elimination of the protein. In familial AD (fAD), the altered Aβ production (increased relative amounts of aggregation-prone longer forms of Aβ, or more aggregation-prone Aβ due to amino acid changes in the central part of the protein) could either be due to missense mutations in the *APP* gene or in the presenilin (*PSEN*) 1 or 2 genes (encoding the active site of γ-secretase). These mutations affect the processing of APP so that longer and/or more aggregation-prone forms of Aβ peptides are made (Goate et al., 1991;Citron et al., 1992;Haass et al., 1994;Suzuki et al.,

1994;Scheuner et al., 1996;Selkoe and Podlisny, 2002;Goate, 2006). Several factors contribute to sporadic AD (sAD), including high age, environmental exposures and genetic risk factors (Dosunmu et al., 2007). The strongest and most replicated genetic risk factor for sAD is the ε4 variant of the apolipoprotein E (*APOE*) gene (Loera-Valencia et al., 2018).

It has been suggested that a conformational change of Aβ into high β-sheet content increases its susceptibility to aggregate from soluble monomers to dimers and further aggregate into insoluble fibrils and plaques (Irvine et al., 2008). Several studies suggest that Aβ dimers and oligomers correlate with clinical symptoms that cause synaptic loss, reduction in long-term potentiation (LTP) and disruption of synaptic plasticity (Murphy and LeVine, 2010). Therefore, Aβ oligomers are associated with neuronal loss and have been suggested to play an important role in AD pathogenesis. However, there is a lot of controversy in this field, in particular since Aβ-targeting drug candidates keep failing in clinical trials (Makin, 2018).

1.2 The amyloid precursor protein

1.2.1 Structure and isoforms of APP

APP is a member of a family of related proteins that also includes amyloid precursor-like proteins (APLP1 and APLP2) in mammals. APLP1 and APLP2 lack the Aβ domain (Sprecher et al., 1993;Wasco et al., 1993). Before APP was discovered, it was referred to as a coagulation factor, nexin-II. However, later it turned out that APP and nexin-II were the same protein (Van Nostrand et al., 1989). The structure of APP can be divided into three domains, a larger N-terminal ecto-domain, a single hydrophobic transmembrane domain and a shorter intracellular C-terminal domain (Kang et al., 1987;Reinhard et al., 2005). APP is a transmembrane glycoprotein encoded by the *APP* gene localised at chromosome 21q21. The mammalian *APP* gene contains 18 exons that are alternatively spliced giving 365 to 770 amino acid-long products. Commonly expressed APP forms are 695, 751 and 770 amino acid-long, named APP695, APP751 and APP770, respectively (Tanzi et al., 1988;Weidemann et al., 1989). APP is expressed in neuronal (brain and spinal cord) and a number of non-neuronal tissues and organs (blood, liver, pancreas, lung, gastrointestinal tract,

testis, prostate glands, breast and placenta). APP695 is the pre-dominant isoform of APP found in neuronal cells (Kang et al., 1987).

Around three decades ago, APP was proven to be the precursor of AD-associated Aβ (Goldgaber et al., 1987;Kang et al., 1987). By now, a large body of literature, reporting results from genetic, biochemical and animal studies, suggests that APP-generated Aβ peptides are responsible for synaptic dysfunction, neuronal loss and eventually cognitive decline (McLean et al., 1999;Selkoe, 2008;Shankar and Walsh, 2009;Palop and Mucke, 2010;Tu et al., 2014;Sadigh-Eteghad et al., 2015;Marsh and Alifragis, 2018). The genetic studies on fAD patients revealed that these individuals have mutations in the *APP*, *PSEN1* or *PSEN2* genes (Schott et al., 2002;Bekris et al., 2010;O'Brien and Wong, 2011;Cacace et al., 2016). These mutations affect the processing of APP, or the sequence of its Aβ domain, so that longer and/or more aggregation-prone forms of Aβ are produced (O'Brien and Wong, 2011).

1.2.2 Processing of APP

APP turnover in neurons is high and it is processed rapidly (Lee et al., 2008). APP is processed by secretases in a step-wise manner, generating both extracellular and intracellular fragments. This processing either leads to the production of Aβ, known as the amyloidogenic pathway, or it precludes Aβ peptide formation in a non-amyloidogenic pathway (Chow et al., 2010).

In the non-amyloidogenic pathway, α-secretase (a disintegrin metalloproteinase, [ADAM]) initiates the cleavage of APP within its Aβ domain, thereby precluding Aβ production. This initial cleavage results in the production of extracellular APP fragment, known as soluble APPα (sAPPα) and C-terminal fragment α (CTFα) of 83 amino acids. Thereafter, the CTFα is cleaved by γ-secretase (a multiprotein complex composed of PSEN1 or PSEN2, nicastrin, Aph-1 and Pen-2), which generates the p3 peptide and an intracellular cytoplasmic domain (AICD) (Chow et al., 2010;O'Brien and Wong, 2011).

In the amyloidogenic pathway, β-secretase (a member of beta-site amyloid precursor protein cleaving enzyme [BACE], in particular BACE1) initiates the cleavage of APP at the N-terminal domain of Aβ. This initial cleavage results in the release of an extracellular APP fragment known as soluble APPβ (sAPPβ) and C-terminal fragment β (CTFβ) of 99 amino acids. Thereafter, the CTFβ is cleaved by γ-secretase (a similar cleavage as CTFα) that generates an

extracellular Aβ peptide and an intracellular cytoplasmic domain (AICD) (Chow et al., 2010;O'Brien and Wong, 2011). The processing of APP is summarised in figure 2. Additionally, there are several other, less well studied APP-processing pathways, *e.g.*, the combined β- and α-pathway (Portelius et al., 2009).

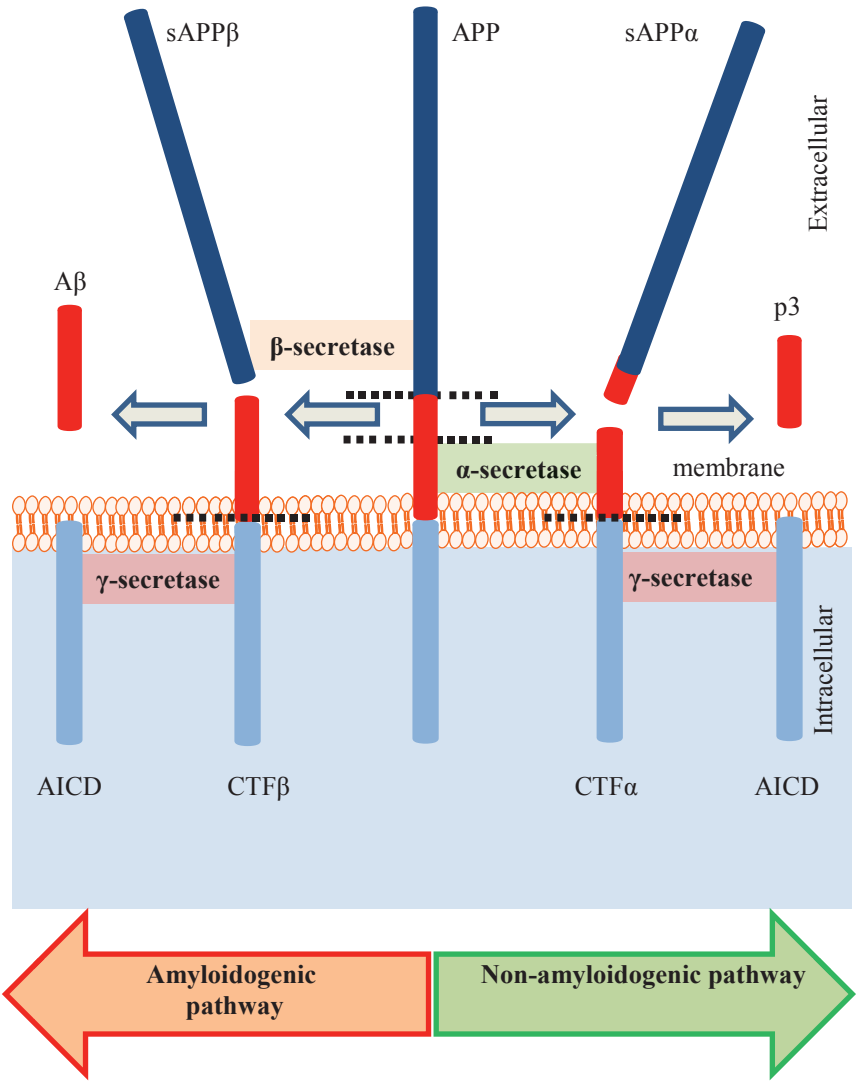

Figure 2 *APP is either processed via a non-amyloidogenic pathway that requires α- and γ-cleavages to generate p3 peptides, sAPPα and AICD fragments (this processing is shown on the right) or processed via the amyloidogenic pathway that requires β- and γ-cleavages and generates Aβ peptides, sAPPβ and AICD fragments (this processing is shown on the left).*

1.2.3 Functions of APP

APP and its metabolites have been extensively studied in relation to AD, but their putative biological functions are less well known. A number of studies highlight the role and function of APP and its cleaved products in cell adhesion, motility and neurogenesis, including neural differentiation, neuro-progenitor cell (NPC) proliferation, neural development, synaptogenesis, neurite formation and guidance (Caille et al., 2004;Nicolas and Hassan, 2014;Stahl et al., 2014).

It has been suggested that APP binds to extracellular matrix (ECM) components such as collagen I, laminin, spondin-1, reelin, glypican, and heparin, β1-integrin and the actin-associated Ena/VASP-like protein that helps in cell adhesion and motility (Small et al., 1994;Young-Pearse et al., 2008;Hoe et al., 2009). APP plays a role in neurite outgrowth and guidance by increasing neurite length and branching (Sosa et al., 2013). Several studies highlight the function of sAPPα in neurogenesis, neuroprotection, memory formation, proliferation, synaptic plasticity and neurite formation (Gakhar-Koppole et al., 2008;Demars et al., 2011;Chasseigneaux and Allinquant, 2012). A few studies have investigated the properties of sAPPβ, and like sAPPα, it also stimulates microglia leading to production of neurotoxins (Barger and Harmon, 1997;Chasseigneaux and Allinquant, 2012). It has also been suggested that sAPPβ, but not sAPPα, induces neuronal differentiation (Freude et al., 2011). A study reported that both, sAPPα and sAPPβ decrease cell adhesion and increase axon outgrowth via the extracellular-signal-regulated kinase (ERK) activation (Chasseigneaux et al., 2011). It is suggested that sAPPα or sAPPβ can be used to stimulate the generation of NPCs and neurons from human embryonic stem cells (Demars et al., 2011;Freude et al., 2011).

APP has multifaceted role in synaptic physiology and development (Zou et al., 2016), and it is highly expressed in growth cones during development (Sabo et al., 2001;2003). In the brain, APP expression peaks during postnatal development (from P1 to P36 in mice). During synaptogenesis and when neuronal connections are formed, its expression increases in pre- and post-synapses (De Strooper and Annaert, 2000;Wang et al., 2009). It has been proposed that APP and its isoforms APLP1 and APLP2 can interact with each other via their heparin-binding domains and form a dimer (Small et al., 1994;Baumkotter et al., 2012). As APP is localised to both pre- and post-synapses, a dimerization across the synapses may result in synapse formation and stabilization (Wang et al., 2009;Baumkotter et al., 2014;Stahl et al., 2014). Further, APP is involved in dendritic spine formation and stability, spine

arrangement, and synaptic plasticity by mediating astrocytic D-serine homeostasis, which is important for synaptic plasticity (Zou et al., 2016;Montagna et al., 2017).

1.3 Biomarkers

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Group, 2001). Biomarkers may be useful in identifying disease risk, disease pathology, therapeutic interventions, diagnosis and prognosis of the disease (Strimbu and Tavel, 2010). A good candidate biomarker for dementia should have high sensitivity, specificity, reproducibility and stability over time. It should be harmless, inexpensive, non-invasive and easily collectable and the data should be reproduced by more than one independent researcher (Humpel, 2011). In the human body fluids, e.g. saliva, blood and CSF, biomarkers may be identified. In the context of neurodegenerative diseases including AD, biomarkers could potentially give information facilitating the diagnosis, prognosis and prediction of a disease (Beach, 2017). The recommendation for potential diagnostic biomarkers for AD suggest that these should have greater than 80% specificity for AD and for differentiating AD from other forms of dementia (Report, 1998).

1.3.1 Biomarkers of AD

The pathophysiological changes in AD can be monitored by brain imaging and cerebrospinal fluid (CSF) analysis (Sperling and Johnson, 2013;Blennow and Zetterberg, 2018a;Hampel et al., 2018). The last few years have also seen very promising developments regarding blood-based biomarkers (Hampel et al., 2018).

1.3.1.1 Imaging biomarkers

AD pathophysiology can be diagnosed by aid of neuroimaging (Johnson et al., 2012). Structural magnetic resonance imaging (MRI) and computed tomography (CT) are used to measure brain volume. Brain atrophy and

neurodegeneration can be measured using structural MRI scans, this is recommended in the evaluation of all patients with suspected cognitive problems to support a clinical AD diagnosis and to exclude other causes (Scheltens, 2009;Harper et al., 2014). Structural MRI has high resolution to distinguish between grey and white matter boundaries and the MRI scans can differentiate between different neurodegenerative diseases and AD variants, at least in the dementia phase of the disease (Frisoni et al., 2010). In AD, the most severe brain atrophy is observed in hippocampal structures, which reflects the loss in hippocampal volume and correlates with the severity of the cognitive dysfunction (Murphy et al., 1993;Gosche et al., 2002).

Functional and molecular neuroimaging, utilising fluoro-deoxy-D-glucose (FDG) metabolism probes and amyloid positron-emission tomography (PET), may help in AD diagnosis (Mosconi et al., 2009;Mosconi and McHugh, 2011). Brain metabolism can be studied by using FDG, a glucose analogue in combination with PET. FDG quantification allows assessing the brain glucose metabolism in the cerebral cortex in a region-specific manner, which correlates with neuronal and synaptic activity. A decrease in glucose metabolism is observed in AD-affected brain regions (De Santi et al., 2001;Mosconi et al., 2009).

