Candidate biomarkers for synaptic pathology:
neurogranin, neuroligin and neurexins in neurodegenerative disorders

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Alla mia famiglia

"*Nothing in life is to be feared, it is only to be understood.*
Now is the time to understand more, so that we may fear less".

Marie Skłodowska-Curie

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ABSTRACT

Synapses are small units of the nervous system containing neurotransmitters and a multitude of proteins. They are organized in pre- and postsynaptic compartments that directly and indirectly interact to integrate and transmit signals between neurons. Synapses represent the site of memory formation and cognitive abilities, and as such, are primarily affected in neurodegenerative diseases such as Alzheimer's disease (AD), the leading cause of dementia in the elderly. Synaptic degeneration has been described as an early event in AD and as a closer correlate to the degree of disease severity and cognitive decline than e.g., amyloid-β (Aβ) deposits, a core pathological hallmark of AD. Therefore, synaptic proteins are regarded as potential biomarkers for the detection of early pathological changes in AD and for tracking disease progression and cognitive decline. They can be detected in body fluids such as cerebrospinal fluid (CSF), both as fragments or as full-length proteins. The study of changes in protein concentration and fragmentation patterns can improve our understanding of neuropathological changes affecting synapse integrity and allow assessing the potential of those proteins or their fragments as biomarkers for synaptic pathology.

The overall aim of this thesis was to investigate the proteolytic processes affecting different synaptic proteins in the human brain and CSF in the context of neurodegenerative diseases, primarily AD, using immunoassays in combination with mass spectrometry (MS)-based proteomics.

Specifically, we investigated the synaptic proteins neurogranin (Ng), neurexins (NRXN) and neuroligins (Nlgn). Ng is a postsynaptic protein supporting synaptic function and memory formation. Previous investigations have found Ng levels to be decreased in the brain and increased in the CSF of AD patients, both as fragments and full-length protein, suggesting potential as synaptic biomarker in AD. However, the mechanisms behind Ng fragmentation and the relevance of these fragments in health

and disease are not fully understood. Our studies of Ng-cleavage revealed calpain-1 and prolyl endopeptidase (PREP) as enzymes yielding Ng peptides, which had previously been found in CSF. The increase of Ng peptide levels in CSF suggests that calpain-1 and PREP activity and/or expression are increased in AD. Furthermore, we identified Ng in CSF as fragments, monomers, oligomers and higher molecular weight complexes. On average, the C-terminal fragments represented about 50% of the total Ng ELISA signal, and for the first time, we were able to immunoprecipitate N-terminal Ng fragments. The study also highlighted the presence of a heparin-binding motif on Ng, which could describe a way for the C-terminal and full-length Ng to be exported across the neuronal plasma membrane.

Presynaptic NRXNs and postsynaptic Nlgns are synaptic adhesion proteins, which bind across the synaptic cleft and take part in synapse formation and stabilization. They have previously been suggested to be potential targets for the toxic Aβ oligomers in AD, which disrupt Nlgns/NRXNs interactions and alter their functions at the synapse. Moreover, synaptic dysfunction in both AD and neurological diseases like schizophrenia and autism has been associated with genetic modifications of NRXNs and Nlgns. We found Nlgn1 levels to be decreased in brain tissue from the parietal and temporal cortices of AD cases. The most pronounced decrease in Nlgn1 levels, however, was observed in the frontal cortex from cases with primary tauopathies, warranting further investigation of the role of Nlgn1 in this group of diseases. Interestingly, we did not observe any change in Nlgn1 levels in the CSF of AD patients. To gain a deeper understanding of the changes of Nlgns and NRXNs in AD and further assess their potential as biomarkers, we then developed a targeted parallel reaction monitoring MS method for their simultaneous quantification. Expanding on our previous results, we did not find any changes in the CSF from patients in different stages of AD, suggesting that these proteins do not reflect synaptic dysfunction in AD.

In conclusion, the studies in this thesis provided novel knowledge about the processing of the synaptic proteins Ng, Nlgns and NRXNs and the groundwork for future investigations into the role of these proteins in AD and other neurodegenerative diseases. Furthermore, they describe novel tools to monitor synaptic dysfunction and improve our understanding of those proteins as biomarkers in neurodegenerative diseases.

Keywords: synaptic dysfunction, Alzheimer’s disease, biomarkers, neurogranin, neurexins, neuroligins, cerebrospinal fluid, brain tissue

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Sammanfattning på svenska

Alzheimers sjukdom (AS) är en neurodegenerativ sjukdom som drabbar mer än 50 miljoner människor världen över, ett antal som förväntas öka. AS tillhör inte det normala åldrandet utan leder till minnesnedsättning samt svårigheter att planera eller lösa problem, minskad eller dålig bedömningsförmåga och förändringar i humör och personlighet som i ökande grad påverkar patientens dagliga liv och slutligen leder till demens. Hittills finns det inget botemedel och diagnosställningen försvåras ofta av co-morbiditet och en lång preklinisk fas som kännetecknas av patologiska förändringar som startar många år innan de kliniska symtomen uppstår. Av dessa skäl kan prognostiska och diagnostiska biomarkörer vara av stor betydelse för att möjliggöra en mer exakt och tidig diagnos som underlättar potentiella interventioner.

Synapser är kontaktpunkter mellan nervceller och är essentiella för informationsutbytet inom nervsystemet. De innehåller neurotransmittorer och en mängd proteiner som samarbetar och interagerar direkt eller indirekt för att integrera överföra signaler. Synapser är centralt involverade i minnesbildning och kognition, därför uppstår nedsatt hjärnnätverksaktivitet och minne vid synaptisk dysfunktion. Förändringar i synaptisk funktion återspeglas vanligtvis genom förändringar i koncentrationen av synaptiska proteiner. Synapsdysfunktion och -förlust har beskrivits som en tidig händelse vid AS och som starkt korrelerad med sjukdomsgrad och kognitiv försämring. Av dessa skäl studeras nu synaptiska proteiner som möjliga biomarkörer för att upptäcka tidiga patologiska förändringar vid AS och för att följa sjukdomsprogression och kognitiv funktionsnedsättning.

Det övergripande målet med detta doktorandprojekt var att studera dom synaptiska proteinerna neurogranin, neuroliginer och neurexiner i hjärnvävnad och cerebrospinalvätska (CSV) från patienter med AS, men även från patienter med andra neurodegenerativa sjukdomar, i syfte att utvärdera användbarheten av dessa proteiner som biomarkörer för synaptisk dysfunktion vid dessa sjukdomar.

Vi identifierade två enzymer som kan klyva neurogranin och generera neurogranin-fragment som hade tidigare visat sig vara ökade i CSV hos patienter med AS. Vi kunde även visa att olika molekylära former av proteinet finns i CSV, vilket motiverar fler framtida studier för att bättre förstå deras potential som biomarkörer. Våra studier av neuroligin-proteiner visade att neuroligin 1-nivån minskar i olika hjärnregioner hos patienter med AS. Ytterligare undersökningar av proteinet i hjärnprover från en grupp patienter med så kallade primära tauopatier visade en ännu markantare minskning av proteinnivåerna, ett resultat som motiverar fler undersökningar av proteinet vid dessa sjukdomar. Proteinnivåerna var emellertid inte förändrade i CSV hos AS patienter. När vi utvidgade undersökningen till alla neuroliginer och deras bindningspartners vid

synapsen, neurexinerna, hittade vi återigen inga förändringar i CSV från patienter i olika AS-stadier, vilket tyder på att dessa proteiner sannolikt inte återspeglar synaptisk dysfunktion vid denna sjukdom.

Sammanfattningsvis gav studierna i denna avhandling ny kunskap om de synaptiska proteinerna Ng, Nlgns och NRXNs samt deras processering och la grunden för framtida undersökningar av dessa proteiners roll vid AD och andra neurodegenerativa sjukdomar. Dessutom beskriver de nya verktyg för att analysera synaptisk dysfunktion och rollen av dessa proteiner som potentiella biomarkörer vid neurodegenerativa sjukdomar.

Riassunto in italiano

Le **malattie neurodegenerative** sono un gruppo di patologie del sistema nervoso centrale che portano ad un progressivo deterioramento e morte delle cellule neuronali. A seconda del tipo di cellule coinvolte e della regione cerebrale colpita, le malattie neurodegenerative possono manifestarsi con deficit cognitivi, demenza, disfunzioni motorie, disturbi comportamentali e psicologici. Con il termine **demenza** si intende un declino delle funzioni cognitive quali la capacitá di ragionare e ricordare, ad un livello tale che il paziente non riesce piú a svolgere le normali attivitá quotidiane. In Italia, piú di un milione di persone soffrono di demenza.

Fra le varie malattie neurodegenerative, la **malattia di Alzheimer** rappresenta la principale causa di demenza nella popolazione anziana. La malattia ha origini sconosciute, ma ad oggi è noto che il progressivo e patologico accumulo delle proteine **beta-amiloide** e **tau** nel cervello sono due segni caratteristici e necessari per la diagnosi definitiva durante l'esame autoptico. Ad oggi non esistono trattamenti in grado di interrompere la progressione della malattia, ma solo cure palliative per attenuarne i sintomi. Inoltre, la malattia di Alzheimer presenta un decorso lungo e silenzioso, in quanto giá nei 10-20 anni prima della manifestazione dei sintomi, la proteina beta-amiloide inizia ad accumularsi innescando una cascata di eventi che portano alla degenerazione neuronale; tale evento causa, solo dopo molti anni, la condizione clinica. Questo rende la patologia di difficile riconoscimento in fase pre-sintomatica e riduce l'efficacia dei trattamenti, poiché questi vengono iniziati solo ad uno stato giá avanzato della malattia. Per tale motivo la ricerca sta compiendo grandi sforzi al fine di diagnosticare la malattia di Alzheimer nella sua fase precoce. A questo scopo, fortemente ricercati sono **biomarcatori** in grado di identificare segnali precoci di degenerazione neuronale. Idealmente, un biomarcatore é una molecola, generalmente di natura proteica, il cui cambiamento é in grado di predire o diagnosticare una condizione patologica. Diversi fluidi corporei possono essere utilizzati come fonte di biomarcatori, come per esempio il sangue, la saliva o il **liquido cerebrospinale**. Quest'ultimo rappresenta il fluido di elezione per studiare cosa avviene nel sistema nervoso, in quanto si trova a diretto contatto con esso, riempiendo le cavitá del nostro cervello e del canale vertebrale. Il liquido cerebrospinale puó essere prelevato attraverso una puntura lombare ed essere utilizzato come fonte di biomarcatori per malattie neurodegenerative.

Le **sinapsi** sono punti di contatto tra neuroni, essenziali per lo scambio di informazioni nel sistema nervoso. Le sinapsi possono essere viste come un bottone, formate da due parti contrapposte denominate compartimento presinaptico e postsinaptico, separati da una piccola fessura. Le sinapsi comprendono una moltitudine di proteine che cooperano ed interagiscono direttamente o indirettamente tra loro per

la trasmissione e l'integrazione dei segnali. Le sinapsi rappresentano il sito di formazione della memoria e delle capacità cognitive e, come tali, sono principalmente colpite durante le malattie neurodegenerative, come ad esempio il morbo di Alzheimer. La degenerazione sinaptica sembra essere un evento precoce nell'Alzheimer ed è fortemente correlata al grado di demenza e di declino cognitivo. Per questi motivi, le proteine sinaptiche sono studiate come possibili biomarcatori per la rilevazione dei cambiamenti patologici della malattia di Alzheimer negli stadi iniziali, per seguire la progressione del morbo ed il declino cognitivo associato.

L'obiettivo generale di questo progetto di dottorato é stato quello di studiare differenti **proteine sinaptiche**, quali la neurogranina, neuroligine e neurexine**,** in tessuti cerebrali e liquido cerebrospinale di malati di Alzheimer, con lo scopo di valutarne la validità come biomarcatori per la disfunzione sinaptica in questa invalidante malattia.

LIST OF PAPERS

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

I. Becker B, Nazir F H, Brinkmalm G, **Camporesi E**, Kvartsberg H, Portelius E, Bostrom M, Kalm M, Hoglund K, Olsson M, Zetterberg H, and Blennow K. *Alzheimer-associated cerebrospinal fluid fragments of neurogranin are generated by Calpain-1 and prolyl endopeptidase*. Molecular Neurodegeneration, 2018. **13**(1): p. 13-47.

II. Nazir F H, **Camporesi E**, Brinkmalm G, Lashley T, Toomey C E, Kvartsberg H, Zetterberg H, Blennow K, and Becker B. *Molecular forms of neurogranin in cerebrospinal fluid.* Journal of Neurochemistry, 2020. **00**(1): p.1-18

III. **Camporesi E**, Tammaryn L, Johan G, Lantero-Rodriguez J, Hansson O, Zetterberg H, Blennow K, and Becker B. *Neuroligin-1 in brain and CSF of neurodegenerative disorders: investigation for synaptic biomarkers.* Acta Neuropathologica Communications, 2021. **9**(1): p. 9-19

IV. **Camporesi E**, Johanna N, Vrillon A, Cognat E, Hourregue C, Zetterberg H, Blennow K, Becker B, Brinkmalm A, Paquet C, and Brinkmalm G. *Quantification of the trans-synaptic partners neuroligin-neurexin in CSF of neurodegenerative diseases by parallel reaction monitoring mass spectrometry.* Manuscript

LIST OF PAPERS

Papers not included in the thesis

I. **Camporesi E**, Nilsson J, Brinkmalm A, Becker B, Ashton N J, Blennow K, and Zetterberg H. *Fluid Biomarkers for Synaptic Dysfunction and Loss.* Biomarker Insights, 2020. **15**: p. 1-17.

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ABBREVIATIONS

aa	Amino acid
Ach	Acetylcholine
AD	Alzheimer's disease
ADAM1	A disintegrin and metalloproteinase 10
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazo-lepropionic acid
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
Aβo	Aβ oligomers
BACE1	β-site APP cleaving enzyme 1
bvFTD	Behavioural variant frontotemporal degeneration
CAA	Cerebral amyloid angiopathy
CaM	Calmodulin
CaMKII	Calcium-calmodulin dependent kinase II
CBD	Corticobasal degeneration
CBS	Corticobasal degeneration syndrome
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTD	C-terminal domain
CTF	C-terminal fragment
DDA	Data-dependent acquisition
DLB	Dementia with Lewy body
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
fAD	familial AD
FDA	Food and Drug Administration
FDG	[^{18}F]fluorodeoxyglucose

ABBREVIATIONS

FRET	Förster/Fluorescence Resonance Energy Transfer
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
GABA	Gamma-aminobutyric acid
GAP43	Growth-associated protein 43
HCD	Higher-energy collisional dissociation
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IP	Immunoprecipitation
IS	Internal standard
IWG	International Working Group
KO	Knock-out
LB	Lewy-body
LC	Liquid chromatography
LN	Lewy neurites
LTD	Long-term depression
LTP	Long-term potentiation
lvPPA	Logopenic variant primary progressive aphasia
m/z	Mass-to-charge
MAPT	Microtubule-associated protein tau gene
MCI	Mild cognitive impairment
MMP9	Matrix metallopeptidase 9
MMSE	Mini Mental State Examination
MRI	Magnetic resonance imaging
MS	Mass spectrometry
ND	Neurodegenerative disease
NFTs	Neurofibrillary tangles
nfvPPA	Non-fluent variant primary progressive aphasia
Ng	Neurogranin
NIA-AA	National Institute on Aging and Alzheimer's Association
Nlgn	Neuroligin
NMDA	*N*-methyl-*D*-aspartate

NRXN	Neurexin
PA	Pathological aging
PAGE	Polyacrylamide gel electrophoresis
PDD	Parkinson’s disease dementia
PET	Positron emission tomography
pI	Isoelectric point
PiD	Pick’s disease
PKC	Protein kinase C
PPA	Primary progressive aphasia
PREP	Prolyl endopeptidase
PRM	Parallel reaction monitoring
PSD95	Post-synaptic density protein 95
PSEN	Presenilin
PSP	Progressive supranuclear palsy
PSPS	Progressive supranuclear palsy syndrome
PTMs	Post-translational modifications
QC	Quality control
RP	Reverse-phase
sAD	sporadic AD
sAPPα	Soluble APP fragment α
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SPE	Solid phase extraction
SS	Splice site
svPPA	Semantic variant primary progressive aphasia
TBS	Tris-buffered saline
VaD	Vascular dementia

1 INTRODUCTION

1.1 Neurodegenerative diseases

Neurodegenerative disease (**ND**) is a term used to describe a group of diseases characterised by progressive deterioration of neuronal structure and function, including neuronal death, in a process defined as neurodegeneration. Neurodegeneration can occur in different brain regions and affects different cell types depending on the pathology [1]. Although more than one pathological change can be present at the time, NDs have in common deposits of misfolded proteins and are therefore frequently classified as **proteinopathies**. Protein aggregates are found in neurons, but also in other cells, such as glia cells, and can be present both intracellularly and extracellularly. The abnormal aggregation of endogenous proteins can be the result of a mutation in the protein-related gene, or it can be triggered by environmental stressors or aging. Protein aggregation is probably a complex multi-step process, which leads to the formation of a variety of different molecular species. A lot of effort has been made towards the understanding of which molecular species and by which mechanisms they cause the damaging effects in the central nervous system (CNS) [2-5].

NDs can be classified based on the type or types of the abnormal protein involved. Hence we can distinguish amyloidosis, tauopathies, α-synucleinopathies and transactivation response DNA binding protein 43 (TDP-43) proteinopathies as the most common ones [6]. NDs can be also classified based on clinical manifestations, involving cognitive, behavioural and motor domains of the brain. Diagnosis of these diseases can be conclusive only after neuropathological examination of *post-mortem* brain tissue, where the identification of the type and the neuroanatomical distribution of the misfolded proteins is performed. As aforementioned, *post-mortem* investigations have revealed that most of the patients show more than one pathogenic process occurring in their brain at the time of autopsy [6]. Heterogeneity is also found in clinical manifestations, inasmuch only few patients present pure syndromes, with most of them having mixed clinical features. Moreover, comorbidities, which is co-occurrence of more than one pathology at the time, can also be present [7]. Additionally, although typically associated with aging, NDs are characterised by a long prodromal stage, identified as the period preceding the actual manifestation of the symptoms in which pathological changes progressively accumulate in the brain. This means the disease may already be at an advanced stage at the time it actually manifests, thus too late to intervene. Taken together, all these aspects challenge the finding of efficient interventions for NDs. Indeed, to date there is no definitive cure for these diseases and the pharmacological treatments available can only ameliorate the symptoms.

Although Alzheimer's disease was the focus of this thesis, synaptic proteins of interest were also investigated for other NDs, *i.e.*, tauopathies, frontotemporal dementia and dementia with Lewy body.

1.2 Biomarkers for neurodegenerative diseases

Neurodegeneration represents the underlying factor for many debilitating and currently incurable age-dependent disorders. Due to the difficult access to the brain, its complexity and the long prodromal stage that masks the onset of these diseases, biomarkers are sought after to predict, monitor and diagnose neurodegenerative disorders.

A biological marker (**biomarker**) is defined 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" [8]. Based on this definition, biomarkers can serve for different purposes and can be classified as biomarkers for; (1) **screening**, to initially identify who may have the disease or not, (2) **diagnosis**, to establish the presence of a disease and discriminate between different diseases, (3) **prognosis**, to predict who will develop the disease, (4) **staging**, to monitor the progression of the pathology (5) **theragnosis**, to monitor an individual's response to a particular therapy.

1.2.1 Fluid biomarkers

Different fluids of the body can be analysed for identifying or quantitating biomarkers. Cerebrospinal fluid (**CSF**) is the most used fluid for biomarker discovery for neurodegenerative diseases, because of its vicinity and interaction with the brain. CSF is a colourless body fluid mainly secreted by specialized cells of the choroid plexus, which allow for the filtration of plasma, retaining high molecular weight components in the plasma and secreting liquid, salts, and lower molecular weight components of the plasma into the CSF [9]. The CSF compartment surrounds the brain, occupying the subarachnoid space and the ventricular system, and filling the central canal of the spinal cord. CSF is important for brain homeostasis as it provides nutrients as well as waste removal of metabolic products out of the brain. Moreover, it provides hydromechanical protection of the brain from impacts and sudden movements of the skull [10]. The average volume of CSF per person is approximately 150 mL and it is renewed four to five times per day, with an estimated total CSF production of 400 to 600 mL in a day. The turnover rate decreases with age [11]. Thanks to the blood–CSF barrier, which restricts the exchange of molecules and proteins [10], the CSF is isolated from the peripheral vascular system and it represents a useful matrix to study what it is happening in the CNS [12, 13]. A sample of CSF can be taken through a procedure called lumbar puncture, whereby a sterile needle is inserted into the subarachnoid space between the L3/L4 or L4/L5 lumbar vertebrae [14, 15]. The lumbar puncture is

an invasive, although generally safe practise. The most common side effect is post-lumbar puncture headache, especially in young patients, while severe side effects are very rare [16]. New guidelines for CSF sampling and handling have been recently published [17], with the aim of reducing variations between laboratories and increase analytical reproducibility.

Aside from CSF, lot of research is currently ongoing towards the development of **plasma biomarkers**, using blood as source of CNS biomarkers. Blood is of great utility because of the easier accessibility as compared to CSF, although its distance from the brain and contact with the periphery represent potential obstacles for CNS-specific biomarkers.

1.3 Alzheimer's disease

Alzheimer's disease (**AD**) is the most common cause of dementia, accounting for more than 60% of all dementia cases [18]. Dementia is a syndrome defined by loss of cognitive functions, including processes like reasoning, remembering and speaking, to a level that severely impacts on the patient's daily life. AD affects roughly 50 million people worldwide (https://www.alzint.org/about/dementia-facts-figures/dementia-statistics/) and due to increasing age and number of the population, the number of affected individuals will likely increase [19]. Accordingly, also the burden of health expenditure will increase, with the risk of overwhelming health and social services. With this scenario, the importance and need of research on this relentless pathology is of utmost importance [20].

