CSF biomarker panels

Focus on synaptic pathology

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To the little girl who just loved science

“I don’t know”

I tell myself

But maybe

We do know a little bit more

Than yesterday

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ABSTRACT

Fluid biomarkers of neuropathological features are important clinical tools in diagnostics and patient monitoring of neurodegenerative diseases. For the most prevalent cause of dementia, Alzheimer's disease (AD), several biomarkers have been introduced in the clinic reflecting the underlying pathophysiology features of amyloid-β deposition, tau pathology with hyperphosphorylation, and neurodegeneration (ATN). Together the biomarkers have been demonstrated to have >90% sensitivity and specificity for the early stages of AD. However, in the field of biomarker research, one area which has gained recent attention is biomarkers reflective of synaptic pathology (degeneration and dysfunction of the synapse), which is an early and central part of the pathophysiology of many neurodegenerative diseases, including AD, and clinically relevant since synaptic function is the foundation of cognition. Synaptic biomarkers are thus of interest not only in the routine clinical assessment of neurodegenerative diseases to facilitate diagnosis, disease staging, and progression, but especially to monitor the efficacy and endpoints of treatments in drug trials which commonly aim to halt or reduce synaptic damage.

The main goals of this thesis were to develop and optimize methods for quantifying biomarkers of synaptic pathology, to evaluate their potential in AD and across neurodegenerative diseases, and to examine concordance and discordance between biomarker results and other measures of synaptic dysfunction. The work focused on multiplexed mass spectrometric (MS) methods that enable quantification with high specificity and sensitivity of a range of potential biomarkers with different functions and localizations. These methods were then used not only to compare the biomarkers' diagnostic and disease monitoring potential but also to reinforce the credibility of the results of proteins with similar outcomes, *i.e.*, validating general and specific pathological patterns across and within neurodegenerative diseases. A synaptic panel assay was successfully established, quantifying 17 synaptic proteins, including several SNARE proteins, neurogranin, synucleins, neuronal pentraxins, and 14-3-3 proteins. Together with an in-house-established MS assay quantifying SNAP-25 and

synaptotagmin-1, the panel method was used to study synaptic proteins across neurodegenerative diseases in several studies included in this thesis.

One of the main findings is that out of the potential synaptic biomarkers, several of them showed specifically higher concentrations in the AD continuum in contrast to other neurodegenerative diseases. Indicating that higher levels of synaptic proteins are possibly generally a specific feature of AD and thus a marker of AD-specific synaptic dysfunction mechanisms compared with other neurodegenerative diseases. The possible exemption to this seems to be 14-3-3 ζ/δ, of which higher levels across neurodegenerative diseases might indicate that it is a general biomarker of synaptic degeneration mechanisms. Particularly, SNAP-25, neurogranin, and β-synuclein, as well as 14-3-3 ζ/δ, seem to be promising AD biomarkers able to both predict disease progression as well as cognitive decline. For SNAP-25, it was also shown that a newly developed Single molecule array (Simoa) assay for SNAP-25 quantification can be used interchangeably with other previously established methods. Furthermore, this thesis work demonstrated that the neuronal pentraxins are present at lower concentrations across neurodegenerative diseases, indicative of synaptic dysfunction and degeneration mechanisms equally affected across diseases. The neuronal pentraxins were also found to be associated with cognitive status in AD dementia and Parkinson's disease, the latter in which they were also associated with cognitive decline and the progression of motor symptoms and might be useful to predict disease severity. This thesis establishes that the neuronal pentraxins are possible prognostic and monitoring biomarkers for synaptic dysfunction/degeneration that associate with cognitive and motor symptoms across neurodegenerative diseases. Additionally, novel differences in synaptic proteins were found in both parkinsonian disorders and genetic frontotemporal dementia (FTD), with differential synaptic impairment represented by different synaptic proteins. Interestingly, multiple abnormalities were shown in the symptomatic patients with MAPT mutations indicating specific synaptic dysfunction in regard to the underlying proteinopathy found in each genetic FTD mutation. The results demonstrate that differential patterns of synaptic protein alterations across neurodegenerative diseases exist, probably due to differences in synaptic pathology mechanisms.

In conclusion, several of the studied synaptic proteins show promise as possible complements to other CSF and imaging markers as diagnostic, prognostic, stage, or monitoring biomarkers of cognitive decline and synaptic pathology. Furthermore, this thesis provided novel insight into synaptic pathology in neurodegenerative diseases. A better understanding of the mechanistic pathways of synaptic dysfunction across and between diseases may thus contribute to improving diagnostics and potentially also to the development of new therapeutic strategies targeting said pathways. The work included in this thesis demonstrates the importance of MS-based biomarker discovery,

allowing for the simultaneous quantification and exploration of multiple biomarkers leading to knowledge that can drive the development of biomarkers as well as new, highly precise methods and increase the availability of biomarker quantification.

Keywords: Mass spectrometry, synaptic dysfunction, CSF biomarkers, neurodegenerative diseases

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SAMMANFATTNING PÅ SVENSKA

Biomarkörer för neuropatologiska förändringar är viktiga kliniska verktyg inom diagnostiken för neurodegenerativa sjukdomar. För den vanligaste formen av demens, Alzheimers sjukdom (AD), har flera biomarkörer introducerats på senare tid som återspeglar de underliggande patologiska förändringarna så som proteindeposition av amyloid-β och tau. Tillsammans har de visat sig ha 80–90 % känslighet och specificitet för de tidiga stadierna av AD. Fortsatt forskning inom området har uppmärksammat potentialen av biomarkörer som reflekterar förlust och dysfunktion av nervkopplingar (synapser), något som är en tidig och central del av många neurodegenerativa sjukdomar, inklusive AD. Detta beror främst på det starka sambandet mellan synaptisk förlust och graden av kognitiv nedgång, då synaptisk funktion är grunden för kognition. Synaptiska biomarkörer är därav av intresse, inte bara i den kliniska rutinmässiga bedömningen av neurodegenerativa sjukdomar för att ställa diagnos och bedöma kognitiv nedgång, utan särskilt för att övervaka effektiviteten av behandlingar i läkemedelsprövningar som vanligtvis syftar till att stoppa eller minska synaptisk skada.

Huvudmålet med denna avhandling var därför att utveckla och optimera metoder för kvantifiering av biomarkörer som reflekterar synaptisk dysfunktion. Delvis för att utvärdera deras potential inte bara i AD utan även inom andra neurodegenerativa sjukdomar, men även studera om synaptiska sjukdomsmekanismer skiljer sig mellan de olika sjukdomarna. Fokus i forskningsarbetet var utveckling och tillämpning av masspektrometriska (MS) metoder, då de möjliggör simultan kvantifiering med hög specificitet och känslighet av en rad potentiella biomarkörer med olika funktioner och lokaliseringar. Detta gjorde det möjligt att inte bara jämföra potentialen hos potentiella biomarkörer utan även förstärka trovärdigheten för resultaten av proteiner med liknande resultat, dvs. validering av allmänna och specifika patologiska mönster inom och emellan olika neurodegenerativa sjukdomar. Under doktorandprojektet utvecklades framgångsrikt en synaptisk panelanalys som kvantifierar 17 synaptiska proteiner, inklusive flera SNARE-proteiner, neurogranin, synukleiner, neuronala pentraxiner och medlemmar av 14-3-3-proteinfamiljen. Tillsammans med en etablerad MS-analys som kvantifierar SNAP-25 och synaptotagmin-1, gjorde denna metod det möjligt att studera dessa synaptiska proteiner inom neurodegenerativa sjukdomar i flera kliniska studier som ingår i denna avhandling.

Ett av de viktigaste resultaten av denna avhandling är att av de potentiella synaptiska biomarkörerna visade sig nästan alla ha specifikt högre koncentrationer i AD. Möjligtvis indikerar detta att högre nivåer av synaptiska proteiner generellt är en specifik egenskap hos AD och därmed en markör för AD-specifika synaptiska dysfunktionsmekanismer jämfört med andra neurodegenerativa sjukdomar.

Ett undantag verkar vara 14-3-3 ζ/δ, för vilken högre proteinnivå förefaller vara en allmän biomarkör för synaptiska sjukdomsmekanismer vid neurodegenerativa sjukdomar, dock särskilt påverkade i AD-patologi. Särskilt SNAP-25, neurogranin och β-synuklein samt 14-3-3 ζ/δ verkar vara lovande AD biomarkörer som både kan förutsäga sjukdomsprogression och kognitiv nedgång. För SNAP-25 visades det också att en nyutvecklad Single molecule array (Simoa)-analys för kvantifiering av SNAP-25 kan användas med samma prestanda som den nuvarande MS-metoden. Dessutom demonstrerades det att de neuronala pentraxinerna är närvarande i lägre nivåer inom neurodegenerativa sjukdomar, vilket tyder på synaptiska dysfunktion och sjukdomsmekanismer som allmänna hos neurodegenerativa sjukdomar. De neuronala pentraxinerna fanns vara associerade med kognitiv status vid AD-demens och även vid Parkinsons sjukdom, där de också var förknippade med kognitiv försämring såväl som utvecklingen av motoriska symtom, vilket kan göra dem användbara för att förutsäga sjukdomssvårighetsgrad. Arbetet visar således att de neuronala pentraxinerna är möjliga prognostiska biomarkörer samt kan förutspå försämring i kognitiva och motoriska symtom hos neurodegenerativa sjukdomar. Dessutom upptäcktes skillnader hos synaptiska proteiner i andra sjukdomar så som progressiv supranukleär pares och multipel systematrofi såväl som i genetisk frontotemporal demens. Resultaten visar att synaptiska proteiner är differentiellt förändrade beroende av sjukdomstyp, förmodligen på grund av skillnader i underliggande sjukdomsmekanismer.

Slutsatsen av dessa studier är flera av de studerade synaptiska proteinerna visar lovande resultat som möjliga komplement till andra biomarkörer som diagnostiska, prognostiska, stadium och/eller övervakningsbiomarkörer för kognitiv nedgång och synaptisk patologi. Dessutom gav studierna i denna avhandling ny insikt inom underliggande förändringar och mekanismer hos neurodegenerativa sjukdomar. En bättre förståelse av dessa mekanismer av synaptisk dysfunktion kan således bidra till att förbättra diagnostiken och potentiellt även till utvecklingen av nya terapeutiska strategier. Detta arbete påvisar även vikten av MS-baserad biomarkörforskning, vilket möjliggör simultan kvantifiering och utforskning av flera biomarkörer, något som leder till kunskap som kan driva utvecklingen av nya biomarkörer såväl som metoder och öka tillgängligheten för diagnostik.

LIST OF PAPERS

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

I. **Johanna Nilsson,** Johan Gobom, Simon Sjödin, Gunnar Brinkmalm, Nicholas J. Ashton, Johan Svensson, Per Johansson, Erik Portelius, Henrik Zetterberg, Kaj Blennow, Ann Brinkmalm. *Cerebrospinal fluid biomarker panel for synaptic dysfunction in Alzheimer's disease.* Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring. 2021. 13(1), e12179.

II. **Johanna Nilsson***, Katheryn AQ Cousins*, Johan Gobom, Erik Portelius, Alice Chen-Plotkin, Leslie M Shaw, Murray Grossman, David J. Irwin, John Q Trojanowski, Henrik Zetterberg, Kaj Blennow, Ann Brinkmalm. *Cerebrospinal fluid biomarker panel of synaptic dysfunction in Alzheimer's disease and other neurodegenerative disorders.* Manuscript.

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VI. **Johanna Nilsson**, Ann Brinkmalm, Sebastian Palmqvist, Wagner Brum, Alexa Pichet Binette, Shorena Janelidze, Nicola Spotorno, Erik Stormrud, Johan Gobom, Henrik Zetterberg, Kaj Blennow, Oskar Hansson. *Cerebrospinal fluid biomarker panel for synaptic dysfunction in a broad spectrum of neurodegenerative diseases.* Manuscript.

Papers not included in the thesis:

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Eleni Gkanatsiou*****, **Johanna Nilsson***, Christina E Toomey, Agathe Vrillon, Hlin Kvartsberg, Erik Portelius, Henrik Zetterberg, Kaj Blennow, Ann Brinkmalm, Tammaryn Lashley, and Gunnar Brinkmalm. *Amyloid pathology and synaptic loss in pathological aging*. Journal of Neurochemistry. 2021:159.2 258-272.

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Bjørn-Eivind Kirsebom, Grit Richter, Kaja Nordengen, Dag Aarsland, Geir Bråthe, Betty Tijms, Pieter J. Visser, **Johanna Nilsson**, Per Selnes, Milica G. Kramberger, Bengt Winbla, Knut Waterloo, Berglind Gísladóttir, Kaj Blennow, and Tormod Fladby. *Stable CSF Neurogranin and BACE1 levels differentiate predementia Alzheimer's Disease patients.* Brain Communications. 2022: Accepted

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ABBREVIATIONS

AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Adaptor proteins
AP2B1	AP-2 complex subunit β
APOE	Apolipoprotein E
APP	Amyloid precursor protein
AUC	Area under the curve
Aβ	Amyloid β
$A\beta_{40}$	Aβ peptide 1-40
$A\beta_{42}$	Aβ peptide 1-42
bvFTD	Behavioral phenotype of frontotemporal dementia
C9orf72	Chromosome 9 open reading frame 72
Cav2.2	N-type voltage-gated calcium channel
CBD	Corticobasal degeneration
CBS	Corticobasal syndrome
CJD	Creutzfeldt-Jakob disease
CSF	Cerebrospinal fluid
CU	Cognitively unimpaired
DAT1	Dopamine transporter
DLB	Dementia with Lewy bodies
ELISA	Enzyme-linked immunosorbent assays
ESI	Electrospray ionization

EWS	Ewing's sarcoma
FAD	Familial Alzheimer’s disease
FBS	Frontal behavioral-spatial syndrome
FDG	[^{18}F]-fluorodeoxyglucose
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma protein
GABA	Γ-aminobutyric acid
GCI	Glial cytoplasmic inclusions
GDI-1	Rab GDP inhibitor alpha
GGT	Globular glial tauopathy
GRN	Progranulin
HCNP	Hippocampal cholinergic neurostimulating peptide
HPLC	High-performance liquid chromatography
IP	Immunoprecipitation
IS	Internal standard
LB	Lewy bodies
LBD	Lewy body disease
LC	Liquid chromatography
LN	Lewy neurites
LTD	Long-term depression
LTP	Long-term potentiation
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment

MDS	Movement disorder society
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSA	Multiple system atrophy
naPPA	Non-fluent/agrammatic variant of primary progressive aphasia
NfL	Neurofilament light chain
NFT	Neurofibrillary tangles
NIA-AA	National Institute on Aging and the Alzheimer's Association
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
ADRDA	Alzheimer's Disease and Related Disorders Association
NINDS	National Institute of Neurological Disorders and Stroke
SPSP	Society for PSP
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NPTX1	Neuronal pentraxin-1
NPTX2	Neuronal pentraxin-2
NPTXR	Neuronal pentraxin receptor
NSF	N-ethylmaleimide sensitive fusion protein
OR	Odds ratio
PD	Parkinson's disease
PDD	Parkinson's disease dementia

PEBP-1	Phosphatidylethanolamine-binding protein 1
PET	Positron emission tomography
PiB	^{11}C-Pittsburgh compound B
PIGD	Postural instability and gait difficulty
PMCA	Protein misfolding cyclic amplification
PPA	Primary progressive aphasia
PRM	Parallel reaction monitoring
PSD	Post-synaptic density
P-tau	Phosphorylated tau
P-tau$_{181}$	Tau phosphorylated at threonine on amino acid residue 181
P-tau$_{231}$	Tau phosphorylated at threonine on amino acid residue 231
RBD	Rapid-eye-movement sleep behavior disorder
Rho	Spearman's correlation coefficient
ROC	Receiver operating characteristic curve
RPG	Resofurin β-D-galactopyranoside
RT-QuIC	Real-time quaking-induced conversion
SBG	β-D-galactopyranoside streptavidin
Simoa	Single molecule array
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF attachment protein receptors
SPE	Solid-phase extraction
SPECT	Single-photon emission computed tomography
SYT-1	Synaptotagmin-1
TAF15	TATA-binding protein-associated factor 15

TDP-43	43 kDa transactive response DNA-binding protein
TMT	Tandem mass tag
t-SNARE	Target SNARE
T-tau	Total tau
UPDRS	Unified Parkinson's disease rating scale
UPS	Ubiquitin-proteasome system
VaD	Vascular dementia
VAMP-2	Vesicle-associated membrane protein-2
v-SNARE	Vesicle SNARE

1 INTRODUCTION

Neurodegenerative diseases are a heterogeneous group ranging from common multi-factorial dementias to rare monogenic inherited errors of metabolism. As a whole, they cause worldwide morbidity and mortality. Many are age-dependent disorders that are becoming increasingly prevalent, partly because the world population's median age is steadily rising [1] – resulting in a sizeable socioeconomic burden and, most importantly, devastating consequences for patients and their relatives. They are debilitating, mostly incurable diseases characterized by progressive damage to the central and peripheral nervous system. The damage commonly takes the form of loss of neurons, their structure, or their functions.