Amyloid PET scans were first possible using a ^{11}C-labelled amyloid tracer (Pittsburgh Compound B) which has high binding affinity to fibrillar Aβ (Klunk et al., 2005). This tracer has therefore significantly higher cortical retention in AD patients compared with controls (Lockhart et al., 2005;Ye et al., 2005). Recently, ^{18}F-labelled amyloid tracers have been introduced, including florbetaben, florbetapir and flutemetamol. These have longer half-lives than ^{11}C-labelled tracers and are thus easier to work with in clinical imaging studies (Wolk et al., 2018).

Tau PET tracers may be used to quantify paired helical filaments (PHFs) in human brains, which correlates with disease stage and tracks disease progression (Dani et al., 2016). A number of tau PET tracers were recently developed and validated. As an example, 18F-flortaucipir has been suggested to discriminate between AD and other neurodegenerative diseases and its retention is a strong *in vivo* measure of the total tau burden in the AD brain (Ossenkoppele et al., 2018;Smith et al., 2018).

1.3.1.2 CSF biomarkers

To detect AD pathophysiology, $A\beta_{1-42}$, T-tau and phospho-tau (P-tau) are routinely used as core CSF biomarkers (Blennow, 2017). In 1995, CSF $A\beta_{1-42}$ concentration measured by enzyme-linked immunosorbent assay (ELISA) was shown to be lower in AD patients compared with cognitively normal control individuals (Motter et al., 1995). Thereafter**,** similar findings were reported in several studies, irrespective of assay used (Andreasen et al., 1999;Flirski and Sobow, 2005;Olsson et al., 2005;Mattsson et al., 2009). A large meta-analysis showed that CSF $A\beta_{1-42}$ concentration is reduced by 40-50% in CSF from AD patients compared with age-matched controls (Olsson et al., 2016). This reduction is believed to be the result of sequestration of $A\beta_{1-42}$ in plaques in the AD brain (Blennow et al., 2001;Blennow, 2004).

Microtubule-associated protein tau is localised to neuronal axons (Biswas and Kalil, 2018). It has been suggested that tau protein aggregation in the brain is associated with neurodegeneration (Musi et al., 2018). In AD, truncated and hyperphosphorylated tau is an important component of the PHFs that constitute the neurofibrillary tangles and neuropil threads. Tau hyperphosphorylation affects the ability of the protein to bind to and stabilize microtubules, which is associated with axonal degeneration (Grundke-Iqbal et al., 1986a;Grundke-Iqbal et al., 1986b). In AD CSF, tau protein concentrations are increased, which likely reflects increased secretion of both T-tau and P-tau from AD-affected neurons (Sato et al., 2018). This may in turn correlate with or predict AD-type neurodegeneration and tangle formation (Blennow et al., 2010).

CSF $A\beta_{1-42,}$ T-tau and P-tau have high diagnostic accuracy and are recommended in the diagnostic research criteria for AD (Dubois et al., 2014;Jack et al., 2016). The core AD biomarkers are shown in figure 3. However, novel AD biomarkers could further enhance our knowledge on molecular mechanisms involved in AD pathophysiology (Blennow and Zetterberg, 2018a). An early event in the AD process is synaptic dysfunction and degeneration and CSF biomarkers for synaptic damage may be altered early in the disease process (Arendt, 2009;Thorsell et al., 2010).

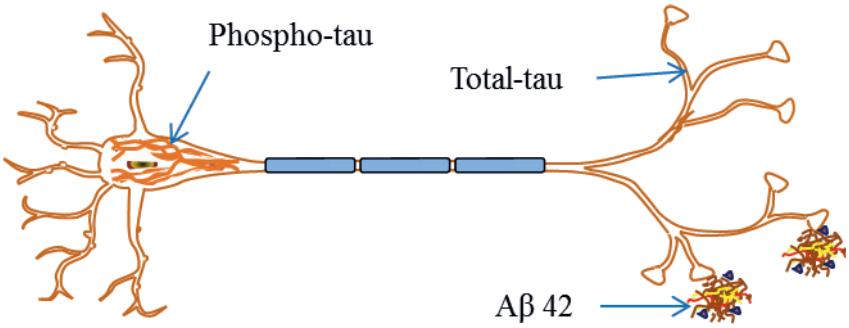

Figure 3 *Sketch of a neuron depicting the core AD biomarkers, including total-tau, phospho-tau and Aβ42.*

1.4 Synapse biology

Synapses are the functional units in neuronal communication in the CNS. They undergo several chemical, electrical and structural changes during learning processes and are built up by the pre- and post-synaptic terminals of two connected neuronal units (Abbas et al., 2018). Synapse formation requires an axon to find its target and interaction between several cell-adhesion molecules to form the physical connection points that build up the synapse (Andreae and Burrone, 2018). Neurotransmitter release is regulated by specialised pre-synaptic proteins, while post-synaptic receptors at the dendritic spine receive the signals and as a consequence a myriad of downstream molecular events takes place (Jahn and Fasshauer, 2012;Sudhof, 2013).

1.4.1 Synaptic pathology in AD

A number of studies suggest that synaptic degeneration and dysfunction are core features of AD pathophysiology, also early in the disease process (Masliah et al., 2001;Selkoe, 2002;Scheff et al., 2007;Arendt, 2009). Further, AD patients have fewer synapses than age-matched cognitively normal controls, and synapse loss correlates more strongly with cognitive decline than plaque and tangle pathology (DeKosky and Scheff, 1990;Terry et al., 1991;Sze et al., 1997). Biomarkers for synaptic activity, dysfunction and/or synaptic loss could thus be useful to detect

neurodegeneration onset and measure its intensity in AD (Blennow and Zetterberg, 2018b).

1.4.2 Synaptic proteins in CSF

Synaptic protein concentrations are generally low in CSF, making it difficult to measure them accurately. However, in the late 1990s, it was possible to identify some proteins in CSF from various synaptic compartments, including pre-synaptic membrane synaptosomal-associated protein-25 (SNAP-25), pre-synaptic vesicle protein synaptotagmin (SYT), pre-synaptic growth-associated protein-43 (GAP-43), the dendritic protein neurogranin (NRGN) and others (Davidsson et al., 1996;Davidsson et al., 1999;Sjogren et al., 2000). Although NRGN, GAP-43, SYT and SNAP-25 were identified in CSF, it was more lately that novel antibodies and sensitive ELISA and mass spectrometry (MS) methods were developed to quantify them in CSF samples (Thorsell et al., 2010;Brinkmalm et al., 2014;Kvartsberg et al., 2015a;Öhrfelt et al., 2016;Sandelius et al., 2018b). Some of the synaptic proteins that have been detected in CSF and examined in AD are illustrated in figure 4.

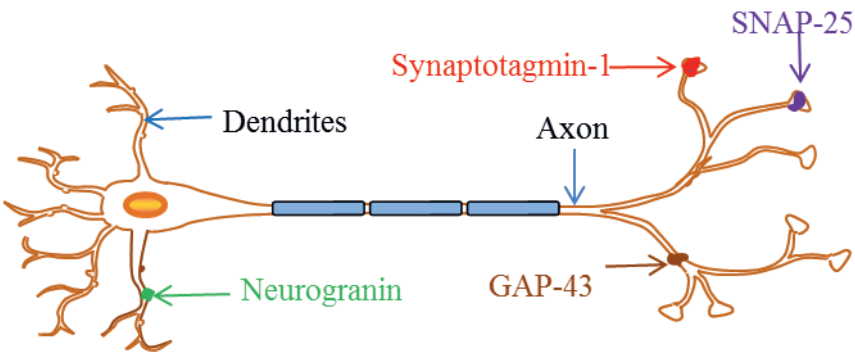

Figure 4 *Sketch of a neuron indicating cellular localisation of the pre-synaptic proteins, including synaptotagmin-1, SNAP-25 and GAP-43 and a post-synaptic protein, neurogranin. These synaptic proteins have been reported to be secreted at higher concentrations into CSF of AD patients as compared to age-matched controls.*

1.4.2.1 Synaptosomal-associated protein-25

SNAP-25, a pre-synaptic protein, is an important component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). The SNARE complex is required in various biological functions including neuronal development. SNAP-25 is a membrane-bound protein that is localised to nerve terminals in the brain. SNAP-25 helps in synaptic communication by docking of the synaptic vesicles to the cell membrane; therefore assisting in synaptic vesicle exocytosis (Greber et al., 1999;Zylbersztejn and Galli, 2011;Cupertino et al., 2016). It is suggested that interactions between SNAP-25 and SYT-1 play a role in vesicle-priming and in neurotransmission (Schupp et al., 2016).

SNAP-25 levels are reduced in the cortex of AD compared with control brains (Davidsson and Blennow, 1998), whereas a significant increase of SNAP-25 in CSF was observed in AD dementia and prodromal AD (Brinkmalm et al., 2014). A recent study concluded that CSF concentrations of SNAP-25 and the SNAP-25/Aβ42 ratio are increased at the early clinical stage of AD, suggesting that CSF concentrations of SNAP-25 and SNAP-25/Aβ42 ratio could be diagnostic and prognostic biomarkers for the earliest symptomatic stage of AD (Zhang et al., 2018).

1.4.2.2 Synaptotagmin-1

Synaptotagmins are a family of synaptic vesicle proteins, and the mammalian family has 17 members, eight of which bind calcium, including SYT-1 (Chen and Jonas, 2017). SYT-1 is localised in the pre-synaptic plasma membrane and is suggested to play a role in endocytosis and exocytosis, synapse function, neuronal development, axonal differentiation and activity-induced remodelling of synapses (Sudhof and Rizo, 2011;Inoue et al., 2013;Baker et al., 2015;Inoue et al., 2015). SYT-1 is a calcium sensor and detects a rise in intracellular calcium ions following an action potential, allowing vesicles to fuse with the pre-synaptic terminals, thereby initiating exocytosis (Sørensen et al., 2003;Xu et al., 2009).

A study, using Chinese hamster ovary (CHO) cells, rat pheochromocytoma cells (PC12) and mouse primary neurons, reported that SYT-1, SYT-2 and SYT-9 interact with APP. It showed that overexpression of SYT-1 increased APP-CTFs, sAPP levels and secreted Aβ levels. On the other hand, knocking down SYT-1 caused a reduction of secreted Aβ, including Aβ40 and Aβ42, which

suggests that SYT-1 regulates Aβ generation (Gautam et al., 2015). Two other studies have suggested that SYT-1 interacts with PSEN1 and regulates synaptic Aβ (Kuzuya et al., 2016;Zoltowska et al., 2017).

In AD brain, SYT-1 levels are reduced in cortical areas (Geppert et al., 1994), indicating synaptic degeneration. Using immunoprecipitation (IP) followed by mass-spectrometry (MS) to monitor SYT-1 in CSF revealed an increase in SYT-1 concentrations in AD dementia and prodromal AD (Öhrfelt et al., 2016). These results make CSF SYT-1 a promising biomarker for synaptic dysfunction and degeneration in AD.

1.4.2.3 Growth-associated protein-43

GAP-43 is also known as neuromodulin, B-50, P-57, F1 and pp46. It is expressed at high levels in the prenatal stage followed by reduced expression during maturation of the central nervous system (CNS) (Benowitz and Routtenberg, 1997;Casoli et al., 2001). In mature brain, GAP-43 is predominantly expressed in the cerebellum (granule cells but not Purkinje cells), neocortex, entorhinal cortex, hippocampus and olfactory bulb (Meberg and Routtenberg, 1991;Casoli et al., 2001).

GAP-43 is a pre-synaptic axonal protein that is highly expressed in neuronal growth cones, where it assists in synaptogenesis, but it is absent from dendrites and myelinated axons (Ramakers et al., 1992;Carriel et al., 2017). A number of studies indicate that GAP-43 is involved in growth cone formation, synaptic plasticity, neurite outgrowth and filopodia formation (Aigner and Caroni, 1993;Grasselli et al., 2011). Sensory neurons that lack GAP-43 in their growth cones are devoid of f-actin and show deficits in adhesion, spreading and branching of axons (Aigner and Caroni, 1993;Benowitz and Routtenberg, 1997). Further, GAP-43 is suggested to be involved in synaptic plasticity and LTP, which is reflected by alterations in GAP-43 levels and phosphorylation (Routtenberg et al., 2000;Denny, 2006).

GAP-43 levels are markedly decreased in the frontal cortex and in the hippocampus in AD (Davidsson and Blennow, 1998;Bogdanovic et al., 2000;Masliah et al., 2001). In addition, a recent explorative proteomics study found increased CSF GAP-43 concentration in AD patients (Remnestal et al., 2016). These results were recently corroborated using a novel GAP-43 ELISA

(Sandelius et al., 2018a); strong correlations of GAP-43 concentration with amyloid plaques and neurofibrillary tangles were seen, and the increase in CSF GAP-43 concentration was surprisingly AD-specific and not seen in other neurodegenerative disorders (Sandelius et al., 2018a).

1.4.2.4 Neurogranin

NRGN is also known as RC3, canarigranin, B-50-immunoreactive C-kinase substrate (BICKS) and p17. It is a 78 amino acid-long post-synaptic protein (Represa et al., 1990). NRGN is a member of calpacitin family that includes GAP-43, peptide protein 19 (PEP-19), Igloo and sperm protein 17 (SP17). One of the characteristic features of these proteins is their binding to calmodulin (CaM) at low intracellular Ca^{2+} levels and release from CaM at high concentrations, and they thereby take part in calcium signalling and synaptic plasticity (Gerendasy and Sutcliffe, 1997;Zhong et al., 2009). NRGN expression is altered in some diseases such as schizophrenia, vitamin A deficiency, hypothyroidism and AD, where it may be implicated in cognitive impairment (Iniguez et al., 1993;Kovalevich et al., 2012).