Alois Alzheimer, a German psychiatrist and neuroanatomist, described the first case of AD in 1906. The pathology was named after him a few years later [21]. Despite the few scientific resources available at that time, he was able to detect in the brain deposits of proteins such as amyloid plaques and neurofibrillary tangles, which are still nowadays recognised as the major pathological hallmarks of AD. More than a century of research has also revealed that along with these main pathological hallmarks gliosis, neuroinflammation [22-24] and vascular dysfunction [25, 26] are also present in the brains of AD patients, demonstrating the complex nature of this disease. AD is characterised by a progressive loss of cognitive functions and macroscopically by a massive brain atrophy, which is a consequence of neuronal degeneration and loss. This process does not occur all at once, but AD is a continuum, with the first pathological changes in the brain starting to appear more than 20 years before the symptoms become overt [27]. In this continuum, three broad phases can be distinguished [28] starting with a **preclinical phase**, where the affected individual can function normally but starts to exhibit abnormal biomarkers and measurable brain changes. This initial phase is followed by a mild cognitive impairment (**MCI**) phase, where the first cognitive symptoms start to appear, like short-term memory impairment, although not severe enough to impact on the patient's daily activity performance. The disease is then inexorably progressing to the **dementia stage**, with impaired communication, disorientation, changes in behaviour and poor judgment, ultimately leading to the inability to perform the everyday activities. It is mostly at these later stages that the patients start to seek medical help. Currently, no disease-modifying treatments exist for AD, and the clinical trials thus far have had a very high failure rate [29]. This is mostly attributed to the fact that patients are treated only at an advanced stage, probably when the disease is already at its irreversible phase. Therefore, recent efforts are focusing on delaying dementia in people who are in the preclinical phase. To this aim, biomarkers for the identification and stratification of patients are of high importance and need [30].

1.3.1 Pathology

1.3.1.1 APP and Aβ

Amyloid beta (**Aβ**) peptides derive from the proteolytic processing of a transmembrane protein called amyloid precursor protein (**APP**), localized in many tissues and especially at neuron synapses [31]. APP has been described as important for neuronal migration and as a trophic factor. Yet the exact physiological role is not well understood [31]. Structurally, APP has a large glycosylated extracellular part, a single membrane-spanning domain and a short cytoplasmic domain. Different isoforms are present, but the most abundant isoform in the brain is the 695 amino acid long APP form. APP undergoes subsequent cleavages, via two possible pathways [32] (Fig.1). In the first one, called the **non-amyloidogenic pathway**, the initial cleavage at the extracellular domain is performed by a α-secretase, resulting in the release of a soluble APP fragment α (sAPPα). The C-terminal fragment (CTF-α or C83) is then cleaved by a γ-secretase, yielding a P3 fragment, so called because of its size of about 3 kDa. In the second pathway, called the **amyloidogenic pathway**, APP is cleaved first by β-secretase, which generates sAPPβ. Then, the CTF-β (or C99) is cleaved by the same γ-secretase, leading to the generation of Aβ peptides. In both cases, we have a release of an intracellular domain, which may translocate to the nucleus and eventually act as gene expression regulator [33].

Aβ peptides are normally produced during APP cell metabolism, but in AD, we have an imbalance between production and clearance of APP cleavage products, which leads to Aβ accumulation. Aβ peptides produced through the amyloidogenic pathway are hydrophobic and prone to aggregate (Fig.1). They can form dimers, oligomers, fibrils that can subsequently form big insoluble aggregates called plaques, which are found in the brain parenchyma (extracellularly) of AD patients. Which form is the most toxic one is still unclear [34], although the soluble Aβ oligomers (Aβo) have been widely regarded as the most toxic ones, especially for synapses [35]. Interestingly, the P3 peptide of the non-amyloidogenic pathway, which has been shown to be non-synaptotoxic, does not form oligomers, but aggregates directly into filaments [36].

1.3.1.2 Proteases involved

The major **β-secretase** of neurons is the β-site APP cleaving enzyme 1 (BACE1), an aspartic protease primarily localized presynaptically [32]. Its cleavage activity on APP, followed by the action of γ-secretase, produces $A\beta_{1-43}$, $A\beta_{1-42}$, $A\beta_{1-40}$, $A\beta_{1-38}$ and similar fragments. $A\beta_{1-40}$ is usually the most abundant, but $A\beta_{1-42}$ is the one mostly increased in AD brain. They show distinct chemical properties, with the 1-42 Aβ peptide considered the most amyloidogenic one, inasmuch it aggregates more rapidly

than the 1-40 peptide [37]. In addition, the 1-43 peptide is highly prone to aggregation [38].

The main **α-secretase** in neurons is a disintegrin and metalloproteinase 10 (ADAM10). Its cleavage activity on APP, followed by γ-secretase, generates harmless species. **γ-secretase** is a transmembrane complex consisting of at least four proteins: presenilin 1 or 2 (PSEN1, PSEN2), acting as catalytic subunits, presenilin enhancer 2 (Pen-2), nicastrin and anterior pharynx defective-1 (Aph-1).

Recently, new proteolytic pathways have been described, such as the one involving the so-called "eta" or **η-secretase** [39]. This newly identified secretase cuts far N-terminal of the β-secretase site and it produces different fragments of about 92 to 108 amino acid length, called Aη peptides. They also appear to be synaptotoxic impairing synaptic plasticity and neuronal activity [40, 41]. Other fragments have also been described [42, 43], leading to a heterogeneity of Aβ peptides. Perhaps, more than one toxic species act in concert; therefore, it would be of importance to characterise all of them precisely to understand the pathophysiological mechanisms concerning APP.

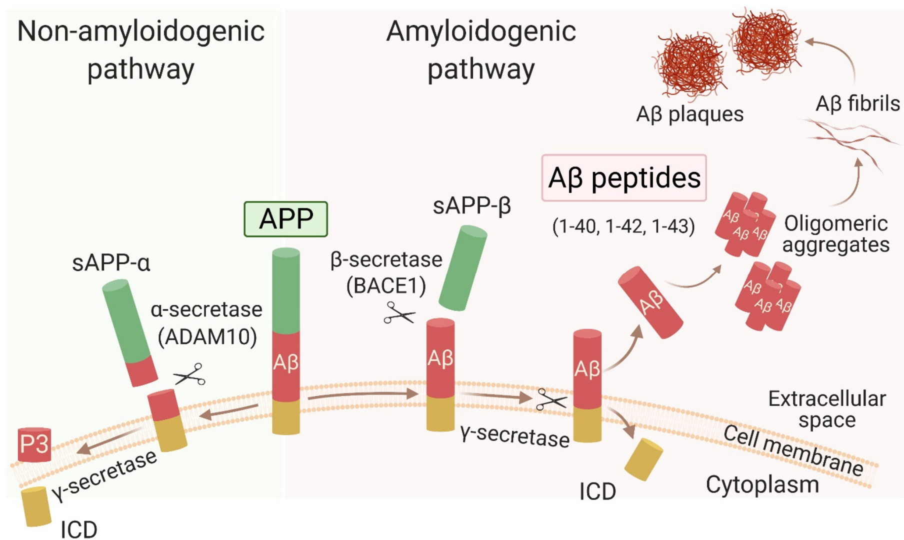

Figure 1. *Schematics of the two APP proteolytic processing pathways. ICD= intracellular domain. Created with BioRender.com*

1.3.1.3 Tau protein and tangles

The second neuropathological hallmark that defines AD pathology is the presence of intraneuronal fibrillary tangles (NFTs) of hyperphosphorylated microtubule-associated protein tau (MAPT or simply **tau**).

Physiologically, tau is synthesised in the cell body and then transported to axons, with minor amounts found in dendrites and nuclei. Tau in axons associates with microtubules, promoting their assembly and stability [44]. Recent studies investigating tau functions in neurons also associate tau with axonal transport and synaptic plasticity, although these new functions are still a matter of debate [45]. The degree of phosphorylation regulates the biological activity of tau protein. In AD brain, the protein becomes abnormally phosphorylated, thus inhibiting its activity to promote microtubule assembly. Hyperphosphorylated tau is the major component of NFTs [46] (Fig. 2). Tau undergoes a number of phosphorylations and other post-translational modifications (PTMs) after its synthesis. Phosphorylation and truncation are the most studied, even though it remains unclear which one is the trigger for aggregation. Emerging studies describe tau as a protein with prion-like properties. This encompasses the ability of the protein to be released extracellularly and to spread to neighbouring cells where it induces the same pathological conformation to a protein of the same kind, acting as a template [47, 48].

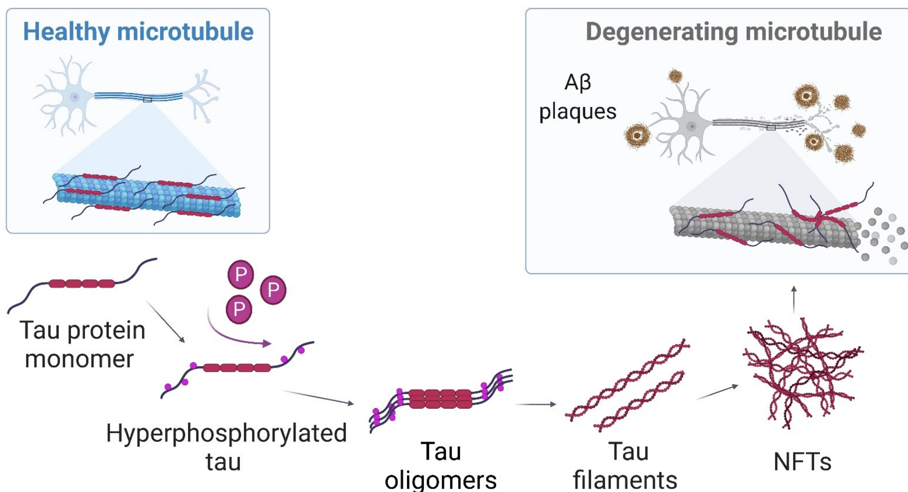

Figure 2. *Tau hyperphosphorylation and consequent microtubules dysfunction. Created with BioRender.com*

1.3.2 Hypotheses on disease manifestation

In AD, Aβ plaques and aggregates of tau appear at different times in different regions of the brain (Fig. 3). The exact sequence of events is not well established, yet, as it is not understood how and whether they interact or influence each other. According to the "**amyloid cascade hypothesis**" Aβ drives the disease and is accumulating in the brain due to an imbalance between production and clearance [49]. Pieces of evidence that led to the hypothesis are the mutations (described below), which are causative of the disease and the fact that people with Down syndrome, carrying an extra copy of the APP gene caused by trisomy of chromosome 21, develop AD, probably because they produce more Aβ [50]. Nevertheless, this hypothesis is largely debated because of different reasons. 1. Many mouse models overexpressing Aβ develop plaques but do not show neuronal loss or memory impairment. 2. The investigation of Aβ depositions with recently introduced amyloid imaging techniques showed some cases of cognitively normal patients with high Aβ deposits and AD patients with low Aβ burden. 3. All the clinical trials aiming at reducing Aβ depositions so far did fail [51]. Moreover, the Aβ plaque burden does not correlate with the degree of cognitive decline as well as the number and the regional distribution of NFTs do. Due to these controversies, a **tau hypothesis** has also been proposed. This hypothesis is founded on the basis that tau better correlates with clinical features of dementia in AD and tau pathological accumulation seems to appear even before Aβ accumulation [52, 53]. Again, another study showed that Aβ plaques enhanced tau aggregation and tau-seeded pathology [54]. Moreover, in the cascade of events, other factors may have an important role: microglia-driven inflammation, oxidative stress, vascular pathologies, bacterial/viral infections. It is thus evident that we do not have a clear picture yet, despite the many studies trying to understand the sequence of events and the relationship between plaques and tangles. AD is a complicated and heterogeneous disease that needs to be further investigated.

1.3.3 Genetics and risk factors

Most AD cases occur for unknown causes and are cases of so-called sporadic AD (**sAD**) or late-onset AD, where the disease usually presents itself after the age of 65 years. There is also an early-onset AD or familial AD (**fAD**) form, although it accounts for less than 1% of all cases [55, 56]. Autosomal dominant mutations in one of the genes encoding for APP, PSEN1 and PSEN2 cause this hereditary form of the disease. In these cases, the disease becomes manifest much earlier and it progresses faster (https://www.alzforum.org/early-onset-familial-ad). Missense mutations, leading to mutated amino acid sequence of the presenilins, lead to the production of longer Aβ peptides, with more hydrophobic and self-aggregating properties. Individuals carrying these mutations have an increase of $A\beta_{1-43}$, $A\beta_{1-42}$ ratio against $A\beta_{1-40}$ [51].

There are also other mutations increasing the risk of developing AD. Among those, the apolipoprotein E allele ε4 (***APOE ε4)*** is the major genetic risk factor [57]. In the CNS, apoE is a protein primarily produced by astrocytes and is responsible for the transport of cholesterol and other lipids to neurons and between cells. Three single-nucleotide polymorphisms lead to a different combination of the amino acid cysteine (Cys) and arginine (Arg) at position 112 and 118, thus resulting in the three common isoforms of the protein, apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158) and apoE4 (Arg112, Arg158). Thus, six possible genotypes exist: ε2/ε2, ε3/ε3, ε4/ε4, ε2/ε3, ε3/ε4 and ε4/ε2. The ε3 allele is the most common, while the ε2 is the least common, but considered protective [58]. Having the ε4 allele is a great risk for AD and it has been estimated that having one *APOE* ε4 allele increases the risk about 3 times and having two *APOE* ε4 alleles up to 12 times [57]. Moreover, carrying the ε4 allele reduces the age of onset of AD. The amino acid variations in the apoE isoforms change the protein structure and modify the lipid-binding and receptor-binding affinity. This has implications for Aβ clearance, where apoE plays a role, with apoE4 showing the lowest binding affinity compared to apoE3 and apoE2 [59]. This so-called loss of function has been connected to the higher Aβ burden in *APOE* ε4 carriers [60]. Moreover, the less efficient transport of cholesterol by apoE4 has also been connected to loss of synaptic integrity and decreased neurogenesis, impaired lipid/cholesterol metabolism and damaged vascular function [61]. Given the high prevalence of apoE4 in AD patients, the apoE protein and its targets and interactors are investigated as possible therapeutic targets for AD [62].

Despite genetics, also aging, in concert with other environmental factors, like education, physical activity, lifestyle, and other pathologies such as obesity, hypertension, depression and cardiovascular diseases increase the risk for developing AD [60]. On the other hand, gene mutations with quite opposite effects have also been described, such as the protective effect of a coding mutation **A673T** in the APP gene. In this mutation, the amino acid substitution, which is close to the BACE1 cleavage site, reduced by approximately 40% the formation of amyloidogenic peptides *in vitro* [63].

1.3.4 Diagnosis and diagnostic criteria

As previously mentioned, the gold standard for diagnosing AD is a *post-mortem* histopathological examination. The investigation takes advantage of the fact that pathological accumulations of Aβ and tau in AD brain follow a stereotypical pattern [64]. Thus, different scoring systems to determine the presence, distribution and amount of plaques and tangles in the brain have been developed. **Thal phases** (0-5) are used to assess Aβ plaque spreading throughout the brain [65], while **Braak stages** (0-IV) describe the distribution of tau pathology [66, 67]. Additionally, the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) is a scoring system for

neuritic plaques, which ranks their density in different regions of the neocortex. These three classification methods can be combined forming the **ABC** system, which describe those pathological aspects after an autopsy [68]. Amyloid-β deposits can also be found in the walls of small and medium cerebral blood vessels and might originate from a type of vascular disease called cerebral amyloid angiopathy (**CAA**). Amyloid deposits indicative of CAA can be found in more than 80% of AD patients [69, 70] and can be similarly staged in the brain using its relative scoring system [71].

In a clinical setting, diagnosis of AD is initially still mostly based on clinical symptoms, guided by diagnostic criteria from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [72-74].

Clinical investigation of patients is performed using cognitive tests, which can be used to evaluate suspected cognitive impairment. Among those, one of the most widely used test is the Mini Mental State Examination (**MMSE**) [75]. The patient undergoes a 30-point questionnaire where questions concerning orientation, memory, concentration, language, and ability to calculate are evaluated. Usually, a score under 24 identifies a cognitive disorder. Anyway, a score of 30 does not completely rule out dementia and a lower score then requires further evaluations, inasmuch the test can not readily discriminate between AD and other dementias. In this scenario, it becomes of importance to combine cognitive and neuropsychiatric investigations with other diagnostic tools, such as biomarkers, to be able to classify patients and to offer a clearer diagnosis.

A lot of effort has been made to better define the pathology, classify patients and discriminate between AD and other dementias, and this has resulted in new research diagnostic criteria. In 2007, thanks to progress in *in vivo* biomarkers, the International Working Group (**IWG**) introduced them for the first time for defining AD. Now, both episodic memory impairment and abnormalities in at least one of the biomarkers are needed to define AD [76]. Increased brain amyloid retention on positron emission tomography (PET), structural brain changes visible on magnetic resonance imaging (MRI), decreased $A\beta_{1-42}$ together with increased total-tau (t-tau) or phospho-tau (p-tau) in CSF, or an autosomal dominant mutation are now recommended for assessment of AD pathology *in vivo*. In 2011, new guidelines from the National Institute on Aging and Alzheimer's Association (NIA-AA) defined AD as a continuum that includes three stages: a preclinical stage with no symptoms; a middle stage of MCI; and a final stage marked by symptoms of dementia, Alzheimer's dementia [27, 74]. In 2014, IWG updated the research diagnostic criteria for AD, refining them to the new set of **IWG-2** criteria [77]. The CSF fluid biomarkers and PET amyloid imaging biomarkers are now defined as diagnostic, thus specific in identifying Aβ pathology at any point of the AD continuum. On the contrary, MRI is considered more a biomarker for monitoring progression, but not specific for Aβ pathology, thus MRI has been removed from the algorithm. Although not yet implemented in the clinic, the new framework

changed the definition of AD, which is now based on biological changes rather than clinical symptoms [78].

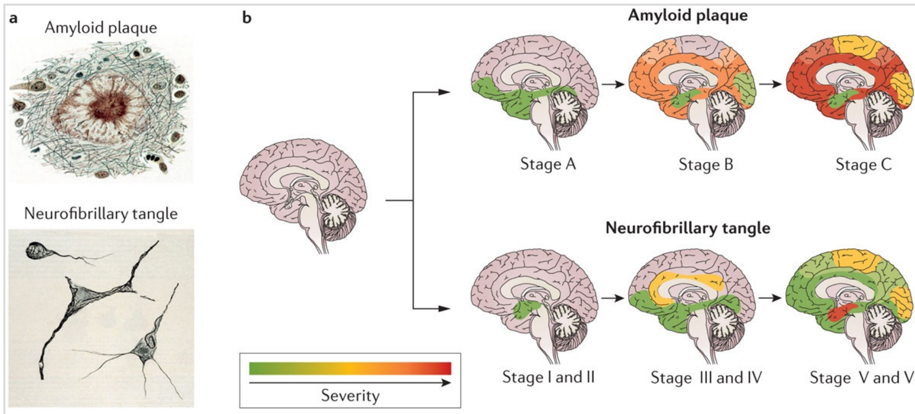

Figure 3. *The pathological progression of AD in respect to amyloid plaque and tau NFTs deposition. Reprinted by permission from Springer Nature: Springer Nature, Nat. Rev. Dis. Primers, (Alzheimer's disease, Masters, C. L. et al.), Copyright (2015).*

1.3.5 Biomarkers

Currently, AD is defined by three biomarkers: **$A\beta_{1-42}$**, **p-tau** and **t-tau**. These core biomarkers can be measured in CSF, and in human brain using PET imaging, which provides a clearer view of the pathology in living individuals. Moreover, MRI is utilised to determine the degree of brain atrophy [78].

1.3.5.1 Fluid biomarkers

In an AD brain, the $A\beta_{42}$ peptide accumulates in the form of fibrils and plaques with the consequent decrease of its levels in CSF, which serves as an indicator of Aβ pathology. However, $A\beta_{42}$ levels also depend on total physiological Aβ production. Therefore, the $A\beta_{42}$ values are often normalized using $A\beta_{40}$, the most abundant Aβ peptide in CSF, as its levels do not change in AD patients. Thus, the ratio **$A\beta_{42/40}$** is usually preferred to $A\beta_{42}$ alone, inasmuch it corrects for individuals with atypical high or low production of Aβ, giving a better separation between AD and controls [79]. In addition, the ratio has a high concordance with amyloid PET [80]. T-tau and p-tau measurements in CSF monitor tau protein changes and NFTs formation. Both are increased in AD CSF but have different meanings. **T-tau** refers to the sum of all the forms of tau detectable by the particular immunoassay used (*i.e.* full-length tau and mostly N-to mid-region fragments of tau) and an increase in its CSF levels reflects the intensity of neuronal and axonal degeneration. However, t-tau is not specific for AD as abnormal levels are also found when the damage occurs due to other causes like

brain trauma, stroke, and Creutzfeldt-Jakob disease (CJD) [81, 82]. On the contrary, **p-tau**, referring to tau phosphorylation at threonine 181, it is index of the hyperphosphorylated state of the protein and NFTs formation in the brain, and it is specific for AD, being able to differentiate AD from other dementias like FTD and DLB [83]. The combined use of these biomarkers increases the sensitivity and specificity in discriminating patients with AD from healthy elderly individuals to 80-90% [84]. Moreover, new combinations of these biomarkers with imaging biomarkers are investigated to maximize their use in reliable detection of other phases of the AD continuum, like MCI [85].

Recent advances in ultrasensitive methodologies have made possible the measurement of these core biomarkers also in blood [86, 87]. Moreover, more tau phosphorylations are being studied and quantified, in both CSF and blood, with the hope to better characterise the sequence of events occurring in AD and to increase diagnostic accuracy at different stages of the disease [88, 89].

1.3.5.2 Imaging biomarkers

The possibility to visualize pathological changes in the brain of living patients provides important temporal and spatial information, which is central to disease staging and complementary to the information provided by fluid biomarkers.

Structural **MRI** is predominantly used for assessing brain volume and atrophy as measures of neurodegeneration, which is most pronounced in the medial temporal lobes of AD patients, already at prodromal stages [90].

For the mapping and quantification of AD-associated pathophysiology, several **PET** tracers sensitive to Aβ and tau aggregates are now available. The [^{11}C]Pittsburgh compound B ([^{11}C]PiB) was introduced first in 2002 and is the most established tracer for Aβ plaques in the brain. However, its short half-life (20 min) pushed towards the development of fluorinated tracers with longer half-life (110 min). To that end, [^{18}F]florbetapir, [^{18}F]flutemetamol and [^{18}F]florbetaben have been designed and are AD diagnostic modalities now approved by the Food and Drug Administration (FDA) and used in clinical practice and trials. In comparison with Aβ plaques, AD-typical paired helical filament tau aggregates are more difficult targets for PET tracers, as these are mainly located intracellularly and exhibit a complex ultrastructure. Thus, the development of tau-sensitive tracers proved more challenging and the most established tau tracer, [^{18}F]flortaucipir, was only recently (2020) FDA-approved for clinical use [91]. However, its "off-target" binding issues can exacerbate accurate tracer uptake quantification, which is why a second generation of tau tracers with somewhat improved binding properties is now under evaluation [92]. Tau tracers are highly desirable tools as tau shows stronger associations with disease progression and cognitive impairment compared to Aβ plaques.

Glucose is the main metabolic substrate for energy formation in the brain. [^{18}F]fluorodeoxyglucose (FDG) is a PET tracer measuring glucose metabolism in the brain, which decreases only mildly in healthy aging but is substantially and focally decreased with synaptic dysfunction and neuronal degeneration [93]. FDG PET is a valuable tool to accurately detect typical spatiotemporal patterns of glucose hypometabolism in both MCI and AD patients and to differentiate AD from other dementia disorders [94].