Classification of neurodegenerative diseases can be performed based on the disease's primary clinical features, such as dementia or parkinsonism [2]. However, mixed clinical features are common, and few patients present with so-called pure syndromes. The diagnostic gold standard is thus neuropathological evaluation at autopsy since disease-specific pathological changes are typically present, affecting different brain regions and cell types. Therefore, classification can also be performed based on pathology, such as the anatomical distribution of the neurodegeneration (*e.g.*, frontotemporal, extrapyramidal, or spinocerebellar) or on the primary molecular abnormality. Many neurodegenerative diseases are proteinopathies, characterized by the aggregation and deposition of misfolded proteins. However, it is critical to note that there is high overlap not only in clinical features but also in pathological processes occurring in the different diseases, as well as a large incidence of comorbidity resulting in an intricate complexity in the classification and study of neurodegenerative diseases.

One major hurdle in the field of neurodegenerative disease is the aforementioned shared pathologies between the numerous diseases as well as progressive changes, which typically lead to insidious onset and a long prodromal phase, which together confounds clinical diagnosis until advanced stages where treatments have low clinical utility [3]. Another is the inadequate tools for patient monitoring at early disease stages for possible drug benefits during drug development projects at clinical trials [4]. A significant part of the continuous research in the field focuses, therefore, on biomarkers (biological markers) of disease, which are laboratory measurements used as indicators of physiological or pathogenic processes [5]. Biomarkers can be utilized for a number of objectives, including recording the very early stages of disease (screening), increasing confidence of diagnosis (diagnostic), predicting disease outcome (prognostic), predicting therapy response (predictive), and monitoring progression or therapy response (monitoring). Different body fluids can be utilized for biomarker quantification, with cerebrospinal fluid (CSF) being the most common for

neurodegenerative diseases. CSF's proximity to the brain parenchyma makes it an optimal fluid for the quantification of biomarkers reflecting disease processes. CSF biomarkers, in general, have a long track record since they were first introduced over 100 years ago, improving patient care and expanding the understanding of disease mechanisms [6]. However, it is only in the last 20 years that real progress has been made for CSF biomarkers of neurodegenerative diseases, demonstrated for Alzheimer's disease (AD), the most prevalent neurodegenerative disease, where CSF measures of the hallmark lesions of the disease, *i.e.*, amyloid-β peptides and phosphorylated tau species, have been implemented in the diagnostic criteria [7]. Although, generally, there is still a far way to go in the field.

One area which has gained recent attention is biomarkers of synaptic pathology (degeneration and dysfunction of the synapse), which is an early and central part of the pathophysiology of many neurodegenerative diseases [8-10]. Biomarkers of synaptic pathology are also of clinical relevance due to the strong association between synaptic degeneration and the degree of cognitive decline since synaptic function underlies cognition [11]. Synaptic biomarkers are thus of interest not only in the routine clinical assessment of neurodegenerative diseases to facilitate diagnosis, disease staging, and progression, but especially to monitor drug trial endpoints and treatment efficacy which commonly aim to halt or reduce synaptic damage.

In conclusion, the main goal of this thesis was thus to develop and optimize methods for the quantification of biomarkers of synaptic pathology. To evaluate their potential not only in AD but also across neurodegenerative diseases to find concordance and discordance in synaptic dysfunction.

1.1 ALZHEIMER'S DISEASE

As previously mentioned, AD is the most common neurodegenerative disease and cause of dementia [1]. In fact, two-thirds of all cases of dementia are caused by the disease. The total number of cases was approximated to be 50 million people worldwide in 2018 (Sweden - 100 000 people). Since age is the most significant risk factor for AD, rising longevity is expected to triple this number by 2050. AD is the 7th leading cause of death (2019), causing significant social and economic costs (trillion US dollars per year). The term Alzheimer's disease was initially coined in 1910 after the German physician Alois Alzheimer, who was the first to describe and document a patient case report of the disease [12]. The patient experienced severe cognitive disturbances, unpredictable behavior, aphasia, disorientation, and delusions. Furthermore, the *post-mortem* examination revealed brain atrophy and characteristic changes in its internal structures in the form of neuronal tangles and extracellular aggregates. Today, these are recognized as defining hallmarks of AD.

1.1.1 PATHOLOGY

A major characteristic of AD is large-scale neurodegeneration, loss of neurons and synapses, leading to brain volume loss (atrophy) [13]. Predominately, the atrophy affects the medial temporal lobe structures and cortical areas of the brain, giving rise to enlarged ventricles and widened sulci. AD is characterized by the anomalous aggregation and accumulation of hyperphosphorylated tau and amyloid-β (Aβ) in intracellular neurofibrillary tangles (NFT) and extracellular plaques, respectively (Fig. 1). Furthermore, AD is marked by a lengthy pre-clinical phase, with neurodegeneration and accumulating pathological changes estimated to begin 2-3 decades prior to symptom onset [14].

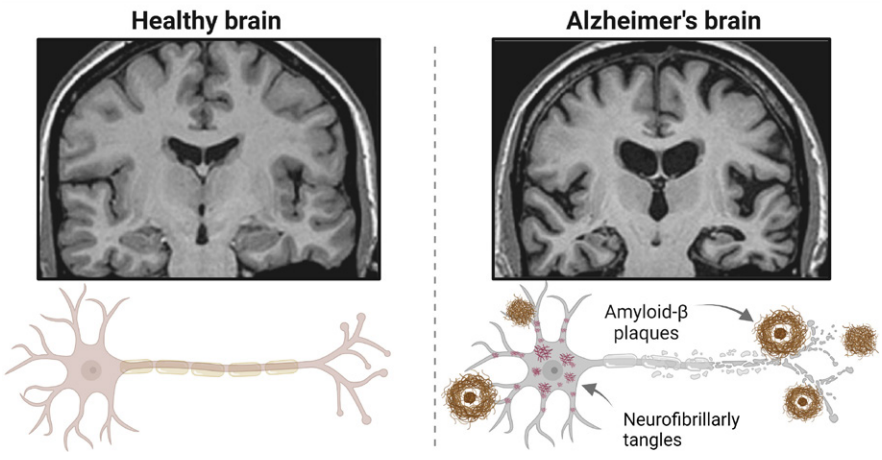

***Figure 1**. Schematic of Alzheimer's pathology. Created with Biorender.com*

Aβ peptides are formed by the sequential enzymatic cleavage through the amyloidogenic pathway of the amyloid precursor protein (APP), a transmembrane glycoprotein with essential neuronal functions, by β- and γ-secretases [15]. Depending on β- and γ-secretase, Aβ peptides with varying lengths are produced, with truncations both at the C- and N-terminal (Fig. 2). The produced peptides have hydrophobic properties and are prone to aggregation into multimers such as oligomers and fibrils that subsequently form the big insoluble aggregates of extracellular plaques. The most studied of the Aβ species is the Aβ peptide 1-42 ($Aβ_{42}$) since this form has been found to be the main plaque component, but the most abundant is the Aβ peptide 1-40 ($Aβ_{40}$), which is also less prone to aggregation. Evidence of toxicity has been found for Aβ regardless of form. However, growing consensus indicates soluble oligomeric Aβ as the primary noxious one, promoting tau phosphorylation, apoptosis, synaptic dysfunction, and degeneration, as well as disruption of the cytoskeleton [15].

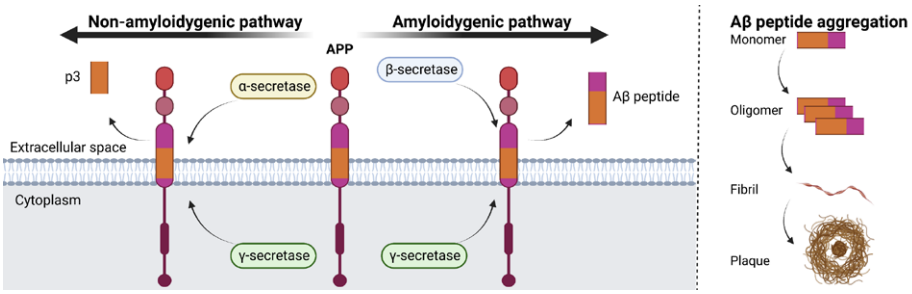

Figure 2. *APP proteolytic pathways (left) and amyloid-β peptide aggregation (right). Created with Biorender.com*

Tau is an axonal protein with six different isoforms in the CNS, whose main function is to bind and stabilize microtubules [16]. Its biological activity is regulated by phosphorylation, where abnormal hyperphosphorylation causes detachment of the protein leading to microtubule disassembly (Fig. 3). Similarly to Aβ peptides, hyperphosphorylated cytosolic free tau is prone to aggregation, clustering together into insoluble fibrils that subsequently form NTFs. Unlike Aβ, tau accumulation under pathological conditions occurs intracellularly in the soma and dendrites. Both soluble oligomeric tau and NFTs have been found to be neurotoxic through a number of pathways [16, 17].

The initial formation of Aβ plagues and NFTs occur in different brain regions, with formation initiated in the neocortex and subcortical areas, such as the hippocampus and amygdala, respectively. They then spread in opposing patterns, with the Aβ plagues spreading into the subcortical regions and the NFT into the neocortex. At post-mortem examination, two scoring systems – Braak and Thal staging are utilized to determine the spread of tau and amyloid pathology, respectively, throughout the brain.

However, 20-40% of non-demented elderly have been found in neuropathological studies to reach the threshold of plagues and tangles to be classified erroneously as AD. Furthermore, only 30% with a definite AD diagnosis were found to have pure Alzheimer's pathology at *post-mortem*.

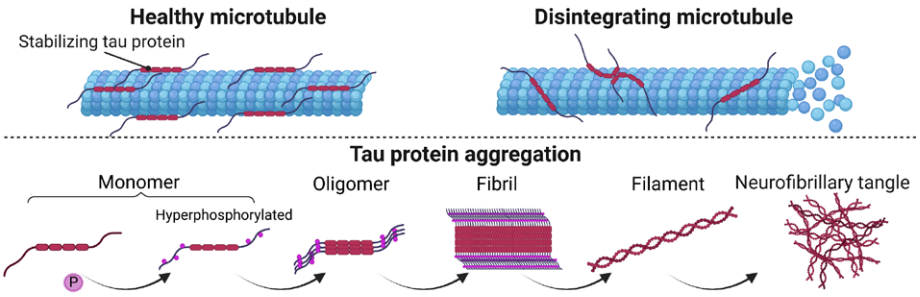

***Figure 3**. Tau aggregation due to hyperphosphorylation and ensuing microtubule dysfunction. Created with Biorender.com*

1.1.2 ETIOLOGY

Less than 1% of all cases are familial AD (FAD), an early-onset hereditary form of the disease caused by autosomal dominant mutations in genes connected to increases in Aβ production [18]. The genes include *APP* as well as *PSEN1* and *PSEN2,* which codes for catalytic subunits of γ-secretase. However, most cases are so-called sporadic AD with no known cause, usually present after the age of 65 years old [19]. Major genetic risk factors include the apolipoprotein E (*APOE*) allele ε4, which increases the risk 3- and 15-fold when being heterozygous and homozygous carriers, respectively [20]. On the other hand, the ε2 allele of the same gene is protective in comparison [20]. The ApoE proteoforms have different receptor- and lipid-binding capabilities, indicated to, in an isoform-specific manner, affect several cellular functions, including Aβ clearance, lipid metabolism, neuroinflammation, and synaptic plasticity [21]. However, the underlying reason for the connection between the *APOE* variants and AD is still under debate. Even though the etiology of AD is not fully known, there are many hypotheses. The main is the amyloid cascade hypothesis, which postulates that Aβ is the primary driver of the disease [22]. The hypothesis states that increased production or decreased clearance of Aβ causes plaque formation, leading to a series of events including NFT deposition and neuronal death resulting in AD. The substantial evidence in support includes the FAD gene heredity link to Aβ metabolism, and Down's syndrome, which has a high incidence of early AD, carries an extra copy of the *APP* gene. However, arguments against the hypothesis exist, such as that clinical trials targeting amyloid pathology have shown a lack of clinical benefit. Alternative theories include tau being the main driver, but also inflammation [23] and oxidative stress [24] have been implicated.

1.1.3 CLINICAL PRESENTATION & DIAGNOSTIC CRITERIA

Prominent symptoms of AD are gradual memory dysfunction and cognitive decline. Early signs include mild memory impairments, particularly episodic memory, commonly experienced concurrently with neuropsychiatric symptoms such as depression. As the disease progress, the symptoms worsen, with executive functions deterioration, including impaired judgment, decision-making, and orientation. Instrumental functions are also affected during disease evolution with impairments to speech (aphasia), skilled and learned movement (apraxia), and recognition (agnosia).