1.4.2.4.1 Expression and subcellular localisation

NRGN is expressed in the cerebral cortex, amygdala, caudate-putamen and the hippocampus (Represa et al., 1990;Alvarez-Bolado et al., 1996). NRGN is also highly expressed in platelets and moderately in B-lymphocytes, and low expression can be detected in lung, spleen and bone marrow. However, NRGN expression has not been observed in glial cells (Glynne et al., 2000;Gnatenko et al., 2003;Diez-Guerra, 2010). A study on rat telencephalon suggested a bi-phasic *NRGN* messenger ribonucleic acid (mRNA) expression (early and juvenile) during development. NRGN protein expression was first detected on embryonic day 18 (E 18) in the piriform cortex and the amygdala at low levels. Its expression increased markedly at birth (postnatal stage-P1), followed by high expression levels postnatally throughout the development, coinciding with synaptogenesis when NRGN expression was detected in the neuronal soma and in the dendrites (Represa et al., 1990;Watson et al., 1992;Alvarez-Bolado et al., 1996).

A number of studies highlight the importance of thyroid hormone (specifically T_3 but not T_4) for adequate NRGN expression, indicating that hypothyroidism leads to reduced NRGN expression (Munoz et al., 1991;Iniguez et al., 1992;Iniguez et al., 1993;Kovalevich et al., 2012). In addition, reports from studies on rats and mice suggest that NRGN expression is reduced in aged and vitamin A deficient rodents, which may cause cognitive deficits. However, these deficits can be cured by supplementing with retinoic acid (Husson et al., 2004;Kovalevich et al., 2012). These reports highlight the significance of thyroid hormones and vitamin A as regulators of NRGN expression. Therefore, *in vitro* cellular models where NRGN expression is investigated, the constituents in media must be supplemented with thyroid hormone and vitamin A.

1.4.2.4.2 Structure of neurogranin

NRGN was first detected in rat forebrain (Watson et al., 1990) and then purified from bovine brain (Baudier et al., 1991). NRGN in brains from humans, rats, mice, and caprine mammals had very similar amino acid sequence, distribution and biochemical properties. In all these animals, NRGN binds CaM in the absence of Ca^{2+} and may be phosphorylated by protein kinase C (PKC) (Huang et al., 1993;Piosik et al., 1995). The *NRGN* gene is located on chromosome 11q24 and is 12.5 kbp long, consisting of four exons and three introns. Exons 1 and 2 encode the full-length 78 amino acid protein, while exons 3 and 4 contain the untranslated 3' sequences (Sato et al., 1995;Martinez de Arrieta et al., 1997).

Circular dichroism studies suggest that NRGN is unfolded in the absence of CaM or protein kinase C (PKC). The central sequence of NRGN protein has abundant hydrophobic and basic amino acids, forming an amphipathic α-helix (Cox et al., 1985;Crivici and Ikura, 1995;Gerendasy et al., 1995). This region is highly conserved and referred as the "IQ" domain (I_{33}QXXXRGXXXR_{43}), which adopts α-helical conformation (Bahler and Rhoads, 2002). CaM interacts with NRGN via the IQ domain in the absence of Ca^{2+}, stabilising the α-helical conformation. NRGN binding to CaM is affected by high Ca^{2+} concentrations or the phosphorylation of serine by PKC (S36 highlighted in red) in I_{33}QA**S**FRGH MAR_{43} (Baudier et al., 1991;Ran et al., 2003).

NRGN has a collagen-like domain that mostly consists of glycines and prolines. This site may be a region for collagenase digestion (Watson et al., 1994). The rat NRGN has four cysteine residues outside the IQ domain, while humans have three. These residues may be oxidised by nitric oxide or other oxidants to form intramolecular disulphide linkages, which results in a reduced binding affinity for CaM and less phosphorylation by PKC (Sheu et al., 1996;Ran et al., 2003). The human NRGN protein sequence with the IQ domain highlighted in green and the collagen-like domain highlighted in yellow is shown in figure 5.

```
                10                  20                  30                  40
MDCCTENACS  KPDDDILDIP  LDDPGANAAA  AKIQASFRGH
                50                  60                  70                  78
MARKKIKSGE  RGRKGPGPGG  PGGAGVARGG  AGGGPSGD
```

Figure 5 *The NRGN protein sequence is shown; amino acids highlighted in green indicate the IQ domain, while those in red show conserved amino acids in the IQ domain. Amino acids highlighted in yellow represent the collagen-like domain.*

1.4.2.4.3 Functions of neurogranin

NRGN is highly expressed at the postsynaptic compartment of synapses and regulates Ca^{2+}/CaM-signalling in dendritic spines (Zhabotinsky et al., 2006;Kubota et al., 2007;Zhong et al., 2009). It has been suggested that NRGN and PKC expression coincide during cortical synapse development and dendritic growth, which indicates a role of NRGN in synapse formation (Represa et al., 1990;Alvarez-Bolado et al., 1996).

NRGN has been implicated in synaptic plasticity. LTP and long-term depression (LTD) have a common pathway at excitatory synapses that is dependent on *N*-methyl-D-aspartate (NMDA) receptor activation and the concentration of Ca^{2+}/CaM. When the intracellular concentration of Ca^{2+} is high, Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is activated. This leads to signalling that increases the insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) in post-synaptic cell membranes, leading to LTP. In contrast, lower intracellular Ca^{2+} concentrations cause activation of the phosphatase calcineurin, which leads to LTD (Malenka and Bear, 2004;Zhong and Gerges, 2010). Altogether, NRGN plays a pivotal role in regulation of synaptic plasticity and function.

There are several studies that highlight the role of NRGN in cognition (Miyakawa et al., 2001;Wu et al., 2002;Huang and Huang, 2012). A study conducted on NRGN knockout (KO) mice (NRGN -/-) indicated that these were normal in general health, as well as with regard to sensory and motor functions, compared to wild-type mice. However, NRGN KO mice had behavioural deficits in the Morris water maze test, deficits in spatial navigation and learning and increased anxiety. These observations suggest a role for NRGN in hippocampus-mediated interactions between stress and performance (Miyakawa et al., 2001). Another study on NRGN KO mice (NRGN -/-) showed that PKC and cyclic adenosine monophosphate (cAMP)-dependent protein kinase signal transductions were attenuated, which led to defects in phosphorylation of cAMP response element-binding protein (CREB) (Wu et al., 2002).

1.4.2.4.4 Disease implication

Several studies have highlighted altered NRGN expression during cognitive impairment, aging, hypothyroidism and vitamin A deficiency, as stated in section 1.4.2.4.1. Several studies have revealed associations between the *NRGN* gene and schizophrenia (Ruano et al., 2008;Stefansson et al., 2009;Steinberg et al., 2011;Shen et al., 2012).

NRGN plays an important role in the regulation of synaptic plasticity and function, a feature that is impaired in AD. NRGN is highly expressed in associative cortical areas in normal human brain, while the expression is reduced in cortex and hippocampus, reflecting synapse loss (Davidsson and Blennow, 1998;Reddy et al., 2005). Several studies have shown increased CSF NRGN concentration in AD patients compared with cognitively normal age-matched controls (Thorsell et al., 2010;Kvartsberg et al., 2015a;Portelius et al., 2015;Wellington et al., 2016). CSF NRGN increase is surprisingly specific to AD and is not seen in other neurodegenerative diseases, including frontotemporal dementia, Lewy body dementia, Parkinson disease and others (Wellington et al., 2016;Portelius et al., 2018). CSF NRGN concentration has been implicated as a predictor of cognitive decline in individuals at increased risk of AD dementia (Headley et al., 2018).

1.5 Stem cell-derived cortical neurons as a model system

Stem cells are clonogenic cells that have the potential of self-renewal and multi-lineage differentiation (Reya et al., 2001). Stem cells are unspecialised cells that have the potential to differentiate into several different cell types during development. These cells may also serve as a repair system. In some organs, for example the bone marrow, stem cells regularly divide to repair and replace cells that are turned over (Bianco et al., 2001). In other organs, *e.g.*, the heart, stem cells divide only under specialised conditions (Blau et al., 2001;Beltrami et al., 2003).

Previously, two main types of stem cells were considered: embryonic stem (ES) cells and somatic stem cells. ES cells are pluripotent and have the capacity to differentiate into all cell types emerging from all the three germ layers (ectoderm, mesoderm and endoderm). Somatic stem cells, on the other hand, have limited plasticity and proliferative capacity and are therefore, multipotent rather than pluripotent. They give rise only to the cell type already present in the tissue of their origin. As an example, bone marrow stem cells give rise to hematopoietic cells (Blau et al., 2001;Passier and Mummery, 2003) and neuroepithelial progenitor cells give rise to adult neural stem cells (Gage, 2000). In this thesis, we have used stem cell-derived cortical neurons as a model system to study some of the AD-associated proteins.

1.5.1 Human induced pluripotent stem cells

In 2006, Yamanaka and his colleagues identified factors that could reprogram adult somatic cells to a pluripotent state. The resulting cells are known as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Mouse iPSCs were first reported (Takahashi and Yamanaka, 2006), but human iPSCs (hiPSCs) were soon to follow (Takahashi et al., 2007).

Yamanaka and his colleagues genetically modified the adult human dermal fibroblasts by overexpressing four factors (also known as the Yamanaka factors): Oct3/4, Sox2, Klf4, and c-Myc to generate the hiPSCs (Takahashi et al., 2007). These factors are highly expressed in ES cells (Liu et al., 2008b). The results suggested that hiPSCs are similar to ES cells in several ways. For example, hiPSCs can differentiate into ectoderm, mesoderm and endoderm, and they have

similar morphology, proliferation and gene expression characteristics, as well as similar surface antigens and telomerase activity (Takahashi et al., 2007).

1.5.2 Cerebral cortex and cortical neurons

The cerebral cortex is an important part of the mammalian CNS, which is involved in cognition, sensory perception and motor control (Rubenstein, 2011;Lodato and Arlotta, 2015). The neo-cortex is the largest part of the cerebral cortex and is highly organized with complex neuronal cells (Molyneaux et al., 2007), while the rest of the cerebral cortex (mainly the olfactory system and the hippocampus) is called the allo-cortex (Posimo et al., 2013).

The cerebral cortex contains two major classes of neurons, interneurons and projection neurons (Parnavelas, 2000). Interneurons connect locally in the cortex and are mostly inhibitory GABAergic neurons, while projection neurons send their axons to distant brain areas and are excitatory glutamatergic neurons. The projection neurons have a triangular shape, and are called “pyramidal cells” and function as a transmission between the cortex and other regions of the brain (Greig et al., 2013). The excitatory projection neurons are generated from progenitors in the ventricular and sub-ventricular zones (VZ and SVZ, respectively). There are different type of progenitors that contribute to corticogenesis, including radial glial progenitors and intermediate progenitors (Molyneaux et al., 2007;Greig et al., 2013).

The progenitor population has different morphological properties. For example, radial glial cells regulate the thickness of the cortex and serve as a scaffold for newly born neurons (Rakic, 1971). They also give rise to outer radial glial cells and intermediate progenitors (Noctor et al., 2001). As new neurons are born during development, the old neurons migrate away from the VZ and form a pre-plate. New neurons migrate into the pre-plate and divide it into the marginal zone and the sub-plate. This eventually forms a cortical plate between the sub-plate and the marginal zone as shown in figure 6. Cortical neurons organize themselves in a stereotyped temporal order. Early born cortical neurons are destined first and populate deeper cortical layers (layer VI, followed by layer V), followed by later born neurons that populate the outer layers (layer IV followed by layer II/III) (Greig et al., 2013). The schematic representation of six cortical layers is shown in figure 7.

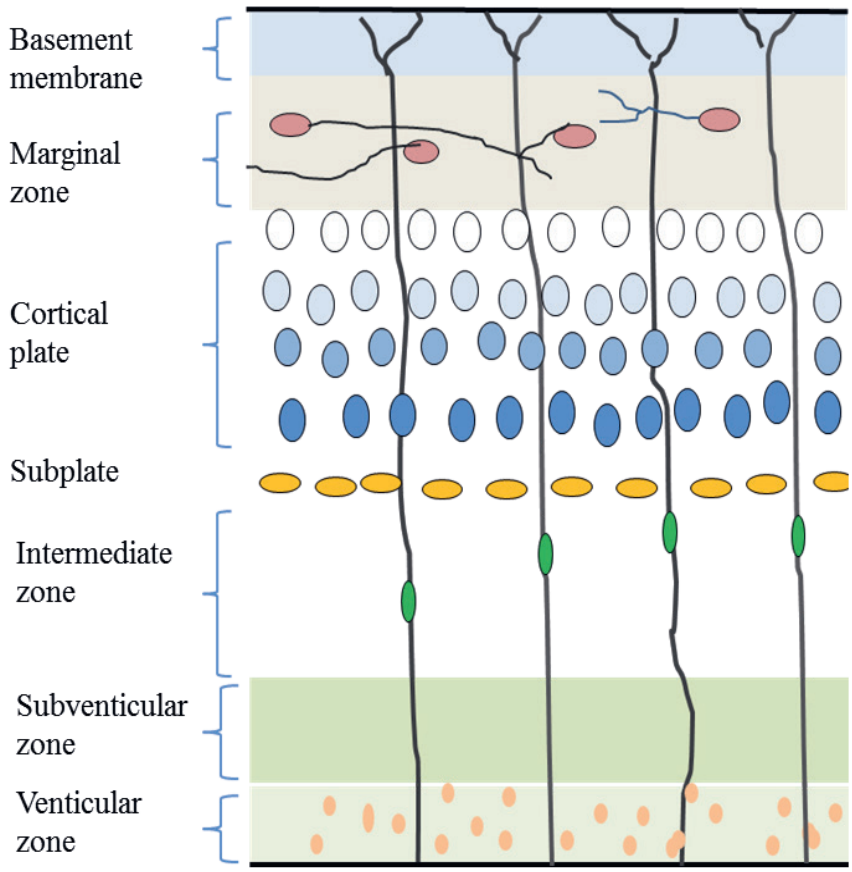

Figure 6 *Schematic representation of corticogenesis in human brain during development.*