Even though imaging biomarkers are of great utility as they combine spatial and quantitative information, performing a PET scan is laborious and requires expensive equipment, thus it is not always applicable. Moreover, currently imaging biomarkers cannot detect very early stages of disease [95]. Fluid biomarkers are cheaper and easier to use.

1.3.5.3 Biomarker classification framework for AD

The above-mentioned biomarkers have been proposed for use in clinical diagnosis and as inclusion criteria for treatment trials. A new framework to describe AD in terms of different biomarker profiles, called **A/T/N**, has been recently been put forward with the aim to provide a classification system easier to read [96]. In this format, "**A**" stands for amyloid and refers to the value of Aβ measured in CSF as Aβ42 and amyloid deposition in brain measured by PET. "**T**" reflects the value of the tau neurofibrillary tangles, as CSF p-tau and tau PET, while "**N**" includes biomarkers for neurodegeneration or neuronal injury, represented by FDG-PET, CSF total tau or structural MRI. In this system each category is rated as positive (+) or negative (-). Thus, A+/T+/N+ identifies a typical AD profile. This system is flexible to be expanded by the addition of new biomarkers if they become available.

1.3.6 Management

As of today, there is no cure for AD, which means a way to stop the onset of neuropathological changes or the subsequent neurodegenerative processes. The available medications only treat or modify the symptoms. FDA approved drugs for the treatment of AD are cholinesterase inhibitors, for example donepezil, galantamine and rivastigmine, and the *N*-methyl-*D*-aspartate (NMDA) receptor modulator/antagonist memantine [97].

Cholinesterase inhibitor treatments are based on the so-called cholinergic hypothesis [98], according to which the loss of cholinergic activity, identified with the loss of acetylcholine (ACh), is closely related to learning and memory impairment. Thus, this class of drugs aims to decrease the extrasynaptic metabolism of ACh. ACh is an essential neurotransmitter for cholinergic neurons and it plays an important role in memory and attention. Although its mechanism of action is not fully understood, ACh is reduced in AD. ACh is synthesized by the enzyme choline acetyltransferase,

then released upon stimulation into the synaptic cleft where it exerts its actions by binding to different receptors. ACh activity is then terminated by the enzyme acetylcholinesterase that hydrolyzes the ACh back into acetate and choline. With the use of cholinesterase inhibitors, ACh hydrolysis is inhibited, thus ACh level at the synaptic cleft is increased and synaptic transmission promoted.

Memantine, has a different mechanism of action as it antagonizes the binding of glutamate to NMDA receptors, thus blocking the channel to the passage of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions [99]. NMDA receptors are ionotropic glutamate channels taking part in synaptic plasticity and memory functions. In AD, Ca^{2+} dyshomeostasis causes synaptic hyper-excitation, which leads to an increased release of glutamate, which in turn overactivates NMDA receptors. This is detrimental for the cell and causes synaptotoxicity. Blocking the over activation of the NMDA receptors with a reversible antagonist has beneficial effects on cognition and memory.

The use of these drugs significantly ameliorates the cognitive symptoms, with the maximum efficacy in the first years of therapy and reduce the need of nursing care. The combination of donepezil and memantine has also been approved for AD treatment with positive synergic effects, and the use of galantamine together with memantine has also been suggested [100]. Antipsychotic drugs can also be used to treat behavioural changes in AD patients. Despite the many ongoing clinical trials [101], no new disease-modifying treatment for AD has been approved since 2003. However, recently developed antibodies targeting different forms of Aβ, such as **aducanumab** and **BAN2401**, appear as promising candidates to tackle AD pathology [102].

1.4 Tauopathies

The presence of tau aggregates in the brain, without significant Aβ pathology, defines a group of progressive neurodegenerative disorders, so-called tauopathies [46]. As tau pathology is the main contributing factor of this heterogeneous group of diseases, they are classified as primary tauopathies, to distinguish them from secondary tauopathies, where tau aggregates are present but together with other neuropathological changes [45]. AD, for instance, is the most common secondary tauopathy.

Also for tauopathies, formal diagnosis can only be obtained at neuropathological examination, as no specific biomarkers are yet available. While all tauopathies share the presence of tau aggregates, these aggregates have distinct characteristics and are different from the NFTs found in AD. Thus, tauopathies can be classified based on the morphology and location of tau aggregates, and on the most prevalent tau isoforms [103]. Based on neuropathological examination and clinical presentation, primary tauopathies constitute a major class of frontotemporal lobar degeneration (**FTLD**), namely FTLD-tau. FTLD-tau includes diseases like corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and Pick's disease (PiD). It is important to highlight that the current terminology distinguishes the underlying molecular pathology from the clinical syndromic presentation, as different pathologies can reflect in the same clinical syndrome and vice versa, several distinct clinical syndromes can be related to the same pathologic entity. As a result of this distinction, the clinical equivalent of FTLD is termed frontotemporal dementia (**FTD**), and FTD clinical syndrome can be due to PSP or CBD pathology. The understanding of clinicopathological associations is a major issue and it stands at the basis for improving ante-mortem diagnosis [104].

The *MAPT* gene encodes for the tau protein, which, because of alternative splicing, can originate six possible different isoforms. These isoforms can contain zero, one or two amino-terminal inserts (termed 0N, 1N and 2N, respectively) and 3 or 4 carboxy-terminal microtubule-binding repeat domains (termed **3R** or **4R**, respectively) [45]. These isoforms are expressed in equal amounts in the adult human brain. In AD, both 3R and 4R tau isoforms are equally expressed. However, a differential immunoreactivity of 3R and 4R specific antibodies to tau pathological inclusions, revealed an imbalance of these isoforms in other tauopathies. For instance, PiD is predominantly a 3R tauopathy, while PSP and CBD are classified as 4R tauopathies [105].

Most of these disorders have unknown aetiology; however, more than 40 pathogenic mutations in the *MAPT* gene are known, leading to inherited forms of FTLD tau-driven disease. The age of onset varies depending on the specific mutation, but generally is between 45 and 65 years of age. This tells us that tau can cause neurodegeneration independently of Aβ [106].

1.4.1 Clinical features and neuropathology

FTD is a term used to describe a group of neurodegenerative disorders primarily affecting the frontal and temporal lobe and is characterised by behavioural, language, motor and cognitive impairment [107]. FTD is the second most common form of early-onset dementia and the third leading form of dementia after AD and DLB. Clinically FTD can be described by two main syndromes: behavioural variant frontotemporal degeneration (bvFTD), and a primary progressive aphasia (PPA). PPA refers to a group of neurodegenerative clinical syndromes with prominent language impairment and can be categorized in (i) non-fluent (nfvPPA), (ii) semantic (svPPA) and (iii) logopenic (lvPPA) variants. Additionally, amyotrophic lateral sclerosis can coexist with FTD (referred to as FTD-ALS) and atypical parkinsonian syndromes can also be associated [108]. A considerable overlap exists between the clinical, neuroanatomical, genetic, and pathological characteristics of FTD, which make the diagnosis difficult, especially at an early stage and for the different subtypes.

Approximately 70% of all FTD cases is represented by bvFTD, which is thus rated as the most prevalent form of presentation of FTLD. Due to the absence of definitive biomarkers, bvFTD diagnosis is still dependent on clinical diagnostic criteria, of which a second revised version was established in 2011 by the International Behavioural Variant FTD Consortium [109]. These criteria divide the diagnosis in "possible", "probable" and "definite" bvFTD and improve the diagnostic sensitivity for early stages of the disease [110]. The diagnosis starts with the patient showing progressive deterioration of behaviour or cognition, and diagnosis of "possible" bvFTD is assigned if signs of behavioural disinhibition, loss of manners, early apathy, early loss of empathy or sympathy, dietary changes and deficits in episodic memory and visuospatial functioning appear. Three or more of these symptoms need to be present. Diagnosis of "probable" bvFTD adds investigation of frontal or anterior atrophy using imaging modalities (*e.g.*, MRI) to a pre-existing possible bvFTD conclusion. A "definite" bvFTD diagnosis is reached when the patient meets criteria for possible bvFTD, but with the presence of histopathological confirmation of FTD, *i.e.* upon autopsy, and/or evidence of a known pathogenic mutation.

If language impairment is at the forefront, then the patient is diagnosed with PPA. The following classification in semantic, nonfluent or logopenic variants of PPA is based on the language features impaired and the suspected neurological localization imputed to the symptoms. svPPA is associated with bilateral anterior temporal lobe atrophy, while atrophy in nfvPPA is more prominent in the left hemisphere. As for bvFTD, all PPA variants reach diagnosis for "definite" pathology when there is a histopathologic confirmation and/or evidence of a known pathogenic mutation [111].

Clinically, FTD also frequently overlaps with three additional neurodegenerative diseases, such as corticobasal degeneration syndrome (CBS), progressive supranuclear palsy syndrome (PSPS), and ALS.

CBS is an atypical parkinsonian syndrome now also recognized as a cognitive disorder, as it usually presents cognitive deficits before the onset of motor symptoms [112]. CBS with underlying tau pathology constitutes a disease entity, namely CBD. However, CBS can be the clinical transduction of other pathologies [113], thus the two terms should not be used interchangeably. The diagnostic criteria for CBD [114] indicate five clinical syndromes accepted as clinical manifestations, including probable and possible CBS and a progressive supranuclear palsy-like syndrome (PSPS). However, these diagnostic criteria lack specificity and together with the lack of biomarkers, it is difficult to recognise if a CBS patient presents a CBD or a non-CBD pathology.

CBD is a rare disorder, of mainly unknown causes; however, some mutations, as for example in the *MAPT* gene and the progranulin gene [113], have been connected to CBS phenotypes. CBD pathology presents variable involvement of frontal, temporal, and parietal cortices. At a microscopic level, CBD is a 4R tauopathy which shows tau inclusions in neurons and glia and extensive thread-like pathology at neuropathological investigation. The main histopathological feature characterizing CBD is tau accumulation in astrocytes, called astrocytic plaques, which is used to differentiate CBD from PSP, which is instead characterised by tufted (with filamentous aggregates) astrocytes. Tau inclusions in oligodendroglia, called coiled bodies, are also present, but much more frequent in PSP than CBD. Moreover, they present a different morphology. Additionally, the presence of so-called ballooned neurons is highly suggestive of CBD pathology, whereas they are rare or absent in PSP.

PSP is a neuropathologically defined disease entity and together with CBD, constitutes one of the most common 4R tauopathy [103]. PSP encompasses a spectrum of heterogeneous clinical phenotypes involving cognition, behavioural disturbances, language, and a variety of movement disorders. Thus the original diagnostic criteria [115] have been expanded to also include phenotypic variants [116], in an attempt to diagnose the disease at an earlier stage, especially giving the current lack of biomarkers. PSP referred to as Richardson's syndrome (PSP-RS) is the clinical phenotype reflecting the movement disorder, named by whom first described it in 1964 [117]. The other clinical phenotypes include, among others, PSP-parkinsonism, the most common among the variants; PSP-speech language presenting similar symptoms to nfvPPA dementia with primarily frontal characteristics or with speech apraxia; and PSP-corticobasal syndrome (about 10% of the cases) [118]. Thus, cognitive manifestations associated with PSP might overlap with CBD and FTD symptoms. PSP is generally a sporadic disease, but very rare familial forms of PSP have been recognized, also associated with mutations in the *MAPT* gene [119]. As mentioned above, PSP shows NFTs and pretangles in neurons, together with filamentous aggregates in astrocytes (tufted astrocytes) and in oligodendrocytes.

Pick's disease (**PiD**) is a 3R tauopathy characterised by spheric neuronal inclusions named "Pick bodies" [120]. The disease predominantly manifests with frontotemporal cortical atrophy, but also in the basal ganglia and white matter. As for the other tauopathies, the term PiD only refers to neuropathological confirmed cases, as it can manifest with a clinical syndrome of CBS, bvFTD and also PPA variants [121].

1.4.2 Biomarkers

To date, no fluid biomarkers are available for the diagnosis of primary tauopathies. The CSF AD core biomarkers do not change across tauopathies [122], although they might be useful to distinguish FTD from AD patients [119]. CSF neurofilament light (NfL), a marker of neuronal damage now also quantified in blood [123-125], has been shown to be increased in PSP compared to PD and DLB [119]. FTD fluid biomarkers have been recently reviewed by Swift *et al.* [126]. Imaging modalities such as MRI and PET, to evaluate patterns of atrophy and pathological changes, hold promises for further future uses, especially now that more and more tau PET-ligand are becoming available [127]. For example, FDG-PET, assessing hypometabolism, and volumetric MRI, measuring the grey matter atrophy, can be very useful in FTD [128].

1.5 Dementia with Lewy body

Dementia with Lewy body (**DLB**) is the second most common form of age-associated dementia, accounting for more than 20% of all cases [129]. The neurodegenerative disorder was named after Friedrich Henrich Lewy who described it for the first time in 1912 [130]. Neuropathologically, DLB is characterised by the intraneuronal accumulation of aggregated protein α-synuclein, which forms the so-called Lewy-body (LB), and Lewy neurites (LN) in neuronal processes. However, LB and LN are also present in other neurodegenerative diseases like Parkinson's disease (PD) and Parkinson's disease dementia (PDD), to mention the most common ones. As aggregates of α-synuclein are the main pathological feature, this class of diseases is also defined as α-synucleinopathies.

α-Synuclein is a presynaptic protein primarily involved in regulating the fusion and clustering of vesicles to the presynaptic plasma membrane, an essential step for neurotransmitter release, as well as for synaptic vesicle recycles [131].

1.5.1 Clinical features and neuropathology

Diagnostic criteria for probable and possible DLB at dementia stage [132] or prodromal stage [133] are based on the existence of cognitive impairment or cognitive fluctuations associated to parkinsonian symptoms, dysautonomia or sleep disorder. Core clinical features are well summarized in Outeiro *et al.* [134]. According to the consensus criteria, the relative temporal onset of cognitive and motor symptoms can

be used to distinguish between DLB and PDD. If dementia occurs first or concomitantly with parkinsonism, then a DLB diagnosis should be considered, while the term PDD should be used when neuropsychiatric and cognitive symptoms occur later, in the context of well-established PD. Accordingly, only one of the cardinal motor features like bradykinesia, resting tremor, or rigidity is required for DLB, while at least two are required to diagnose PD. Imaging and electrophysiological biomarkers are included in the diagnostic criteria, but not mandatory, and are divided in indicative and supportive, depending on their specificity and availability [132].

DLB is mainly a sporadic disease with unknown aetiology, although some reports of occurrences in families with a history of dementia and DLB have been reported, as well as increased risk susceptibility for some genes, like for example *APOE* ε4 and some mutations in the APP gene [135]. This highlights the overlap and similarities that DLB shares with AD. Indeed, at *post-mortem* examination, approximately 50% of the patients also show high levels of AD neuropathological changes [132] and other way around, some degree of LB pathology can also be found in AD diagnosed cases. The presence of α-synuclein aggregates and distribution in the brain can be staged according to the relative criteria for pathological assessment [136]. Neuropathologically, DLB and PDD are very similar, with DLB probably showing less severe neuronal loss in the substantia nigra and a higher rate of AD pathology and widespread cerebral atrophy [137, 138]. This slightly different propagation pattern might be reflected in clinical diversity between DLB and PDD, while the AD pathology probably accounts for the cognitive symptoms. A recent histopathological investigation of 16 DLB and 52 PDD brains showed more prominent concurrent CAA pathology in DLB, a characteristic that could be used to better discriminate between these two pathologically close diseases [139].

No disease-modifying treatments are available for DLB, although some patients show better control of motor disturbances with levodopa. New possible pharmacological interventions are being explored [140].

1.5.2 Biomarkers

As previously mentioned, a series of indicative and supportive biomarkers are in use [132], although there are no diagnostic biomarkers yet. These biomarkers are mainly represented by imaging modalities (MRI, PET, electroencephalography (EEG) and single-photon emission computed tomography (SPECT)). The combination of FDG-PET and MRI shows to be useful in discriminating CBD from FTD and AD [141]. α-Synuclein quantified in CSF or blood (both plasma and serum) shows variable and inconsistent results (reviewed in our recent review [142]). Neuronal-derived vesicles from blood might represent an alternative to avoid peripheral α-synuclein contamination [143].

1.6 Synapses and dendritic spines in physiology and pathology

In the nervous system, a **synapse**, from Greek “coming together”, is the structure that allows the transmission signal to pass between two neurons or from a neuron to the target cell, by means of neurotransmitters.

There are many different types of synapses in the brain [144]. Synapses can be described as small buttons (less than a micrometer in diameter) organized in a presynaptic compartment, represented by the axon terminal of a neuron, and a postsynaptic compartment, where the signal is transmitted. Among two neurons, synapses can be found between an axon terminal and; (i) another axon (axoaxonic), (ii) the soma of another neuron (axosomatic) or (iii) a dendrite (axodendritic). Moreover, there are also synapses that end on a blood vessel and secrete directly into the blood stream (axosecretory), or on another axon terminal (axosynaptic), or with no connection to cellular structure, secreting into the extracellular fluid (axoextracellular).

Based on their transmission modality, chemical and electrical [145] synapses can be distinguished. **Electrical synapses** provide a direct electrical coupling between two cells, which allows the direct passage of ions and signalling molecules. The connection is mediated by gap junctions, pores that allow for the passage of a very rapid, passive and bidirectional electric potential. In contrast, **chemical synapses** do not allow a direct passage of the signal, but the action potential in the presynaptic neuron leads to the release of a neurotransmitter, a chemical messenger that diffuses across the synapse and binds to channels and receptors on the postsynaptic side, triggering a signal. In a chemical synapse, the pre- and postsynaptic cells are separated by a synaptic gap or cleft (~20-25 nm). In these synapses, the passage of an electric potential is slow and unidirectional. Both types of synapses are required, as electrical synapses transfer the signal very quickly, allowing groups of cells to act in unison, and chemical synapses allow neurons to integrate information from multiple presynaptic neurons, determining whether the signal will be propagated further or not.

Most of the synapses in our body are represented by chemical synapses. Chemical synapses can be further divided in **excitatory** and **inhibitory** (Fig. 4). They contain different sets of molecular and cellular components, which are reflected in distinct functional properties and plasticity rules. Excitatory synapses use glutamate as neurotransmitter, while inhibitory synapses use gamma-amino butyric acid (GABA) as major neurotransmitter. Excitatory synapses are the main representatives [146]. While inhibitory GABAergic synapses are mainly located on dendritic shafts, dendritic spines are the primary location of excitatory synapses. **Dendritic spines** are small membrane protrusions from the dendritic shaft containing receptors and postsynaptic density components. They contain all the receptors and signalling pathways for signal

integration and action potential generation [147]. Binding of a neurotransmitter to its target receptor can either allow ions to pass through a channel or activate a G-protein. Activation of a G-protein on the postsynaptic membrane leads to activation of a second messenger, which can have different effects like opening ion-channels, or initiate transcription of new proteins. In the human brain, billions of neurons interact and communicate between each other through trillions of synapses. Neurons respond differently depending on which type of information they receive, thus taking part of an extremely complex signalling system [148].

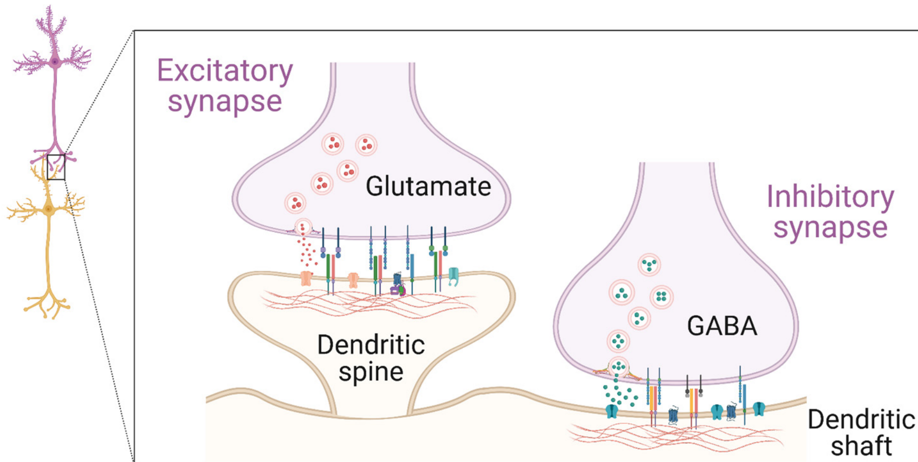

Figure 4. *Schematic representation of an excitatory and inhibitory synapse between two neurons. Created with BioRender.com.*

1.6.1 Synapses in physiology

Synapses are formed during the pre- and postnatal period of life, reaching a maximum number during the first years of age, which is then refined during adolescence where almost half of the synapses are eliminated through a physiological process called pruning. Synapses that survive to adulthood are the ones steadily conserved, although synapse formation and elimination continues, to a certain extent, throughout life [149].

During synaptogenesis, a contact between the presynaptic axon and the postsynaptic dendrite must first be made [150]. This initial contact appears to be mediated by trans-synaptic adhesion molecules. Numerous cell adhesion molecules have been involved in synapse development including, integrins, cadherins, and members of the immunoglobulin (Ig) superfamilies, neural cell adhesion molecules (NCAM), nectins, neuroligins, neurexins, synaptic cell adhesion molecules

(SynCAMs), neuronal pentraxins, and ephrins, among others. These molecules are also defined as synaptogenic, since they contribute to the “genesis” or formation of the synapse [151]. Then, pre- and postsynaptic proteins, which seem to be already present in neurons before synapses are formed, are transported to the sites of assembly between axons and dendrites. Fundamental for neuronal circuit formation are astrocytes, the most abundant glial cells in the brain [152]. Astrocytes have a direct contact with neurons and synapses and play a key role in synapse assembly and maturation, as well as synaptic elimination [153]. The importance of astrocytes for synapses developed into the concept of “tripartite synapse”, a functional unit defined by the contact between two neurons and an astrocyte [154].