The first consensus criteria for diagnosis of AD was established in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and relied on the clinical symptoms [25]. In 2011, updated criteria by the National Institute on Aging and the Alzheimer's Association (NIA-AA criteria) and separate sets of diagnostic guidelines for the symptomatic stages of AD, mild cognitive impairment (MCI) [26] and dementia [7], were created. A clinical diagnosis of possible or probable AD can be set in accordance with the medical history and a battery of examinations and evaluations, both physical, neurological, and neuropsychological, confirming the presence of dementia and excluding other etiologies using selective ancillary testing. A range of cognitive tests exists to evaluate cognitive impairment in patients with dementia, and one of the most conventional tests is the Mini-Mental State Examination (MMSE) [27]. The test is composed of a 30-point questionnaire that evaluates orientation, memory, concentration, and language, as well as the calculation ability of the patient, where a score below 27 commonly indicates cognitive impairment. However, a definite diagnosis of AD can only be set after *post-mortem* pathological confirmation (gold standard), establishing the presence of NFTs and amyloid plaques [7].

Biomarkers

Due to the scientific progress in CSF measures as well as in imaging, biomarker evidence has also been added to the diagnostic research NIA-AA criteria of possible and probable AD dementia [7]. At the time, recommendations were also created for so-called preclinical AD, a stage of AD without overt symptoms but with abnormal AD biomarkers [28]. As the field has continued to advance rapidly, in 2018, NIA-AA presented further updated research criteria that define AD as a pathologic process, which can be identified *in vivo* by biomarkers including imaging and fluid as valid proxies for the neuropathologic changes occurring in AD [29]. The criteria define AD as a continuum that can be characterized utilizing the ATN biomarker framework, where the biomarkers are arranged into those reflective of amyloid-β deposition ("**A**"), pathologic tau ("**T**"), and neurodegeneration ("**N**") [30]. The system classifies each category as positive or negative, where a typical AD profile is identified as A+/T+/N+.

As neurodegeneration is not specific to AD, N biomarkers of neurodegeneration are generally suggested to be used to stage severity similarly to cognitive symptoms, unlike A and T, which reflect specific neuropathological changes that define AD.

Currently, biomarkers of Aβ deposition include CSF measures of low levels of $A\beta_{42}$ and cortical amyloid positron emission tomography (PET) ligand binding (*e.g.*, ^{11}C-Pittsburgh compound B (PiB), [^{18}F]-florβpir, [^{18}F]flutemetamol, and [^{18}F]florβben) [29]. Low levels of $A\beta_{42}$ are believed to reflect reduced secretion due to retention and inclusion of the hydrophobic peptide in amyloid plaques [31]. In order to increase biomarker performance of $A\beta_{42}$, the ratio of $A\beta_{42}$ over $A\beta_{40}$ levels is commonly used, as $A\beta_{40}$ is believed to be a measure of the total Aβ production allowing for compensation of individual variations.

Similarly, for tau pathology, biomarkers include cortical tau PET ligand binding (*e.g.*, [^{18}F]flortaucipir) as well as high CSF levels of different phospho-forms of tau (p-tau) [29]. The p-tau toolbox is undergoing current rapid expansion, with new potential p-tau biomarkers being developed. The classical AD biomarker, however, is p-$tau_{181,}$ tau phosphorylated at threonine on amino acid residue 181, where high levels of the marker are believed to reflect increased tau phosphorylation in the brain [31]. Lastly, biomarkers of neurodegeneration include atrophy visualized by anatomic magnetic resonance imaging (MRI), hypometabolism as shown by [^{18}F]-fluorodeoxyglucose (FDG) PET, or high CSF total tau (t-tau) levels [29]. FDG PET is a valuable tool to differentiate AD from both healthy aging as well as other dementia disorders by estimating the typical spatiotemporal patterns of hypometabolism in AD by utilizing radiolabelled glucose and measuring its uptake [32]. Levels of t-tau believed to reflect neuronal tau secretion and injury allude to the measurement of tau, regardless of isoform, modification status, and if it is full-length tau or a fragment [31]. Unlike p-tau, high levels of t-tau are not specific to AD. In fact, they are also found in acute conditions (*e.g.*, stroke and brain trauma) [33, 34] and disorders with rapid neurodegeneration (*e.g.*, Creutzfeldt-Jakob disease (CJD)) [35]. Another potential biomarker for neurodegeneration found in high levels in AD, which is increasingly recognized and believed to reflect axon-neuronal damage, is the cytoskeletal protein neurofilament light chain (NfL) [36].

Imaging allows for the possibility of gaining important temporal and spatial information for staging purposes but is laborious and expensive. Changes in the concentrations of the core AD CSF biomarkers of $A\beta_{42}$, t-tau, and p-tau occur early in the course of the disease. The combined use of the three has been demonstrated to have 80-90% sensitivity and specificity for prodromal AD [37]. A large part of the current biomarker research is focused on moving the biomarkers to blood, a biofluid more accessible than CSF, in light of recent advances in ultrasensitive methodologies [38].

An overview of some of the currently available biomarkers for AD is visualized in Figure 4, including advantages and disadvantages with each measurement.

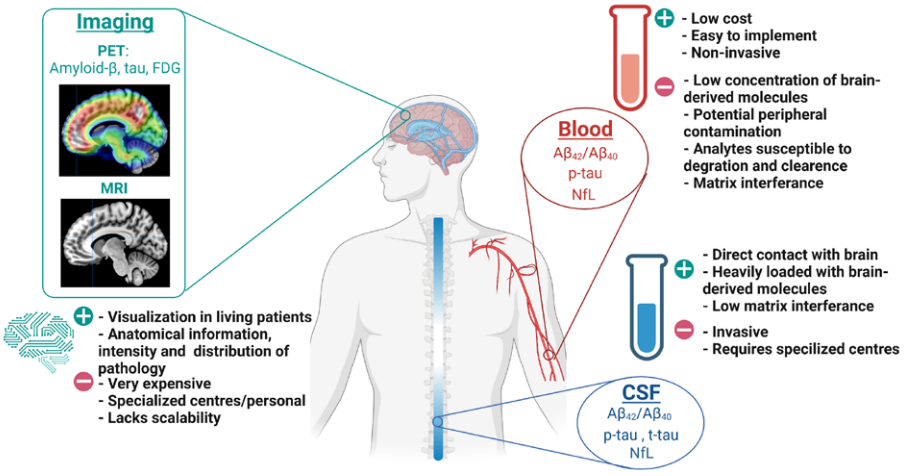

Figure 4. *Advantages and disadvantages of fluid and imaging biomarkers. Curtesy of Juan Lantero Rodriguez. Created with Biorender.com*

Management and treatment

Despite the many clinical trials, no disease modifier treatment is currently available for AD. Only symptomatic treatments of the cognitive symptoms exist, including acetylcholinesterase inhibitors (*e.g.,* donepezil and galantamine) and N-methyl-D-aspartate (NMDA) agonists (*e.g.,* memantine) [39]. However, several past and present clinical trials include anti-amyloid and anti-tau immunotherapy, utilizing antibodies to target the underlying pathology, both in the form of soluble oligomers and insoluble plaques and NFTs. In June 2021, the U.S. Food and Drug Administration approved aducanumab (Aduhelm™), a human immunoglobulin-γ-1 monoclonal antibody targeting soluble and insoluble forms of aggregated Aβ as the first disease-modifying treatment of AD in the USA [40]. However, the drug has not been approved for use in Europe by the European Medicines Agency (EMA), and post-approval studies of clinical efficacy are needed. However, suggested recommendations for the appropriate use of aducanumab state that before receiving treatment, AD pathology should first be confirmed using imaging or CSF biomarkers [41]. This demonstrates the importance of biomarkers in the treatment strategies for AD to ensure proper use and cost-effectiveness.

1.2 FRONTOTEMPORAL DEMENTIA

Initially, frontotemporal dementia was first used as a term by Arnold Pick in 1892 to describe a patient experiencing lobar atrophy, aphasia, and presenile dementia [42]. Today, FTD is the clinical term of a heterogeneous group of dementias which are the second most common cause of early-onset (<65 years) dementia cases after AD and third in late-onset (>65 years) where its surpassed by dementia with Lewy bodies [43]. The estimated prevalence is 150-220 per million people, with 75% of the FTD cases occurring before 65 years of age [44].

1.2.1 PATHOLOGY

Similar to the heterogeneity found in the clinical presentation of FTD, there is also considerable heterogeneity in the underlying neuropathology (Fig. 5) [45]. The pathological counterpart of FTD is frontotemporal lobar degeneration (FTLD), characterized by frontal and temporal neuronal loss, a relatively consistent feature of FTD (~80%). FTLD is thus a term commonly used to define pathological conditions that mainly present as clinical FTD but can also be found in other syndromes. The current neuropathological subgroup classification of FTLD is based on the presence of proteinaceous aggregations or lack thereof [46].

One of the major subgroups consisting of 40-45% of all FLTD cases, presents with tau inclusions (FTLD-tau). This group of primary tauopathies can be further subdivided based on the occurrence of specific tau species in the inclusions – mainly the presence of either three or four microtubule-binding repeats or both. These primary tauopathies include progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), globular glial tauopathy (GGT), and Pick's disease [47]. Both PSP and CBD will be further introduced during the section on parkinsonian disorders (Page 12) due to overlap in the clinical and pathological presentations. The second significant subgroup (50-60% of FTLD cases), FTLD-TDP, contains pathological inclusions of 43 kDa transactive response DNA-binding protein (TDP-43) that are tau-negative and ubiquitin-positive. The group can also be further divided into subtypes; A, B, and C, depending on distinct patterns of the pathological inclusions and predominant lesion type(s) [46]. Further, there is FTLD-FUS, a minor subgroup (5-10%), containing inclusions consisting of fused in sarcoma protein (FUS). The group is also referred to as FTLD-FET since it was shown that the inclusions in FTLD-FUS also comprise the two other FET proteins, TATA-binding protein-associated factor 15 (TAF15) and Ewing's sarcoma (EWS) [48]. Both TDP-43 and the FET proteins are nucleic acid-binding proteins involved in several parts of gene regulation processes. The protein has not been identified in a rare subgroup, FTLD-UPS, which presents with mutations in the CHMP2B gene and inclusions positive for ubiquitin-proteasome system markers (UPS). In another, called FTLD-NI, no inclusions are present [46].

1.2.2 ETIOLOGY

FTD is a common familial disease (hereditary), with 30-50% of cases reporting a previous family history, but the heritability differs between FTD variants, with TDP-43 proteinopathies being the most frequent cause [45, 47]. In fact, 10-27% of the cases display an autosomal dominant mode of inheritance, highlighting the importance of genetics in the etiology of FTD. Responsible for almost all cases of familial FTD (60%) are mutations in the genes of either, in order of prevalence, chromosome 9 open reading frame 72 (*C9orf72*), progranulin (*GRN*), or microtubule-associated protein tau (*MAPT*) [49]. Rare genetic causes (<5% of FTD cases) include, among others, mutations in *VCP*, *OPTN*, *TARDP*, *SQSTM1, CCNF, CHCHD10, CHMP2B, TBK1, TIA1,* and *FUS*. However, the known genes do not account for all familial cases, suggesting that there are other disease-causing genes yet to be discovered. Figure 5 gives a simplified overview of the complex landscape of subtypes present in FTLD pathology and FTD etiology.

1.2.3 CLINICAL PRESENTATION & DIAGNOSTIC CRITERIA

There are two major clinical phenotypes of FTD. A behavioral phenotype (bvFTD) typically displays disinhibition, apathy, abnormal appetite, loss of empathy, and obsessive-compulsive behavior [43]. The second phenotype primarily affects language abilities, coined primary progressive aphasia (PPA). PPA is characterized either by impaired expressive language and single-word comprehension deficits (semantic variant), impaired speech and agrammatism (non-fluent/agrammatic variant), or impairments in word retrieval and repetition (logopenic variant). As the phenotypes of FTD progress, their symptoms commonly converge, and they tend to develop global cognitive impairment and motor deficits such as parkinsonism.

The most recent diagnostic criteria for bvFTD, proposed by the international consortium in 2011, include three levels of diagnostic certainty for a diagnosis of bvFTD: definite, probable, and possible [50]. For a diagnosis of definite bvFTD, requirements entail the existence of the clinical syndrome with either pathological or genetic verification of FTLD. For a diagnosis of possible bvFTD, the criteria require the presence of three out of six clinically discriminating symptoms. At the same time, a diagnosis of probable bvFTD can be made if functional disability and characteristic neuroimaging also are present.

Diagnostic criteria for PPA and its variants require basic features of gradual progression of impairment in language that should be prominent and isolated [51]. For a clinical diagnosis of a specific variant, characteristic features should be present. When supported by neuroimaging changes in line with those formerly associated with each PPA variant, a higher confidence level is given. A definitive diagnosis can only

be confirmed by pathology or genetic evidence. To study the FTD syndrome in living subjects concerning the complex heterogeneity of underlying pathology, studies of familial cases are thus favorable, allowing for insight due to the linear relationship commonly found between pathology and genetic mutations.

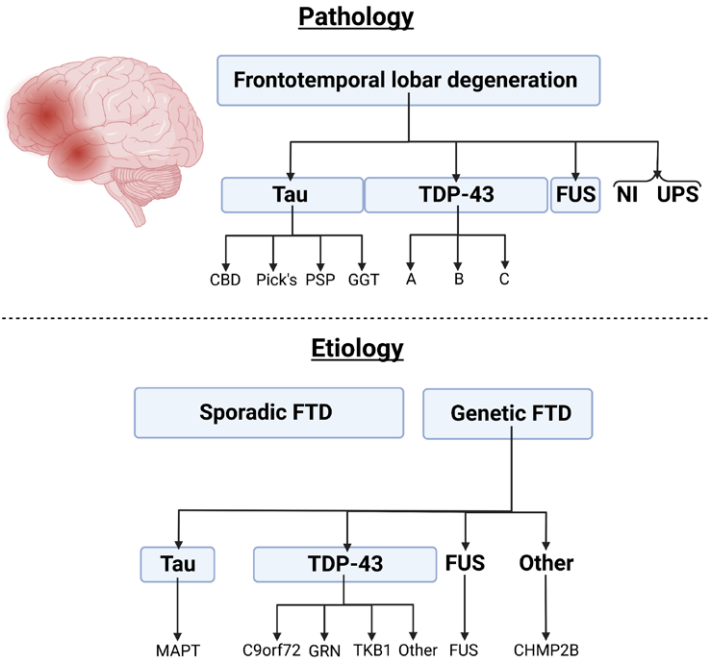

Figure 5. *Simplified illustration of the complex landscape of subtypes present in FTLD pathology and FTD etiology. Created with Biorender.com*

Biomarkers

There is a lack of fluid biomarkers to aid the diagnosis of FTD. However, the core AD biomarkers can be used to rule out an AD diagnosis [52]. Markers of neurodegeneration, especially, NfL, have shown promise as a prognostic biomarker and for differentiation of non-neurodegenerative disorders. Progress is also being made in finding specific CSF biomarkers for identifying pathogenic mutations, which is less expensive than genetic screenings. For example, CSF progranulin for *GRN* and dipeptide repeat proteins for *C9orf72* mutation carriers. On the other hand, imaging, including MRI and FDG PET, are much more practical, identifying atrophy and disproportionate hypometabolism in the frontal and temporal lobes [43].

Management

There are no approved disease-modifying drugs available for FTD. Treatment of FTD is based chiefly on modifying behavioral symptoms with low doses of atypical antipsychotics and antidepressants, such as selective serotonin reuptake inhibitors [43].