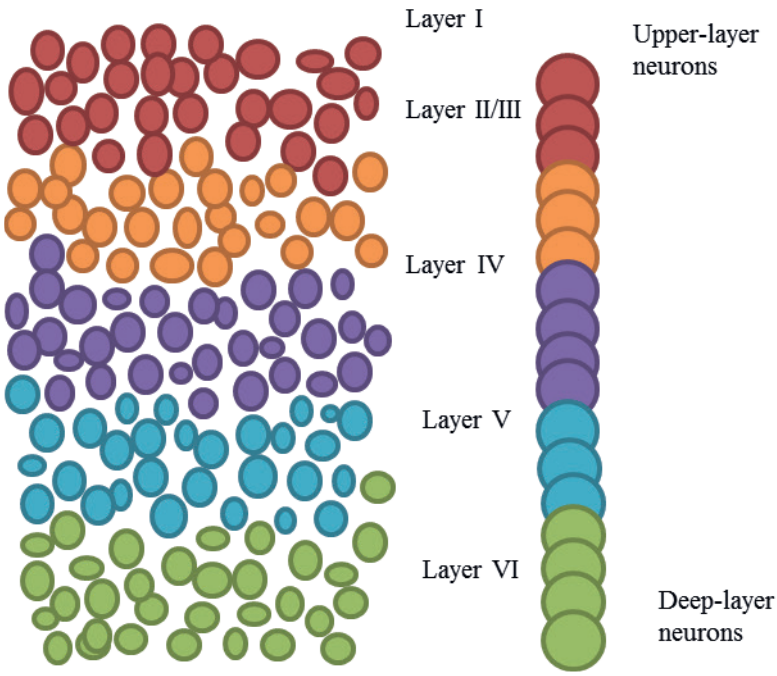

***Figure** 7 Schematic representation of cortical neuronal layers. Deep layer neurons are born first (layer VI) followed by layer V, IV, III and II. Upper layer neurons are born later and form outer layers.*

1.5.3 iPSC-derived cortical neurons

There are different methods to generate cortical neurons from hiPSCs, including methods described by Shi *et al.* for the generation of cortical neurons from mono-layer cultures (Shi et al., 2012a). Lancaster *et al.* developed cerebral organoids with various brain regions (Lancaster et al., 2013) and Paşca *et al.* developed cortical spheroids (Paşca et al., 2015). However, in this thesis, we have used a protocol described by Shi *et al.* (Shi et al., 2012a), which has the advantage that it apparently recapitulates *in vivo* development. Further, the cells are cultured in mono-layers, where most of the cells are in direct contact with the cell-culture media. The neuronal proteins are secreted into the cell culture medium and can therefore be detected using immunochemical and mass spectrometric methods. In contrast, in three-dimensional models, all cells are not in direct contact with the cell culture medium and therefore, some proteins may

not reach it. Thus, the Shi *et al.* model matched our experimental paradigm to study expression and secretion of neuronal proteins.

In 2009, Chambers *et al.* reported the successful generation of neuronal rosettes directly from pluripotent stem cells by dual-SMAD inhibition. The inhibition of the dual-SMAD pathway inhibits bone morphogenetic protein (BMP)- and transforming growth factor-β (TGF-β)-signalling in hiPSCs. It reduces the pluripotency of stem cells (Xu et al., 2008) and suppresses the trophoblast lineage (Xu et al., 2002) and the mesodermal and endodermal lineages (D'Amour et al., 2005;Laflamme et al., 2007), and favours the formation of the primitive ectoderm (Chambers et al., 2009).

The dual-SMAD inhibition approach was later improved by Shi *et al.*, when they demonstrated that stimulating retinoid-signalling together with dual-SMAD inhibition forced the lineage towards forebrain identity by generating cortical neurons. ES cells and hiPSCs were both used to generate cortical neurons (Shi et al., 2012a;Shi et al., 2012b). The protocol generates cortical neurons in the same temporal order as observed *in vivo,* which includes the appearance of deep-layer neurons first and upper-layer neurons last. Human cortical neurogenesis is reported to span around 100 days (Caviness et al., 1995), while the Shi *et al*. protocol extends at least 90 days. Further, it generates cortical neurons with functional excitatory synapses that have the ability to fire action potentials. Since the neurons are cultured in mono-layers, it is relatively easy to observe the morphological changes and protein localisations during differentiation as compared to cultures of cortical spheroids and cerebral organoids. Finally, the collection of conditioned cell culture media is possible for biomarker quantification.

2. Aims

2.1 General aims

The overall aim of this thesis was to investigate the expression and secretion of selected Alzheimer-associated biomarkers in a newly adapted model of stem cell-derived cortical neurons. For one of the markers, neurogranin, we further investigated its processing and examined its molecular forms in CSF.

2.2 Specific aims

Paper I

To characterise the cell model and to investigate the step-wise processing of APP during differentiation of hiPSCs to cortical neurons

Paper II

To investigate the expression and secretion of synaptic proteins during hiPSCs differentiation to cortical neurons

Paper III

To identify the enzymes required for the processing of NRGN, yielding truncated peptides that are secreted at increased concentrations into CSF from AD patients

Paper IV

To identify the molecular forms of NRGN in CSF and to determine the ratio of C-terminal NRGN to total-NRGN in CSF

3. Methods

The results obtained in this thesis are based on a variety of methods as described in detail in each paper; the key methods are described briefly as follows.

3.1 Ethical permits

Human iPSCs used in papers I and II were de-identified cells, which were covered under the ethical permit from the regional ethical review board in Gothenburg (2014-731). The experiments on mouse brain extracts performed in paper III were approved by the regional animal ethics committee in Gothenburg (2013–103). CSF samples used for method development in paper IV were terminally de-identified samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden (approved by the regional ethical review board in Gothenburg, August 11, 2014).

3.2 Stem cell culture

Papers I and II are based on hiPSC-derived cortical neurons. Three hiPSC lines of different origins were used in this thesis; Con1 hiPSC line originating from fibroblasts (Sposito et al., 2015), A2B hiPSC line originating from chondrocytes (Boreström et al., 2014) and BJ1 hiPSC line originating from fibroblasts (Bergström et al., 2016). These lines were obtained from our collaborators at University College London, United Kingdom, and the Sahlgrenska Academy, University of Gothenburg, Sweden, respectively. hiPSCs were cultured and maintained using standard procedures and cell culture media for each cell line, as detailed in papers I and II. In this thesis (papers I and II), we differentiated hiPSCs to cortical neurons.

3.3 hiPSCs differentiation to cortical neurons

To differentiate hiPSCs to cortical neurons, we followed a protocol described by Shi *et al.* (Shi et al., 2012a) with some modifications, as described in papers I and II. Briefly, hiPSCs were cultured to full confluence in mono-layers followed by initiation of neural induction using neural maintenance media (NMM) (the

constituents of which are described in papers I and II), further supplemented with mouse Noggin-CF chimera and SB431542 to inhibit SMAD proteins.

By inhibiting SMAD proteins in hiPSCs, BMP- and TGF-β-signalling are inhibited, which reduces the pluripotency of stem cells (Xu et al., 2008) and suppresses the trophoblast lineage (Xu et al., 2002), as well as the mesodermal and endodermal lineages (D'Amour et al., 2005;Laflamme et al., 2007), and favours the formation of the primitive ectoderm (Chambers et al., 2009). Further, the supplementation of cell culture media with retinoic acid is required for efficient cortical induction and cortical neuron generation (Bibel et al., 2004;Siegenthaler et al., 2009). Cells are maintained for 10-12 days in neural induction medium to initiate corticogenesis followed by supplementation with fibroblast growth factor-2 (FGF-2). FGF-2 expands the neuronal rosettes; however, removal of FGF-2 promotes neurogenesis in this protocol. The cortical neuronal cells are maintained for at least two months to achieve mature cortical neurons. In paper I, cortical neuronal cells were maintained for at least 120 days, and in paper II, cortical neuronal cells were maintained for at least 150 days. This protocol generates cortical neurons in a step-wise manner; first hiPSCs are differentiated to neuro-progenitors, which is followed by the appearance of immature cortical neurons mostly expressing deep layer neurons. Finally, mature cortical neurons appear mostly representing upper layer neurons. Astrocytes also appear when mature cortical neurons are formed. The hiPSCs differentiation process to cortical neurons is summarised in figure 8.

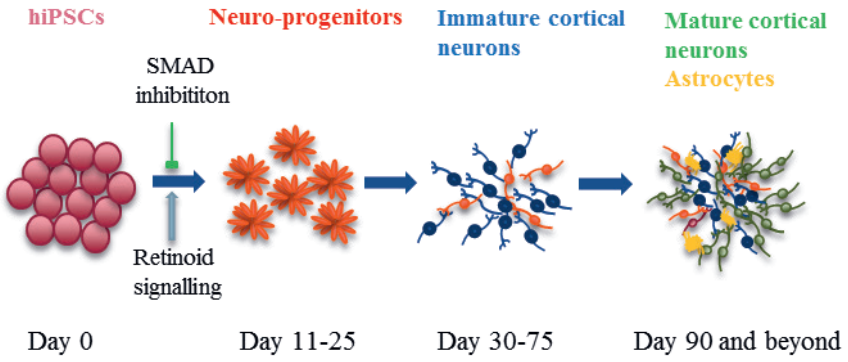

Figure 8 *Schematic representation of the hiPSCs differentiation process to cortical neurons. During differentiation, stem cells develop into neuro-progenitors, immature neurons and then mature cortical neurons. Astrocytes appear late during this differentiation process.*

There are several advantages with preparing cortical neurons from hiPSCs. The hiPSCs can be used to study the development of human cortical neurons *in vitro* and are easy to manipulate and study. A variety of human neurological disease-specific *in vitro* models can be created using hiPSC-derived neurons from patients with genetically determined neurodegenerative disease. One of the biggest advantages of using hiPSC-derived neurons is that the results may be more likely translated to humans since hiPSCs originate from human stem cells and they have many characteristics of human *in vivo* neurons. Although there are several advantages of using hiPSCs to model neurodegenerative diseases, there are also disadvantages that need to be considered. It may be difficult to model diseases with a long latency period, such as AD. AD needs a decade or more to develop pathological signs but it is practically challenging to culture cells for long periods to mimic the pre-clinical phase of disease. In addition, the disease progression in the patient may be vastly different from the phenotype observed in a cell culture dish, mainly due to lack of environmental factors that accelerate the disease progression. Further, several hiPSC-derived models generate a single purified lineage-committed cell type. This excludes the interactions of the cell type that is affected in the patient (*e.g.* neurons in AD) with other cell types present in physiological environment (*e.g.* astrocytes, microglia and oligodendrocytes in AD).

3.4 Complementary DNA

In papers I and II, complementary DNA (cDNA) was prepared from hiPSC-derived cortical neurons during different stages of differentiation, summarised in section 3.3. cDNA is synthesised from mRNA by reverse transcription (RT). An enzyme, RT, uses RNA as a template and a primer complementary to the RNA to synthesize the cDNA, which is then amplified by polymerase chain reaction (PCR) (Kohara, 2001). The preparation of cDNA and its detection by highly sensitive PCR enables the detection of very low levels of mRNA in a sample. One of the drawbacks with this method includes its inability to amplify promoters and operators, as well as intron sequences; therefore, it is not suitable for regulatory studies. Further, RT-PCR is not a quantitative method and it only allows to determine if the mRNA is expressed or not in the cells.

3.5 Quantitative PCR

To quantitate and compare the expression of genes, quantitative PCR (qPCR) was performed in papers I and II. In paper I, mRNA levels of the *APP* gene, as well as mRNAs of markers of stem cells, neuro-progenitors and cortical neurons, were quantified. In paper II, mRNA levels of synaptic proteins including *NRGN*, *GAP-43*, *SYT-1* and *SNAP-25* were quantified in different cortical neuronal differentiation stages.

Average cycle thresholds (C_T:s) of housekeeping genes; *RPL-27*, *RPL-30* and *HPRT-1* were used as endogenous controls. All primers used in this thesis are described in papers I and II. The relative quantities were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). In brief, the C_T value of the average of the endogenous genes was subtracted from the C_T value of the target gene to get ΔC_T values. Thereafter, ΔC_T values were compared to the highest ΔC_T value to get $\Delta\Delta C_T$. The formula for fold change used was $2^{-\Delta\Delta CT}$.

qPCR using fluorescence gene expression assays involves both unlabelled primers and the reporter probes containing a dye at the 5' end and a quencher molecule at the 3'end of the probe. At the beginning of the qPCR, denaturation is initiated to separate the two strands of DNA and at this stage the fluorescence from the reporter dye at the 5' end is absorbed by the quencher at the 3' end. In the next step, the primers and the probe anneal to their target sequences followed by the polymerisation of new strands by DNA polymerase. DNA polymerase has an endogenous 5' nuclease activity and when it reaches the probe, the reporter dye is separated from the quencher; as a result, fluorescence intensity is generated. With increase in the number of cycles more reporter dye is released resulting in increased fluorescence which is proportional to the dye released (Hancock et al., 2010).

One of the several advantages of qPCR is that several gene assays can be performed from a small cDNA sample. Therefore, qPCR is known for its potential of high throughput. The results obtained are relatively easy to quantify and less susceptible to subjective interpretation compared with other RNA-detecting techniques, such as RNA fluorescence *in situ* hybridization (RNA-FISH). However, the major drawback of qPCR is that cells are destroyed and cannot be morphologically examined, which is possible with RNA-FISH. It is important to keep in mind that RNA expression does not always correlate with protein expression. Therefore, to support our findings at mRNA levels, we have also performed immunoblotting in this thesis.

3.6 Western blot

In papers I and II, the western blot technique was used to examine APP and synaptic protein expression during neuronal differentiation. In paper III, the technique was used to detect full-length NRGN and its CALP1-cleavage products. In paper IV, western blot was used to detect NRGN and its cleavage products in CSF fractions collected after size exclusion chromatography (SEC) to examine its molecular state.

In western blot, the proteins are first denatured and then separated using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE resolves proteins based on size, meaning that smaller proteins migrate faster through the gel, whilst larger proteins migrate slower. The separated proteins are blotted onto a membrane (in our case we used nitrocellulose) followed by blocking of the membrane to prevent unspecific binding of the detector antibodies. After blocking, the membranes are incubated with primary antibodies and unbound antibodies are removed by washing. The primary antibodies are directed towards specific epitopes of the target protein. Thereafter, secondary antibodies conjugated with some form of signal generator (in our case horseradish peroxidase [HRP]) are used to bind to the primary antibodies. A developing solution containing luminol, an HRP substrate that becomes chemiluminescent upon oxidation (Mahmood and Yang, 2012), is added. The luminescence is detected by a light-sensitive camera and converted to images in which the luminescence intensity can be quantified by densitometry (image J software). Western blot of housekeeping proteins may be used to normalise the signals.