Once synapses are formed, activity-dependent processes and predetermined genetic developmental stimuli act in combination to mediate synapse maturation. The maturation of a synapse involves structural and functional changes, *e.g.*, enlargement in synapse size and increased release of neurotransmitter receptors [147]. Events like synaptic formation, maturation and elimination can be defined as synaptic plasticity, which is at the basis for processes like adaptation, learning, and memory [155]. Synapses are thus plastic structures that can undergo changes. Two of the most often described models for synaptic plasticity are termed long-term potentiation (LTP) and long-term depression (LTD) [156]. LTP can be defined as an activity-dependent strengthening of the synapse. In a glutamatergic synapse, the α-amino-3-hydroxy-5-methyl-4-isoxazo-lepropionic acid (AMPA) receptors and the NMDA receptors are the main glutamatergic receptors at the postsynaptic side. They are permeable to different ions; particularly NMDA receptors are permeable to Ca^{2+} [157]. LTP is generally associated with recruitment of more AMPA receptors and dendritic spine growth. More AMPA receptors increase the excitatory current, which in turn renders the synapse more likely to fire on its next activation. Alternatively, low levels of synaptic stimulation can activate NMDA receptors to produce LTD, with removal of postsynaptic AMPA receptors and loss of spines [158]. There are different forms of LTP and LTD, governed by the release of different neurotransmitters, activation of different postsynaptic receptors and different secondary messengers. However, it is generally accepted that incorporation of receptors at the synaptic membrane make the synapse stronger and more likely to fire, and vice versa [159].

Ca^{2+} ions have a central role in synaptic functioning [160]. In response to an action potential, Ca^{2+} influx at the presynaptic terminal triggers neurotransmitters release. Ca^{2+} entry into the postsynaptic cell controls dendritic excitability, both increases and decreases in synaptic efficacy, and gene expression. Depending on the amount of Ca^{2+} influx and the subsequent signalling cascade activated, either LTP or LTD is induced [161, 162].

1.6.2 Synapses in pathological conditions

Synapses represent the site for signal transmission and integration of subsequent responses, thus central in neuronal circuit communications. Synapse abnormalities are now recognized as the basis of numerous neurological disorders, including those associated with aberrant neural development and neurodegeneration [163]. Pathological conditions also seem to affect different synapse subsets and, possibly, specific brain circuits. For example, a balance between excitation and inhibition is at the basis for proper brain function, and an imbalance may underlie several neurological diseases like autism and schizophrenia. This imbalance could also be the results of a neurodegenerative process, as PD is an example of brain circuits where excitation and inhibition balance is altered [164].

Different mechanisms possibly leading to synaptic dysfunction have been described during neurodegenerative diseases, although many questions remain unresolved. Synapse dysfunction and loss are central events in AD [165]. The number of synapses in the brain decreases during normal aging. However, the synapse-to-neuro ratio is significantly lower in the brain of AD patients compared to age-matched individuals without AD [166]. Synapse loss seems most severe close to Aβ plaques and diminishes with distance from them [167]. Quite the opposite, soluble Aβo seems to be the responsible for synaptotoxicity [168, 169]. However, how Aβ leads to synaptic loss is not clear yet [170]. Possible ways are the Aβ stimulation of a mitochondrial apoptotic pathway, Aβ triggers Ca^{2+} influx, causing excitotoxicity, and stress-related signalling pathways in neurons [171].

Together with Aβ, also for abnormal tau several mechanisms for synaptotoxicity have been described. Animal models of tau pathology show early synaptic loss prior to neuronal death [172]. In human AD brain, the missorting of tau into dendrites represents one of the early signs of neurodegeneration, probably disrupting the actin cytoskeleton and consequently dendritic stability and functions [173, 174]. As for Aβ, soluble oligomeric tau is the species considered responsible for the initial synaptic damage, as these aggregates are small and can travel from one cell to another and pathologically interact with a variety of cell proteins [175]. Tau toxicity can be exerted through different pathways [176]. Moreover, microglial cells and astrocytes seems to play an important role in initiation and progression of tau-associated neurodegeneration [172].

Synapses have been described as the primary site of pathology also in α-synucleinopathies. Small α-synuclein aggregates, oligomers and protofibrils, seem to be present before the larger LB and LN aggregates are deposited [177]. These smaller aggregates are deemed responsible for presynaptic dysfunction, altering vesicle docking and fusion with the presynaptic membrane and consequently neurotransmitters release. This leads to dopaminergic and cholinergic transmission impairment. Other pathogenic events caused by α-synuclein aggregates have been

described [131], of which mitochondrial damage [178] and membrane disruption [179] are of relevance. For both tau oligomers [48] and α-synuclein oligomers [131] a "prion-like" behaviour has been suggested.

1.6.3 Synaptic biomarker landscape

More and more studies have been shown that synaptic degeneration is an early event in AD, and loss of synapses precedes cognitive impairment [180-182]. Synapse loss also correlates better with cognitive decline and the severity of dementia, than NFTs and Aβ plaques [165]. Moreover, synaptic degeneration also appears to be an early sign underlying pathological changes and cognitive decline in other NDs [183-186].

Several synaptic proteins have been investigated in CSF as possible synaptic biomarkers [142]. However, despite the many research efforts none of the biomarker candidates are in use in research setting. New imaging modalities, like the PET tracer [^{11}C]UCB-J targeting the synaptic vesicle protein 2A (SV2A) [187, 188] and PET tracers for AMPA receptors [189, 190] are being developed and now investigated. A combination of fluid and imaging biomarkers will be highly beneficial to the study of synaptic pathology during NDs. However, despite these available tools, we still diagnose the disease based on clinical symptoms and at a time when it is already probably too late to intervene, due to the long prodromal stage and comorbidities. Therefore, a deeper investigation of synaptic biomarkers is desirable, as biomarkers for synaptic activity, dysfunction and/or loss could be highly valuable to detect the neurodegeneration onset and measure its intensity and progression, both in AD and other NDs [191]. Being able to measure changes in synaptic proteins would also shed light onto pathological mechanisms occurring during neurodegeneration, as different synaptic proteins could be affected differently.

1.7 Proteins investigated in this study

1.7.1 Neurogranin

Neurogranin (Ng), named after its granular appearance in immunocytochemical studies [192], is a 78 amino acid (aa) long postsynaptic protein, important for synaptic function and memory formation. In the CNS Ng is abundant in the cerebral cortex, hippocampus and amygdala, whereas is practically absent in the thalamus and cerebellum [193]. In the brain, Ng is expressed in neurons where it localises in distal parts of the dendrites and dendritic spines, but not in inhibitory synapses [194] and glial cells [193]. In the periphery, Ng is expressed at low levels in the lung, spleen, and bone marrow (Human Protein Atlas). High levels of Ng expression have been found in platelets [195].

Ng in humans, rat and mice has a highly conserved amino acid sequence as well as distribution and biochemical properties. Ng presents a central well-conserved region abundant in hydrophobic and basic amino acids, which is referred to as "IQ motif" ($\underline{I}_{33}$QXXX$\underline{RG}$XXX$\underline{R}$XX$\underline{I}_{46}$), essential for binding to calcium-binding protein calmodulin (CaM) and phosphatidic acid (see below). In the IQ motif a serine at position 36 represents a phosphorylation site for protein kinase C (PKC), which is the main kinase responsible for Ng phosphorylation. The region C-terminal to the IQ domain (aa 48-78), mostly consisting of glycines and prolines, represents a collagen-like domain. Outside the IQ domain, human Ng contains three cysteine residues, which can be oxidized by nitric oxide and other oxidants to form intramolecular disulphide bonds. These oxidations attenuate Ng binding affinity for CaM and may represent another alternative mechanism for the regulation of intracellular levels of CaM, other than phosphorylation [196]. Ng in the cell is essentially unstructured, but the IQ domain adopts an α-helical conformation upon its binding with CaM [197].

Ng has an important role in synaptic plasticity, which seems to be put in place through the regulation of CaM availability. CaM is the major calcium-binding protein in eukaryotic cells [198]. In the proposed model, at a resting state Ng binds to calcium-free CaM via its IQ domain. Upon neuronal excitation, activation of NMDA receptors cause a high Ca^{2+} influx into the postsynaptic compartment. This leads to PKC activation, Ng phosphorylation, and consequent release of CaM from Ng-binding. CaM is now free to activate downstream signalling pathways, such as the calcium-calmodulin dependent kinase II (CaMKII), provoking phosphorylation of AMPA receptors with subsequent translocation to the plasma membrane [193] (Fig. 5). More AMPA receptors translocated to the plasma membrane are an indication of synaptic potentiation and LTP, as previously described. On the contrary, if the increase in Ca^{2+} is small, CaM activates another pathway, the CaM-dependent protein phosphatase calcineurin, which in turns regulates LTD [198]. Therefore, Ng phosphorylation/dephosphorylation is of central importance as it allows CaM to be

free to exert its functions, one of which promote LTP [199]. Thus, Ng regulatory mechanisms on the availability of CaM pose the protein as central in balancing LTP and LTD processes [193, 200]. Supportive of this model, reduced Ng concentration in aged mice brain is related to CNS dysfunction [201] and Ng knockdown in mouse models leads to reduced spatial and motor learning and LTP [202]. Conversely, overexpression of Ng resulted in improved cognition and LTP [203-205].

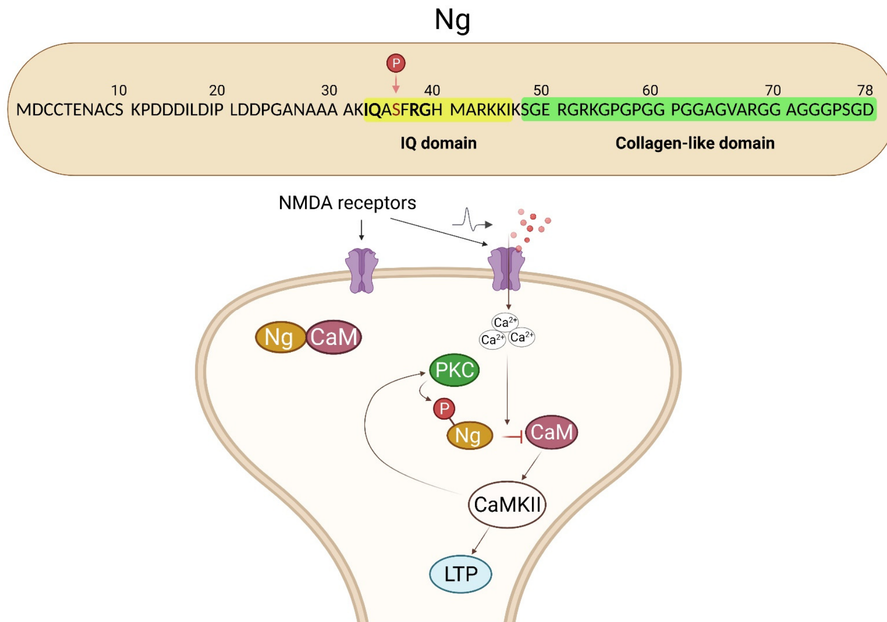

Figure 5. *Ng protein sequence and schematics of Ng mechanisms in the postsynaptic compartment.* Ca^{2+} *entry upon synaptic activation, weakens the affinity of CaM for Ng. Free CaM can activate the CaMKII leading to downstream effects like LTP and increased expression of PKC, which in turn phosphorylate Ng, further preventing its binding to CaM. Created with BioRender.com*

1.7.1.1 Ng as synaptic biomarker

Because of Ng's important role in cognition and memory and its neuronal-specific expression, the protein has been extensively studied as biomarker for synaptic dysfunction and loss. Initial investigations in brain, showed that Ng was decreased in AD as compared to controls [206]. These studies have been subsequently replicated [207]; moreover, the protein has been studied in CSF of various neurodegenerative diseases (reviewed in [142]). Ng appears significantly increased in CSF of AD patients, already at the MCI stage, as compared to controls [208, 209]. This decrease in brain and increase in the CSF could reflect synaptic dysfunction and breakdown. A recent

meta-analysis [210] showed that Ng CSF levels could discriminate between MCI and AD patients from a control population. The constant and apparently specific change of CSF Ng levels in MCI and AD, have led to the proposal of using Ng as new synaptic biomarker for improving diagnosis, and for disease progression monitoring in the AD continuum [209, 211].

Ng is also measurable in blood (plasma) [212, 213], although no difference between AD and control samples has been observed, probably due to Ng expression in the periphery overwhelming CNS-derived Ng in the measurement. A suggested alternative is to measure Ng in blood neuronal-derived exosomes, where the protein levels appear to be decreased in MCI and AD when compared to control [214].

1.7.2 Neuroligins

Neuroligins (Nlgn) are a family of postsynaptic cell adhesion proteins. Human Nlgn is expressed from five genes, with Nlgn-1, -2 and -3 predominantly expressed in the CNS. Nlgn1 is localized at excitatory synapses, while Nlgn2 at inhibitory synapses [215, 216]. Nlgn3 has been found in both [217]. The Nlgn3 and Nlgn4 genes in humans are localized to the X-chromosome. Nlgn4 (or Nlgn4-X) is the least studied of the Nlgn family. In the human brain, Nlgn4 has been found to be predominantly expressed in the cortex, localized in excitatory synapses where it seems to play a role in excitatory synaptic transmission. However, further investigations are needed to clarify its function [218]. The fifth gene encodes for Nlgn4, called Nlgn4-Y (rarely Nlgn5) as it is located on the Y chromosome. Nlgn4-X and Nlgn4-Y have a high sequence homology, thus are often refer to by one name, Nlgn4 [219]. When comparing their sequence, Nlgn1, -3 and 4 are more similar to each other than to Nlgn2. All the proteins are type I transmembrane proteins presenting a large extracellular domain, a helical transmembrane region, and a short cytoplasmic C-terminal domain (CTD). On the extracellular domain, Nlgn2 and -3 are alternatively spliced at single position, referred to splice site A (SS#A), to distinguish it from a second splice site, SS#B, which only occurs in Nlgn1 [220]. Alternative splicing is important for the proteins, as it regulates the strength of binding with the presynaptic counterparts neurexins (NRXN), to regulate their downstream events [221]. The proteins are proteolytically cleaved by metalloproteases at their extracellular domain and by γ-secretase at the CTD. The structure of Nlgns is shown in Fig. 6.

Nlgn1 is the most studied of the Nlgn family. Nlgn1 is a ~94 kDa protein that harbours a large extracellular domain, consisting of 695 residues, which presents six glycosylation and three disulfide bonds. Nlgn1 is important for synapse development and function [222], and both the extracellular parts and the CTD have distinct roles (see below) [223]. The extracellular domain undergoes proteolytic cleavage mediated by ADAM10 [224] or matrix metallopeptidase 9 (MMP9) [225] or probably both, leading to the release of a soluble extracellular fragment. The cleavage seems to

happen in an activity-dependent manner upon synaptic activation, with increased synaptic activity leading to increased cleavage [224-226]. Its cleavage seems to weaken the synapse by decreasing presynaptic transmitters release [227]. Moreover, the cytoplasmic domain of Nlgn1 is phosphorylated at threonine 739 by CaMKII [228] following synaptic activity. This phosphorylation is specific for Nlgn1 and modulates Nlgn1 surface expression, probably rendering the protein more available to protease cleavage [229]. The remaining membrane-tethered C-terminal fragment is subsequently cleaved by γ-secretase, and can successively promote cofilin phosphorylation, thus leading to actin stabilization, linking Nlgn1 to spinogenesis and changes in spine morphology [230]. Additionally, the C-terminal fragment contains a PDZ recognition system [231] which binds to postsynaptic scaffolding proteins like post-synaptic density protein 95 (PSD95) [232], a key scaffolding protein of glutamatergic synapses, important to recruit receptors and channels such as NMDA and AMPA receptors to structure the synapse [233-236]. Moreover, Nlgn1 has been shown to be necessary for LTP [223, 237, 238]. This is reflected in neuroligin-knockout (KO) mouse models showing a decrease in LTP [239], while an increased number of glutamatergic synapses as well as increased synaptic activity are seen upon overexpression of the protein [240, 241].

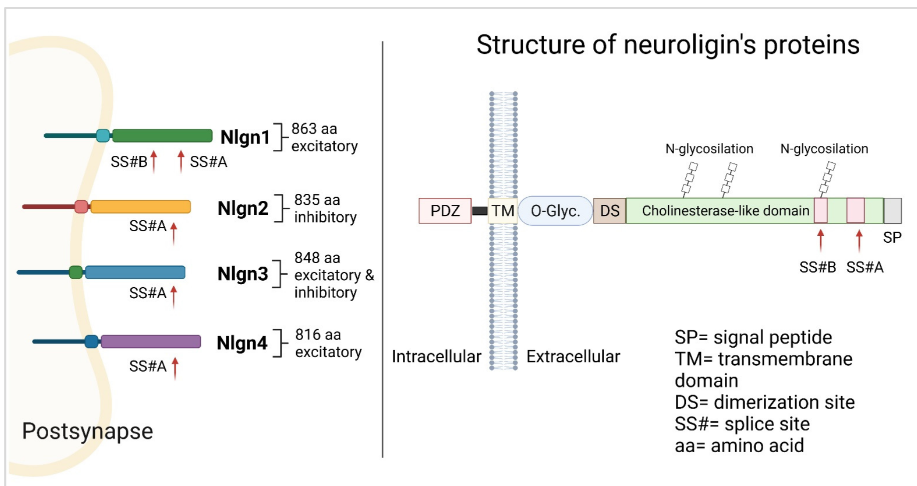

Figure 6. *Structure and location of Nlgn proteins. Created with BioRender.com*

Nlgn2 has a central role in the organization of inhibitory synapses [241]. Experiments using both neuronal cultures and transgenic mice demonstrated that Nlgn2 overexpression selectively increases the function of inhibitory but not excitatory synapses [242, 243] and KO mice showed unaltered synapse number but dysfunctional and altered in postsynaptic component [244]. The C-terminal intracellular domain of Nlgn2 interacts with the postsynaptic complex at GABAergic synapses, which

includes the γ-aminobutyric acid type A (GABAA) receptors, ligand-gated chloride channels that mediate fast inhibitory synaptic transmission, as well as scaffolding proteins like gephyrin and others [245] that serve to anchor GABA receptors and regulate the GABAergic transmission [246]. Nlgn2 is mainly found as homodimer, with no evidence of heterodimers with Nlgn3 [247]. **Nlgn3** can be subject to proteolytic cleavage regulated by PKC signalling in response to synaptic activation. Nlgn3 proteolytic processing also affects Nlgn1 when the latter is found in heterodimers with Nlgn3. Thus, Nlgn3 cleavage can influence Nlgn1 activity and reduce synapse strength [248]. However, little is known on Nlgn3 activity and function.

1.7.3 Neurexins

NRXNs are a family of cell adhesion proteins expressed at the presynaptic site of neural cells. NRXNs exhibit a more complicated domain structure and higher diversity than Nlgns. In humans, three different genes encode for NRXN-1, -2 and 3. These genes present two different promoters, which give rise to a long α-form and a short β-form of the proteins. Further, the proteins present five SS# available for protein modification of α-NRXNs and two SS# for β-NRXNs, which can originate more than a thousand of variants [249]. The extracellular part of α-neurexins contains six LNS (laminin-neurexin-sex hormone binding globulin) domains and three epidermal growth factor-like (EGF) domains. The shorter β-neurexins present an identical CTD with only one LNS domain followed by a unique 37 histidine-rich residue. All NRXNs are N-glycosylated and contain O-glycosylation in the LNS-6, near the transmembrane region [249]. α-NRXNs seem more abundant than β-NRXNs [250]. NRXNs structure is shown in Fig. 7.

Contrary to Nlgns, which only bind to NRXNs, the NRXNs show extracellular binding to other ligands besides Nlgns, and the extensive alternative splicing appears to dictate binding rules [251, 252]. On the cytoplasmic side, NRXNs associate with and recruit synaptic ligands involved in neurotransmitter release. In particular, NRXNs seem needed for the precise clustering of Ca^{2+}-channels necessary for the proper function of the release machinery [253]. After all, NRXNs were discovered as receptors for α-latrotoxin, a neurotoxin from the black widow spider that causes massive neurotransmitter vesicle release from the presynaptic terminals [254]. NRXNs undergo proteolytic processing operated by metalloproteases at the extracellular domain and by γ-secretase at the remaining CTF [255]. Their proteolytic cleavage is activity-dependent and important for their function, although, it is not clear, how they operate at the synapses and if α-NRXNs and β-NRXNs have overlapping or different functions. NRXNs studies are hampered by the many splice variants and the lack of antibodies.

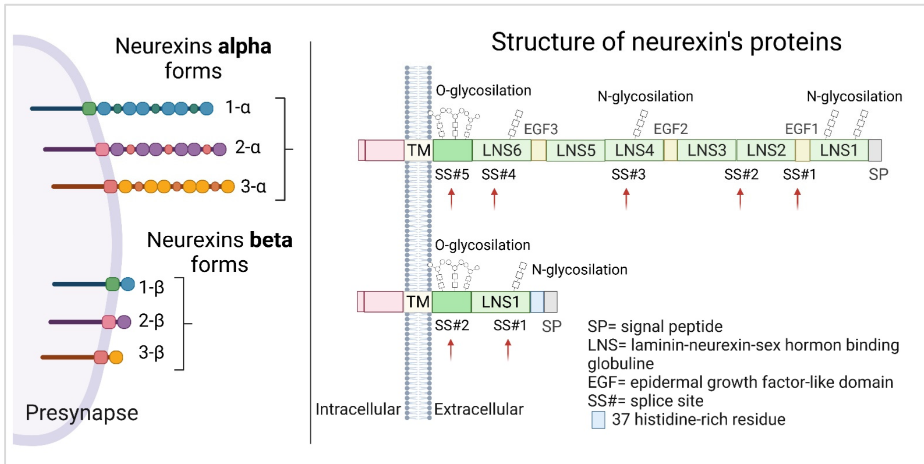

Figure 7. *Molecular diversity of α- and β-NRXN proteins. Created with BioRender.com*

1.7.3.1 NRXNs-Nlgns interaction

Nlgns were initially identified as endogenous ligands of NRXNs [256]. Through their interaction, Nlgns and NRXNs define and stabilize synapses, probably by determining whether a synapse will be excitatory or inhibitory [257]. Alternative splicing regulates their binding and the distribution of Nlgns and NXRNs at the synapse, thus regulating their function. For instance, the presence of the insert in SS#B of Nlgn1 limits the binding of the protein to only β-NRXNs, which lack the insert in SS#4. [258]. The insert of NRXNs in SS#4 promote differentiation of inhibitory synapses, while lack of the SS#4 insert induces differentiation of excitatory synapses, as binding to Nlgn1 is favoured [259]. Nlgns and NRXNs interact across the synaptic cleft in the presence of Ca^{2+}, forming a transsynaptic bridge that stabilises the two terminals of the synapse [260]. Studies of Nlgn-NRXN binding revealed that they form heterotetramers, which involve a Nlgn dimer binding to two NRXNs. The Nlgns can form homodimers, *e.g.*, Nlgn1 binding to Nlgn1, or heterodimers, *e.g.*, Nlgn1 dimerising with Nlgn3. Dimerisation of Nlgns seems to be essential for their synaptogenic activity [261].