1.3 PARKINSONIAN DISORDERS

Parkinsonism is a group of neurological diseases clinically characterized by slow movements (bradykinesia), rigidity, tremor, and other changes such as cognitive and autonomic dysfunction. The most common form is **Parkinson's disease** (PD), with a prevalence of 1000-2000 per million people, making it the second most frequent neurodegenerative disease [53]. A hallmark of PD is the pathological intracellular aggregation and accumulation of α-synuclein in the form of Lewy bodies; PD is thus considered to be a synucleinopathy. Approximately 50-80% of all PD cases will progress and experience cognitive decline leading to the development of **Parkinson's disease dementia** (PDD) [54]. An atypical form of parkinsonism is **dementia with Lewy bodies** (DLB). DLB is as well pathologically identified by the presence of Lewy bodies, which, however, develop early cognitive symptoms [55]. DLB is the second most frequent cause of dementia (20%) after AD and has an estimated prevalence of 4000 per million [56]. PD, PDD, and DLB belong to a spectrum of **Lewy body disease** (LBD).

Other rare atypical forms of parkinsonism, ranked by prevalence (ca 5-10% of parkinsonism cases), are – **progressive nuclear palsy** (PSP, prevalence; 50 per million [57]), **multiple system atrophy** (MSA, prevalence; 50 per million [58]), and **corticobasal degeneration** (CBD, prevalence; 10 per million). However, there is a great discordance in prevalence rates among estimations due to clinical overlap and disease heterogeneity leading to a high rate of misdiagnosis among atypical parkinsonian diseases.

1.3.1 PATHOLOGY

Parkinsonian diseases are generally defined by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (Fig. 6). Still, their type of pathological inclusions differ despite overlapping clinical presentations.

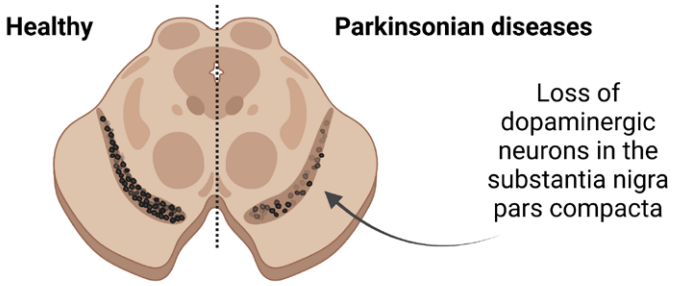

Figure 6. *Illustration of the degeneration of dopaminergic neurons in the substantia nigra pars compacta found in parkinsonian diseases. Created with Biorender.com*

As previously mentioned, the LBDs are synucleinopathies, where intracellularly aggregated α-synuclein are found in pathological inclusions in the form of Lewy bodies (LB) and Lewy neurites (LN) (Fig. 7) [55]. In PD, the inclusions are confined to the limbic regions as well as the brainstem, while in PDD and DLB patients, the inclusions are also present in the neocortex [59]. Of note, AD-comorbidity is further a frequent feature of LBD (<10% PD, 40% PDD, >70% DLB) believed to contribute to the cognitive decline found in DLB and PDD [60]. In fact, ~50% of DLB cases present with enough co-pathology for a secondary neuropathological diagnosis of AD. PDD and DLB are neuropathologically very similar. However, it's suggested that DLB shows a less severe degeneration in the substantia nigra and a higher burden of LB pathology in limbic and neocortical regions in addition to the higher rate of AD pathology. MSA is similar to the LBDs a synucleinopathy. However, MSA presents primarily with a type of inclusions known as glial cytoplasmic inclusions (GCI), aggregated α-synuclein in oligodendrocytes with a typical distribution in the cerebellum, pons, and basal ganglia [61]. For more on α-synuclein and its function, see the section on the synuclein family (Page 28).

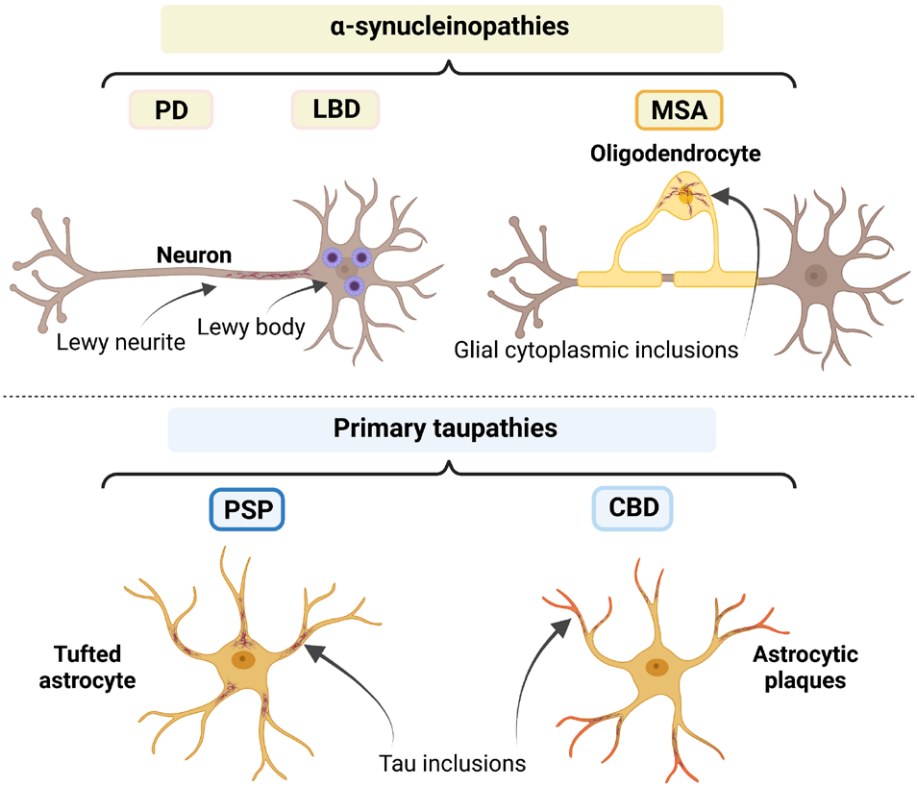

***Figure 7**. Schematic of the pathological inclusions present in the different α-synucleinopathies; PD, LBD, and MSA, as well as in the tauopathies; PSP and CBD. Created with Biorender.com*

PSP and CBD, on the other hand, are primary tauopathies characterized by tau protein inclusions, predominantly anti-4R immunoreactive, in neurons in the form of NTFs but also glia (Fig. 7). In the early stages, they both primarily affect the basal ganglia. Still, they differ in distribution and spread patterns, where cortical and white matter (neocortex) regions are more affected in CBD. Deep gray matter regions (brainstem) are more affected in PSP [62-64]. Furthermore, they also differ in their glial lesion types, where the pathological hallmarks of CBD and PSP are in the form of astrocytic plaques and tufted astrocytes, respectively.

1.3.2 ETIOLOGY

The majority of PD cases are sporadic, with only 5-15% of all cases being familial [65-67]. Twenty-three genes have been found to cause PD potentially, all named PARK in the order they were identified. The foremost monogenetic mutations found in autosomal dominant PD are *SNCA (PARK1 & PARK4)* and *LRRK2 (PARK8)*, where the point mutation in the α-synuclein gene *SNCA* was the first discovered, albeit rare. In contrast, mutations in *LRRK2* are the single most frequent cause of inherited PD [66, 67]. In autosomal recessive PD, the most critical monogenetic mutations are connected to mitochondrial homeostasis; *DJ-1* (*PARK7)*, *PINK1 (PARK6)*, and *PRKN (PARK2),* which are rare but are responsible for a substantial proportion of early-onset PD. Genetic risk factors include loci in *GBA1*, which encode for the lysosomal enzyme glucocerebrosidase, as well as *MAPT, SNCA,* and *LRRK2* [65, 68]. For DLB, the pathogenic causative mutations are rarer than in PD but with a more common frequency of genetic risk factors such as *GBA1* and the *APOE* ε4 allele [55].

Similarly, MSA, PSP, and CBD are considered non-familial or sporadic neurodegenerative diseases, although rare reports of familial cases have been described [61, 69]. Identified genetic risk loci are suggested to be the same for MSA as for DLB, while for the tauopathies, PSP and CBD, *MAPT* is a major risk factor.

1.3.3 CLINICAL PRESENTATION & DIAGNOSTIC CRITERIA

PD is a complex progressive disease characterized by motor symptoms including tremor, stiffness, slowness, and imbalance, and non-motor symptoms including a lost sense of smell and rapid-eye-movement sleep behavior disorder (RBD) (Fig. 8) [54, 60]. The non-motor symptoms typically occur during the preclinical or prodromal stage, while cognitive impairment is a common complication of late-stage disease, PDD, corresponding to poor prognosis. The first formal and most utilized diagnostic criteria for PD have been set by the United Kingdom Parkinson's Disease Society Brain Bank [70]. The criteria state that bradykinesia should be present in addition to another sign of either rigidity, rest tremor, or postural instability. Additionally, three or more supportive prospective criteria, such as unilateral onset, disease progression,

persistent side asymmetry, positive dopaminergic response, or clinical course longer than ten years, should be found. Furthermore, the absence of exclusion criteria is required, such as secondary causes including repeated strokes or toxic agent exposure, but also symptoms more indicative of atypical Parkinson's diseases and negative dopaminergic therapy response.

In order to evaluate the impairment and disability present in PD, rating scales have been implemented; the most widely used is the Unified Parkinson's Disease Rating Scale (UPDRS) [71]. The scale design is constituted of four components, namely, motor experiences of daily living, non-motor experiences of daily living, motor complications, and motor examination. Individual sub scores of the UPDRS scale include items such as scores of tremor as well as postural instability and gait difficulty (PIGD).

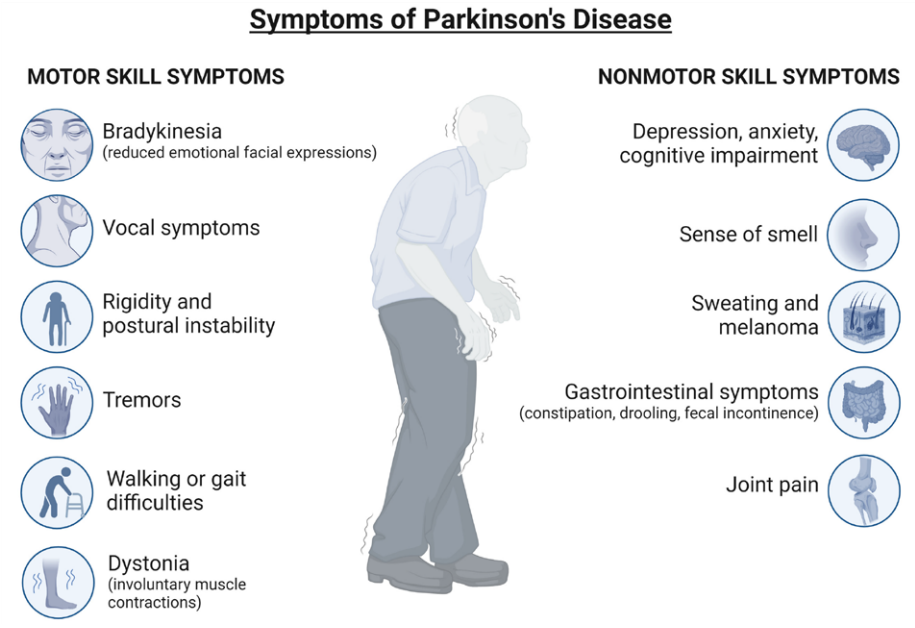

Figure 8. *Illustration of the wide range of motor and non-motor symptoms of Parkinson's disease. Created with Biorender.com*

DLB does not only overlap in neuropathology with PDD but also in clinical features and only differs by an arbitrary rule of distinction that the cognitive impairment starts within 12 months of the onset of motor symptoms [55, 72]. However, 25% of DLB patients never develop parkinsonian symptoms; if they do, they may be very mild. Like PD, DLB patients also commonly present with RBD, as it is associated with an underlying synucleinopathy, and the symptoms may precede the cognitive decline. Other key characteristics are visual hallucinations and alertness, and attention

variations. The consensus criteria of 2017 for clinical and pathologic diagnosis of DLB [73] established by the DLB consortium states that in addition to cognitive impairment, two of these core features (parkinsonism, hallucinations, fluctuations, or RBD) are enough for probable and one for possible DLB.

There is high variability in clinical presentations for MSA, and the disease is thus often divided into two different clinical phenotypes [74]. Cases displaying predominant parkinsonism, MSA-P, such as bradykinesia with tremor, rigidity, or postural instability, or cases presenting with predominant cerebellar ataxia, MSA-C, such as limb ataxia, cerebellar dysarthria, or cerebellar oculomotor dysfunction. All MSA patients, however, present with autonomic failure – such as urinary, erectile, and bowel dysfunction or orthostatic hypotension, which according to the Gilman criteria [74], supports a diagnosis of probable MSA in combination with negative dopaminergic therapy response and cerebellar syndrome. Parkinsonism or cerebellar syndrome with suggested autonomic dysfunction only supports a diagnosis of possible MSA.

In PSP, the clinical presentations are even more diverse than for MSA, with multiple phenotypes where the definition is continually undergoing revision [75]. Key features of PSP are early falls and gait instability, distinguishing the disease from other parkinsonian disorders. Other hallmarks include vertical supranuclear palsy and progressive dementia. Criteria for the diagnosis of PSP were established by the National Institute of Neurological Disorders and Stroke and Society for PSP (NINDS-SPSP) in 1996 [76]. The criteria relied on the presentation of postural instability, vertical supranuclear gaze palsy, and during the first year of onset of symptoms for a diagnosis of probable PSP, with excellent specificity. However, as this mainly captured the phenotype of PSP referred to as Richardson's syndrome, with low sensitivity for other subtypes or early presentations, the criteria were revised in 2017 by the International Parkinson and Movement Disorder Society (MDS)-endorsed PSP Study Group [77]. The revised criteria divide symptoms into four core functional domains: ocular motor dysfunction, postural instability, akinesia, and cognitive dysfunction, wherein in each domain, three characteristic clinical features were specified and stratified by levels of certainty (suggestive of, possible, or probable). The resulting 12-unit grid is then used to combine different symptoms present in a patient resulting in the PSP predominance type on a specific level of certainty. Predominance types include as well as Richardson syndrome of; postural instability, ocular motor dysfunction, parkinsonism, progressive gait freezing, CBS, frontal presentation, and speech/language disorder.

CBD is a neuropathological confirmed diagnosis that was first described with clinical features of the corticobasal syndrome (CBS), the two – the pathological entity and the phenotype have thus historically been used interchangeably [78]. CBS presents with

both cortical and extrapyramidal signs, such as cortical sensory deficits, apraxia, and alien limb phenomena, as well as asymmetrical Parkinsonism, dystonia, and myoclonus, respectively. However, clinicopathological studies have shown that CBS often may be caused by pathologies other than CBD, such as AD and PSP. Similarly, CBD may clinically present with different subtypes, and current diagnosis criteria [79] identify four phenotypes; frontal behavioral-spatial syndrome (FBS), non-fluent/agrammatic variant of primary progressive aphasia (naPPA), and PSP syndrome, as well as CBS which is the most frequent. For a clinical research diagnosis of probable CBD the criteria require – FBS or naPPA with at least one feature of CBS or either a clinical phenotype of probable CBS.