Western blot is a useful method to detect protein expression and to give a crude estimate of protein quantity (the method may be characterised as semi-quantitative). The main advantage of western blot is its high sensitivity; as little as 0.1 ng of a protein can typically be detected on blots. In addition, the method may give information not only on full-length proteins, but also peptides and truncated fragments of the proteins. One of the disadvantages of western blot is cross-reactivity of antibodies that may bind to off-target proteins and show false positive results. Another disadvantage is that cells have to be lysed to release their protein content; thus, the method cannot give any information on subcellular localisation of the target protein. In contrast, immunocytochemistry (ICC) can be used to localise the protein expression.

3.7 Silver staining

In paper III, we performed silver staining to detect NRGN and its truncated peptides after digestion with CALP1. After performing SDS-PAGE, gels were processed for silver staining. Silver staining is a technique used to detect proteins and peptides in gels. It is performed in a step-wise manner. First, the proteins are fixed in the gel using acetic acid and ethanol solution. The fixed proteins are then stained using a silver salt, along with sensitizing chemicals. Finally, the protein pattern is revealed by reducing the silver ions bound to the proteins to metallic silver, which gives a brown-black colour (Chevallet et al., 2006). A "stopping" solution is used to stop the reaction and prevent excessive background. The intensity of brown-black colour correlates with the amount of protein present in a band.

The main advantage of silver staining is its high sensitivity. Silver staining detects low protein levels (less than 1 ng per band). SDS-PAGE gel is often fixed with formaldehyde during silver staining; however, this reduces the down-stream applications of the gels (excised bands that have been fixed like this are for example not useful for mass spectrometric [MS] analysis). However, there are protocols where gels are not fixed with formaldehyde but these protocols compromise with the sensitivity of silver staining.

3.8 Coomassie staining

Coomassie staining was performed in paper III to detect NRGN proteins and peptides. After performing SDS-PAGE, gels were processed for Coomassie staining.

The Coomassie dye is anionic and forms complexes with proteins through ionic interaction, as well as van der Waals and hydrophobic interactions. The dye colour, initially reddish brown, changes to blue when the dye is complexed with protein. These interactions do not covalently modify the proteins. The gel is de-stained in water, removing the less strongly bound dye, thereby reducing the background staining. The intensity of the blue bands correlate with the amount of protein present in a band (Sasse and Gallagher, 2009).

Coomassie staining is simple and quick to perform as compared to silver staining and it is compatible with MS. However, the sensitivity is lower compared to silver staining.

3.9 Immunocytochemistry

Immunocytochemistry (ICC) is an antibody-based method to detect the expression and localisation of proteins in cells. In papers I and II, we performed ICC to detect the expression and localisation of neuronal transcription factors, markers for cortical neurons and synaptic proteins. In ICC, the first step involves fixation of cells. In this thesis, we have used paraformaldehyde (PFA) and ice-cold methanol to fix the cells. Thereafter, cells are permeabilised to enable antibodies to penetrate the cell membrane and bind to the intracellular proteins. This is followed by blocking of unspecific binding sites and then by incubation with primary antibodies, which is followed by incubation with fluorescently conjugated secondary antibodies that produce fluorescence when excited (Maity et al., 2013). Nuclear staining was performed using nuclear-specific dyes. In this thesis we have used 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei. The slides are then mounted using ICC mounting media before performing immunocytochemical analysis using a microscope.

ICC is performed on fixed and permeabilised cells, therefore the morphology of the cell is conserved unless the fixation affects it. Therefore, antibodies directed against specific antigens can be used to determine both the cellular expression and localisation of the target proteins. The disadvantage may be antibody cross-reactivity with other proteins. Generally, the images captured after ICC staining cannot distinguish between full-length protein and peptides.

3.10 Confocal microscopy

ICC samples were analysed using confocal microscopy. In paper I, some of the neuro-progenitor, cortical and synaptic markers were identified using confocal microscopy. In paper II, synaptic protein expression and localisation were detected using confocal microscopy.

Confocal microscopy uses fluorescence optics where laser light is focused on a single spot at a defined depth within the sample; the fluorescent light is emitted at exactly this point. The fluorescent light from the illuminated spot enters the

light detector, whereas, the out of focus light is prevented from entering the light detector by a pinhole. The specimen is scanned in a raster pattern that leads to the formation of a single optical plane. A three dimensional image can be created by scanning several optical planes and stacking them together (z-stack) using suitable software. Confocal microscopy have better resolution as compared to conventional epi-fluorescent microscopy (Swaim, 2010).

3.11 Enzyme-linked immunosorbent assay (ELISA)

In paper II, ELISA was performed to measure concentrations of NRGN and GAP-43 in conditioned cell-culture media. In paper IV, ELISA was performed to measure NRGN concentration in CSF. There are various ELISA methods, such as direct ELISA, indirect ELISA, sandwich ELISA and competitor/inhibitor ELISA. However, we have only used the sandwich ELISA format in our studies. The sandwich ELISA requires two antibodies, the capture and detection antibody, and both antibodies should be specific for the protein of interest and should have different epitopes. The first antibody (capture antibody) is coated at the bottom of a well of the ELISA plate (polystyrene plate with modified surface to maximize adsorption of proteins/peptides). Free binding space in the ELISA wells are then blocked by *e.g.* albumin or casein. The sample is then added followed by the addition of the detector antibody, either conjugated to HRP or other enzymes such as alkaline phosphatase. In this thesis, we have used a sandwich ELISA, where the detector antibody was unlabelled. Therefore, a secondary HRP-conjugated detection antibody was added. The detection solution contains a substance, tetrametylbenzidine (TMB), which reacts with peroxidase, producing a soluble blue reaction product. This product is converted to a yellow colour through the addition of sulphuric acid, which can be measured at 450 nm in an ELISA spectrophotometer. The intensity of the coloured product is proportional to the amount of protein present in a sample and can be quantified when compared to the signal intensities in a standard curve of known protein concentrations (Gaastra, 1984;Aydin, 2015). One of the main advantages of ELISA as compared to other immunoassays is the absolute quantification of proteins in a sample. The analytical sensitivity depends on the antibodies and other factors, but ELISA often has a limit of quantification in the low-medium (10-100 pg/mL) levels.

3.12 Electrochemiluminescent immunosorbent assay

In paper I, we used the Meso Scale Discovery (MSD) platform to quantify specific APP peptides. MSD works according to the same principles as ELISA but differs slightly in the way the signal is generated. MSD assays use a ruthenium-based electrochemiluminescent molecule (SULFO-TAG) that is conjugated to the detection antibodies. This method uses electrical potentials, which when applied to the plate electrodes leads to currents in the samples resulting in light emission by the SULFO-TAG labels. The emitted light intensity correlates with the amount of peptide/protein in the sample. Each peptide/protein can be quantified when compared to the standard curve of known protein concentrations (Oh et al., 2010). Several capture antibodies can be coated to small spots in a single well and thus several peptides/proteins can be quantified from a single sample in a well in a multiplexed assay.

3.13 Patch clamp

In paper I, electrophysiological experiments were performed on the cells to determine the maturity of neurons during hiPSCs differentiation to cortical neurons. The patch clamp technique is useful in investigating ion channels, electrical properties and functional connectivity of neurons. In paper I, we performed whole-cell patch clamp recordings during neuronal differentiation. The whole-cell patch clamp technique involves a glass micropipette that makes a patch with the neuronal membrane. The membrane patch is disrupted by applying suction to create a high resistance seal, known as a gigaohm seal. This allows the electrical and molecular access to the intracellular space.

With whole-cell patch clamp, the voltage-clamp mode and current-clamp mode can be used. In current-clamp mode, the current is kept constant and changes in voltage are monitored (*e.g.*, changes in membrane potential), and action potentials can be recorded. While keeping the voltage constant in voltage-clamped mode, changes in the current can be measured, for example, excitatory post-synaptic currents and inhibitory post-synaptic currents (Kornreich, 2007).

One of the drawbacks of whole-cell patch-clamp is that only one cell at a time can be patched. If the cell population is heterogeneous (*e.g.*, if the cells have differential maturation stages in the dish), a large cell sample must be patched, which may be time-consuming and practically difficult. As an alternative or

complement, the bio-circuit microelectrode array plates or calcium imaging can be used.

3.14 Lactate dehydrogenase assay

In paper II, we performed the lactate dehydrogenase (LDH) assay to monitor release of LDH into cell culture medium as a marker of cytotoxicity. LDH is a cytosolic enzyme that is released to the extracellular environment when the plasma membrane is damaged. The released LDH can be quantified by an enzymatic reaction in which LDH converts iodonitrotetrazolium to formazan so that a red colour develops, which can be measured at 490 nm by standard spectroscopy (Kumar et al., 2018). The amount of formazan produced correlates with the amount of released LDH into the culture media.

Measuring LDH in cell culture media is reliable and quick method. The limitation of LDH assays is that serum and some samples contain LDH. However, in our experimental set-up cell culture media was devoid of serum and there was no detectable LDH activity in media not exposed to cells.

3.15 Immunoprecipitation

In papers I and II, we performed immunoprecipitation (IP) to isolate APP peptides and SYT-1 and SNAP-25 proteins from conditioned cell culture media. It is a method used to separate a specific analyte (often a protein of interest) from a mixture in complex samples, for example cell culture media, plasma or CSF, by the use of antibodies.

IP involves the immobilization of specific antibodies on magnetic beads or any other support material (*e.g.,* agarose particles) followed by incubation with the samples. Thereafter, the protein of interest bound to the antibodies on the magnetic beads is isolated by magnetic separation of the beads from the samples, followed by extensive washing to remove non-specific binding (Kaboord and Perr, 2008). The protein of interest can then be eluted and used in any down-stream analyses, such as MS or western blot.

3.16 Mass spectrometry

In paper I, MS analyses were performed to determine the secreted APP peptides during hiPSCs differentiation to cortical neurons. In paper II, MS analyses were performed to determine the secretion of SYT-1 and SNAP-25 proteins to cell culture media. In paper III, we used MS to analyse CALP1 cleavage products. MS is an analytical method used to determine the molecular mass of a compound. It can be used to analyse a variety of molecules, for example, atoms, peptides, proteins and protein complexes. The basic principle involves the ionization of the analytes and separation of ions from each other on the basis of their mass-to-charge ratio (m/z). Therefore, a mass spectrometer has three main components, namely an ion source, a mass analyser and a detector. The ion source ionizes the analyte, which is transferred into gas-phase ions. The ions are subjected to electric and magnetic fields, where they are accelerated and separated according to their m/z ratios in the mass analyser and finally detected by image current detector or an impact detector.

In MS, different ionization techniques can be used including hard ionization and soft ionization techniques. Hard ionization techniques, including electron ionization, result in higher fragmentation of ions. Soft ionization, including electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), generates little fragmentation of ions. The soft ionization techniques can be used to analyse peptides and proteins (Matthiesen and Bunkenborg, 2013).

Mass spectrometers can be equipped with different mass analysers, which have different properties with respect to mass accuracy, speed, m/z resolution and fragment ion analysis (*e.g.*, time-of-flight (TOF), quadrupole and orbitrap spectrometers). Some mass spectrometers can be equipped with several types of analysers, so-called hybrid instruments (*e.g.*, Q Exactive) (Matthiesen and Bunkenborg, 2013). In this thesis, we employed MALDI/TOF using the UltraFleXtreme instrument in paper I, liquid chromatography followed by mass selection/mass separation using Q Exactive in paper II and paper III.

There are several advantages of using MS, including the large number of analytes that can be measured in one sample. With MS, the relative abundance of various peptides of a protein can be quantified and, by the use of isotope-labelled standards, absolute quantification may be achieved. MS is highly specific to the molecular identity but sometimes this specificity may result in loss of

recognition, *e.g.*, if a target peptide is post-translationally modified so that it does not appear at the expected mass.

3.17 FRET protease assay

In paper III, we used Fluorescence Resonance Energy Transfer (FRET) peptides to identify NRGN-cleaving enzymes. FRET is a phenomenon that relies on the distance-dependent interaction between excited states of two dye/fluorescent molecules. Generally, the excitation of a donor dye/fluorescent molecule is transferred to an acceptor dye/fluorescent molecule. This interaction takes place over greater distances than interatomic distances; however, the two dye/fluorescent molecules should be in close proximity. The donor molecule emits fluorescent light that overlaps in wavelength with the absorption of the acceptor, which then emits fluorescence at a longer wavelength. In our study, the acceptor dye only absorbed the fluorescence, without becoming fluorescent itself ("quencher"). Only when the peptide sequence separating the donor and quencher dyes is cleaved, the quenching effect of the acceptor is released, so that the donor dye becomes more fluorescent. The increase of fluorescence can be taken as a measure of peptide cleavage. This is the basis of the protease assay for detecting cleavage of a peptide linking a fluorescence donor and a quencher (Zauner et al., 2011).

3.18 Ultrafiltration

In papers III and IV, ultrafiltration was performed. Ultrafiltration involves a porous membrane to separate molecules in fluids on the basis of size and shape. Ultrafiltration membranes are available in different pore sizes, from 0.002 to 0.1 micrometres, corresponding to molecular weight cut-offs (MWCOs) of approximately 10 to 100 kilo Daltons (Zumstein, 1998).

The molecules that are larger than the membrane pores are retained at the membrane surface and are therefore concentrated in the "retentate" which is the remaining liquid in the sample reservoir. The size selectivity of ultrafiltration membranes is expressed as MWCO.

3.19 Size exclusion chromatography

Size exclusion chromatography (SEC) was used in papers III and IV to obtain fractions of proteins and peptides separated by size. A variety of molecular weight standards were used to ascertain the size of the proteins in various fractions including blue dextran (to indicate void volume), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and aprotinin (6.5 kDa). SEC separates molecules based on size, when they pass over the SEC resin. SEC resin contains a porous matrix of spherical particles that ideally do not bind the molecules. After sample application to the column, molecules that are larger than any of the resin particle pores elute first. Proteins that are smaller than some of the particle pores can diffuse into those particles and reside there for a while before exiting. The molecules that are smaller than the smallest resin particle pores can enter any particle pore and therefore also have the highest chances of being delayed during the migration along the column; therefore, they are eluted last. The isolated fractions can be purified, analysed or further used in down-stream processes (Irvine, 2000).