1.7.3.2 Functions of NRXNs-Nlgns

Initially NRXNs and Nlgns were deemed essential for synapse formation. Expression of Nlgn1 in non-neuronal cells induces presynaptic differentiation [262, 263], and similarly expression of NRXNs induces postsynaptic differentiation [264]. While these experiments showed that the proteins could initiate synapse formation, Nlgns [222], α- [265] and β-NRXNs [266] triple KO mice models can still form

synapses, although they appear immature and deficient in synaptic transmission. Therefore, although general overexpression of the proteins *in vivo* and *in vitro* [267-269] shows an increase in synapse numbers, subsequent studies revealed that they are not strictly essential for it [257, 270], proposing instead that NRXNs and Nlgns are required for the assembly of a proper functional synapse and are essential for synapse efficacy and plasticity, as previously anticipated.

NRXNs [271] and Nlgns [272] are also expressed in astrocytes, where they control astrocyte morphogenesis interacting with NRXNs on the neuronal synapse, which in turn promotes synaptic growth into mature and active units. Astrocytic NRXN-1α seems required for normal AMPA-receptor-mediated synaptic responses and for LTP [271]. Although these studies are relatively new and much has to be discovered on the function of NRXNs and Nlgns in astrocytes, as previously mentioned, they highlight the close link between astrocytes and synapses during synapse formation and development.

1.7.3.3 NRXNs-Nlgns in pathology

Nlgns and NRXNs have been connected to neurodegenerative and neurological disorders. Nlgn1 has been shown as target of Aβo both *in vivo* and *in vitro*. Interaction with Aβo has also been shown for NRXNs [273, 274]. Aβo interfering with the Nlgn-NRXN interaction is a proposed mechanism for synaptic dysfunction in AD [275, 276]. Additionally, a recent study [277] showed a reduction of Nlgn1 protein in hippocampus of AD patients, which was modulated by Aβ load. Moreover, a genetic study in rodents showed that Aβ fibrils induce epigenetic alteration in *NLGN1* promoter region, altering *NLGN1* gene transcription and impairing glutamatergic synapses and memory in the brain [278].

Mutations in the *NLGN2* gene have been found in schizophrenia [279], while mutations in the *NLGN3* [280], *NLGN4* [281] genes have been connected to autism spectrum disorders. Nlgn2 has also been shown decreased in CSF of prodromal AD and AD patients [282].

Gene mutations of NRXNs have also been connected to autism and schizophrenia [283]. NRXN-1α, NRXN-2α and NRXN-3α proteins have been found altered in CSF samples of MCI and AD patients [282, 284].

NRXNs and Nlgns are a class of cell adhesion proteins primarily involved in synaptic function whose activity and regulatory mechanisms are highly interconnected. However, their mechanisms of action at the synapse and their implication in diseases and pathological processes need further investigation.

2 AIM

2.1 General aim

The overall aim of this PhD thesis was to investigate changes in concentration and fragmentation pattern of synaptic proteins during neurodegenerative diseases, particularly AD, both in brain tissues and CSF. Additionally, their usefulness as candidate biomarkers to monitor synaptic dysfunction in AD is described. The focus was on the postsynaptic spine compartment of glutamatergic synapses, which is a particularly vulnerable and pathophysiologically relevant neuronal structure. The study primarily focused on Ng and Nlgn1, and subsequently expanded to the other components of the Nlgn family and the transsynaptic binding partners NRXNs.

2.2 Specific aims

1. To identify the enzymes responsible for Ng processing, leading to the C-terminal fragments increased in CSF of AD patients

2. To identify the molecular forms of Ng in CSF and to determine the abundance of C-terminal peptides to total-full-length Ng in CSF

3. To identify changes in Nlgn1 levels in brain of neurodegenerative diseases and study the protein in CSF of AD and control subjects, to evaluate its synaptic biomarker’s utility

4. To develop a targeted method for the simultaneous quantification of Nlgns and NRXNs in CSF, in order to assess protein changes during neurodegenerative diseases

3 MATERIALS

3.1 Ethical approval

All the studies conducted in this thesis involving human and animal samples were performed in accordance with the declaration of Helsinki, the current European Law (Directive 2010/63/EU), as well as the local ethical review board and the guidelines at the University of Gothenburg or the guidelines at the university or facility where the samples were collected or the experiments conducted (for the different ethical approvals, please refer to the respective papers).

The following general rules applied: the lowest possible sample volumes were used. Samples were coded and patients' information were only available to authorized people. All the results are reported as a group study and not as individual test results.

3.2 Samples used in this thesis

My research projects entailed work with mainly human samples, such as CSF and brain tissue. In **paper I,** brain samples from wild type mice were also used in accordance with the Swedish Animal Welfare Agency rules.

3.2.1 Human brain samples

In-house de-identified human brain samples were used in **paper III**, study one, in compliance to the ethical declaration from the Nederlands Brain Bank, which can be found online at https://www.brainbank.nl/media/uploads/file/Ethical-declaration.pdf. In the same paper, human brain samples for study two and three were obtained from the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology, University College London. All the subjects signed an informed consent to donate their brain for scientific research after death. The brains were collected as fast as possible and, once at the brain bank, they were divided into the two hemispheres; generally, one hemisphere is fixed and used for neuropathological diagnosis and immunohistochemistry and the other one is used for sample collection. In **paper II**, pooled TBS fractions of the same brain materials were utilised in the study.

3.2.2 Cerebrospinal fluid

CSF sample pools, often termed quality controls (QC), were prepared and used for protein analysis and assay validation in **paper II** and **IV**, respectively. To create CSF pools, samples from our clinical routine Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, were used; the patients underwent the lumbar puncture as a routine analysis at the geriatric clinic. After analysis, samples were stored

for two months in case there were a need for repeated analysis or further investigations. After this period, only de-identified leftover samples were used, following a procedure approved by the Ethics Committee at the University of Gothenburg (EPN 140811). In **paper II** and **IV** small cohorts of anonymized clinical samples, biochemically defined AD and non-AD controls, were obtained from the Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, and utilised in the studies. In **paper III**, CSF samples were obtained through our collaboration with the Department of Clinical Sciences Malmö, Lund University and were part of the Swedish BioFINDER study (http://biofinder.se/). In **paper IV**, the CSF samples were collected at the Cognitive Neurology Center, Fernand Widal Lariboisière University Hospital, Paris, from patients visiting because of a cognitive complaint. The Bichat Hospital Ethics Committee of Paris Diderot University approved the study.

4 METHODOLOGY

4.1 Brain protein extraction

Brain protein extraction is a multi-step procedure by which proteins are extracted from different cell compartments based on their solubility in the extraction buffer. The tissue is homogenized by disruption, such as cutting and smashing, usually carried out in the extraction buffer of interest. Different buffers are used depending on the nature of the protein, and whether it is located extracellularly, intracellularly of whether it is membrane-bound. If soluble extracellular proteins are the target, then hydrophilic buffers, such as Tris-buffered saline (TBS), are used. If the proteins of interest are membrane-bound, then detergents that help breaking the attachment to the phospholipid cellular membrane need to be added (*e.g.*, Triton-X 100). The buffer often contains protease inhibitors to prevent protein degradation and loss due to enzymatic digestion. Centrifugation, carried out at different rates and time, is then needed to remove undissolved pieces of tissue and organelles. The supernatant, containing the proteins of interest is aliquoted and stored at – 80 °C prior to analysis.

In this thesis, soluble TBS extract containing protease inhibitors was chosen for the study of the proteins of interest, with the assumption that it contains many of the soluble proteins also found in CSF.

4.2 CSF sampling

CSF was sampled by lumbar puncture according to standardized procedures [15]. Lumbar puncture was performed between the L3/L4 or the L4/L5 vertebral interspace and the CSF was collected in polypropylene tubes (Fig. 8). CSF samples were centrifuged at 2000 × g for 20 min at + 4 °C to remove cell debris and the supernatant was subsequently aliquoted in new tubes and stored at – 80 °C pending analysis.

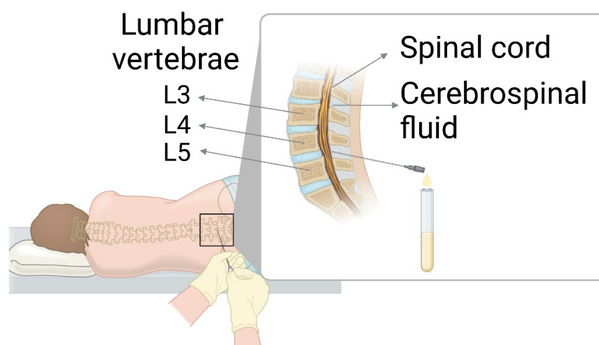

Figure 8. *Lumbar puncture procedure. Created with BioRender.com*

4.3 Antibody-based assays

4.3.1 Gel protein electrophoresis and western blot

Electrophoresis is defined as "the transport of charged molecules through a solvent by an electric field". Gel electrophoresis can be used as a purification technique prior to mass spectrometry (MS) analysis or can be the first step for a western blot.

During gel **electrophoresis**, the sample is loaded into a solidified gel forming a porous matrix in where proteins migrate and separate. Typically, polyacrylamide gel electrophoresis (PAGE) is used. Gels are made with different acrylamide concentration, depending on the size of the target protein. In the gel, larger proteins migrate slower due to obstruction by the gel matrix, while smaller proteins migrate faster; thus from the top to the bottom of the gel we find proteins from higher to smaller molecular weight, respectively. A ladder or molecular weight marker is loaded in a separate lane, thus the molecular weight of the protein of interest can be estimated. The electrophoresis can be performed under denaturing or non-denaturing conditions, the latter also called native gel. Denaturing gel electrophoresis is often used for protein size determination, whereas native gel electrophoresis is usually used to identify protein complexes or enzyme activity. A typical denaturing agent is sodium dodecyl sulphate (SDS) which disrupts protein's tertiary structure and charges proteins and peptides negatively. By the application of an electrical field, proteins in the gel migrate from anode (-) to cathode (+).

Proteins separated in the gel can be directly visualized by staining the gel by, *e.g.*, Coomassie blue stain, or Silver stain. Subsequently, stained bands of interest can be excised, and **in-gel digestion** can be performed during sample preparation for mass spectrometric identification of proteins. This technique comprises several steps performed on the proteins contained in the gel pieces, such as destaining, reduction and alkylation, proteolytic cleavage and, finally, extraction of the generated peptides. For a more detailed description of protein digestion, see 4.6.2.

Western blot is a widely used technique in molecular biology, which allows the separation and detection of proteins from a complex mixture. Western blot requires three main steps: electrophoresis, protein transfer, incubation with antibodies and detection. After electrophoresis, proteins are transferred from the gel to a solid membrane, to which they bind by adsorption or can be additionally fixed by paraformaldehyde. The membrane is then blocked to prevent unspecific binding from the primary antibody. The membrane is then incubated, typically with a primary and a secondary antibody. The primary antibody specifically recognizes proteins of interest. The secondary antibody binds to the primary antibody and is usually labelled with a molecule for generation of a detection signal, for instance horseradish peroxidase (HRP). HRP can react with a substrate (luminol) leading to a chemiluminescent

reaction with release of energy in form of light. The emitted light can be captured by a charge-couple device (CCD) camera or on an X-ray film. ImageJ (freely available software at https://imagej.nih.gov/ij/download.html), is used for quantitation via image analysis of the detected bands. In the past, western blot was mainly used as qualitative technique. However, band signal intensity is, to a certain extent, proportional to protein concentration and therefore western blot data can be interpreted as semi-quantitative. The high sensitivity of western blot is one of the main advantages of this technique, as down to 0.1 ng of a protein can be detected. Moreover, it provides visual information of full-length and possible fragments of the protein. On the other hand, off target bindings of the antibody might lead to false-positive results. Samples can be loaded neat, like CSF or brain homogenate, or can be first enriched using, for instance, immunoprecipitation.

In **paper I**, western blots are used for Ng fragments detection after *in vitro* digestion with calpain enzyme. A native gel overlay with enzyme substrate is also performed for the identification of enzymatic activity. In **paper II**, western blots under denaturing and non-denaturing conditions were used to study the different forms of Ng in CSF. In **paper III**, western blot is the method used for Nlgn1 quantification in human brain samples and CSF. In-gel digestion followed by MS is also used as an antibody-free method to confirm the presence of the Nlgn1 protein and the specificity of the antibodies in the human brain homogenate and CSF samples.

4.3.2 Enzyme-linked immunosorbent assay

ELISA is an immunoassay that relies on antibodies to detect a target antigen using the specificity of their interaction. There are different types of ELISA, direct, indirect, competitive, but the most used is the so-called sandwich format (Fig. 9). In this setting, an antigen is captured between two different antibodies. Having two different antibodies targeting the antigen increases the specificity of the assay. The assay is typically performed in a 96 well plate. The so-called capture antibody is immobilized on the surface of the wells of the plate. Then the remaining binding sites of the wells are blocked with blocking solutions (high concentrations of proteins, such as bovine serum albumin (BSA) or milk proteins). The capture antibody is then exposed to the target protein, capturing it. A second antibody, so-called detection antibody, is added for the recognition of the antigen. In a sandwich ELISA system, either monoclonal or polyclonal antibodies can be used as capture and detection antibody. Monoclonal antibodies recognize a single epitope and are generally preferred as capture antibody inasmuch they increase the specificity of the assay and are typically available in purified form, facilitating immobilisation. A polyclonal antibody recognizes different epitopes on the same antigen and it is often used as the capture antibody when the aim is to pull down as much of the antigen as possible. Typically, the two antibodies have

a different epitope on the antigen. Between each step, the plate is washed with a mild solution to remove any excess of proteins or antibodies non-specifically bound.

To detect the antibody-antigen complex, different strategies can be used. To reduce background signal, the capture antibody is often directly conjugated with a molecule, such as biotin, which strongly reacts with streptavidin-conjugated HRP added subsequently. A chromogenic substrate, *i.e.* 3,3' ,5,5'-tetramethylbenzidine (TMB), is then added, on which the HRP enzyme acts, producing a quantifiable colour change. The intensity of the colour produced in a given time period is proportional to the amount of HRP bound, hence to the target analyte concentration. The reaction is stopped by adding an acidic solution (sulphuric acid) which lowers the pH inactivating the HRP enzyme and changes the colour to yellow. The colorimetric reaction is measured and quantified using an absorbance spectrophotometer.

An in-house sandwich ELISA was used in **paper II** for detection of Ng peptides before and after immunodepletion of individual CSF samples. Moreover, core AD biomarkers were measured with commercially available ELISA kits from INNOTEST (**paper II**) or EUROIMMUN (**paper III**) according to the manufacturer's instructions.

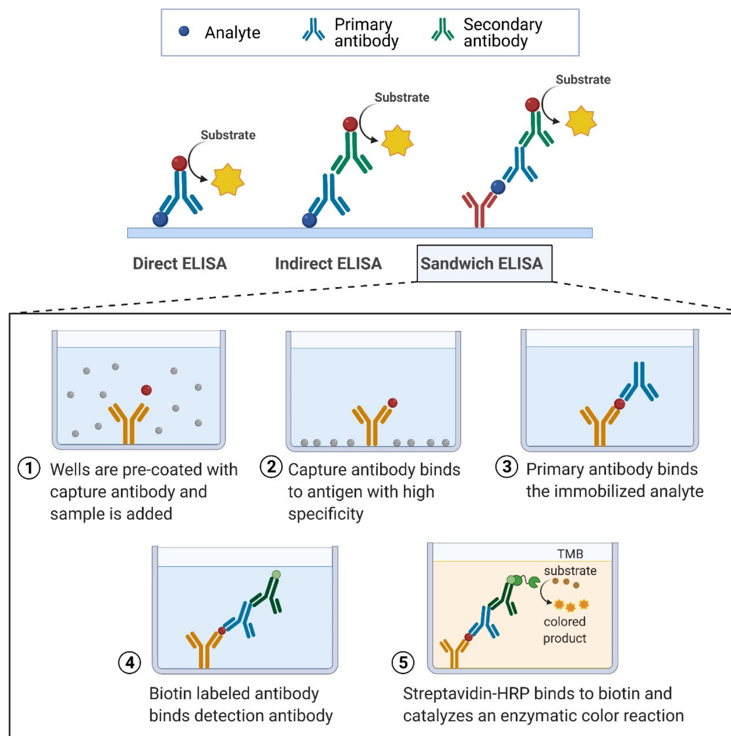

Figure 9. *Schematic of the different ELISA methods with focus on the sandwich format. Created with BioRender.com*

4.3.3 Immunoprecipitation

IP is an immunomethod used to purify and enrich an analyte from a complex matrix, such as CSF or brain homogenates. Thus, IP is often viewed as a pre-purification step. There are many different IP protocols. The one described here refers to the experimental setting used in the studies included in this thesis. First, the antibody is coupled with a solid support represented by small spherical magnetic beads, pre-coated with a species-specific IgG antibody. A capture antibody of the same species is then immobilized and, optionally, covalently bound to the beads. Free binding sites on the beads, not occupied by the immobilized antibody, are then removed in a blocking step. Subsequently, the blocked antibody beads are incubated with the sample, typically overnight at +4 °C for antigen recognition. The day after, a series of washes is performed before elution of the analyte from the beads-coupled antibody. Elution can be performed using different elution buffers. If the IP is followed by western blot analysis, the captured peptides/proteins of the sample is eluted with sample buffer and heated at 70 °C for 10 minutes. If the IP is followed by MS analysis, the sample is eluted with a formic acid solution and dried overnight in a vacuum centrifuge ("speedvac"). During the different steps, the magnetic antibody beads are retained and separated from the sample liquid with the help of a magnet. A schematic of IP steps is shown in Fig. 10. IP is used in **paper II** to immunoprecipitate Ng and its peptides from CSF.

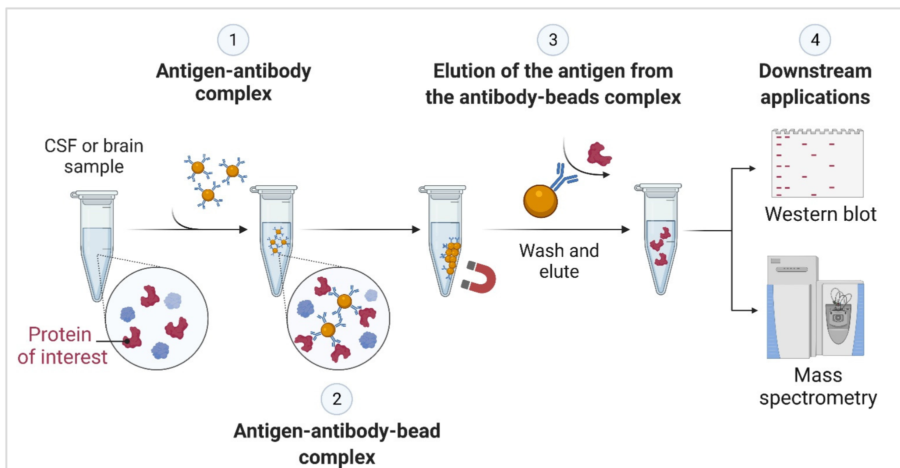

Figure 10. *Schematic of the IP procedure. 1) The beads are coupled with the antibody, 2) the antibody-coupled beads are incubated with the sample for antigen binding, 3) washing steps separate the beads from the immunodepleted sample. Subsequently the antigen is eluted from the antibody-beads complex, 4) the purified analyte is ready for downstream application. Created with BioRender.com*

4.4 FRET technology

Förster Resonance Energy Transfer (FRET), named after the German scientist, is a physical phenomenon of energy transfer between two light-sensitive molecules, called chromophores. The energy transferred is non-radioactive and when the chromophores are fluorescent, we refer to it as Fluorescence Resonance Energy Transfer. FRET can be utilised in an assay system composed of a donor fluorophore and an acceptor, separated by a short distance from each other. The donor fluorophore is put into an excited electronic state via an exciting light beam and transferring its energy to the acceptor. In the assay we used in paper I, the acceptor did not transmit emission further, but quenched it, resulting in no signal when FRET occurred. Therefore, in this distance-dependent physical process, when donor and acceptor are in close proximity (1-10 nm), no emission is observed. When donor and acceptor are brought apart, the excited fluorophore is no longer quenched and an increase in fluorescence occurs, which is visible in the UV or visible spectrum (Fig. 11). To study enzymatic cleavage, quenched FRET peptides were designed. These peptides present a donor and a quenching group (acceptor) on either side of the peptide sequence containing a protease cleavage site. When an enzyme cleaves the FRET-peptide into two fragments, fluorescence is no longer quenched and is emitted, proportional to the amount of peptide hydrolysed. With this technique, enzyme processing, both proteolytic and inhibitive activity, *in vivo* can be studied. This technology has been used in **paper I** to identify Ng peptide cleaving enzymes and to study cleavage sites along the protein sequence.

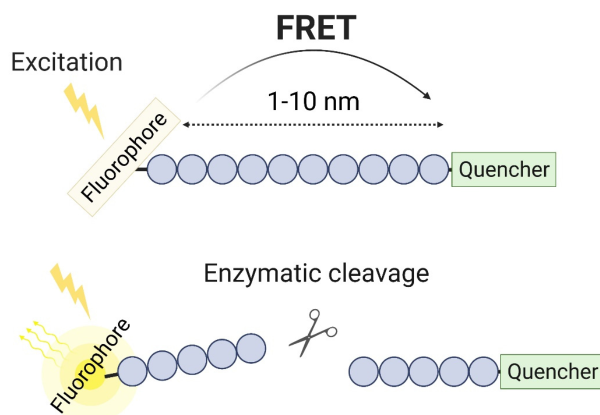

Figure 11. *FRET technology applied to peptides. Enzymatic cleavage can be detected by emission of a fluorescent signal, which occurs when the fluorophore and the quencher are no longer in close proximity. Created with BioRender.com*

4.5 Chromatography

Chromatography is defined as the process of separation of individual components of a mixture, based on their relative affinities towards stationary and mobile phases. Typically, the sample is flowing through a stationary phase, carried by a mobile phase. In size exclusion chromatography (SEC) molecules in solution are separated according to their size, with largest molecules eluted first and smaller molecules eluted last. The column is packed with fine, porous beads which can be of different nature, such as consisting of agarose, or polyacrylamide. The pore sizes of these beads determine the optimal separation range of the macromolecules. Advantages of this method are separation of molecules at native conditions (non-denaturing buffers) which does not change the biological activity of the proteins being separated. Moreover, sample loss can be minimal if binding of the solute to the stationary phase can be kept small. On the other hand, the initial sample volume is diluted in a larger volume of final eluate. The resolution of SEC is often less than with other chromatographic techniques (*e.g.*, ion-exchange, reverse-phase (RP) chromatography). Thus, the technique is generally combined with others that further separate molecules by other characteristics, such as pH, charge, and affinity for certain compounds.