Biomarkers

Dopamine transporter single-photon emission computed tomography (SPECT) scans with [^{123}I]ioflupane, also known as DatScan or (^{123}I)FP-CIT, are commonly used to confirm an uncertain parkinsonian disorder diagnosis by identifying the presynaptic dopamine neuronal dysfunction. It is reported to be highly accurate, with 98%-100% sensitivity and specificity, in detecting nigrostriatal cell loss. The DatScan works by demonstrating reduced uptake in the basal ganglia of the radioactive dopamine transporter tracer [80]. However, when the clinical examination shows unequivocal parkinsonism, the imaging typically contributes minimally to the diagnostic assessment and cannot be used for the differential diagnosis among parkinsonian disorders. In that case, MRI may be more helpful identifying specific patterns of atrophy [81, 82].

There are no current CSF biomarkers in use, but biomarkers of the underlying pathology such as α-synuclein and tau are being explored. For the tauopathies, CBD and PSP have shown varying but primarily negative results for the currently available tau biomarkers developed for AD. Studies on t-tau and p-tau have shown normal healthy, or marginally higher levels than controls [83]. Similarly, studies exploring total CSF α-synuclein as a potential biomarker of α-synucleinopathies (PD, DLB, and MSA) have found inconsistent results with low clinical utility and only slightly lower concentrations indicated than in controls [10]. However, the inconclusive and disappointing results of α-synuclein and tau as diagnostic biomarkers for parkinsonian disorders might be due to targeted protein forms.

Recent success has been found for the measurement of pathogenic α-synuclein aggregates in CSF using protein misfolding cyclic amplification (PMCA) assays) or aggregation assays (*e.g.*, real-time quaking-induced conversion (RT-QuIC). Studies have shown promising results with 80-95% specificity and sensitivity for PD, MSA, and DLB from other neurodegenerative diseases such as AD and healthy controls. Of note, when it comes to biomarkers, both imaging and fluid, DLB is a particular case

due to the high presence of AD co-pathology, and an AD profile is thus a common feature that influences the clinical diagnostic accuracy of said biomarkers [84, 85].

Management

Presently, no pharmacologic therapies delay or prevent any of the parkinsonian disorders. For PD, there are many symptomatic therapies available [54]. For the motor symptoms, the treatments are primarily dopamine based. Beneficial initial treatments include levodopa (precursor to dopamine) preparations, monoamine oxidase-B (MAO-B) inhibitors, and dopamine agonists. For atypical parkinsonian disorders, levodopa is as well used for movement symptom treatment. However, patients typically display a transient or poor response which is thus commonly included as a diagnostic criterion [86].

1.4 THE SYNAPSE

The brain's most prominent and abundant feature is the synapse, the point of contact at which an impulse is transmitted from one nerve cell to the next. As the functional unit of the nervous system, the 100 trillion synapses function to interconnect the 86 billion neurons of the brain [87]. During the course of life, synapses are constantly formed (synaptogenesis) and eliminated (synaptic pruning), creating an equilibrium and ensuring proper connectivity throughout the whole brain (this is sometimes called synaptic homeostasis). The synapse exists in a wide variety of shapes and locations. The vast majority of synapses in the human brain exist as a pre-synaptic axonal bouton of a neuron connecting to the dendritic spine of another (axodendritic synapse) (Fig. 9). Other types include, but are not limited to axoaxonic (axon to axon), axosomatic (axon to soma), and dendrodendritic (dendrite to dendrite) synapses.

There are two main modalities of synapses based on their mode of transmission; electrical and chemical (Fig. 9). The electrical synapse provides a direct connection between adjacent neurons mediated by gap junctions, *i.e.*, pores, which are comprised of clusters of intercellular channels [88]. The gap junctions allow for the direct passage of small molecules and electrical currents leading to rapid bidirectional electrical potential. They are thus common in neural systems that require the fastest possible response, such as defensive reflexes. The chemical synapse, on the other hand, comprises the pre-synaptic terminal from which the neurotransmitters are released upon the arrival of an action potential, the synaptic cleft (15-20 nm) of which they transverse, and the post-synaptic terminal at which they finally bind to the receptors from which the signal then further propagates [89]. In contrast to the electrical, the chemical synapse's signaling is thus slow and unidirectional. However, unlike electrical, the chemical synapses can amplify and transform the signals. Of the two modalities, chemical synapses are the most common of the two modalities, but both types and their interactions are necessary for normal brain function and development.

Chemical synapses can further be classified into excitatory and inhibitory, based on if they increase or decrease, respectively, the probability of action potential [90]. The major neurotransmitter of excitatory synapses is glutamate. Glutamatergic synapses are the brain's most common kind of synapses and are primarily located on dendritic spines. They are recognized by the asymmetrical form of their synaptic junction and a large post-synaptic density (PSD). The PSD, on the other hand, is a protein-dense specialization constituted of receptor, scaffold, and signaling proteins linked to the postsynaptic membrane. The major neurotransmitter of inhibitory synapses is γ-aminobutyric acid (GABA), which has symmetrical synaptic junctions and small PSD that are primarily located on dendritic shafts.

Studies have shown that the synapse is a plastic entity; it's not static but changes over time [91, 92]. The definition of synaptic plasticity specifically entails the activity-dependent modification, enhancing (long-term potentiation, LTP) or reducing (long-term depression, LTD), the strength and or efficacy of synaptic transmission. It can be divided into short-term, which lasts tens of milliseconds to a few minutes, and long-term, typically lasting minutes to hours. Synaptic plasticity consequently allows the brain to change and adapt to new information. Long-term synaptic plasticity is thus, for most models, believed to be the foundation of memory and learning. Furthermore, it may occur at excitatory or inhibitory synapses as well as through presynaptic and postsynaptic mechanisms and depend on the involvement of several synaptic proteins. The most extensively studied and, therefore, classic example of long-term synaptic plasticity is observed in the CA1 region of the hippocampus and is induced by the activation of N-methyl-D-aspartate receptors (NMDAR) of glutamatergic synapses (Fig. 9).

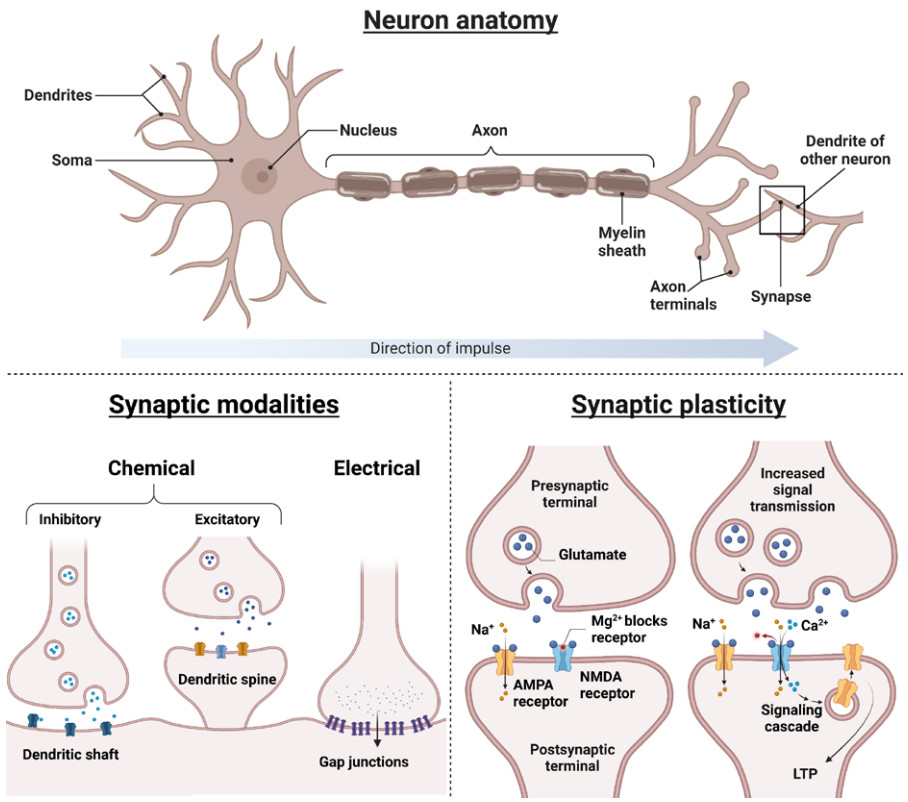

Figure 9. *Illustration of the neuron, its parts, and different synaptic modalities and synaptic plasticity. Created with Biorender.com*

When NMDARs are activated by high-frequency transmission, there is a sudden influx of calcium ions which induces a signaling cascade mediated by interaction with calmodulin and downstream activation of CaM kinase II. The kinase phosphorylates the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), promoting expression, insertion, and stabilization of the AMPARs at the PSD. Since the AMPAR mediates fast synaptic transmission, this, in turn, leads to increased synaptic strength, *i.e.*, LTP. Morphological changes that accompany LTP are dendritic spine growth and spine enlargement. On the other hand, low transmission levels may lead to pruning and spine shrinkage through the induction of LTD, caused by the dephosphorylation of AMPAR and, in turn, receptor internalization via endocytosis.

1.4.1 SYNAPTIC DYSFUNCTION

Imbalance and perturbance of the homeostasis of the synapse and its function result in decreases in synapse numbers and morphology changes such as spine shrinkage (Fig. 10) [93]. Increasing documentation has shown that dysfunction of the synapse is a significant factor in several neurodevelopmental disorders [94], including bipolar disorder, autism, and schizophrenia, as well as neurodegenerative diseases. For many neurodegenerative diseases, including all covered in this thesis, synaptic dysfunction and synaptic degeneration are considered a hallmark and part of the central pathophysiology [8-10]. This fact has been especially documented in AD, where a decrease in synaptic density has been found to surpass 40% in some brain regions [95, 96]. The synaptic dysfunction and degeneration also occur earlier in the disease progression and are more severe than neuronal loss [97]. Additionally, synapse degeneration correlates more robustly with cognitive decline than the numbers of Aβ plaques or NFTs, indicating that it represents a possible vital diagnostic and therapeutic target [11, 98].

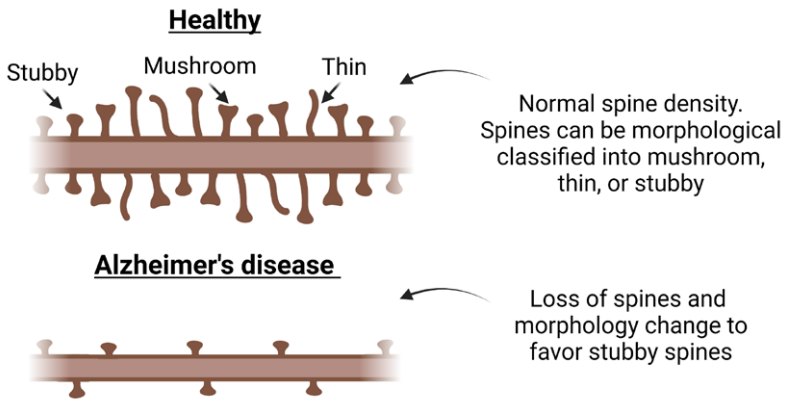

Figure 10. *Illustration of spine dysfunction in Alzheimer's disease. Created with Biorender.com*

As described earlier, during AD, toxic protein accumulation occurs, often at the synapse, causing modifications to synapse structure and composition, leading to impaired synaptic plasticity. With shrinking distance to Aβ plaques, the severity of synaptic dysfunction has been found to be increased. However, as previously mentioned, soluble Aβ oligomers found surrounding the Aβ plaques in a halo formation are believed to be the primarily synaptotoxic form, not the plaques themselves [99]. How the Aβ oligomers affect the synapse is presently quite unclear. Still, multiple pathways have been suggested and documented at both the pre-and postsynaptic compartment through indirect and direct interactions with receptors and other components (for reviews, see [91, 100, 101]). One of the mechanisms involves interaction with NMDARs or other postsynaptic receptors, causing increased calcium concentrations leading to synapse dysfunction and removal by inhibiting LTP. Other mechanisms include the activation of the downstream classical complement pathway leading to accentuated synaptic engulfment by phagocytic microglia.

Similarly, pathogenic tau forms found in both primary and secondary tauopathies have been indicated to cause synaptic dysfunction through several mechanisms [102]. One of the suggested mechanisms of hyperphosphorylated oligomeric tau, after mislocalization to the dendritic synapse, includes impaired synaptic plasticity and LTP by inhibiting trafficking or anchoring of AMPARs and NMDARs [103]. Pathological tau is also found presynaptically and may interfere with neurotransmitter release by associating with synaptic vesicles and their regulatory proteins [104]. However, tau is also believed to contribute to dysfunction through loss of its physiological function, which causes microtubule disintegration and, as a consequence, impaired axonal transportation of synaptic cargo, including mitochondria [105].

Synaptic dysfunction also plays a major role in α-synucleinopathies believed to be mediated by synaptic accumulation of α-synuclein [106]. In fact, in PD, 50% of known causative genes and risk factors have synaptic functions [107]. Yet again, α-synuclein oligomers and protofibrils have been identified as the most toxic species instead of the inclusions. Overexpression of α-synuclein has been implicated in impairing several steps of exocytosis and endocytosis, such as inhibiting SNARE-mediated fusion [108] and reclustering of synaptic vesicles [109], respectively. Additional implicated toxic mechanisms include negative modulation of dopamine by reducing dopamine transporter activity and inhibiting dopamine synthesis enzymes (*e.g.*, tyrosine hydroxylase) [106, 110]. Taken together, toxic accumulation of α-synuclein may impair neurotransmitter exocytosis, neuronal communication, and dopamine homeostasis. Interestingly, for both tau and α-synuclein, prion-like propagation and spreading trans-synaptically has been suggested, creating an intimate link between disease progression and synaptic function [111].

Lastly, dysfunction of FUS and TDP-43 might likewise impair synaptic function by regulation of synaptic plasticity through RNA transport and local translation [112]. Thus, there are multiple paths to synaptic dysfunction present in neurodegenerative diseases, which all converge at the synapse regardless of pathology. This fact highlights the need for further studies of synaptic dysfunction as a central pathway occurring prior to neurodegeneration with close ties to the underlying pathogenesis, where the synapse is one of the primary sites of pathology.