There are various chromatographic methods to separate proteins but keeping in mind the intended down-stream application, we preferred SEC. It yields a good separation of large molecules from the small molecules in small eluate volumes. In many cases, it does not result in sample loss, since, ideally, the solutes do not interact with the stationary phase. In this thesis, we used ion exchange chromatography and gel electrophoresis followed by western blot as down-stream applications.

3.20 Recombinant protein expression

In paper III, we expressed recombinant neurogranin protein in bacteria using the pET SUMO expression system, and starting with the neurogranin ORF clone RC201209 (Origen) as a template. In short, the general principle for recombinant protein expression involves a first step in which the cDNA is inserted into a plasmid using restriction enzymes and DNA ligase that produce a recombinant DNA. The recombinant DNA was then transduced into bacteria; for example, *Escherichia coli* and selected using an antibiotic resistance gene. The DNA was then transcribed and translated into the protein of interest. After production of the desired protein, the bacterial cells were lysed to release the protein along

with several other products. Thereafter, the protein of interest was purified and used in down-stream processes (Rosano and Ceccarelli, 2014).

3.21 Epitope mapping

Epitope mapping was performed in paper IV using binding measurements between an immobilised antibody and peptides (in mobile phase) on a Biacore instrument. Biacore monitors interactions quantitatively and in real time by determining mass changes of captured molecules on a sensor surface utilizing the surface plasmon resonance (SPR) phenomenon. The SPR response correlates with the change in mass concentration close to the surface (Safsten, 2009). Only peptides that contain an epitope for the immobilized antibody are bound by the antibody, while the others are not retained and do not give a binding response signal. Using peptides staggered in sequence as probes, one can then narrow down the epitope region along the sequence, as long as the epitope is not affected by the shortened sequence of the peptides or by formation of non-native secondary structures of the shorter peptides in comparison to the native protein.

3.22 Statistical analysis

In paper I, one-way analysis of variance (ANOVA) was used with batches and days as factors followed by Tukey's post hoc analysis. Batch was regarded as a random factor and day as a fixed factor. mRNA and protein expressions were analysed from four separate differentiations (n=4) up until d120 and media were analysed from five separate differentiations (n=5) up until d120. In paper II, two-way ANOVA was used with batches and days as factors followed by all pairwise comparisons between days using Tukey-Kramer method. Batch was regarded as a random factor and day as a fixed factor. mRNA and protein were analysed from four separate differentiations (n=4) up until d150, and thereafter in two separate differentiations (n=2) up until d210, and media were analysed from five separate differentiations (n=5) up until d150, thereafter from three separate differentiations (n=3) up until d210. For correlations between *NRGN* mRNA (fold change) and secreted NRGN concentrations or GAPDH densitometry and secreted synaptic protein concentrations, Spearman's rank correlation was used. In paper IV, unpaired t-test was performed to determine the statistical significance between the differences of NFL-21 (control) and SC-514922 monoclonal antibody after immunodepletion followed by neurogranin ELISA.

Immunodepleted SC-514922 mean values were compared using one-way ANOVA followed by Tukey post-hoc analysis. The results shown in paper I, II and IV are given as means and standard errors of the mean (SEM). In all papers, statistical significances were defined as $p<0.05$*, $p<0.01$** and $p<0.001$***.

4. Results and discussion

Although hundreds of studies have highlighted the significance and importance of Alzheimer-related biomarkers using human *post-mortem* brains, CSF, rodents and *in vitro* cellular models, the possibility to identify, investigate and validate them using *in vitro* models have often been hampered by the cellular models lacking human origin. Further, the research on AD biomarkers "in a dish" requires very sensitive methods to detect biomarkers in cell-conditioned media.

Recent advances in cellular modelling techniques and improvements of detection methods to measure AD-related biomarkers, both intracellularly and in fluids, provide novel approaches to increase our knowledge on what these biomarkers reflect. In, 2007, Yamanaka and his colleagues, first reported the generation of hiPSCs (Takahashi et al., 2007). Since then, several human cellular models have been developed, including neuronal models.

hiPSC-derived cortical neuronal models were developed gradually. A number of protocols for differentiation of hiPSCs to cortical neurons have been reported. However, in this thesis, we have used one protocol, described by Shi *et al.* (Shi et al., 2012a). In paper I, we used the neuronal model to study the expression and processing of APP during differentiation to cortical neurons.

Synaptic dysfunction and degeneration are considered early events during neurodegeneration, including AD. Although Aβ and tau are the core AD biomarkers, some synaptic proteins are released into CSF, both physiologically in healthy individuals and at higher concentrations in some neurodegenerative diseases. Among these synaptic proteins are NRGN, GAP-43, SYT-1 and SNAP-25, which are reported to be secreted at increased concentrations into CSF of AD patients as compared to controls (Thorsell et al., 2010;Brinkmalm et al., 2014;Öhrfelt et al., 2016;Sandelius et al., 2018a). These synaptic proteins are under consideration to be included in the panel of core AD biomarkers, alongside CSF T-tau, P-tau and Aβ42. However, at this stage, they are regarded as potential AD biomarkers. In paper II, we have used the same cellular model to study the expression and secretion of some of the AD-related synaptic proteins during differentiation to learn more about mechanisms underlying their release.

One of these potential synaptic biomarkers, NRGN, is reported to increase specifically in CSF of AD patients as compared to other investigated dementias (Wellington et al., 2016;Portelius et al., 2018). Its potential clinical utility was

the reason for our focus to learn more on its release and processing. In paper III, we identified the enzymes that process NRGN to generate its most abundant C-terminal fragments. In paper IV, we additionally determined the molecular forms of NRGN present in CSF.

The studies that are presented in this thesis were aimed at developing methods and generating data to increase the interpretability of biomarkers related to AD. The models and results may facilitate future studies on novel candidate biomarkers for AD-related processes.

4.1 Paper I

APP and its processed products have several physiological functions that are important for neuronal development and adult brain plasticity. There is a sequential processing of APP, which leads to the formation of a variety of Aβ peptides and other APP fragments. In this study, we performed a detailed analysis of APP expression and processing during hiPSCs differentiation to cortical neurons.

hiPSCs were differentiated to cortical neurons for 120 to 150 days. During the differentiation process, cells were characterised for stem cell, neuronal, cortical and astrocytic markers. Simultaneously, APP expression and processing were investigated. One of the main findings was that APP is expressed throughout the differentiation process, but that it is differently processed by the APP-cleaving enzymes, α-secretase (ADAM-10), β-secretase (BACE-1) and γ-secretase (PSEN-1), at different developmental stages.

APP expression was relatively unchanged during the differentiation process, as shown in figure 9. However, its processing was altered during differentiation. During the stem cell stage, neither sAPP nor Aβ was secreted into the cell culture media. This could be due to low mRNA levels of *BACE-1* and *PSEN-1,* although the levels of *ADAM-10* mRNA were relatively high. Another possibility could be that the essential co-localisation of APP with its secretases is lacking in stem cells (Haass et al., 2012).

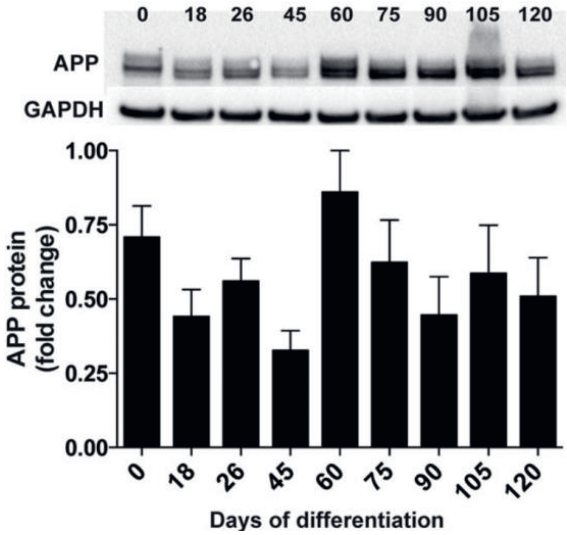

Figure 9 *Intracellular APP expression as detected with western blot. APP expression remained relatively unchanged as compared to APP-generated peptides (Bergström et al., 2016).*

In neuro-progenitors, only secretion of sAPPα, as well as the shorter Aβ peptides Aβ1-15, Aβ1-16, Aβ1-17, and Aβ1-19, was detected (see figure 9 for details). This suggests that APP was mainly cleaved via a non-amyloidogenic pathway by α- and β-secretase during this stage. Further, *ADAM-10* remained stable and *PSEN-1*mRNA levels increased slightly, whereas *BACE-1* was expressed at low levels at this stage.

In cortical neurons, sAPPβ was secreted. Further, secretion of the short peptides decreased, whereas the longer Aβ peptides including Aβ1-38, Aβ1-40 and Aβ1-42 increased gradually over time. The increase in long peptides suggests an increase in concerted β- and γ-secretase activities that shifts APP-processing towards the amyloidogenic pathway as neurons mature (figure 10C and 10D). In mature neurons, APP was still stably expressed (figure 9), while *BACE-1* expression was the highest, as compared to stem cells or neuro-progenitors, while the expressions of *ADAM-10* and *PSEN-1* remained stable.

Altogether, the Aβ peptide patterns during differentiation suggest that APP is processed via the non-amyloidogenic pathway in neuro-progenitors, where, α- and β-secretases were active and these might have the necessary co-localisations to cleave APP to generate short Aβ peptides. Amyloidogenic APP-processing

first appeared in the neuronal stage, when β- and γ-secretases were active and might have their necessary co-localisations to cleave APP to generate the long Aβ peptides that are associated with AD.

The long Aβ peptide, Aβ42, is one of the major constituents of AD plaques and its concentration is increased in AD brain and decreased in AD CSF (because of retention of the protein in the AD brain) (Motter et al., 1995). In parallel with studies examining the association of long Aβ peptides with AD, a number of studies have also highlighted the role and function of APP and its cleavage products in normal cellular processes, including neural differentiation, neuro-progenitor proliferation, motility, synaptic plasticity, neurite formation and guidance, etc. (Caille et al., 2004;Nicolas and Hassan, 2014;Stahl et al., 2014). The differential cleavage pattern suggests a role for various APP products during different stages of neuronal differentiation.

There are some shared advantages and limitations in papers I and II, as the same differentiation protocol was used (Shi et al., 2012a). These are therefore described in the next section (Paper II).

In summary, paper I adds new information to the existing literature regarding the processing of APP, that is, APP is stably expressed throughout differentiation but its processing is altered in subsequent stages of neuronal differentiation. Importantly, the amyloidogenic pathway is associated with mature cortical neurons. Future studies should examine whether this is relevant to synaptic homeostasis in mature neurons and if the process, if deregulated by mutations or Aβ aggregation, may play a role in synaptic degeneration in AD.

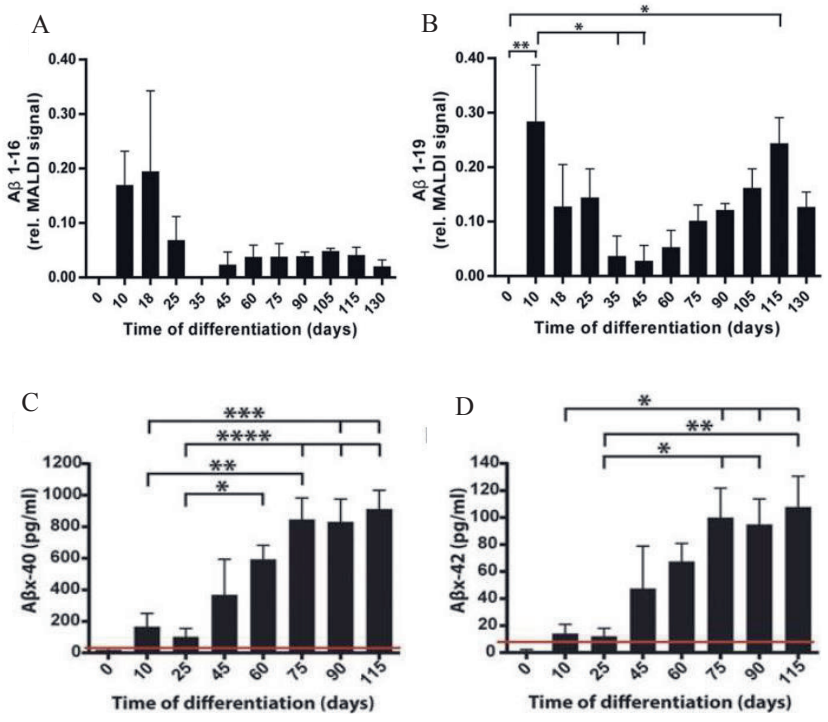

Figure 10 *APP processed Aβ peptides shift during neuronal differentiation as detected in conditioned cell culture media. (A) and (B) show the detection of short Aβ peptides during early neuronal differentiation using IP-MS. (C) and (D) indicate the shift towards longer Aβ peptides during later neuronal differentiation using MSD platform (Bergström et al., 2016).*

4.2 Paper II

In this study, we used the same cellular model as described in paper I to examine the temporal changes in the expression and secretion of a number of synaptic proteins during the differentiation to cortical neurons. For our study, we selected the AD-associated synaptic proteins, NRGN, GAP-43, SYT-1 and SNAP-25. These proteins are known to be secreted at increased concentrations into CSF from AD patients as compared to age-matched healthy controls (Sjogren et al., 2001;Thorsell et al., 2010;Brinkmalm et al., 2014;Öhrfelt et al., 2016). In addition to the characterisation presented in paper I, we further characterised the

hiPSC-derived cortical neurons using the protocol described by Shi *et al.* (Shi et al., 2012a).

hiPSC-derived cortical neurons were maintained over a period of at least 150 days (up to 210 days), which encompasses the different stages of neuronal development. The differentiation of hiPSCs to cortical neurons was characterised further by performing ICC. On the basis of cell morphology and ICC shown here and gene expression, ICC and electrophysiology shown in paper I, hiPSCs differentiation to cortical neurons was divided into four stages, (i) stem cell (d0), (ii) neuro-progenitors (d11 to d25), (iii) immature cortical neurons (d30-d75) and (iv) mature cortical neurons (d90 and beyond).