In ion-exchange chromatography, the separation is based on the charge of the protein in the separating buffer. At the isoelectric point (pI) the protein charge is zero. Bringing the pH above and below the pI will charge the proteins negatively and positively, respectively. In an anion-exchange column, the column is packed with positively charged resin that will bind negatively charged protein at high pI. Then proteins can be eluted in two ways; the salt concentration in the elution buffer is gradually increased, or the pH of the solution is gradually decreased. In the first way, the negative ions in the salt solution compete in the binding to the resin. In the second way, proteins gradually become positively charged, thus detaching from the resin. The tightness of the binding between the molecules and the resin is proportional to the strength of the negative charge of the substance.

Ion exchange chromatography and SEC are used in **paper I** to prepare enzyme-enriched fractions from mouse brain extract to use in the FRET assay. SEC is also used in **paper II** to fractionate the different Ng species present in CSF.

4.6 Mass-spectrometry based proteomics

The word proteomics refers to the large-scale study of the proteome, the entire set of proteins expressed and modified by a cell, tissue, or organism. Proteomics usually refers to studies of proteins carried out with the use of MS and database searching. Proteomic studies include investigation of protein modifications, interactions and localization, key elements of protein functions, and is of high importance for the understanding of molecular basis of cellular processes in both heath and disease. This in turn is a starting point for identification of molecular targets, drug development and biomarker discovery, which represent some of the applications of proteomics. Proteomic studies of the proteome of synapses have represented a turning point in elucidating synapse complexity and diversity [285].

4.6.1 Sample preparation

Sample preparation prior to MS analysis is a critical step, which has to be chosen depending on the biological matrix in use and the abundance and chemical characteristics of the proteins of interest. One aim is to reduce the complexity of the sample, as high abundant proteins can mask the signal of low abundant ones, and increase the sensitivity of the analysis. This, combined with chromatographic separation techniques, offers highly sensitive and reliable measurement of the protein(s) of interest, which can reach the attomolar range [286]. At the same time, every step added before the actual analysis might results in loss of analyte and also increase variability. As mentioned previously, gel electrophoresis and IP are two techniques that can be used to purify the target proteins from the matrix and are often used in combination with MS analysis. However, IP relies on the availability of antibodies and is not the ideal choice when targeting many proteins.

4.6.2 Protein digestion

Protein digestion refers to the process of cutting down proteins into smaller pieces, peptides, which size is more suitable for the MS analysis. Protein digestion can be achieved using different proteolytic enzymes, among which trypsin is the most commonly used. Trypsin is a serine protease which hydrolyses proteins at the C-terminal side of the amino acid lysine (K) and arginine (R), except when they bound C-terminally to a proline. Trypsinated peptides always contain a K or an R at their ends, which are easy to protonate, facilitating MS analysis. Trypsin has an optimal operating temperature of 37 °C, but the optimal time of the trypsination can vary. As trypsin typically requires a pH close to neutral to be active; the reaction is stopped by acidification with acidic buffers (*e.g.*, formic acid). Trypsin is often modified to avoid auto-digestion, and associated to other proteases, as for example Lys-C, to increase

proteolytic efficiency on the C-terminal side of lysine. These strategies aim to reduce the degree of the so-called missed cleavages, yielding peptides that are not fully digested. If the protein of interest contains disulfide bonds (S-S), the samples are typically reduced and alkylated prior to trypsination.

Protein digestion can be performed in-gel (as mentioned previously 4.3.1) or in-solution. When performed in-gel, the proteins are in the gel matrix. To make them accessible to the trypsin, the gel is first cut into pieces and dried. Subsequently the gel pieces are re-hydrated with the digestion buffer containing trypsin. The gel pieces swell by absorbing the buffer and trypsin diffuses into the gel matrix.

In this thesis, trypsination was carried out overnight (≈ 14-16 h) both in-gel (**paper I-III**) and in-solution (**paper II-IV**) settings.

4.6.3 Solid phase extraction

Solid phase extraction (SPE) is a chromatographic technique where solid particles (stationary phase), usually packed in a cartridge-type devise, are used to chemically separate the components of the samples, which elute with the mobile phase. Depending on the material used for the stationary phase, ion exchange, reverse phase or mixed-mode extractions can be performed.

In **paper IV**, the Oasis-HLB 96-well μElution Plate, 2 mg Sorbent per Well, 30 μm (Waters Co., Milford, MA, USA) was used. This format employs the most commonly used reverse-phase (RP) extraction setting, where the stationary phase sorbent consists of a non-polar packing material and the sample is loaded and eluted in a polar liquid phase, *e.g.*, water and methanol. In this setting, the packing material consists of special hydrophilic-lipophilic balanced (HLB) polymers which offers the advantage of being water-wettable, suitable to retain a variety of compounds and not drying out. SPE can be performed using an extraction manifold (vacuum station) which allows the simultaneous preparation of many samples, in four different steps; 1) the cartridge is conditioned with methanol, and then equilibrated with water, 2) samples are loaded, usually in a polar solvent, and adsorbed onto the stationary phase 3) samples are washed with water to flush away unwanted compounds like salt, sugars, and impurities 4) samples are eluted with a less polar solvent, *i.e.*, methanol. SPE is mainly used for cleaning up the samples from salt and detergents, which leads to sample purification and reduction of sample matrix, with the final aim of increasing the performance of the MS analysis.

4.6.4 Liquid chromatography

LC, specifically high-performance liquid chromatography (HPLC), is a chromatographic technique that can be coupled to MS to facilitate analysis of complex samples. HPLC differs from the previously described chromatography techniques in using high-pressure (50-400 bar) to pass the mobile phase through the column. Ultrahigh-performance LC (UHPLC) columns also exists, which are typically packed with smaller adsorbent particles and can reach higher pressure (over 1000 bar). These characteristics increase resolving power (the capability to distinguish between compounds), throughput and sensitivity when separating complex mixtures.

As previously described, RP chromatography is the most commonly used approach. Molecules are injected and carried by the mobile phase onto the stationary phase where they get separated based on their polarity, structural characteristics and interaction with the two phases. The stationary phase is made non-polar by the addition of carbon chains (*e.g.*, C8, C18) covalently bound to the surface of silica particles. The most hydrophilic molecules will be eluted first, and the most hydrophobic ones will interact more strongly with the hydrophobic stationary phase which retains them. Elution of these molecules is achieved by the addition of organic (less polar) solvent, *e.g.*, acetonitrile or methanol, to the aqueous mobile phase. If the composition of the mobile phase changes over time during separation, we have a gradient elution system. The use of a gradient represents an efficient way to separate mix of molecular compounds still using a minimal volume, which in turns increases the instrument loading capacity. The mobile phase is pumped at a steady rate, typically using nanoflow or microflow in proteomics analyses. Nanoflow (nanoliter per minute) offers high sensitivity and it is generally used in exploratory studies, inasmuch the time of analysis is longer. Microflow (microliter per minute) is more suitable for high-throughput targeted purposes because faster and more robust, although less sensitive. LC can be performed separately (off-line) or can be directly connected to the mass spectrometer, *i.e.*, on-line.

Microflow RP-HPLC has been used in **paper IV**. Different gradient settings were tested to optimize peptide separation; a broken gradient utilizing increasing amount of acetonitrile, from 0 to 40%, was judged to be best.

4.6.5 Mass spectrometry

MS is a sensitive analytical technique used to separate and detect molecules contained in a sample based on their molecular mass. In a general MS workflow, molecules are brought from a solid or liquid phase into the gas phase, ionized and separated based on their mass-to-charge (*m/z*) ratio and finally detected. MS can be

used for identification and quantification of a variety of molecules, to study PTMs and molecular structural characteristics. To achieve this, a mass spectrometer is composed of three major parts: the ion source, the mass analyser and the detector. Different technologies are used and combined in a mass spectrometer. In this thesis, a Q Exactive instrument was used in **paper I**, **II**, and **IV**, while an Orbitrap Fusion Tribrid mass spectrometer (both from Thermo Fisher Scientific Inc.) was used in **paper I** and **III**. Both these mass spectrometers are so-called hybrid instruments consisting of more than one type of analyser (see 4.6.5.4).

4.6.5.1 Ion source: electrospray ionization

In the ion source, molecules are transferred to gas-phase ions. Electrospray ionization (ESI) offers the advantage of being a soft, non-destructive ionization technique, with very little molecular degradation occurring, preserving molecule characteristics like PTMs and, under special conditions, protein complexes. Moreover, it can be directly coupled to an LC-system, since they both use liquid samples.

In ESI, the sample is dissolved into a volatile, polar solvent, which is flowing through a capillary needle. Under the application of a high voltage between the needle and the nozzle, the inlet to the mass spectrometer, the liquid emerging from the needle assumes a conical shape called Taylor cone. Charged droplets are detaching from the tip of the Taylor cone due to the strong electric field. At the same time, a warm flow of nitrogen gas surrounding the needle further assists desolvation and droplet shrinkage. The nozzle is also heated, to further facilitate the formation of single ions in gas-phase [287]. However, the exact mechanisms from charged droplets to ion formation is not entirely understood yet. ESI can be run in positive or negative ion mode, where molecules are charged positively or negatively. In positive ionization mode formic or acetic acid are often added in the solvent to aid protonation of the analyte molecules.

4.6.5.2 Mass analysers

Ions created in the ion source are then transported to the mass analyser, a device able to separate ions according to their m/z. In a Q Exactive instrument both a quadrupole and an orbitrap mass analyser are employed.

A quadrupole consists of four rods with hyperbolic inner surfaces. Opposite rods are connected in pairs so that they have the same potential, while the other pair have the opposite potential with the same magnitude, thus creating a quadrupolar field. The quadrupole uses a combination of direct-current (DC) and radio frequency (RF) potentials to filter the ions entering from the source. At given values of DC and RF only ions with a particular m/z range will have a stable trajectory and travel parallel to

the rods, and finally pass through the analyser. Ions that have different *m/z* will not have a stable trajectory and will collide with the rods or exit between them, thus being filtered out (Fig. 12 A). Quadrupole mass analysers have good sensitivity and high dynamic range but comparatively low mass accuracy and mass resolution.

The orbitrap is an ion trap comprising one outer and one central electrode, which are barrel-shaped (Fig. 12 B). Utilizing an electrostatic field between the inner and outer electrodes, ions that enter the orbitrap start to oscillate around the central electrode, and at the same time they also oscillate in the axial direction, along the electrode axes (Fig. 12 B). Different ions oscillate at different frequencies, which are inversely proportional to the square root of their *m/z*. Compared with a quadrupole, the orbitrap provides lower capacity and sensitivity, but it offers very high resolution and mass accuracy.

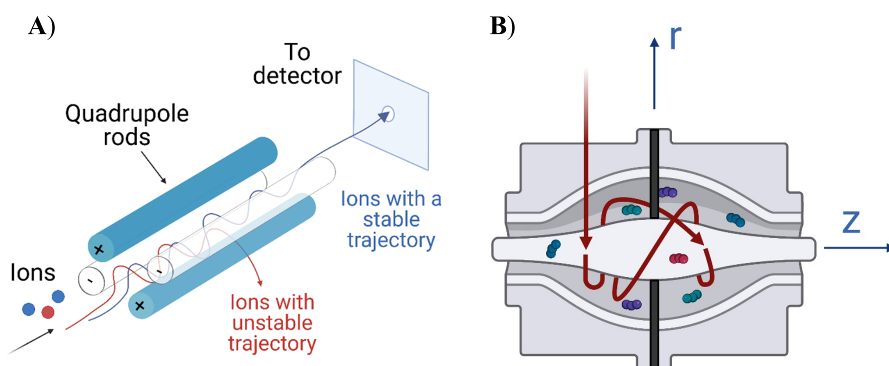

Figure 12. *Schematic representation of **a**) quadrupole and **b**) an orbitrap. Created with BioRender.com*

4.6.5.3 Detector

In the orbitrap, the signal is recorded as image current (or image charge). The outer electrode is split in two, separated by an insulator, and is also used as detector. The ions induce a small current at every passage close to one of the outer electrodes. The image current is then amplified and transformed to a frequency spectrum using Fourier transformation. Then mass spectra can be obtained using the relationship between frequency and *m/z*.

4.6.5.4 Hybrid quadrupole/orbitrap technology

In a hybrid instrument different types of mass analyser are combined. The Q Exactive hybrid instrument (Thermo Fisher Scientific) is represented in Fig. 13. This

technology combines a quadrupole and an orbitrap to obtain a highly accurate and efficient instrument.

Mass spectrometers can be operated to acquire "full-mass" scans (MS), which means the instrument records mass spectra for all analytes (precursors) in the set, generally wide, mass range. Full scan analysis can detect a large number of peptides, but the identification ability is very limited, as two peptides with very similar mass cannot be distinguished. Therefore, full mass scans are normally accompanied by tandem mass analysis (MS/MS), where selected precursor peptides are fragmented to produce ions that can be used to identify the precursor. This is of high importance as the fragmentation pattern of two peptides with very similar mass is in most cases different and can be used to distinguish between the two. MS/MS thus allows obtaining structural information and identification of proteins.

MS/MS analysis is achieved in a Q Exactive instrument using the quadrupole (**1**) as a mass filter for selected precursor ions based on their *m/z* (Fig. 13). The ions are then sent to the so-called higher-energy collisional dissociation (HCD) cell (**2**) where they collide with nitrogen gas molecules causing them to be excited, finally leading them to fall apart into fragment ions. Subsequently, these fragment ions are collected in the C-trap (**3**) and from here injected into the orbitrap (**4**) for analysis and detection [288]. Thus, with this setting, selected ions in the quadrupole (MS) are further fragmented (MS/MS) to get structural information and identify peptides by database searching. Finally, to achieve protein identification, mass spectra from MS/MS are matched against databases containing all human protein sequences, where different probabilistic scoring algorithm (*e.g.*, Mascot) are used for protein identification.

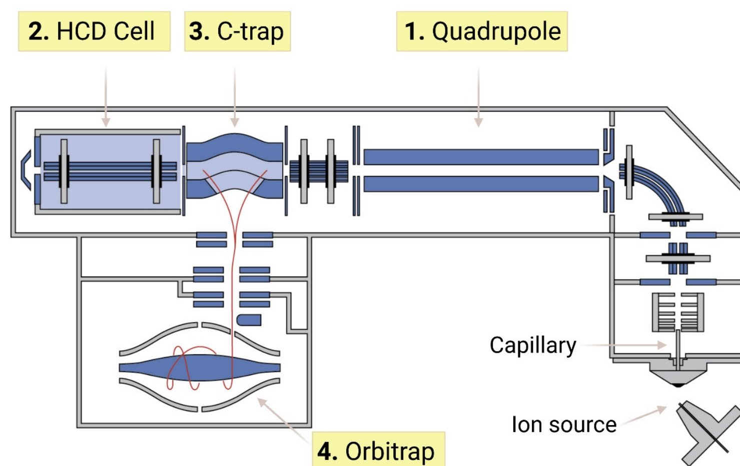

Figure 13. *Schematic of the Q Exactive hybrid instrument. With the courtesy of Thermo Fisher Scientific.*

For exploratory studies, so-called data-dependent acquisition (DDA) is generally used. In DDA, the selection is based on which ions are above the set intensity threshold, thus only those ions will be further selected for MS/MS analysis. Nevertheless, DDA often provides the key information prerequisite to build following targeted experiments. When operated in targeted mode, the instrument only acquires mass spectra of the selected masses of interest. Targeting only the ions of interest increases sensitivity and quantitative performance, thus this mode is favourable for quantitative, hypothesis-driven studies.

4.6.5.5 Parallel reaction monitoring

PRM is a targeted mass spectrometric method where precursor ions of interest are selected for fragmentation. In PRM, all fragments from the precursor ion are monitored simultaneously, *i.e.*, in parallel, without the need of previous selection of which fragment ions to monitor (Fig. 14). PRM assays are usually performed using a hybrid instrument, combining the narrow isolation of precursor ions in the quadrupole and the high resolution of, for example, the orbitrap, which enables distinction of ions with similar *m/z*. Thus, this method offers high dynamic range, relatively high sensitivity and very high specificity [289].

In order to quantify proteins or peptides of interest in targeted MS assays, stable isotope-labelled peptides can be used. These peptides are enriched with ^{13}C and ^{15}N that causes a shift in their mass but the chemical characteristic will be identical to their endogenous counterpart. Therefore, these heavy-labelled peptides will co-elute and ionize the same way as the endogenous (light) ones. Tryptic peptides C-terminally $^{13}C/^{15}N$ labelled at lysine or arginine were used in **paper IV**. Skyline, a quantitative analysis software (MacCoss Lab Software), was then used for peak area integration and quantification of the identified peptides. The software allows for manual inspections of the peak and refinement of the transitions from the precursor ions. The peptide quantification is based on the ratio of the summed signal from the selected fragments of the unlabelled endogenous peptides over the signal of the heavy-labelled standards [290].

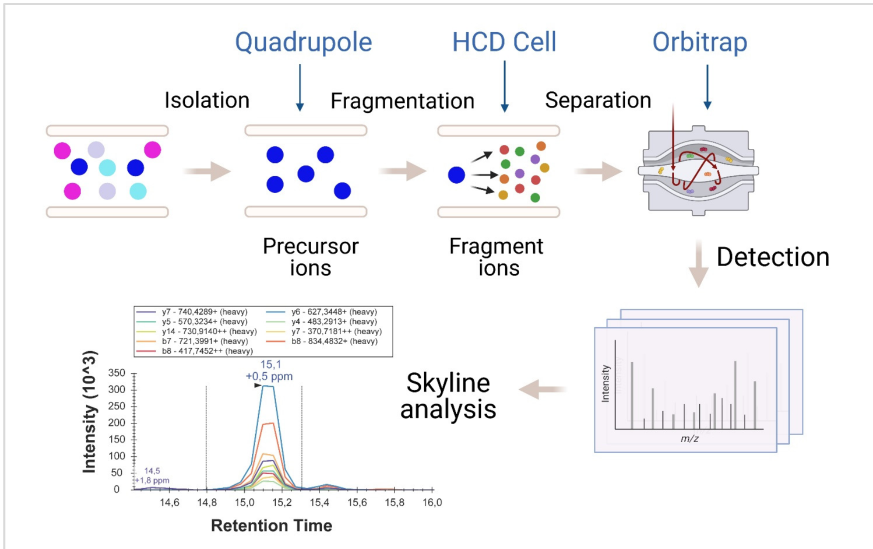

Figure 14. *Schematics of the PRM method. Created with BioRender.com*

4.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, USA) and SPSS, version 26 (IBM corp., Armonk, NY, USA) software. After testing for normality using Shapiro-Wilk test, either parametric or non-parametric tests were employed depending on data distribution. Unpaired *t*-test with Welsh correction was used to assess differences in normally distributed data between two independent groups. Otherwise, Mann-Whitney U and Kruskal-Wallis tests were used for comparing two or more groups, respectively. Kruskal-Wallis tests were followed by Dunn's test for multiple comparison when appropriate. Sex, age, and gender were investigated as covariates. Analysis of covariance was used to investigate group differences adjusting for age effects. Associations between biomarkers were investigated using Spearman's rank correlation. All tests were two-sided, and a probability of $p \leq 0.05$ was defined as statistically significant, unless significance levels were adjusted for multiple comparison using Bonferroni correction.

5 RESULTS AND DISCUSSION

5.1 Paper I

5.1.1 Rationale

The synaptic protein Ng has been studied as synaptic biomarker for AD; many studies in mouse brain [206] and human brain [207] showed a decrease of the protein in AD compared to controls, while the protein has been found elevated in CSF of AD patients compared to controls [291]. Ng changes in CSF appear to be specific for AD, and are not apparent in other neurodegenerative diseases [208, 292]. One exception is CJD in which the levels of Ng rise high, but in CJD many CNS proteins can be found elevated in CSF, most likely due to the severe destruction ongoing in CJD brain [293]. In CSF, Ng was found as both full-length protein [291] and fragments [211], with Ng C-terminal fragments appearing to be the major species present.

Ng is a postsynaptic CaM binding protein involved in LTP-LTD of dendritic spines through modulation of intracellular Ca^{2+} concentration. Ng has an important role for synaptic function and cognition and in neuronal plasticity [193, 205]. Ng KO mice exhibited behavioural abnormalities and impaired learning and memory [202], while overexpression of the protein could rescue the negative effect of Aβo on synaptic transmission in rat hippocampal slice cultures. In CSF, increased Ng concentration correlated with the rate of cognitive decline and disease progression [211, 294]. Therefore, there are reasons to believe that normal levels of Ng are important for maintaining proper cognitive functions.

Ng cleavage might be the first event affecting its function, leading to Ng-diminished activity, with Ng fragments subsequently ending up in the CSF. Given that its enzymatic cleavage might be of importance in understanding Ng's roles in physiology and pathology, the aim of the study was to identify the enzymes yielding the cleavage pattern seen in CSF.

5.1.2 Study

It was known from previous in-house studies that Ng fragments in the CSF frequently had been generated by a cleavage in the IQ domain of Ng, and at between aa 75 and aa 76. Therefore, fluorigenic quenched FRET probes, represented by Ng sequences containing the theoretical cleavage sites were designed and used for testing candidate enzymes. The quenched FRET probes chosen contained Ng39-51 and Ng31-40 peptides, encompassing the IQ domain and the C-terminal Ng70-78 peptide. The initial screening was carried out using a probe from the central IQ region, Ng39-51. The enzymes initially tested were chosen based on reported elevated activity during AD. Of the enzymes tested, *i.e.*, BACE1, TACE1, IDE and calpain-1, only the latter

could cleave the probe and was thus selected for further investigations. As shown in Fig. 15, calpain-1 could cleave Ng39-51 and Ng31-40 but not the quenched peptide Ng70-78, near the C-terminus of Ng.

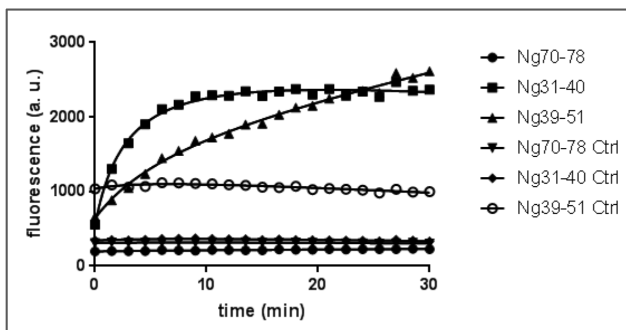

Figure 15. *Quenched FRET experiment showing the probes utilised for testing the calpain-1 activity. Ng39-51 and Ng31-40 showed an increase in fluorescence indicating cleavage by capain-1. The C-terminal quenched peptide Ng70-78 did not show any increase in fluorescence. Ng39-51 Ctrl, Ng31-40 Ctrl and Ng70-78 Ctrl were quenched peptides used as background controls, thus in assay buffer but without calpain-1.*

Calpain-1 cleavage activity was then confirmed exposing the enzyme to full-length recombinant Ng. Initially the Ng recombinant fusion protein containing a tag (Ng-Myc-DDK) was used. Thereafter, to exclude the interference of the tag with the enzymatic activity, full-length Ng was expressed in a SUMO expression vector system which allowed complete removal of the fusion protein part. The cleavage mix was visualized using Coomassie-Silver stained SDS PAGE gel and western blot. In Fig. 16a, lane 1, full-length Ng gave a band of ~12 kDa, while digestions in lanes 2, 3 and 4 contained a major cleavage product corresponding to the ~5 kDa band, together with a second, faint ~6.5 kDa band in lane 2 and 3. When probing the blot with a C-terminal (NG36) antibody (panel b), we could confirm that the ~5 kDa band corresponded to the C-terminal half of Ng. Probing the blot with an N-terminal antibody (NG-H6) showed a different pattern of N-terminal generated peptides (panel c, lane 2-3), which were completely cleaved using higher calpain-1 concentration (panel c, lane 4). A more thorough investigation of calpain-1 cleavage sites was carried out analysing the cleavage mix with the use of MS. Two main cleavage sites between Ng37-38 and Ng42-43 were identified, in line to the peptide pattern seen in CSF. This set of experiments confirmed that calpain-1 cleaved Ng close to the molecule's centre, generating several N- and C-terminal fragments. However, calpain-1 could not cleave the quenched FRET probe Ng70-78, which on the one hand showed that quenched

probes can discriminate cleaving activities among enzymes, and on the other hand that other enzyme (s) might be involved in the C-terminal Ng75-76 cleavage.