1.5 SYNAPTIC BIOMARKERS

To quantify synapse dysfunction and degeneration in living patients, biomarkers reflecting the synaptic pathology in CSF are in demand. Not only in the routine clinical assessment of neurodegenerative diseases to facilitate diagnosis, disease staging, and progression, but also to monitor the efficacy and endpoints of treatments in drug trials. It has been around 30 years since the first studies detecting synaptic proteins in the CSF emerged [113]. Since then, several synaptic proteins have been studied as potential synaptic degeneration biomarkers, such as the presynaptic proteins synaptosomal-associated protein 25 (SNAP-25) and synaptotagmin-1 (SYT-1) and the postsynaptic protein neurogranin. The CSF levels of which all have been shown to be increased in AD [114-116]. However, there is a great number of unexplored synaptic proteins that can be potential biomarkers of synaptic dysfunction. For example, at the synaptic cleft, only the process of neurotransmitter release through synapse vesicle exocytosis is controlled by a large number of different proteins.

In an explorative CSF proteomic study using tandem mass tag (TMT) multiplex quantification for the comparison of AD with controls, 1991 proteins were detected and identified, of which at least 74 were identified as synaptic proteins [117]. Of those, 17 were determined to be of interest as potential synaptic CSF biomarkers but, at present, had been mostly unexplored. The proteins had a wide range of functions and localizations and included SNARE proteins, adaptor protein complex proteins, rab accessory regulatory proteins, neuronal pentraxins, 14-3-3 proteins, phosphatidylethanolamine-binding protein 1 (PEBP-1), and synucleins. In this thesis, the focus lies on those 17 synaptic proteins as well as SNAP-25 and SYT-1.

Table 1 gives an overview of the proteins and their synaptic function and localizations based on evidence and curated synaptic gene ontologies resources (missing γ-synuclein, some 14-3-3 members, and PEBP-1) [118]. In the following sections, however, each protein is discussed individually in relation to the literature, including other suggested functions, their connection to the synapse and pathology, and their current status as biomarkers.

Table 1. *Synaptic proteins and some of their functions and localizations according to SynGO.*

Protein	Biological process	Cellular component
NPTX2	❖ Neurotransmitter receptor localization to postsynaptic specialization membrane ❖ Regulation of postsynaptic neurotransmitter receptor activity	❖ Extrinsic component of postsynaptic specialization membrane ❖ Synaptic cleft
NPTX1	❖ Neurotransmitter receptor localization to postsynaptic specialization membrane ❖ Postsynaptic density assembly ❖ Regulation of postsynaptic neurotransmitter receptor activity	❖ Synaptic cleft
NPTXR	❖ Neurotransmitter receptor localization to postsynaptic specialization membrane ❖ Regulation of postsynaptic specialization assembly ❖ Regulation of postsynaptic neurotransmitter receptor activity	
14-3-3 ζ/δ	❖ Regulation of synapse maturation ❖ Synaptic target recognition	❖ Postsynaptic specialization
β-synuclein	❖ Synaptic vesicle endocytosis	❖ Presynaptic cytosol
GDI-1		❖ Presynaptic cytosol
Neurogranin	❖ Postsynaptic modulation of chemical synaptic transmission	❖ Postsynaptic membrane
AP2B1	❖ Postsynaptic neurotransmitter receptor endocytosis ❖ Synaptic vesicle endocytosis	❖ Extrinsic component of presynaptic endocytic zone membrane
Complexin-2	❖ Regulation of synaptic vesicle fusion to presynaptic active zone membrane	❖ Presynapse ❖ Postsynapse
Syntaxin-1B	❖ Synaptic vesicle exocytosis ❖ Synaptic vesicle fusion to presynaptic active zone membrane	
Syntaxin-7		❖ Integral component of synaptic vesicle membrane
SNAP-25	❖ Presynaptic dense core vesicle exocytosis ❖ Synaptic vesicle exocytosis	❖ Anchored component of presynaptic membrane ❖ Extrinsic component of presynaptic membrane
SYT-1	❖ Calcium-dependent activation of synaptic vesicle fusion	❖ Integral component of synaptic vesicle membrane
VAMP-2	❖ Synaptic vesicle docking ❖ Synaptic vesicle exocytosis	❖ Integral component of synaptic vesicle membrane

1.5.1 THE NEURONAL PENTRAXINS

The neuronal pentraxins; neuronal pentraxin-1 (NPTX1), neuronal pentraxin-2 (NPTX2), and the receptor (NPTXR), belong to the phylogenetically conserved pentraxin family. The proteins are present in both the pre-and postsynaptic compartment, where NPTX1 and NPTX2 are secreted glycoproteins, and their receptor, NPTXR, is anchored to the plasmatic membrane. The protein family is all recognized by their cyclic multimeric structure (penta) and by the His-x-Cys-x-Ser/Thy-Trp-x-Ser/Thy (where x is any amino acid) sequence - the so-called pentraxin signature [119]. The neuronal pentraxins are presynaptic proteins that associate and form heteromultimers and have been shown to be important for recruitment during exocytosis of AMPA-type glutamate receptors by binding and clustering the receptors (Fig. 11) [120, 121]. The expression of NPTX1 and NPTX2 are oppositely regulated in response to a reduction of neuronal activity [122, 123], where the expression of NPTX1 is induced while NPTX2 is reduced. This has led to the speculation that they might also have opposing functions, where NPTX1 and NPTX2 have been implicated in negatively regulating and promoting, respectively, synaptic plasticity at excitatory synapses. NPTX1 has also been found to participate in synaptic pruning by induction of the classical complement cascade [124]. The proteins thus play vital roles in synaptic function and plasticity.

In connection with pathology, NPTX2 has been specifically implicated in PD. Its mRNA expression is substantially upregulated (substantia nigra and frontal cortex), and it colocalizes with α-synuclein in Lewy bodies. Similarly, NPTX1 was reported to be upregulated, while NPTXR, on the other hand, had downregulated mRNA expression [125]. Interestingly, in AD brains, NPTX2 expression has been found to be downregulated, and NPTX1 upregulated or unchanged [123]. However, increased expression of both has been connected with different neurotoxicity pathways. Increased expression of NPTX1 has been associated with mediation of amyloid-β neurotoxicity [126], apoptotic neuronal death [127], and, as previously mentioned, synaptic pruning through activation of the complement cascade. As previously mentioned, NPTX2, on the other hand, has been found to be synaptogenic [122, 123]. Still, increased NPTX2 protein expression has also been implicated in meditating highly selective non-apoptotic cell death of dopaminergic neurons [125, 128]. Furthermore, in a mouse model, NPTX2 overexpression was found in the striatum after levodopa treatment, which was found to contribute to the development of levodopa-induced dyskinesia [129].

Several studies have reported that the pentraxins have reduced CSF levels in AD in comparison with controls [130-137], and it has been reported that the levels seem to follow a constant linear decrease from controls to MCI and lastly to AD [130]. There are fewer studies on other neurodegenerative diseases, but reports of lower pentraxin

concentrations also exist in PD, PSP, MSA, CBS, and DLB, utilizing both explorative and targeted measures [138-141]. Correlations with cognitive measures such as MMSE have also been found more robustly than other synaptic biomarkers. In PD, the pentraxin CSF levels have also been found to correlate with α-synuclein levels.

1.5.2 THE 14-3-3 PROTEIN FAMILY

The 14-3-3 protein family constitutes seven proteins; alpha (α), epsilon (ε), eta (η), gamma (γ), theta (θ), zeta (ζ), and sigma (σ) [142, 143]. Phosphorylated forms of α and ζ exist; β (beta) and δ (delta), respectively, and the proteins are thus commonly denoted alpha/beta (α/β) as well as zeta/delta (ζ/δ) to encompass both proteoforms. The protein family is present throughout the body but is so highly abundant in the brain that they constitute 1% of the brain's total soluble fraction. The family forms homo- and heterodimers and has been found to have many binding partners (>100) with interactions mainly dependent on phosphoserine- or threonine. They are thus associated with wide modulation abilities affecting a range of neuronal functions. However, their functions are still largely unknown in detail. Some of the 14-3-3 proteins are particularly enriched in the synaptic compartments [144], and several studies have explored their potential function in transmission and plasticity, of which they seem to be an important modulator [142], especially 14-3-3 ζ/δ [145]. Indicated synaptic functions include the modulation of N-type voltage-gated calcium channel (Cav2.2) activation [146] and the regulation of surface delivery of glutamate receptors (*e.g.*, NMDAR) [147, 148] at the presynaptic site and postsynaptic site, respectively, leading to long-term potentiation (Fig. 11).

The protein family has been known for a long time to be established biomarkers of CJD [143]. But more recently, they have been highlighted to generally have a strong connection with neurodegeneration. In fact, it has recently sparked an investigation if the 14-3-3 proteins could be potential drug targets [149, 150]. The protein family is genetically and functionally linked to AD, and several explorative proteomics studies have suggested several of the 14-3-3 protein family members as potential biomarkers [151-153]. However, very few studies have quantified them in AD in a targeted setting. The exception of the family is 14-3-3 γ protein which was recently found to be higher in the CSF of AD and FTD patients in comparison with controls [154]. Furthermore, the 14-3-3 proteins colocalize in neurofibrillary tangles and interact with the key AD pathology protein tau [155-157]. Interestingly, there is a strong connection between the 14-3-3 protein family and tau and its phosphorylation [149]. Of the proteins in the family, the ζ/δ isoform has been pointed out to be especially connected. Similarly, in PD, they are present in Lewy bodies and interact with the three major onset and progression proteins; LRRK2, α-Synuclein, and Parkin [158].

1.5.3 THE SYNUCLEIN FAMILY

The synuclein family is composed of three small soluble members; α, β-, and γ-synuclein [159]. Of the three, only α and β are enriched in the brain, specifically in presynaptic terminals. At the same time, γ-synuclein has wide-spread peripheral expression but is still found in enhanced levels in the brain but with a broad neuronal localization. What they do have in common, on the other hand, is a highly conserved α-helical lipid-binding motif with a structural resemblance to apolipoproteins. The function of the synucleins is, however, still largely unknown, especially for β- and γ-synuclein, but they are believed to have likely roles involved in the regulation of synaptic plasticity. All three synucleins have been indicated in the regulation of synaptic vesicle endocytosis [160], and α-synuclein has been indicated to play a critical role in the clustering of synaptic vesicles and synaptic vesicle exocytosis (Fig. 11) [161]. These functions do correlate with their lipid-binding biochemical characteristics. α-synuclein's mechanistic effect on synaptic vesicle exocytosis is suggested to be through the binding of vesicle-associated membrane protein-2 (VAMP-2), contributing to the maintenance of SNARE-complex assembly [162, 163]. The protein also regulates dopamine neurotransmission by associating with dopamine transporter (DAT1) [164]. Recently, the other two members were suggested to affect synapse function by modulating the binding of α-synuclein to synaptic vesicles [165]. The synucleins' molecular roles seem thus complementary but not functionally redundant.

All synucleins have been repeatedly linked with neurodegenerative diseases [161]. In particular, pathogenic α-synuclein aggregates into toxic forms of oligomers, fibrils, and large intracellular aggregates of LBs, LNs, and GCIs of synucleinopathies and is as well found in amyloid plaques in AD [161, 166]. Additionally, the association with α-synuclein and DAT1 is a likely contributor to the specific vulnerability of dopaminergic neurons in synucleinopathies [164]. γ- and β-synuclein, on the other hand, are found to colocalize with α-synuclein in non-LB inclusions in PD, LBD, and FTD, as well as having altered mRNA expression found in AD [167-169]. Interestingly, in contrast, β-synuclein has been implicated in having anti-aggregation properties capable of inhibiting α-synuclein aggregation, causing some studies to label β-synuclein as neuroprotective. Oeckl *et al.* were the first to study all three synucleins in parallel in neurological disorders finding increased CSF concentrations in AD and CJD in comparison with controls [170] but no alterations in other neurological disorders. An observation for β-synuclein which have been repeatedly confirmed [171]. Additionally, for β-synuclein, the increase in the CSF has repeatedly been demonstrated at the early stages of prodromal AD (MCI) [172]. More recently, it has also been shown that β-synuclein is a potential biomarker in plasma, showing increased levels in the plasma of AD patients compared with controls [173] – something not feasible for the other synucleins due to peripheral expression.

1.5.4 PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN 1

Phosphatidylethanolamine-binding protein 1, also known as Raf kinase inhibitor protein, is a regulatory protein with largely unknown function but is known to play an important modulatory role in protein kinase signaling cascades, including NF-kappa B, MAP kinase, and glycogen synthase kinase-3 [174]. The protein is also a precursor to the hippocampal cholinergic neurostimulating peptide (HCNP) implicated in the induction of acetylcholine synthesis, one of the major neurotransmitters, and enhancement of glutamatergic activity [175]. Further, HCNP has been found to accumulate in Hirano bodies, intracellular aggregates of actin, and actin-associated proteins, which are found in neurodegenerative diseases such as AD [176]. There are few studies of PEBP-1 and even fewer concerning neurodegenerative diseases. Significantly downregulated PEBP-1 protein expression has, however, been reported in the hippocampus of late-onset AD patients [177], and in explorative proteomic studies, it has shown higher CSF protein levels in AD than in controls [178]. Furthermore, as a precursor to HCNP, PEBP-1 might be an interesting potential biomarker of cholinergic dysfunction, which is known to be associated with memory loss and neurodegenerative diseases, not least in AD [179].

1.5.5 THE RAB FAMILY

The rab family and its accessory regulatory proteins, such as **rab GDP inhibitor α (GDI-1),** are major players in vesicle trafficking, including regulating both endocytosis and exocytosis during the synaptic vesicle cycle [180, 181]. Rab activity is tightly controlled by GTP-binding, cycling between an inactive GDP-bound and an active GTP-bound form. GDIs such as GDI-1 work to slow the rate of dissociation of GDP and ensure recycling by membrane dissociating of the rab proteins into the cytosol for shuttling between membranes. Since there are more than 60 rab proteins in the family, GDI-1 is involved in a wide range of vesicle trafficking functions [180, 181]. Several of the rab proteins function at the synapse, including the most abundant rab protein in the brain, which is Rab3a, which is localized at synaptic vesicles and controls synaptic vesicle docking (Fig. 11). Another rab, rab5, is involved in endocytosis; both in the form of presynaptic vesicle exocytosis as well as postsynaptic AMPAR internalization during LTD. Rab8, on the other hand, is involved in the synaptic delivery of AMPAR during LTP.

The rab family and its regulatory proteins also have a strong association with disease since dysregulated trafficking is believed to contribute to the pathogenesis of neurodegenerative diseases such as AD [182]. Implications exist in the regulation of mechanisms such as Aβ internalization, trafficking, and clearance. A loss of GDI-1 in mouse models has been reported to cause impaired memory and plasticity, possibly due to impaired synaptic vesicle recycling, which also has been found as a consequence

[183-185]. However, in contrast, explorative proteomics studies have found upregulated GDI-1 in brain tissue of AD patients [186] as well as higher levels in CSF [178]. Even though GDI-1 has been identified in explorative proteomic studies as a highly significant AD biomarker target [178], it has currently not been explored as a CSF biomarker in targeted settings.