At the stem cell stage, synaptic protein expression was undetectable. When neuro-progenitor cells formed, but neurite outgrowth was not yet observed, NRGN, GAP-43, SNAP-25 and SYT-1 were detected at low levels intracellularly. Thereafter, when immature cortical neurons formed, we observed a gradual increase in synaptic protein expression that coincided with neurite network formation. At this stage, GAP-43 was expressed the highest as compared to neuro-progenitors and mature neurons. It has been suggested that GAP-43 is expressed at increased concentrations in growth cones of developing neurons (Benowitz and Routtenberg, 1997). In agreement with this, we found that GAP-43 was expressed the highest in immature neurons at d60 of differentiation, a stage when the neurons remained in growth phase. Further, at this stage, weak staining was observed for synaptic vesicle glycoprotein-2 (SV-2), a pre-synaptic protein, and post-synaptic density protein-95 (PSD-95), a post-synaptic protein, which indicates that synapses were not yet fully developed. We selected SV-2 and PSD-95 as synaptic proteins to compare the expression and localisation of these commonly used synaptic markers in immature and mature neurons.

When neurons matured, strong and punctate localisations for SV-2 and PSD-95 were observed. During this stage, a complex dense network of cortical connections was established and thick bundles of neurites were formed. A further increase in expression of NRGN, SNAP-25 and SYT-1 was seen, and the expression of these synaptic proteins was highest at this stage of differentiation. Further, punctate staining of NRGN, co-localising with PSD-95 in post-synaptic terminals, was observed in mature neurons. GAP-43 was co-localised with tau in axons, and SYT-1, SNAP-25 and SV-2 were all localised in pre-synaptic terminals. The results are summarised in figure 11. The highest expression of

these synaptic proteins in mature neurons indicates that they have a role in stabilising synapses, as suggested by Pinto *et al*. (Pinto et al., 2013).

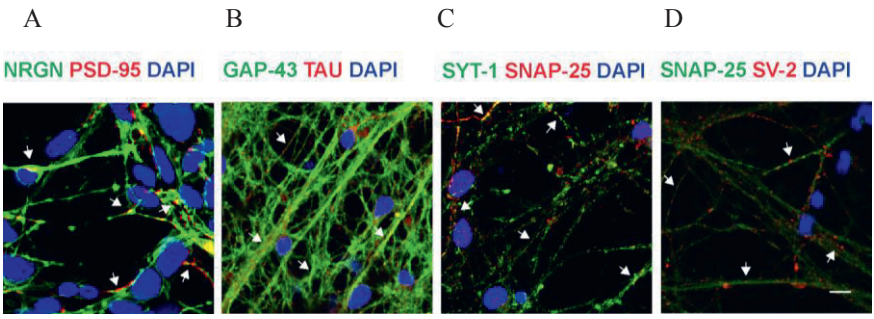

Figure 11 *Localisation of synaptic proteins in mature cortical neurons is shown on d120 of differentiation. (A) NRGN and PSD-95 are co-expressed in the post-synaptic terminals (arrows). (B) GAP-43 and tau are co-stained in axons (arrows). (C) SYT-1 and SNAP-25, pre-synaptic proteins, are stained together in the same compartment (arrows). (D) SNAP-25 and SV-2 pre-synaptic proteins are co-stained in the same compartment (arrows). blue = nuclei (DAPI), Scale bar = 10 μm (Nazir et al., 2018).*

The sensitive methods developed in our lab to detect NRGN, GAP-43, SYT-1 and SNAP-25 in CSF also makes detection of these synaptic proteins possible in cell-conditioned media. During the stem cell stage, only SYT-1 secretion was seen, as detected by IP-MS (figure 12D). In stem cells, intracellular SYT-1 protein expression was not detected, while *SYT-1* gene was expressed at this stage. The result is puzzling, but a possible explanation could be that most of the translated SYT-1 protein may be secreted and therefore no intracellular SYT-1 protein expression could be observed.

In neuro-progenitors, secretion of SNAP-25 was not detected, and a very low secretion of NRGN, GAP-43 and SYT-1 was seen (figure 12). In immature neurons, a gradual increase of these synaptic proteins was observed. When the neurons matured, between d120 and 135 of differentiation, an increased secretion of NRGN, GAP-43 and SNAP-25 was observed, while, SYT-1 secretion peaked on d180 of differentiation. The secretory profile suggests that mature neurons are secreting relatively more of these proteins.

Taken together, this study presents a detailed analysis of the expression and secretory profiles of a number of synaptic proteins. Previously, intracellular expression of the investigated synaptic proteins has been shown using rat and mouse models, or their primary neuronal cell cultures (Alvarez-Bolado et al.,

1996;Sidor-Kaczmarek et al., 2004;Higo et al., 2006;Greif et al., 2013). To our knowledge, the secretion of these synaptic proteins during stem cell differentiation to cortical neurons has not been examined before.

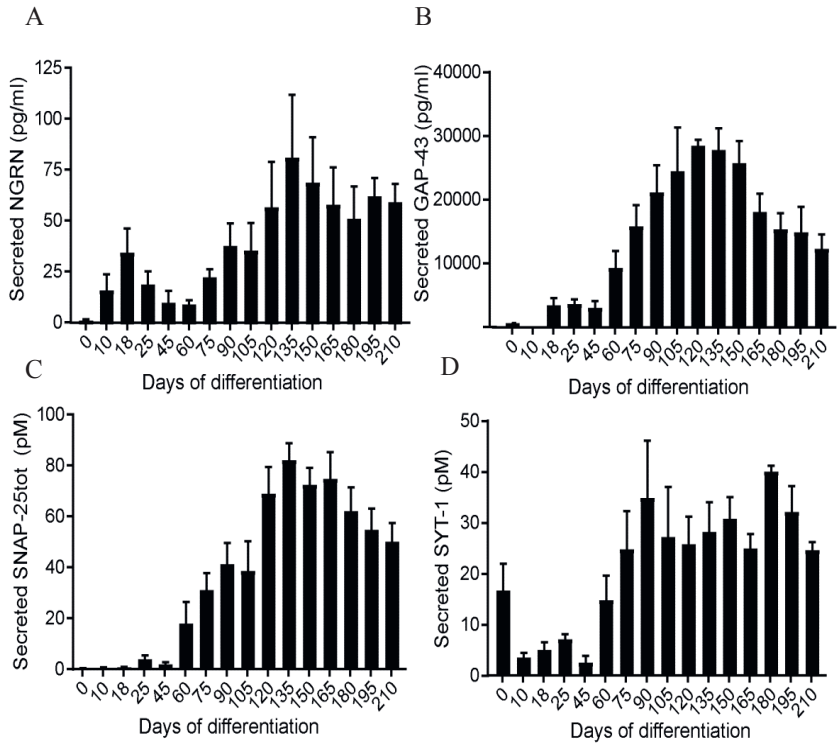

Figure 12 *Synaptic proteins are secreted during stem cell differentiation to cortical neurons as detected in conditioned cell culture media. (A) and (B) show the secretion of NRGN and GAP-43, respectively, as detected by ELISA. (C) and (D) show the secretion of SYT-1 and SNAP-25 as detected by IP-MS (Nazir et al., 2018).*

There are several advantages (common to papers I and II) of using the protocol developed by Shi *et al.* (Shi et al., 2012a) to study AD-related biomarkers *in vitro*. First, this model recapitulates *in vivo* development, in the sense that it generates the cortical neurons in the same temporal order as observed *in vivo*. Second, *in vivo* corticogenesis takes 100 d, while this model takes 90 d to develop all six layers of cortical neurons. There are different variants of protocols to differentiate hiPSCs to neurons, *e.g.*, by growing brain organoids

(Lancaster et al., 2013) or cortical spheroids (Paşca et al., 2015). However, these 3D models have certain limitations that limit their use in our experimental paradigm, including difficulties in accessing the internal structures in organoids or spheroids for imaging purposes. Further, in 3D matrigel models, it is difficult to collect the cell-conditioned media from the cultures and hence, these protocols may hamper the investigation of the processing of APP and secretion of synaptic proteins during differentiation to neurons. Using the Shi et al. protocol (Shi et al., 2012a), APP processing and synaptic proteins expression and secretion were analysed in detail and the results were reproducible using more than one hiPSC origin (fibroblasts and chondrocytes).

Despite having several advantages, these studies also have some limitations to consider, *e.g.*, the heterogeneity of the maturity of individual neurons. The processing of APP and the expression and secretion of synaptic proteins should thus be examined using more than one differentiation protocol in future studies to establish the results. Further, this model generates cortical neurons and astrocytes; there might be differences in APP-processing and synaptic protein expression in neurons and astrocytes, which could not be distinguished using this model. However, the above-mentioned studies add a lot of information to the existing literature and provide a basis for future studies, as suggested in future perspectives.

4.3 Paper III

In paper II, we used a hiPSC-derived cortical neuronal model to study the expression of synaptic proteins that are being evaluated as biomarkers for AD. Here, we selected NRGN since it has been detected at increased concentrations in CSF of AD patients as compared to age-matched controls, both as a full-length NRGN molecule (Thorsell et al., 2010) and as C-terminal peptides (Kvartsberg et al., 2015a). Further, the increase in NRGN concentration is specific to AD as compared to other dementias (Wellington et al., 2016;Portelius et al., 2018).

The mechanism through which NRGN is released into CSF largely as C-terminal peptides is yet unclear; however, the processing of NRGN may shed light on the origin of the C-terminal peptides that appear to be the most abundant forms in CSF (Kvartsberg et al., 2015a). Here, we set out to identify the enzyme(s) responsible for the cleavages that generate the most abundant NRGN forms in CSF (the C-terminal half of the protein).

We utilised three fluorescence-quenched FRET probes to search for enzymes cleaving NRGN in two sequence regions. The probes covered the two regions that are adjacent to the N-termini and the C-termini of the fragments found in CSF. Among several enzymes tested, all of which are known to show elevated activity in AD (BACE1, TACE1, IDE, and CALP1), it was found that only CALP1 cleaved the quenched peptides NRGN 31–40 and NRGN 39–51 (in the middle region of NRGN) but not the quenched peptide Ng70–78 near the C-terminus of NRGN (shown in figure 13).

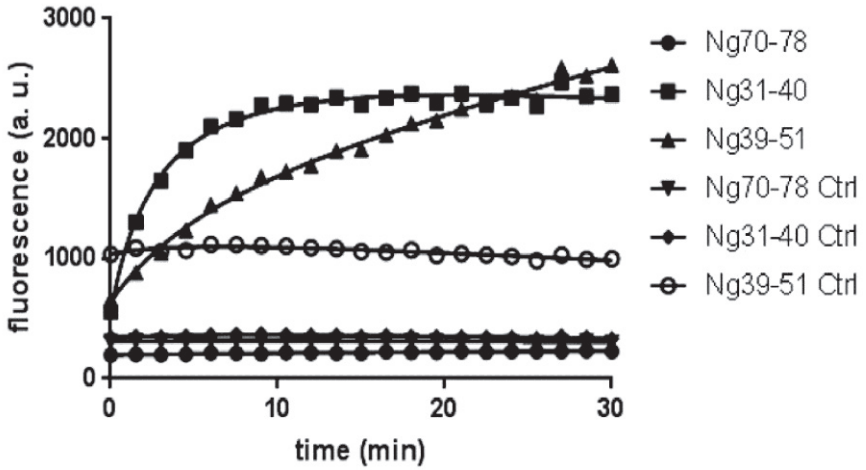

Figure 13 *CALP1 cleaves two quenched Ng peptides Ng31-40 and Ng39-51 (central region) but not the quenched peptide Ng70-78 (C-terminal region).The y-axis indicates fluorescence arbitrary units while the x-axis shows the reaction time in min. Ng39–51 Ctrl, Ng31–40 Ctrl, Ng70–78 Ctrl, quenched peptide in assay buffer without CALP1 (Becker et al., 2018).*

To confirm the cleaving activity of CALP1, full-length NRGN was expressed in the SUMO expression vector. The SDS-PAGE results (figure 14 A) using full-length NRGN and human CALP1 digestion solution show that full-length NRGN is detected at an apparent MW of ~12 kDa in the control sample (lane 1, no added CALP1). The CALP1-containing digestion solutions showed major cleavage products of ~5 kDa and ~6.5 kDa, as detected by Coomassie followed by silver stain (lanes 2-4). Further, we detected the C- and N-terminal peptides in human CALP1-treated samples using an antibody directed against C-terminal NRGN (NG36, immunogen aa 63–75; figure 14B) and the N-terminal antibody SC-514922 (H-6 clone; immunogen aa 1–50; figure 14C). The N- and C-terminal peptides migrate in the same manner as synthetic NRGN 1-42 and

NRGN 43–78 peptides, respectively. This suggests that human CALP1 cleaves NRGN generating C- and N-terminal peptides.