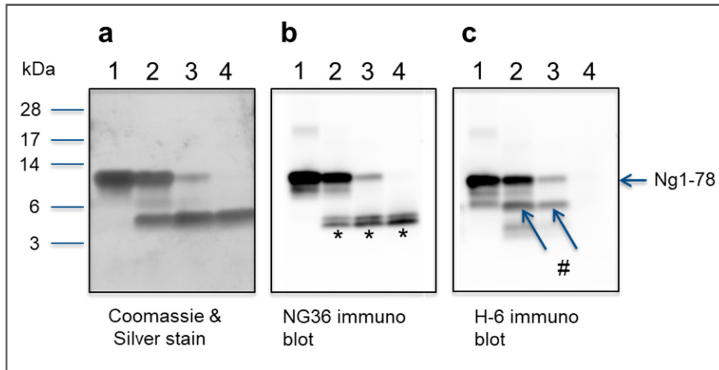

Figure 16. *Detection of C-terminal and N-terminal fragments of Ng in digests by calpain. Digestion products were analysed in reducing SDS PAGE gels (12%). Panel a) Coomassie stain followed by silver stain; Panel b) NG36 immunoblot; Panel c), H-6 immunoblot. Lanes 1, Ng control; lanes 2, 3 and 4, Ng digest with 90-, 30-, 10-fold diluted calpain-1, respectively. The location of the parent Ng1–78 band is indicated on the right near panel c with an arrow, those of the major ~ 5 kDa C-terminal fragments by an * in panel b, and those of the main N-terminal fragment near 6.5 kDa by a # in panel c. Size markers for panel a as indicated.*

As indicated above, several peptides truncated at aa75 that had been previously detected in human brain [207] have also been seen in CSF. This suggests that brain tissue could be a good source for identifying enzymes with C-terminal cleavage activity. Using mouse brain tissue, enzymatically active fractions were identified in a series of chromatographic steps and concentrated by ultrafiltration. The concentrated fractions were then separated on a native gel. Active bands were detected by soaking the gel after the run with the fluorogenic peptide Ng70-78 and visualized by exposing the gel to UV-light. The fluorescent bands were cut out, digested, and analysed with LC-MS/MS. Using this strategy, the prolyl endopeptidase (PREP) enzyme was identified as the main hit.

To confirm that the active fractions had the correct enzymatic activity (generating the correct fragments), they were incubated with the synthetic KKK-Ng 50-78 peptide and the mix was analysed by MS. In the spectrum of one fraction, a strong peak with a mass difference of 259 Da less than the initial substrate KKK-Ng 50-78 appeared (Fig. 17). This peak corresponded to the cleaved product KKK-Ng 50-75, confirming the cleaving activity between aa 75-76. To confirm these results further, using either mouse or human PREP enzyme, longer Ng peptides were digested. Although the efficiency of the cleavage was much lower for these longer peptides, cleavage products

ending at aa75 could still be detected at MS analysis (only about 1%), confirming PREP enzyme activity for both species.

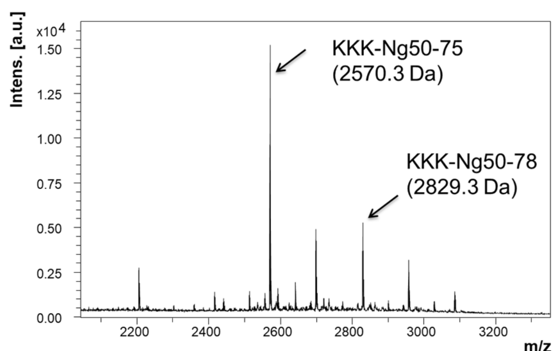

Figure 17. *Active fractions during enzyme enrichment show cleaving activity at Ng75-76 (MALDI-TOF analysis). Synthetic KKK-Ng50–78 was incubated with an active fraction and the reaction mix separated by HPLC. MALDI-TOF analysis of one of the HPLC fractions showed the presence of an [M +H]+ peak at 2570.3 Da which is consistent with a cleavage at Ng75-76 which releases –SGD (mass difference of 259 Da relative to the substrate KKK-Ng50–78).*

5.1.3 Discussion

In this study, we investigated enzymes responsible for Ng processing. We identified that calpain-1 and PREP enzymes can cleave the protein *in vitro*, originating a series of peptides that might explain the fragment pattern previously seen in CSF [211]. Central for Ng function appears to be its well conserved IQ domain, located centrally in the protein sequence, specifically between aa 33-46. However, its mechanism is not fully understood yet, besides that the IQ sequence is the binding region on Ng for binding to CaM. Previous studies in CSF revealed that Ng is present as full-length and C-terminal peptides. N-terminal peptides were not detected [211]. Calpain-1 cleaves Ng at two major cleavage sites, between amino acid 37-38 and 42-43, while PREP cleaves between amino acid 75-76, near the C-terminal end of Ng, and at other positions in Ng as well. The central calpain-1 cleavage sites (between aa 37-38 and aa 42-43) are located in the IQ domain of Ng, which is essential for its binding to CaM. Thus, the cleavage by calpain-1 will most likely interfere with the function of Ng in Ca^{2+}/CaM-dependent signalling for synaptic plasticity. PREP seems able to cleave only shorter fragments of Ng, while its activity was very much reduced on larger fragments of the protein. PREP is an enzyme known to cleave peptides C-terminally after proline [295]. However, it has also been reported that the enzyme preferentially cleaves smaller peptides, with less than 30 aa [296], and requires a conformational

change for its activity [297]. Therefore, suboptimal conditions in the *in vitro* assay and substrate size limitation of the enzyme could account for the low cleavage efficiency that PREP enzyme showed for longer peptides as Ng1-78 and Ng43-78. Taking this into account, we hypothesized a model in which calpain-1 acts first, cleaving Ng and the shorter fragments generated are subsequently cleaved by PREP, and, potentially by other proteases, given that other peptides were also found.

One limitation of this study could be represented by the a priori selection of the enzymes to test for Ng cleavage, based on their suggested involvement in AD pathology. Because of that, other enzymes possibly involved in Ng cleavage may have been disregarded.

Nevertheless, this study is one of the first to identify a possible cleavage pathway supporting the *in vivo* generation of Ng fragments, as detected in CSF. However, further studies should be directed toward a more detailed cleavage site mapping and towards the understanding of where and when the cleavage happens, in relation to AD pathological events. The current study gives insight about how the cleavage of Ng may affect its biological activity and its altered levels in AD brain, creating new knowledge on the protein involvement in the pathophysiology of AD.

5.2 Paper II

5.2.1 Rationale

In our previous work (Paper I) we have shown a possible pathway leading to the fragments seen in CSF. Yet, the extent of Ng fragmentation in physiology and disease is not known. Therefore, with this study we aimed at extending our knowledge on the various molecular forms of Ng in CSF and determine the extent of Ng fragments compared to total full-length of the protein, as they might be used as biomarkers. The detection of the Ng forms present in CSF would be of importance to understand Ng processing and could represent a way to monitor disease progression.

The postsynaptic protein Ng has been studied in brain and CSF. In bovine brain extract, Ng was found as a dimer and higher oligomeric forms [298], as also previous in-house MS investigations on TBS human brain extract showed that traces of Ng oligomers might be present. Recently, a study from our group showed that Ng processing is increased in brain of familial and sporadic AD subjects, and some peptides showed an increased ratio-to-total full-length Ng [207]. The Ng molecular forms found in brain could be reflected in CSF. CSF studies showed that the protein was present as C-terminal fragments, with little amount of full-length Ng, whereas N-terminal fragments were not detected [211].

5.2.2 Study

Aiming at identifying which molecular forms of Ng exists in CSF, CSF pools were initially concentrated and analysed by immunoblots using two C-terminal (NG36 and NG2) and one N-terminal (NG-H6) antibody (Fig. 18). All the antibodies recognized a band ~12 kDa representing the monomeric full-length Ng. Moreover, the antibodies also recognized two other bands around ~35 kDa and ~70 kDa. NG-H6 also recognized an additional band at ~ 55 kDa, which was not detected with the other two antibodies. This band most likely represents a cross-reaction of the NG-H6 antibody with the protein growth-associated protein 43 (GAP43), which also contains an IQ domain as Ng.

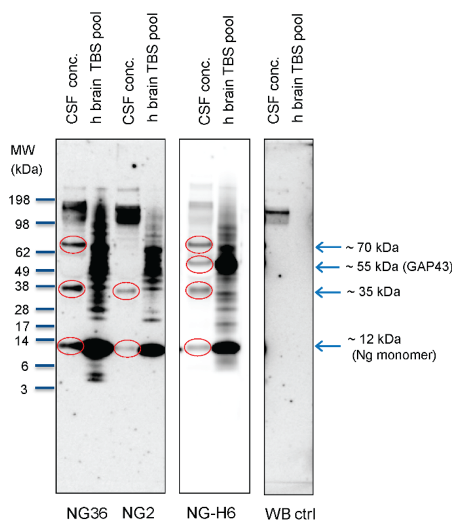

Figure 18. *Concentrated CSF showing Ng as full-length monomer and as higher molecular weight species, denoted by red circles. The TBS human brain samples were used as positive control showing the same monomeric Ng band. Samples were analysed on SDS-PAGE gels at non-reducing conditions.*

These initial findings were confirmed by repeating the experiment with larger amount of concentrated CSF, which were subsequently size-separated using SEC chromatography. The fractions were then analysed by ELISA and by western blot, using both reducing and non-reducing conditions. ELISA analysis showed that Ng was present in different fractions eluting at different molecular weights. The western blot analysis showed different bands at an apparent molecular weight of ~6 and ~12 kDa, corresponding to the C-terminal fragments and the monomeric Ng, respectively, together with a ~35 and a ~70 kDa bands, corresponding to higher molecular forms of Ng. Under reducing conditions, no higher molecular weight bands were detected, indicating that probably disulphide bridges are implicated in the formation of Ng complexes. These series of experiments told us that Ng is present at various molecular form in CSF.

To study these different Ng forms in more detail, subsequently to SEC, fractions of concentrated CSF pools, containing the ~12, ~35 and ~70 kDa bands, were each pooled and analysed by IP-MS. The IP was performed using NG36 and NG-H6 antibodies. Analysis of the ~12 kDa pool led to the identification of Ng and either endogenous N-terminal, C-terminal peptides, and full-length protein were found. Analysis of the ~35 and ~70 kDa pooled fractions only yielded to Ng peptides identification when the samples underwent trypsination prior MS analysis, although only one C-terminal fragment was found at very low concentration. This is possibly due to the low abundance of Ng peptides and the low sensitivity of detection of MS for peptides in this high mass range. The best Ng sequence coverage was obtained when IP-MS was performed using NG36 and NG-H6 antibodies, both individually, and in combination, on the same non-concentrated and non-fractionated CSF pool (Fig. 19).

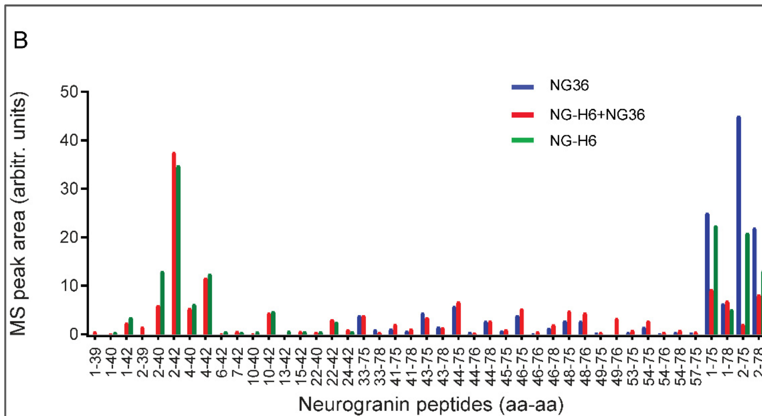

Figure 19. *Ng peptides identified by IP-MS/MS analysis in the non-concentrated CSF pool, using NG36 and NG-H6 antibody separately and as a mixture. Peptides covering the whole Ng sequence were identified, as well as the full-length or near full-length protein. The heights of the bars reflect peak areas of the identified peptides.*

Next, the ratio between Ng C-terminal peptides and total-Ng was determined by ELISA. The available in-house ELISA is denoted as for “total-Ng” and it cannot distinguish between the signal contribution deriving from the C-terminal fragments and full-length Ng. Initially, "total-Ng" was measured using that ELISA in CSF samples from AD patients and controls. Thereafter, aliquots of the same samples were incubated with NG H-6 antibody beads to remove selectively full-length Ng (leaving C-terminal Ng peptides in solution). The NG H-6 antibody binds to the central IQ domain of Ng (approximately at aa 37-39) and can only recognise full-length Ng and fragments of Ng N-terminal of approximately aa 43 (Ng X...42). C-terminal peptides past aa 42 ("Ng43...X") are not binding to NG-H6 [299]. After immunodepletion, the

ELISA signal in those samples was again determined, providing an estimate of the contribution of the C-terminal fragments of Ng to the "total-Ng" signal measured initially. This strategy was applied to 9 non-AD control and 11 AD-like CSF samples, defined so by the AD-core CSF biomarkers measurements. Antibody-coupled bead concentration was optimized to achieve near-complete removal of full-length Ng. The results obtained showed that the samples, on average, did not show any difference in the extent of depletion (in % of total Ng signal) (Fig. 20).

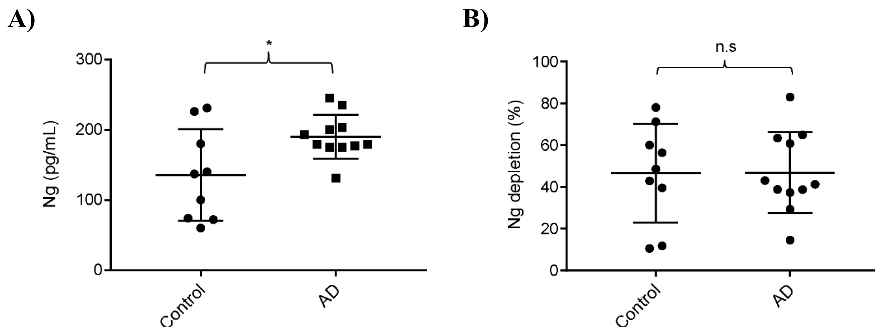

Figure 20. *ELISA measurements of **A)** levels of Ng in control and AD samples before immunoprecipitation. This in-house sandwich ELISA assay is based on two C-terminal Ng antibodies and targets both full-length Ng and C-terminal Ng peptides, without distinguish between them. **B)** Scatter plot showing the loss of signal (in % of total signal) after depletion of full-length Ng. The same signal of C-terminal peptides (of about 50%) was found between the non-AD control group and the AD-like group, with no difference between them.*

Finally, in a separate immunodepletion experiment, using heparin beads and a series of custom-synthesized Ng peptides, a heparin-binding motif was identified on Ng (suggested sequence between aa 43-48). Ng peptides and full-length Ng containing the sequence aa 43...48 bound to the heparin beads, whereas other Ng peptides lacking this sequence did not. This sequence was therefore designated to carry a heparin-binding motif.

5.2.3 Discussion

In this study, we showed that Ng is present in different forms in CSF, as a monomer and as higher molecular weight forms. CSF also contains Ng peptides, according to this and previous studies [208, 211, 300]. Of these fragments, C-terminal fragments accounted on average for approximately 50% of the signal for "total-Ng" (= C-terminal fragments and full-length Ng). However, in this study we also show for the first time the presence of considerable amounts of N-terminal fragments. Additionally, a new N-terminal near-full length species of Ng, Ng2-75 has been found, with so far unknown

functions and unknown origin. All of this opens new possibilities for new studies on the relevance of Ng N-terminal peptides and the development of new N-terminal Ng targeting assays. Analysis of the different fragments also revealed that full-length Ng and C-terminal peptides contain a heparin-binding motif [301], which could represent an export mechanism for Ng into CSF [302]. However, in that case separate mechanisms for transport of N-terminal fragments of Ng need to exist, unless Ng forms escape the cells by mere leakage from damaged cells. Higher molecular weight forms of Ng could represent Ng complexes formed either with other Ng molecules or with other proteins. New interaction partners could play a role in Ng function or secretion and are therefore of interest. However, our analysis using IP-MS could not fully answer the question on the identity of the binding partners in those complexes.

Considering previous results showing increased Ng proteolytic processing in AD brain [207] and increased Ng secretion in CSF of AD patients [211, 300], a better understanding of which Ng forms are present in CSF will increase our knowledge on what forms of Ng are actually targeted in the current ELISA assays and guide future assay development studies. As such, previous studies have primarily identified several C-terminal Ng peptides in CSF [211, 213], which prompted us to identify the ratio between those and total-Ng in CSF. The ratio could provide hints to proteolytic events involved in AD and could be utilised as AD biomarker to follow these events. However, in the samples analysed no differences were found in the ratio of C-terminal fragments to total-Ng.

The small samples size (n=20) of the cohort utilised to determine the ratio of C-terminal fragments to total-Ng, represents a limitation of this study. Moreover, the assay we here finalized to determine the amount of C-terminal peptides considers the total-Ng content as a sum of full-length Ng and C-terminal fragments, but does not take into account N-terminal peptides which might be present as well. Therefore, additional studies using a higher sample numbers and targeting specifically full-length Ng and either C-terminal or N-terminal fragments (ideally by ELISA) would be highly valuable for a complete characterization of Ng fragments in CSF. Limitations in the study of the higher molecular forms of Ng were the low abundance of these species and their high molecular mass, which made their analysis using both western blot and MS challenging. However, concentrating the CSF prior to western blot analysis was a winning strategy to increase the detectability of these species, which could be detected in western blot without prior need of IP.

In conclusion, this study showed that N-terminal, C-terminal fragments and full-length Ng protein are present in CSF. Although quantitation of Ng together with its C-terminal fragments in CSF using ELISA has previously been shown to have good potential in discriminating AD from control samples, the determination of a ratio of C-terminal fragments to total-Ng seems to have no power in distinguishing between the groups. To the present knowledge, no specific cellular functions of Ng fragments are known. The cleavage of full-length Ng, however, will cause loss of the normal function

of Ng. The existence of Ng fragments in brain extracts and in CSF could point to yet unidentified functions of fragments, and therefore, future studies are needed to investigate these fragments more thoroughly. Aside from functional roles in cells, particular Ng fragments may be more suitable as biomarkers for AD than the collective signal from ELISA from "total-Ng", warranting further studies on Ng fragments also in the direction of suitability as biomarkers.

5.3 Paper III

5.3.1 Rationale

Synapse adhesion proteins have a central role in synapse formation, maturation and maintenance of proper synaptic activity [151, 303]. Among them, Nlgns have been thoroughly characterised [222, 269], and studies have connected their alteration with neurological and neurodegenerative diseases [227, 257, 278, 304]. Nlgn1 is the most studied member of the Nlgn family. Nlgn1 is located postsynaptically, specifically expressed at glutamatergic synapses [305], and it is important for LTP and synaptic activity [230, 235, 306]. Aβo have been shown targeting the protein both *in vivo* and *in vitro* [276, 307]. Moreover, a recent study showed reduction of Nlgn1 in hippocampus of AD patients, which was modulated by Aβ load [277]. In this study, we therefore decided to investigate Nlgn1, as Aβ-related neurodegenerative effects might alter its levels and activity in the brain. Different brain regions of AD subjects were investigated. Moreover, a group of primary tauopathies was also included to investigate Nlgn1 brain changes in relation to tau dyshomeostasis. As changes in brain Nlgn1 might be reflected in the CSF, we investigated the protein in also CSF samples of AD and controls.

5.3.2 Study

In order to investigate Nlgn1 in brain tissues, a western blot approach was developed and optimized, using two different monoclonal antibodies both targeting the extracellular part of the protein: the a-Nlgn1 antibody and F-7 antibody. Nlgn1 undergoes proteolytic cleavage that leads to the release of its extracellular domain [224]. Therefore, we targeted this soluble extracellular domain, hypothesizing that it may be detectable in brain TBS extracts. Both antibodies mentioned above were used in the pilot study. However, given that they showed similar results, only one antibody (F-7) was used in the further experiments. The pilot study comprised 4 control samples and 4 samples from patients with sAD, all were brain samples from parietal cortex (Fig. 21 A). Subsequently, a second cohort of brain samples from the temporal cortex was analysed (Fig. 21 B). This second cohort also included familial cases of AD and a group denoted as pathological aging (PA), together with AD and controls. The term PA describes a group of subjects showing abnormal brain deposition of Aβ and tau pathology at autopsy, without any symptoms of dementia throughout life. In both cohorts, analysis of Nlgn1 levels showed a reduction of the protein in AD brains as compared to either controls or PA cases, respectively (Fig. 21).

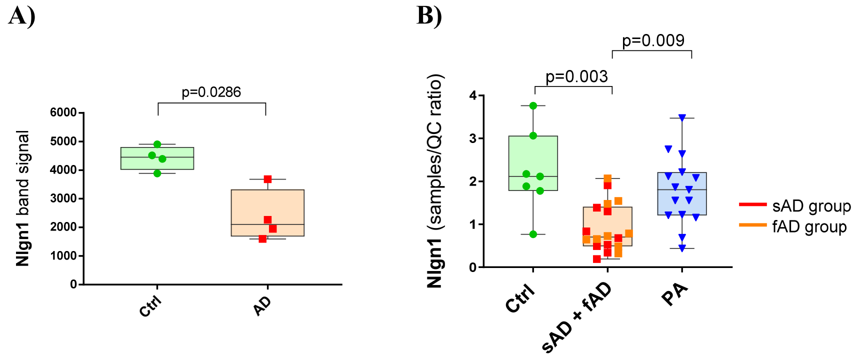

Figure 21. *Reduction of Nlgn1 levels in **A**) parietal cortex and **B**) temporal cortex. TBS brain extracts were analysis using western blot under reducing conditions. In both western blot analyses shown here, the F-7 antibody was used.*

In temporal cortex brains, Nlgn1 levels were also investigated in relation to pathological changes, independently of diagnosis. Nlgn1 showed lower levels with increasing NFTs deposition (indicated by Braak stages). The same Nlgn1 reduction was seen with higher CERAD scores (describing neuritic plaques). No differences in the protein levels were found in relation to Aβ deposition (Thal phases) or in relation to CAA pathology and *APOE* genotype.