1.5.6 ADAPTOR-2 PROTEIN COMPLEX

Synaptic vesicles undergo a trafficking cycle to ensure fast and recurrent rounds of the release of neurotransmitters. The process entails exocytosis, followed by multiple pathways of endocytosis and recycling [187, 188]. The major endocytosis route is clathrin-mediated, where the protein clathrin forms a scaffold and coats the part of the plasma membrane to be endocytosed. However, in order to link the clathrin scaffold to the membrane adaptor proteins (AP) complexes are needed. For vesicle endocytosis at the membrane, this is done by AP-2 (Fig. 11), which is a heterotetrameric protein composed of two large subunits (adaptins; α and β (AP2B1)), a medium subunit (mu), and a small subunit (sigma) like all AP complexes [188]. AP-2 complexes do not only serve a vital function in presynaptic vesicle recycling; clathrin-mediated endocytosis serves to modulate the protein composition of the plasma membrane and transport cargo into the neuron. Another important function of the AP-2 complex is where activity-dependent endocytosis also regulates postsynaptic membrane receptor abundance (*e.g.*, NMDAR-dependent internalization of AMPAR). The protein complex is thus also involved in the mediation of synaptic strength and plasticity [189, 190].

An increasing number of studies report a strong connection between neurodegenerative disease and dysregulation of the endocytic membrane-trafficking pathway [191]. This links AP-2 indirectly with pathology, but several direct links also exist. Among others, several subunits of AP-2, such as AP2B1, have been genetically linked as a potential risk factor for PD [191]; AP2B1 also has a functional interaction with the key PD influencing factor, LRRK2 [192]. As a biomarker, AP-2 is relatively unexplored. Still, a few studies exist on AP2B1, finding no difference in AD but decreased levels in PD [193], as well as decreased levels in LBD compared with controls [141].

1.5.7 NEUROGRANIN

Neurogranin is a small brain-enriched postsynaptic protein present in the cytosol of dendritic spines. The protein contains an IQ motif and is a substrate of protein kinase C, which works to block the binding properties of the IQ region through phosphorylation, thus regulating its function [194]. Its only documented function entails binding calmodulin in response to low calcium levels. The binding leads to the

prolonging of calmodulin and its availability leading to sustained activation of post-synaptic signal transmission (Fig. 11) [115]. In neurogranin mouse knockout models, deficits in spatial memory and synaptic plasticity occur [195], signifying neurogranin's importance in these processes.

As previously mentioned, neurogranin is one of the potential synaptic biomarkers that are currently widely studied. Both targeted mass spectrometric methods and enzyme-linked immunosorbent assays (ELISA) have repeatedly reported elevated CSF concentrations of neurogranin in AD compared with controls [115, 196], also confirmed by several meta-analyses [196, 197]. High CSF levels of neurogranin at prodromal stages have also been shown to be predictive of a more rapid AD progression [198]. The protein has as well been reported to be specifically increased in AD compared with other neurodegenerative diseases [199, 200], with the exception of CJD, which displays even higher levels than AD [201]. Due to the encouraging results of neurogranin as a potential synaptic biomarker in AD in CSF, several studies have tried to explore its functionality in blood. However, likely due to a sizeable peripheral expression, the plasma levels of neurogranin seem to be unchanged in AD compared with controls [202, 203].

1.5.8 SNARE PROTEINS

Presynaptic vesicular exocytosis at the plasma membrane is mediated by a large protein family of soluble N-ethylmaleimide sensitive fusion protein (NSF) attachment receptor (SNARE) proteins [204, 205]. The SNARE proteins can be separated into vesicle (v-SNAREs) and target (t-SNAREs), which are inserted into or linked to the vesicle membrane and nerve terminal membrane, respectively. Together four SNARE motifs assemble and form the trans-SNARE complex, a twisted parallel four-helix bundle that brings the opposing membranes together (Fig. 11). Upon the arrival of an action potential to the presynaptic compartment, voltage-gated ion channels are opened. Calcium influx occurs, which mediates the trans-SNARE complex docking and Ca^{2+}-triggered fusion of the vesicles to the cell membrane during vesicular exocytosis [204, 205]. However, some SNAREs are present as both v- and t-SNARES and can thus be classified based on structural features such as their contribution to the zero ionic layer, the primary position of interlinkage in the core SNARE complex. Proteins contributing an arginine (R) and glutamine (Q) residue are classified as R-SNAREs and Q-SNAREs, respectively. The functionality of most R-SNAREs is as v-SNAREs, and most Q-SNAREs function as t-SNAREs.

Synaptosomal-associated protein 25

SNAP-25 is a presynaptic t- and Q-SNARE protein with two SNARE motifs and is thus a component of the trans-SNARE complex together with syntaxin-1 and VAMP-2, mediating fast exocytosis (Fig. 11) [206]. SNAP-25 does not have a trans-membrane

domain but is membrane-anchored by side chains of palmitoyl covalently bound to amino acid residues of cysteine found in the proteins' central linker domain. The two splice isoforms of the protein; SNAP-25A and SNAP-25B diverge by nine amino acid residues. SNAP-25 function extends beyond exocytosis; at the presynapse it additionally regulates calcium influx by negative modulation of various voltage-gated calcium channels as well as been indicated to participate in slow, clathrin-dependent endocytosis [205]. Even though SNAP-25's function and localization are believed to be mainly found in the pre-synaptic compartment, postsynaptic involvement has also been indicated with functions such as controlling trafficking and receptor exocytosis (*e.g.*, NMDAR).

SNAP-25 was first detected in the CSF a little over 20 years ago [113] and was later confirmed in a post-mortem study to be present in decreased levels in the brain of AD patients [114]. Since then, SNAP-25 has emerged as a promising synaptic biomarker, and for quantification, both conventional immunoassays, such as ELISA, and mass spectrometric methods exist [114, 130, 207, 208]. Developing evidence suggests that SNAP-25 might be specifically associated with amyloid pathology since several studies have found that SNAP-25 is present in higher CSF protein levels in AD in comparisons with controls [114, 130, 207, 208], but no change has been observed in non-amyloid pathologies, such as in FTD [151]. In support, higher SNAP-25 protein levels have been found in cognitively unimpaired people with evident amyloid pathology compared with those without [209]. The exception is CJD [210], in which increased levels of SNAP-25 have also been observed.

Syntaxins

Syntaxins are a conserved protein family of transmembrane t- and Q-SNARE proteins anchored to the membrane via carboxy-terminal tails. The protein family is characterized by their SNARE domain which is a roughly 60-residue-long membrane-proximal coiled-coil domain [211, 212]. The two first syntaxins, syntaxin-1A and syntaxin-1B, were discovered for their involvement in the trans-SNARE complex and function in exocytosis. Even though the two proteins have 84% amino-acid sequence homology and seem functionally redundant, syntaxin-1B has been indicated to be the primary mediator for rapid, induced and spontaneous, synaptic vesicle exocytosis [213]. Similarly to SNAP-25, syntaxin-1 has also been found to bind calcium ion channels, allowing for the close regulation and coupling between calcium influx and neurotransmitter release [214]. The other 13 syntaxins belonging to the family reportedly have various functions and localizations not connected to vesicle exocytosis. One of them is the endosomal syntaxin-7 which is involved in the mediation of endocytic trafficking [215].

Though several links to disease pathology exist, few studies exist on syntaxins as potential biomarkers. Syntaxin-1A has been found to interact specifically with intracellular Aβ oligomers via its SNARE motif, which may cause inhibited SNARE complex formation resulting in impaired exocytosis [216]. Syntaxin-1 (both A and B) has been confirmed to be lower in brain tissue of AD patients [217], and syntaxin-1B has been explored in the CSF, finding decreased levels in preclinical AD [218] but with unchanged levels in AD dementia and FTLD pathology [218-220].

Vesicle-associated membrane protein-2

VAMP-2, otherwise known as synaptobrevin-2, is a brain-specific R- and v-SNARE belonging to the VAMP family of small integral membrane proteins [206]. Similar to syntaxin-1, VAMP-2 contains one SNARE motif preceding its transmembrane region. In addition to its presynaptic function in vesicle exocytosis, the protein has also been demonstrated to be present in the post-synaptic compartment and involved in the trafficking of glutamate receptor subunits (*e.g.*, AMPAR) [221]. The protein levels of VAMP-2 have been confirmed to be decreased in the brain tissue of AD patients [217]. The protein has recently gained interest as a potential CSF biomarker with specifically higher levels in AD pathology [218-220].

Complexins

Complexins are small hydrophilic accessory proteins and are named as such since they bind the SNARE-complex through their α-helical motif. The protein thus serves as a clamp and modulates SNARE function both as an inhibitor of premature release and facilitator of vesicle fusion upon Ca^{2+} influx (Fig. 11) [222, 223]. Out of the four mammalian complexin proteins, two of them are brain-enriched; complexin-1 and complexin-2. Protein levels of both complexins have been shown to be lower in the brains of AD dementia in comparison with non-demented subjects [224]; however, as CSF biomarkers in a targeted setting, they seem not to have been explored. In explorative CSF proteomics studies of AD patients, on the other hand, complexin-2 has been repeatedly identified to be higher than controls [178, 225].

Synaptotagmin-1

SYT-1 belongs to the membrane-trafficking protein family of synaptotagmins and is thus not a SNARE protein but is involved in the control and triggering of the vesicle fusion [226-228]. The protein family is defined by its transmembrane region and the two carboxy-terminal C2-domains, which act as binding domains of Ca^{2+} [222]. Of the family members, SYT-1 is the most studied, and it is vital for fast, synchronous neurotransmitter release via its Ca^{2+} sensitivity as a calcium-sensor vesicle protein found in the synaptic vesicles. Upon calcium influx due to an action potential, SYT-1 binds Ca^{2+} and acts to displace complexin allowing for SNARE-mediated exocytosis (Fig. 11). Including its function in exocytosis, SYT-1 also regulates clathrin-mediated

endocytosis by interacting with adaptor proteins like AP-2 [229]. However, even though its mostly known for its function at the presynaptic terminal, it also has been indicated to modulate exocytosis at the postsynaptic compartment. Studies have reported that it might mediate exocytosis of AMPA receptors enabling LTP [177]. Studies of SYT-1 as a potential biomarker have shown the protein to have changed concentrations in AD compared with controls in both the CSF (increased) [116] and in post-mortem brain tissue (decreased) [96]. Less work has been done on other neurodegenerative diseases, but SYT-1 seems specifically increased in relation to AD pathology [151, 230].

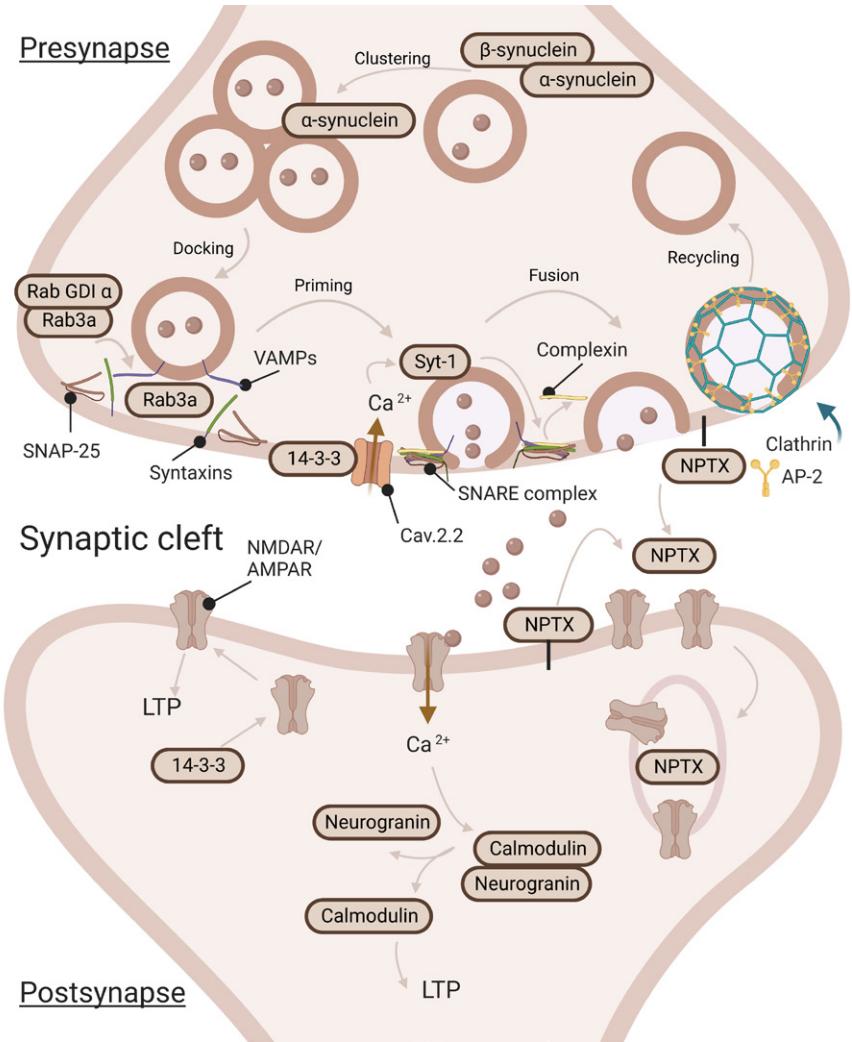

Figure 11. *Illustration of the synapse and some of the suggested synaptic protein's locations and functions. Created with Biorender.com*

2 AIM

2.1 GENERAL AIM

The overall aim of this Ph.D. project was to study both known and new potential synaptic biomarkers that might reflect synaptic dysfunction in neurodegenerative diseases and to assess their usefulness as biomarkers. In order to fulfill this, the general aim included the development of a novel synaptic biomarker panel and employing it, together with established methods, in the investigation of neurodegenerative diseases, particularly AD. The general aim also included the study of the possible presence of synaptic biomarker patterns which can aid in the diagnosis, particularly distinguishing different neurodegenerative diseases with shared pathologies, as well as finding biomarkers that can be potential endpoints in clinical trials.

2.2 SPECIFIC AIMS

Paper I: To develop a multiplex MS method of a panel of several synaptic proteins and investigate their potential as biomarkers for AD.

Paper II: To validate the findings of Paper I in pathology confirmed cases of AD and to expand the study of the synaptic panel in other neurodegenerative diseases.

Paper III: To study the synaptic panel proteins and assess their performance as potential synaptic biomarkers in the spectrum of parkinsonian disorders.

Paper IV: To study the synaptic panel proteins and assess their performance as potential synaptic biomarkers in genetic subtypes of FTD.

Paper V: To compare a novel SNAP-25 Single molecule array (Simoa) assay with the in-house developed IP-MS method and to evaluate their performance. Furthermore, the aim is to examine if SNAP-25 can be quantified in blood with the new Simoa assay.

Paper VI: To study SNAP-25, SYT-1, and the synaptic panel proteins in a large-scale cohort of the AD continuum, including a range of neurodegenerative diseases. In addition, the aim is to assess the synaptic proteins' abilities to predict cognitive decline and progression to dementia in AD.

3 MATERIALS

3.1 STUDY POPULATIONS

All the studies conducted in this thesis included participants who had given their informed consent. Their recruitment and sample collection were performed per approved ethical permission from the local ethical committee. In **Papers I-VI**, CSF samples were utilized, while only in **Paper V** plasma was used. Additionally, de-identified CSF samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, were pooled and utilized for method development and quality controls in all papers. CSF, as well as plasma samples, were collected according to standardized procedures. CSF was collected in polypropylene tubes via lumbar puncture, centrifuged at 2000 × g at 4°C for 10 minutes to remove insoluble material and cells, and stored at –80°C until use. Whole blood was collected in tubes coated with the anticoagulant ethylenediaminetetraacetic acid (EDTA), centrifuged at 2500 × g at 4°C for 10 minutes to obtain plasma by removing the supernatant, and stored at –80°C until use.