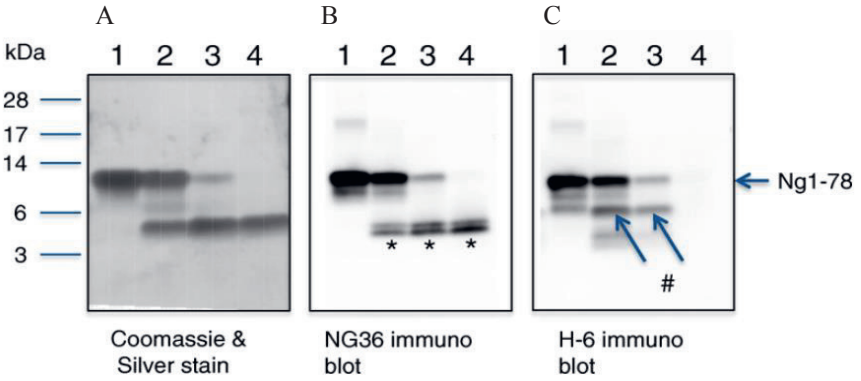

*Figure 14 Detection of C-and N-terminal NRGN fragments in digests by CALP1. (A) shows Coomassie stain followed by silver stain; (B) NG36 immunoblot; (C) H-6 immunoblot. The SDS-reducing gels were loaded with NRGN control (lane 1), digests of NRGN, 90-, 30-, 10-fold diluted CALP1 (lane 2, 3, and 4, respectively). The major ~ 5 kDa C-terminal fragments are indicated by * and those of the main N-terminal fragment near 6.5 kDa are indicated by #. The size markers are shown on the left of panel A (Becker et al., 2018).*

Since CALP1 did not cleave the quenched FRET probe NRGN 70-78, another enzyme (s) may be required for the cleavage between NRGN 75-76. Several C-terminal peptides found in CSF were truncated at aa 75 and NRGN X-75 peptides have been detected in human brain tissue (Kvartsberg et al., 2015a), suggesting that brain tissue may be a suitable source for identifying the C-terminally cleaving enzyme(s). Therefore, we used mouse brain extracts and the C-terminally quenched FRET probe (NRGN 70-78) to detect the C-terminal-cleaving activity. Enrichment of this enzymatic activity by several chromatographic steps and native SDS-PAGE allowed identification of the potential enzyme by LC MS/MS analysis of cut-out and trypsin-digested gel bands. The database searches revealed mouse prolyl endopeptidase (PREP) (Uniprot ID Q9QUR6; EC:3.4.21.26) as the main hit. To ensure that the active fractions from the chromatographic purifications cleaved the last three amino acids at the C-terminus of NRGN, synthetic peptide KKK-NRGN 50–78 was incubated with active fractions. MALDI-TOF analysis showed that C-terminally truncated peptide KKK-Ng50–75 could be generated *in vitro*. This experiment was also performed using recombinant PREP enzyme, either mouse or human

enzyme, and for both species, cleavage between aa 75 and 76 could be confirmed. However, other peptides were also generated by PREP cleavage. To extend these experiments to the larger NRGN or a larger fragment, we incubated full-length recombinant human NRGN 1–78 or the synthetic peptide of human NRGN 43–78 with human recombinant PREP enzyme that resulted in NRGN 1–75 and NRGN 43–75, respectively, albeit at much lower yield (only about 1%).

It has been suggested that CALP1 activation is increased in AD patients (Saito et al., 1993;Liu et al., 2008a). Our results suggest that CALP1 cleaves NRGN either in or around the IQ region, which would prevent NRGN-binding to CaM. As a result, Ca^{2+}/CaM-dependent protein kinase signalling will be disturbed, leading to defective LTP. The reports from the studies on NRGN knockout mice suggest that these mice have problems with spatial memory and altered LTP and LTD (Pak et al., 2000). One may speculate that CALP1 indirectly causes the memory disturbancies in AD patients. While PREP is known to cleave peptides C-terminally after proline (Kalwant and Porter, 1991), it is known to preferentially digest peptides that are smaller than 30 aa, *e.g.*, substance P, and thymosin β4. A proteomic study on hypothalamus tissue extracts from mice dosed with PREP inhibitor S17092 showed that PREP inhibition resulted in lower levels of NRGN 54–75 peptides as compared to vehicle treated mice (Nolte et al., 2009). It should be noted that NRGN contains a number of prolines after which PREP may cleave. Therefore, we hypothesise that NRGN peptides are generated by an initial cleavage of NRGN by CALP1 that leads to the generation of shorter peptides for cleavage by PREP and potentially other proteases. This eventually leads to the generation of C-terminal fragments that are detected in CSF. In short, CALP1 cleaves NRGN in the IQ motif, whilst PREP cleaves after prolines, resulting in the C-terminal NRGN peptides ending at NRGN 75 that are detected at elevated levels in CSF.

This is one of the first studies to identify the enzymes that process NRGN *in vitro*. It sheds light on the importance of CALP1 and possibly PREP activation in AD. It is tempting to speculate that this NRGN fragmentation could play a role in the synaptic dysfunction that is so typical of AD.

One possible limitation of this study is that the potential NRGN-cleaving enzymes that were investigated (as mentioned earlier) were selected on the basis of their suggested involvement in AD pathogenesis. In that way, other potential NRGN-cleaving enzymes may have been neglected. Full-length NRGN is a 78 aa protein and thus larger than the 30 aa proposed to be the upper limit for effective PREP cleavage. Prolonged *in vitro* cleavage of recombinant full-length

NRGN and CALP1-digested fragment (NRGN 43-78) by PREP yielded indeed only small amounts of cleaved products, NRGN 1–75 and NRGN 43–75, respectively. This may reflect non-ideal *in vitro* digestive conditions for the PREP enzyme, or a strict size limitation for PREP activity on NRGN.

Nevertheless, this study adds a lot of information to the existing literature that supports an *in vivo* generation of NRGN fragments as detected in CSF. In future studies, quenched FRET peptides could be used that encompass all NRGN aa sequences. In addition, all the cleavage sites in the NRGN sequence using CALP1 and PREP need to be determined in the future. Further, the enzymatic activity of these enzymes with regard to neurogranin cleavage needs to be studied using cell-based and *in vivo* models. This study should facilitate also future studies aimed at identifying enzymes that may be involved in the processing of other neuronal proteins that are detected at increased concentrations in CSF from AD patients.

4.4 Paper IV

In this manuscript, we aimed at extending earlier in-house data that show that CSF also contains apparently full-length NRGN in addition to the C-terminal fragments (the formation of which we studied in paper III). It was also not clear whether full-length NRGN exists in CSF only as a monomer or also as higher-order structures (dimers, oligomers), as has been reported for extracts from brain (Kvartsberg et al, 2015b). We therefore provide here an initial characterisation of the molecular forms of NRGN in CSF. Extending the characterisation to NRGN fragments, we developed an immunodepletion-ELISA method to estimate the ratio of C-terminal peptides to total NRGN (full-length NRGN and it fragments). This was of particular interest, because a study from our lab had shown that NRGN processing was increased for some endogenous NRGN peptides in AD brain tissue (Kvartsberg et al., 2019). The assay developed will enable future studies to explore whether the ratio of C-terminal NRGN fragments to total NRGN can be utilized as an AD biomarker.

To determine the molecular forms of NRGN, pooled CSF was concentrated by ultrafiltration and SEC was performed. The size-separated fractions were analysed by SDS-PAGE/western blotting for NRGN using NG36 (in-house monoclonal antibody; immunogen Ng63–77) and SC-514922 (clone H-6 monoclonal antibody; immunogen Ng52–63). The results indicated that under reducing conditions, full-length (~12 kDa) and truncated NRGN (~5 kDa) were

detected in CSF using NG36 and SC-514922 monoclonal antibodies. Under non-reducing conditions, NRGN bands corresponding to ~36 kDa and ~60 kDa were also detected, in addition to NRGN fragments and full-length NRGN. These bands indicate that there are additional molecular forms of NRGN in CSF. Using the SC-514922 antibody, a strong band at ~49 kDa was also detected; however, this band most likely corresponded to GAP-43, since SC-514922 cross-reacts with GAP-43.

Under both reducing and non-reducing conditions, a band corresponding to ~12 kDa carried distinct epitopes for NRGN using NG36 and SC-514922 monoclonal antibodies. A faint band detected at ~5 kDa also carried both epitopes for the two NRGN antibodies used here (SC-514922 antibody (IQ domain) and the C-terminal NG36 antibody) and therefore most likely represent NRGN fragments overlapping with the epitopes for these antibodies. Additional, bands, around ~36 and ~60 kDa, were clearly detected using SC-514922 and NG36 monoclonal antibodies in non-reduced samples of CSF. The bands greater than ~12 kDa represented molecular forms that could be NRGN complexed with other proteins and/or higher Ng oligomers. However, under reducing conditions, these bands were strongly decreased in intensity suggesting that these complexes were held together via disulfide bridges.

The possible interaction partners of NRGN in the high molecular weight band species could be CaM (Huang et al., 1993;Gerendasy et al., 1995;Gerendasy and Sutcliffe, 1997) or an as of yet unidentified binding partner. Alternatively, these complexes could be dimers or multimers of NRGN. Arguably, such forms of NRGN could have been artificially generated by oxidation of the cysteine groups of NRGN during the sample preparation for SDS-PAGE. However, the fact that these higher MW complexes eluted at the expected higher MW (relative to monomeric NRGN) in the size exclusion chromatography fractions, ruled out this possibility and showed that such NRGN forms already exist in CSF. Furthermore, a study has shown the presence of dimers of NRGN in human brain extracts using MALDI-TOF (Kvartsberg et al., 2015a).

It has been reported that endogenous C-terminal fragments are identified in CSF (Kvartsberg et al., 2015a;Kvartsberg et al., 2015b). In an attempt to assess the ratio of C-terminal fragments to total-NRGN in CSF, we immunodepleted CSF of full-length and mainly N-terminal NRGN peptides using the SC-514922 monoclonal antibody and measured the remaining NRGN by NG36/NG2 sandwich ELISA which detects “total” NRGN (full-length NRGN and fragments). The comparison of the ELISA signals before and after depletion

showed that the C-terminal peptides comprise about half of the total NRGN species detected. Whether this ratio increases in CSF of AD patients in a manner similar to what has been shown in brain tissue (Kvartsberg et al, 2019) is unknown and requires further investigation.

This is one of the first studies that show the direct detection of full-length NRGN in CSF using immunoblots. A previous study could detect NRGN in CSF but required prior enrichment by IP followed by western blot (Thorsell et al., 2010). We have improved detection sensitivity by concentrating CSF samples by ultrafiltration prior to immunoblotting, by fixing the blots to reduce losses of NRGN, and by using improved NRGN antibodies. This study provides the methodological basis to separate and purify NRGN forms from CSF and to study whether a specific molecular form might increase in AD (or in particular of its stages) compared to control. In future work, the ratio of NRGN fragments to full-length or total-NRGN as a biomarker should be examined. A current limitation is the unavailability of sensitive anti-NRGN antibodies specific to the N-terminus of the protein, which prevented us from designing an assay selective for only full length NRGN.

5. Conclusions

APP is expressed throughout neuronal differentiation but it is processed differently during subsequent stages of differentiation. APP is processed in human neuro-progenitors and neurons, but not in hiPSCs, which suggests that APP-processing is specific for neuronal cells. Further, the increase in APP-processing by β/γ-secretases in mature neurons suggests that amyloidogenic Aβ formation is associated with mature neurons.

Synaptic proteins are expressed in neuro-progenitors at low levels and their expression gradually increases during differentiation to neurons. An increase in the expression of synaptic proteins indicates an increase in neurite outgrowth, synapse formation and establishment of the synaptic connectome. In addition, mature neurons secrete more of synaptic proteins as compared to neuro-progenitors and immature neurons.

CALP1 and PREP cleave NRGN and generate to a large extent the pattern of C-terminal peptides that can be identified in CSF. CALP1 cleaves NRGN in the IQ motif between aa 42 and 43, while PREP cleaves NRGN after prolines in the NRGN sequence. These cleavages generate C-terminal truncations at 75^{th} aa of NRGN which is a common NRGN truncation found in CSF.

In addition to the monomeric form of NRGN and its fragments, several molecular forms of NRGN are present in CSF, which correspond to higher molecular weight forms of NRGN. These represent either NRGN oligomers or NRGN complexed with other proteins in CSF. C-terminal NRGN peptides constitute around 50% of total-NRGN in CSF.

6. Future Perspectives

In this thesis, we have examined the expression and secretion of several AD-associated proteins during hiPSC differentiation to cortical neurons, including APP and its processed peptides, and synaptic proteins, NRGN, GAP-43, SYT-1 and SNAP-25. In the future, it will be interesting to determine: (a) if APP processing is altered during subsequent neuronal stages using AD patient-specific cell lines compared to healthy cell lines; (b) if synaptic protein expression is altered in AD patient-specific cell lines compared to healthy cell lines, and cell lines representing other neurodegenerative disorders. Further, these studies should also be conducted in human-derived cellular models using protocols to directly convert human somatic cells to neurons, with the potential advantage of preserving the biological aging and epigenetic memory of these cells.

In AD, some of the reported synaptic proteins as mentioned in this thesis are secreted at increased concentrations into CSF as compared to age-matched controls. One of these synaptic proteins, NRGN is reported to be specifically associated with AD as compared to other types of dementia investigated (Wellington et al., 2016;Portelius et al., 2018). Therefore, the specific mechanism of secretion of NRGN needs to be explored using a neuronal cellular model derived from AD and control hiPSCs. It would also be of high interest to examine the mechanisms of release in relation to synaptic activity and AD-related exposures, including aggregated Aβ peptides.

In this thesis, we have reported the processing of NRGN by CALP1 and PREP that generate C-terminal peptides of NRGN as detected in CSF. Our approach could be utilised to identify enzymes that may process the N-terminal region of NRGN. Further, studies establishing the detailed map of NRGN cleavage sites need to be conducted.

We have verified the existence of several molecular forms of NRGN present in CSF. The composition of higher molecular weight NRGN complexes (greater MW than ~12kDa) needs to be determined. This should clarify whether they constitute oligomers of NRGN or whether NRGN is complexed with some other proteins. Further, it should be investigated whether a particular form of NRGN is increased in AD as compared to age-matched controls. Some of the current studies are hampered by the unavailability of N-terminal NRGN antibodies

(epitopes at far N-terminal region of NRGN). In the future, efforts are required to develop N-terminal NRGN antibodies.

As indicated in the thesis, the ratio of C-terminal peptides to total-NRGN was ~50% in the CSF pool studied. Some studies report that CALP1 activity is increased during AD progression and that C-terminal truncations of NRGN increase in AD brain (De Vos et al., 2015;Kurbatskaya et al., 2016). The particular role of C-terminal peptides generated after CALP1 cleavages are still unknown. Our preliminary data and the reports from the studies using cell lines indicate that C-terminal peptides may translocate to nuclei, in addition to being secreted. Therefore, the particular role of C-terminal peptides needs to be explored using primary cells or neuronal cell lines. By understanding the role of C-terminal peptides translocating to nuclei, we could add to the understanding of neuronal signalling and its possible changes in the pathogenesis of AD and thus increase the interpretative value of those Ng fragments as biomarkers.

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