Nlgn1 was then investigated in relation to tau changes, using a cohort of brains from tauopathy patients. This cohort included pathologically diagnosed PSP, CBD, and PiD samples, together with AD and controls from frontal grey matter, a relevant region for tau accumulation in tauopathies. The analysis revealed a marked reduction of Nlgn1 protein in PiD and CBD, which could differentiate them from control, but not from AD. PSP did not show a significant difference compared to the other groups, but had the same trend in reduced Nlgn1 levels as observed for the other tauopathies. There was also no difference from AD and control. The analysis for the tauopathy samples is shown in Fig. 22.

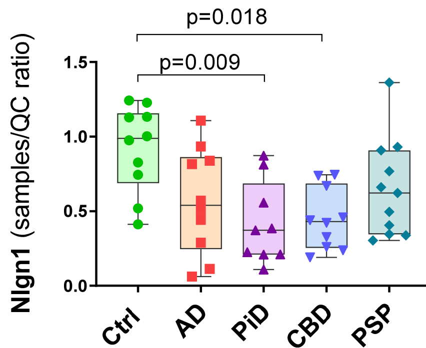

Figure 22. *Nlgn1 reduction in frontal grey matter of tauopathies.*

We then wanted to investigate if the Nlgn1 reduction in AD brain was reflected in CSF of AD patients. Therefore, using the same western blot method, the protein was analysed in CSF of clinically diagnosed AD and healthy controls subjects (different patient cohort as in previous figures). Nlgn1 levels were slightly increased in the AD group as compared to controls, however with high overlap between the groups (Fig. 23 A). When the groups were dichotomized based on Aβ levels, in Aβ+ and Aβ- groups, or using all AD core biomarkers, in AD- and AD+ groups, the difference was lost (Fig. 23, B and C).

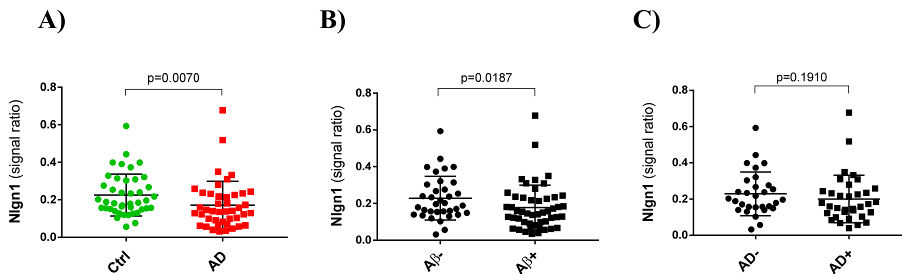

Figure 23. *Nlgn1 levels did not differ in CSF of AD patients as compared to controls. Panel **A)** shows the significant increase of Nlgn1 in the AD group compared to the control group, although high overlap between the groups is present. The difference is abolished when the groups are dichotomized in **B)** Aβ+ and Aβ- based on the ratio* $A\beta_{42/40}$ *and in **C)** AD+ and AD- when also the other core biomarkers p-tau and t-tau are used for group definition.*

Finally, the presence of Nlgn1 in brain extracts and CSF was also confirmed using LC-MS/MS. In-gel digestion and MS analysis of gel bands cut out at the position of

Nlgn1 immunoreactive bands (as determined with a western blot from protein aliquots run on same gel) was performed. Nlgn1 peptides were identified corresponding to the immunoreactive bands, but not in gel samples corresponding to higher or lower molecular weights. This experiment further confirmed the presence of the protein and the specificity of the antibody used.

5.3.3 Discussion

Different from the previous two studies on Ng, here we focused on the postsynaptic adhesion protein Nlgn1. Earlier studies showing a reduction of Nlgn1 levels in hippocampus of AD patients [277] and the possible disruptive interaction of Nlgn1 with Aβo [276] have brought us to hypothesize that the protein might be altered during AD. The detection of such changes could shed light into events leading to synapse dysfunction in AD pathology, and possibly yield new synaptic biomarkers to follow such changes.

According to previous investigations, our results show that Nlgn1 levels are reduced in parietal and temporal cortex of AD patients. For the first time, we also showed that the protein is reduced in frontal grey matter of PSP, CBD and PiD brains compared to control, although Nlgn1 levels could not discriminate between the different tauopathies or between tauopathies and AD. The investigation of Nlgn1 in CSF showed that the protein is present, although the difference seen in AD brains is not reflected here. Nlgn1 levels correlate moderately with t-tau and Ng, but not with p-tau. Moreover, the protein does not correlate with the cognitive decline measured by the MMSE score. These results suggests that Nlgn1 does not represent a promising candidate biomarker for AD pathology in CSF, but instead the protein alteration detected in brain might be indicative of a general synaptic dysfunction and neuronal damage seen in AD. Tau misfolding and aggregation leads to pathological accumulation of the protein in brain which represents the main hallmark for a group of neurodegenerative diseases named tauopathies [103]. The presence of tau aggregates is also necessary for the biological definition of AD, a secondary tauopathy [308]. Currently, no biomarkers are available to distinguish among tauopathies or between them and AD. The marked reduction of Nlgn1 found in frontal grey matter of tauopathies raises the interest for further studies of the protein in CSF of these patients.

The specificity of the antibodies used was verified in western blot with recombinant Nlgn proteins (Nlgn1, -2 and -3) and using MS analysis of excised gel bands at the position of Nlgn1 immunoreactive bands. In the western blots, the Nlgn1 band appeared as a double band at ~80 kDa. However, it was not possible to perform a distinct quantification of the lower and upper band of the Nlgn1 double band due to the intrinsic limited size resolution of the western blot technique. Moreover, the small sample size of the cohort investigated and western blot-inherent difficulties for protein quantitation (low dynamic range; poor precision) need to be addressed in future studies to shed more light on the complex mechanism that regulate Nlgns at the synapse, both

in physiology and pathology. Such studies could be expanded to different fragments of the protein as well as isoforms and PTMs. Other members of the Nlgns family should be investigated as Nlgn2 levels have been reported elevated in CSF of AD patients, also in the prodromal stage [282]. Moreover, Nlgn3 shedding can influence Nlgn1 cleavage when the two proteins dimerise at the synapse [248].

5.4 Paper IV

5.4.1 Rationale

In paper III, we investigated Nlgn1 in brain and CSF of neurodegenerative diseases. However, Nlgn1 is not the only member of the Nlgn family, as five genes in humans encode for the proteins, originating Nlgn2, Nlgn3, Nlng4-X and Nlgn4-Y (or Nlgn5) [229]. Nlgn4-X and –Y share a high sequence homology and are therefore often referred to as Nlgn4 only. Nlgn1-3 are the most abundant members of the Nlgn family in the CNS. Nlgn4, although the least studied of the family, has also been recently described having a role in excitatory synapse transmission in the brain [218] and are essential for synapse assembly and proper function [245, 305] and are essential for synapse assembly and proper function [222, 269]. In our previous study, Nlgn1 was found reduced in brain of AD and tauopathies [309]. However, what happens to the other Nlgns family members?

Nlgns are expressed postsynaptically, and in order to exert their functions, they need to interact with their presynaptic partners, the NRXNs [215, 310]. In turn, NRXNs are a class of synaptic adhesion proteins essential for the assembly and the proper formation and function of the presynaptic part of synapses [311]. Gene mutations of both Nlgns and NRXNs have been described to be involved in the aetiology of synaptic dysfunction in cognitive disorders like schizophrenia and autism [279-281, 312-314]. As for Nlgn1, Aβo interact with NRXNs and disrupt their ability of regulate excitatory synaptic activity [274]. Moreover, the levels of various members of the NRXN family have been found altered in CSF of AD patients [284]. Considering the tight relationship of NRXNs and Nlgns with each other, we aimed at simultaneously studying both families of proteins to assess their changes in CSF of neurodegenerative diseases. The aim was to understand if these proteins are involved in synaptic pathological changes during neurodegeneration and to evaluate their potential as biomarkers to reflect such changes.

5.4.2 Study

To study NRXNs and Nlgns, we developed a targeted MS method for their simultaneous quantification in CSF. The method was developed for quantifying Nlgn1-4 and the most commonly investigated NRXN-1α, NRXN-1β, NRXN-2α, NRXN-3α. β-forms of NRXNs appear to be less abundant than the α-forms [250],

however we added NRXN-1β because the protein has been described as the main binding partner of Nlgn1 [256, 260]. Peptides for each of the proteins were selected either based on previous studies [282, 309, 315, 316] or based on their uniqueness. Importantly, all the peptides belong to the extracellular domain of the proteins, which undergoes a proteolytic cleavage [225, 248, 317, 318], leading to the release of this soluble domain into CSF. Heavy-isotope-labelled internal standards (IS) were used for peptide quantification.

The method was initially tested on a pilot cohort comprising biochemically defined AD and non-AD control CSF samples, with the use of the core AD CSF biomarkers. None of the peptides showed a difference between the two groups (Fig. 24).

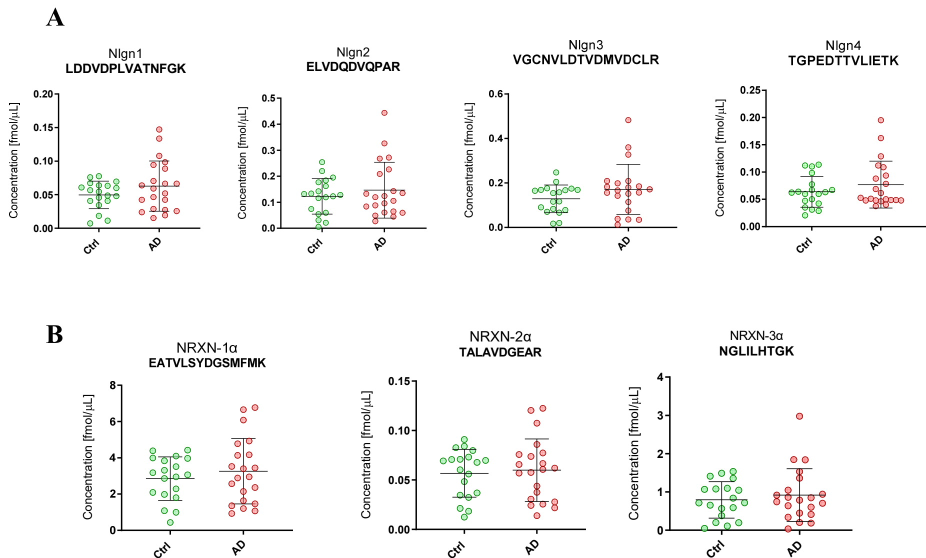

Figure 24. *Nlgns (A) and NRXNs (**B**) group comparison in the discovery cohort. In the figure, only one representative peptide per protein is shown. The bars indicate median with interquartile range.*

To extend this preliminary investigation, a larger cohort was used to study the proteins in the AD continuum. This second cohort consisted of a total of 22 controls, 77 AD, 44 MCI due to AD, 46 non-AD MCI and 28 non-AD dementia samples. In this clinical cohort, patients underwent a thorough neurological investigation. Results were consistent with the previous investigation, and also in this experiment, no difference between the groups was found for all the peptides (Fig. 25). The non-AD dementia group was composed by 13 FTD, 3 vascular dementia (VaD) and 12 DLB cases. When comparing peptides levels across the individual groups, significance differences could not be found.

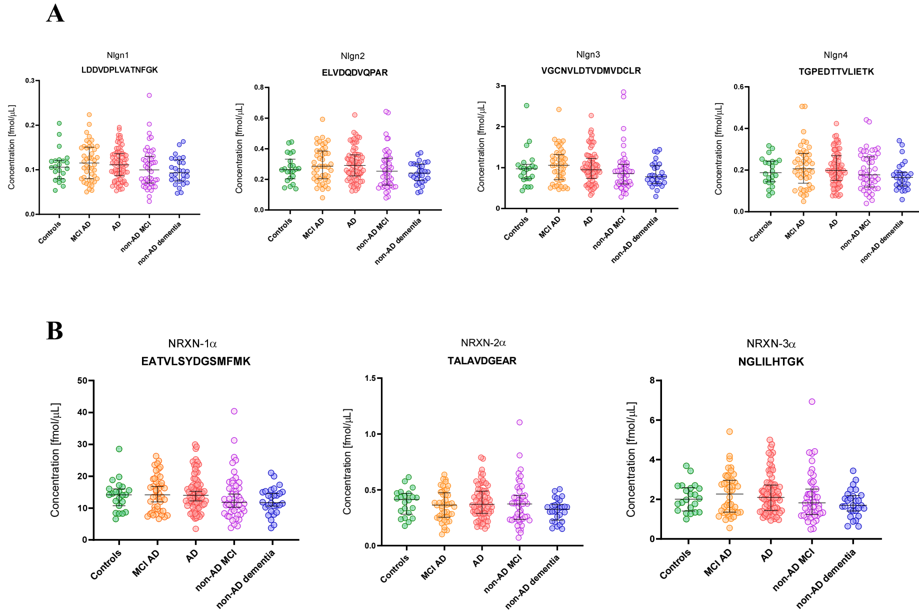

Figure 25. *CSF concentrations obtained by PRM analysis of the NRXNs-Nlgns panel in the clinical cohort. In the figure, only one representative peptide for (**A**) Nlgns and (**B**) NRXNs is shown. The bars indicate median with interquartile range.*

To study the proteins as function of Aβ deposition, the groups were dichotomized based on the $A\beta_{42/40}$ ratio into Aβ+ and Aβ-. However, the peptides did not show any difference in relation to Aβ pathology.

All the peptides, with few exceptions, showed a high correlation between each other across all the clinical groups. They also correlated relatively strongly with $A\beta_{40}$ and $A\beta_{42}$, although they did not correlate with the ratio $A\beta_{42/40}$. Generally, NRXNs and Nlgns moderately correlated with t-tau and to a lesser extent with p-tau. With Ng and GAP43, NRXNs and Nlgns showed a moderate to weak correlation, respectively.

5.4.3 Discussion

In this work, we have developed a targeted MS method for the simultaneous quantification of NRXNs and Nlgns in CSF of neurodegenerative diseases, focusing on their investigations in AD. In contrast to previous studies [282, 315, 316], which found NRXNs at changing levels in CSF of AD patients, already at prodromal stage, here we find that NRXN proteins do not change in CSF of AD or MCI patients. The high dynamism of these proteins at the synapse, the small sample number and small effect size of previous studies [282, 315, 316], and the expression of NRXNs and Nlgns by astrocytes [271, 272], could all have contributed to different levels of these protein in the CSF and possibly explain the deviating results among different studies. Nlgn

proteins also do not show any difference among the groups. Although we did not investigate the other Nlgns and NRXNs in paper III, the results for Nlgn1 in CSF shown here in paper IV are in line to what we have shown previously in paper III, namely a small or no difference in CSF of AD patients [309]. We found a modest correlation between Nlgns and NRXNs and t-tau and Ng; however, the differences for Nlgns and NRXNs between the AD and Ctrl groups did not reach significance in the present study.

NRXN and Nlgn proteins showed a good-to-high correlation between each other, indicating that they might reflect similar events at the synapse. NRXNs and Nlgns undergo proteolytic processing [255] which is very similar as for the APP protein [32]. This might explain the high correlation found with the proteins and the APP proteolytic products $A\beta_{40}$ and $A\beta_{42}$. Correlation of either NRXNs or Nlgns with the other synaptic proteins Ng and GAP43 were found to be weak (Ng) or moderate (GAP43). Ng and GAP43 are intracellular proteins, while NRXNs and Nlgns are transmembrane proteins, in contact with the extracellular space through their extracellular domain. Therefore, shedding and release of these proteins might occur by separate mechanisms and may reflect different events at the synapse, which could be an explanation for the observed low correlations.

In this study, tryptic peptides of NRXN and Nlgn were analysed. The proteins are shedded from the external side of the plasma membrane (defined as ectodomain cleavage [319]), yielding the soluble fragments of the extracellular domains, which we therefore target in CSF. However, different metalloproteases can achieve the cleavage, leading to the release of possible multiple fragments of the same ectodomain. Moreover, NRXN and Nlgn proteins undergo alternative splicing which can originate different protein isoforms. Different cleavage sites and multiple splice variants of the proteins might lead to a variety of endogenous peptides which would be of interest to explore as they might convey more valuable biomarker information, as is the case for the pathologic APP endogenous fragment $A\beta_{1\text{-}42}$.

In conclusion, this is the first study that simultaneously quantified NRXNs and Nlgns in CSF to investigate the proteins during AD. Our panel assay provides a powerful tool to investigate the complex biology that regulates NRXNs and Nlgns, and to understand more about their in physiological processes and in disease.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Synaptic protein dysfunction plays a significant role in the pathology and progression of neurological and neurodegenerative diseases; thus its study is of primary importance to understand specific changes of synaptic proteins and define biomarkers able to detect such changes.

The work presented in this thesis investigated two different categories of synaptic proteins: the intra-neuronal dendritic protein Ng and the transmembrane proteins NRXNs and Nlgns.

We used antibody-based strategies together with MS methods to study **Ng** fragments and species in CSF. We also detected two enzymes that can cleave Ng and generate these fragments identified in CSF. The calpain-1 enzyme performs a cleavage at the IQ domain of Ng. Subsequently, other enzymes can further cleave the generated peptides, such as the identified PREP enzyme. Although this model might explain most of the peptides identified in CSF, the exact cleavage sites and the possible involvement of other proteases need additional research efforts. Our investigation also showed that Ng in CSF is present in the form of complexes, even though the nature of such complexes still needs to be clarified. The identification of possible yet unknown Ng binding partners could shed light into Ng functions at the synapse.

A shift from the full-length Ng to peptides appear to happen in AD brain [207], and this increased production of peptides seems to be reflected in CSF [294, 300, 320]. Moreover, changes appear to be specific for AD pathology and are not seen in other diseases [292, 320-322]. Previous studies mainly identified C-terminal fragments of Ng as the main species in CSF [211, 213], mostly because well binding N-terminal antibodies were not available. Here, we confirmed that C-terminal fragments are present in CSF; however, N-terminal fragments and the full-length protein are also found in significant amounts. The ratio of C-terminal fragments to total-Ng in CSF was not a useful indicator of progression to AD, as it did not improve the separation between AD and control, as also reported in a recent study [323]. Therefore, more investigations are needed to understand whether cleavage of Ng is a causative part of the AD pathogenesis or rather an indicator of the process, and why increased CSF Ng is only seen in AD and in conditions of massive neuronal damage (*e.g.*, CJD [293], traumatic brain injury [324]). Future studies of Ng fragments in brain of other neurodegenerative diseases might help to understand if Ng fragmentation is specific for AD pathology. In addition, it might be of interest to investigate if a particular fragment, other than the sum of C-terminal fragments species nowadays detected by immunoassays, would represent a better biomarker than for instance the APP specific peptides $A\beta_{1\text{-}42}$. Additionally, further work is needed to assess the extent of N-terminal fragments and their potential use as biomarkers, which is nowadays hampered by the

limited choice of antibodies especially for the far N-terminal sequence of Ng. The understanding of Ng proteolytic processing role in the pathogenesis of AD can increase the interpretative value of Ng biomarkers for AD. Longitudinal studies are also needed to understand the time course of appearance and significance of increased Ng peptides in relation to disease progress and state.

Only few studies investigated the potential of NRXN proteins as CSF biomarkers for synaptic pathology and no studies are available for the Nlgns. In this work, an initial specific investigation was conducted on the postsynaptic protein **Nlgn1**, using antibodies targeting its extracellular domain in both human brain and CSF. We found that Nlgn1 protein levels are decreased in parietal and temporal cortex of AD brains. Decreased levels of the extracellular domain of the protein might be due to reduced expression or increased proteolytic cleavage and secretion. However, the use of brain samples imply investigation of proteins at an end stage, when the disease is probably at an advanced stage, thus the decreased level of the protein might have been the consequence of extensive synaptic loss. These changes seen in brain are not reflected in CSF of AD patients. On the other hand, Nlgn1 was not decreased in frontal grey matter of AD patients compared to controls, as it was in the tauopathies group. These results might suggest regional changes of the protein during AD pathology, together with Nlgn1 tau-related changes. Further investigations of Nlgn1 in CSF of tauopathies might be of interest to elucidate the extent of protein changes in relation to tau pathology.

The Nlgn1 investigation was expanded to the other Nlgn family members and their binding partners NRXNs, with the development of a specific MS method for their simultaneous quantification in CSF of neurodegenerative diseases. The MS panel used revealed that also the levels of the other **Nlgn** family members and **NRXNs** are unchanged in CSF of AD patients.

Future studies on Nlgn2, -3 and -4, and of the NRXNs in brain of AD could possibly elucidate if the proteins are not changed at all, or if the changes seen in brain are lost along the way of getting into the CSF, as it seems to be the case for Nlgn1. Nlgn1 and NRXN-2 have been found elevated in plasma neuron-derived exosomes of AD patients [325]. Therefore, it would be of interest to investigate more on the proteolytic processing of these proteins during AD and their transfer from the brain to the periphery. The proteolytic cleavage of NRXNs and Nlgns is described as an active event, which depends on synaptic activity [224, 248, 255, 318]. Cell models could be used to examine the mechanism of release in relation to AD-related exposures, including for instance aggregated Aβ peptides or tau protein. These investigations could lead to a better understanding of NRXNs and Nlgns involvement during neurodegenerative diseases, as well as to the discovery of endogenous peptides connected to pathological events with potential utility as biomarkers.

In conclusion, the constant and robust change of Ng in AD but the unchanged levels of Nlgns and NRXNs might suggest that synaptic pathology in AD is not a generalized

disruption of synapses that leads to an overall change in all synaptic proteins, but it might entail more specific mechanisms, which affects a subset of proteins but not others. The intra- vs. extracellular localisation of a synaptic protein could also be of relevance in that regard. Along the same lines, studies of synaptic dysfunction might help in finding new disease targets to certain pathologic mechanisms, as for example calpain-1. Together with these findings, this thesis also highlights the importance of studying the proteolytic processes of synaptic proteins to better understand their value as biomarkers for synaptic pathology in AD.

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