3.1.1 PAPER I

In **Paper I**, the study population included two cohorts. The pilot composed of neurological controls (n=20) and AD (n=20), biochemically defined based on the AD CSF core biomarker concentrations (cut-offs; p-tau$_{181}$>60 pg/mL; t-tau>380 pg/mL; Aβ_{42}<550 pg/mL) according to the IWG-2 biomarker criteria [231]. The second cohort, on the other hand, consisted of clinically diagnosed AD patients (n=32) diagnosed according to NINCDS-ADRDA [25] as well as healthy controls (n=20).

3.1.2 PAPER II

The study population utilized in **Paper II** included autopsy-confirmed AD (n=63), LBD (n=21), and FTLD (n=53) cases either with a familial form (n=11) or pathologically confirmed by autopsy (n=42). AD pathology was confirmed at autopsy according to established criteria for intermediate or high AD neuropathologic change (ADNC) [232]. FTLD pathology on the other hand was confirmed by associated mutations (*GRN* (n=2), *C9orf72* (n=6), *TARDBP* (n=1), *MAPT* (n=2)) [233] or by either the presence of misfolded TDP-43 or tau [234]. Lastly, LBD pathology was confirmed by the presence of LB [235]. AD-comorbidity (high/intermediate ADNC) was found to be present in one FTLD patient and nine LBD patients. Healthy control subjects (n=48) were included who were cognitively unimpaired (CU), with an MMSE score of less than 28 [236], and AD CSF biomarker-negative (Aβ_{42}>192 pg/mL).

3.1.3 PAPER III

In **Paper III,** the study population constituted of two separate parkinsonian disorder cohorts, a discovery cohort (n=154) from Sahlgrenska University Hospital (Gothenburg, Sweden) and a validation cohort (n=143) from Umeå University Hospital (Umeå, Sweden). The two cohorts included PD (n1=51, n2=101) diagnosed according to the MDS clinical diagnostic criteria [237] or by the UK PD Society Brain Bank criteria [70], CBD (n1=11, n2=3) diagnosed based on Armstrong's criteria [238], PSP (n1=22, n2=21) diagnosed by MDS criteria or by NINDS-SPSP criteria [76], MSA (n1=31, n2=26) diagnosed by Gilman's criteria [239]. Additionally, healthy controls (n1=48, n2=30) included orthopedic patients undergoing lower limb surgery or recruited by advertisements and among relatives. Furthermore, in the validation cohort, a group of AD (n=23) patients fulfilling the Diagnostic and Statistical Manual of Mental Disorders [Fifth Edition] (DSM-5) criteria for AD dementia [240] from BioFINDER-2 was added for comparison.

3.1.4 PAPER IV

For the study population of **Paper IV**, a genetic FTD cohort from the GENFI study was used. The cohort was composed of symptomatic patients who were pathogenic mutation carriers in *GRN* (n=17), *MAPT* (n=12), or *C9orf72* (n=26) and their healthy at-risk first-degree relatives who are so-called presymptomatic mutation carriers in *GRN* (n=23), *MAPT* (n=23), or *C9orf72* (n=31). Additionally, a group of healthy non-carriers (n=61) was also added for comparison.

3.1.5 PAPER V

For the study population in **Paper V**, a cohort containing a subgroup of patients and controls (n=71) from the Translational Biomarkers in Aging and Dementia cohort (TRIAD, McGill University, Canada) was utilized [241]. The cohort included CU and cognitively impaired (CI) participants diagnosed with AD according to NIAAA criteria or a clinical diagnosis of FTD, MCI, or dementia with an unconfirmed diagnosis. The participants were also further classified based on amyloid status (CSF $A\beta_{42/40}$, cut-off≤0.068 [242]); positive (+) and negative (-), which resulted in four groupings: CU-, CI-, CU+, and CI+. Additionally, for CU subjects, an age cut-off >25 years old was set, creating a last fifth group of "Young" individuals. Furthermore, in **Paper V**, to test the Simoa assay's ability to detect SNAP-25 in blood, plasma samples from patients older than 80 years of age (n=32) were utilized.

3.1.6 PAPER VI

For the study population of **Paper VI**, the cohort was composed of participants from the prospective Swedish BioFINDER-2 study [243]. The cohort included CU participants (n=464) and patients with MCI (n=209), AD (n=151), as well as a spectrum of other neurodegenerative diseases (n=156). Patient inclusion criteria included fulfilling the DSM-5 AD criteria [240] for AD, DSM-5 FTD criteria for bvFTD [240], MDS criteria for PD [244], DSM-5 subcortical vascular dementia (VaD) criteria for VaD [240], MDS criteria for PSP [77], Gilman's criteria for MSA [74], Armstrong criteria for CBS [79], criteria for PPA [245], and criteria for DLB [73]. For the CU and MCI participants, a further subdivision was performed based on Aβ-positivity into controls (CU-), preclinical AD (CU+), and prodromal AD (MCI+) if they were Aβ-positive and tau-positive following the NIA-AA research framework [29].

4 METHODOLOGY

Targeted proteomics offers a powerful approach to investigate new potential biomarkers. For validation of biomarker candidates, targeted tandem mass spectrometry (MS) is advantageous since there is no need for antibodies (unless immunoprecipitation (IP) is required for sample enrichment) as compared with immunoassays, and the option of multiplexing is both cost- and time-effective. This thesis uses two targeted MS methods utilizing proteolytic peptides, combining isolation and separation techniques with MS to quantify proteotypic peptides of target proteins. The steps of MS-based proteomics can broadly be subdivided into three parts: sample preparation, sample separation, *e.g.*, liquid chromatography (LC), and detection and quantitation by MS.

In the first method [133, 246], developed in **Paper I** and used with minor modifications in **Papers II, III, IV**, and **VI,** a panel of peptides from several synaptic proteins, including syntaxins, VAMP-2, AP2B1, complexin-2, PEBP-1, synucleins, GDI-1, neuronal pentraxins, and 14-3-3 proteins are measured. Following protein digestion, a broad enrichment technique called solid-phase extraction (SPE) is used, allowing for the simultaneous purification of a wide range of peptides present in the sample. High-performance liquid chromatography (HPLC) and high selectivity MS using parallel reaction monitoring (PRM) or multiple reaction monitoring (MRM) are used in conjugation for quantification. In the second method [114, 247], used in **Paper V** to measure SNAP-25 as well as in **Paper VI** to measure SNAP-25 and SYT-1, the difference lies in the separation technique and the fact that protein digestion is performed after isolation. Instead of SPE, the method utilizes IP as a sample enrichment step of targeted proteins, allowing for an increased detection sensitivity of low abundant proteins.

Prior to or during the sample preparation in both cases, heavy stable isotope labeled peptides are added, as internal standard (IS) is essential to perform reliable quantitation with MS. This also ensures that any analytical confounding factors affecting the target peptide can be disregarded as they will affect the chemically identical IS in the same manner. Additionally, in **Paper V**, Simoa, a semi-automated technique that shares the same principle as a sandwich ELISA, was utilized to quantify SNAP-25 and to compare the newly developed method to the IP-MS approach.

In Table 2, an overview of the synaptic proteins and their respective target peptides utilized for quantification is given and in which paper they were quantified. Of note, in **Paper V**, which quantified two peptides of SNAP-25, the peptides are named by which forms of SNAP-25 they quantify. The first peptide (*e.g.*, AEDADMRNELEEMQR), which targets all SNAP-25 forms containing acetylated N-

termini of SNAP-25, is thus referenced as [Total]. The second peptide (*e.g.*, MLQLVEESK), referenced as [Long], on the other hand, targets only the SNAP-25 forms, which contain acetylated N-termini and, at minimum, the first 40 amino acids.

Table 2. *Synaptic proteins and respective peptides targeted in the different papers.*

Paper	Protein	Accession	Sequence	Position
Panel I				
I-IV & VI	*NPTXR*	O95502	NNYMYAR	[302-308]
↓			LVEAFGGATK	[479-488]
	NPTX1	Q15818	LENLEQYSR	[144-152]
			ETVLQQK	[63-69]
			CESQSTLDPGAGEAR	[89-103]
			LTPGEVYNLATCSTK*	[385-400]
	NPTX2	P47972	VAELEDEK	[177-184]
			WPVETCEER	[419-428]
			ETVVQQK	[68-74]
	14-3-3 ε	P62258	IISSIEQK	[62-69]
			LICCDILDVLDK*	[95-106]
	14-3-3 η	Q04917	AVTELNEPLSNEDR	[29-42]
	14-3-3 θ	P27348	AVTEQGAELSNEER	[28-41]
	14-3-3 ζ/δ	P63104	VVSSIEQK	[61-68]
	β-synuclein	Q16143	EGVVQGVASVAEK	[46-58]
	γ-synuclein	O76070	ENVVQSVTSVAEK	[46-58]
			EQANAVSEAVVSSVNTVATK*	[61-80]
	PEBP-1	P30086	LYEQLSGK	[180-187]
			NRPTSISWDGLDSGK	[48-62]
	GDI-1	P31150	QLICDPSYIPDR	[279-290]
	AP2B1	P63010	NVEGQDMLYQSLK	[880-892]
			IQPGNPNYTLSLK	[905-917]
	Neurogranin	Q92686	KGPGPGGPGGAGVAR	[54-68]
	Complexin-2	Q6PUV4	AALEQPCEGSLTRPK	[84-98]
	Syntaxin-1B	P61266	QHSAILAAPNPDEK	[56-69]
	Syntaxin-7	O15400	EFGSLPTTPSEQR	[72-84]
	VAMP- 2	P63027	LQQTQAQVDEVVDIMR*	[32-47]
Panel II				
II & VI	*SNAP-25*	P60880	AEDADMRNELEEMQR*	[2-16]
			MLQLVEESK	[32-40]
VI	*SYT-1*	P21579	VPYSELGGK	[215-223]

Notes: The position reflects the amino acid sequence of the protein.
**Peptides which are not included in the synaptic panel in all papers.
**N-terminally acetylated.

4.1 SAMPLE PREPARATION

In targeted proteomics, a very important step to enable a sensitive and reliable measurement is the sample preparation which has to be chosen carefully with respect to the target protein and sample matrix. Particularly, reducing the sample matrix complexity is of vital importance, both in the form of highly abundant proteins and other constituents, including salt, lipid, and sugar content which can interfere with the analysis and lead to reduced sensitivity. However, with every added step in the sample preparation, variation is induced, leading to a trade-off between sensitivity and reproducibility as well as throughput. To counteract the induced variation, ISs can be used. Generally, IS should be added to the sample as early in the sample preparation process as possible.

4.1.1 HEAVY STANDARD ADDITION

In targeted proteomics, the addition of stable isotope-labeled peptides as IS is common practice [248]. When analyzing tryptic peptides, the IS regularly consists of synthesized peptides where the C-terminal Lysine and Arginine have been labeled with ^{13}C and ^{15}N. These peptides thus only differ in mass (L, 8 Da; R, 10 Da) but are chemically equivalent in chromatography and ionization properties to the endogenous target peptide. The established synthesis method consists of rapid peptide chain assembly through successive reactions of amino acids on insoluble porous support and is thus aptly termed solid-phase peptide synthesis. Stable isotope-labeled amino acids are commonly produced by the use of microorganisms grown in the presence of highly ^{13}C-enriched CO_2 or highly ^{15}N-enriched ammonia as the only source of carbon and nitrogen, respectively.

In **all papers**, stable isotope-labeled peptides have been utilized as IS, purchased from either Thermo Fisher Scientific (AQUA QuantProHeavy) or JPT Peptide Technologies (SpikeTides L). According to both companies, they deliver peptides with high isotope purity (Thermo >99%, JPT >97%); it is, however, of importance to quality check any possible unlabeled (light) contamination that can otherwise interfere with the quantification [248]. The usage of stable isotope-labeled peptides allows for that pre-analytical confounding factors such as pipetting errors or other variation factors, as well as variation in quantification affecting the target peptide can be significantly decreased and the overall variance in quantification reduced.

4.1.2 PROTEIN DIGESTION

Protein digestion refers to protein cleavage into peptides commonly in sample preparation procedures by enzymes such as trypsin [249]. Trypsin is the most frequently used proteolytic enzyme in proteomics. It is a serine protease causing site-specific cleavage at the carboxyl side of lysine (K) and arginine (R) through hydrolyzation. The optimal operating temperature and pH are 37°C and neutral pH; stopping the digestion process is thus typically performed by the addition of acids to reduce the pH. Furthermore, trypsin is often modified and used in combination with Lys-C, another enzyme with a site-specific cleavage, to reduce autolysis, increase enzymatic efficiency, and minimize missed cleavages at the C-terminal side of lysine. However, the Lys-Cs cleavage reaction mechanism differs from that of trypsin. Additionally, prior to digestion, samples are commonly reduced and alkylated in order to break possible disulfide bonds (S-S) of the target proteins, which otherwise might obscure cleavage sites.

In **all papers**, an MS-grade mix of trypsin and Lys-C (Promega Corporation) is utilized to digest proteins into peptides. In targeted proteomics, protein digestion utilizing trypsin is a vital step to produce peptides with masses suitable for MS analysis [249]. The frequency of arginine and lysine in the human proteome results from the theoretical prediction in proteolytic peptides on average composed of 9 amino acids (with a standard deviation of 15), which is a suitable length for identification by mass spectrometry. Tryptic peptides having a C-terminal basic amino acid (Lys or Arg) are ideal for collision-induced dissociation and most often fragment into complete or semi-complete y-ion series, where especially the high mass y-ions are identity informative.

4.1.3 SOLID-PHASE EXTRACTION

SPE is a standard purification method that uses a stationary phase of solid particles to separate the sample into desired and undesired components based on their physical and chemical properties [133, 246]. SPE commonly utilizes pre-packed plates or cartridges, depending on particle material type; reverse phase, ion exchange, or mixed-mode extractions can be performed. In the case of reverse-phase sorbents, SPE separates the hydrophobic desired components, *i.e.*, proteins in the sample, from the undesired components, *i.e.*, salts and detergents, based on hydrophobicity. The sample is loaded, allowing the proteins to bind to the sorbent and a polar aqueous solvent, *i.e.*, water is used to wash away the unbound undesired components (Fig. 12). Organic solvents such as methanol which is less polar than water, can then be used for elution of the purified desired components in the sample by reducing the hydrophobic interactions with the sorbent. By using a rotary pump or similar extraction manifold to create a vacuum, the plate or column is effectively emptied between each step.

In **Papers I-IV, and VI**, reversed-phase SPE is performed utilizing Oasis 30 µm HLB 96-well µElution Plates (Waters Corporation) according to the manufacturer's instructions with minor modifications. The packaging material is composed of a copolymer consisting of a balanced amount of hydrophilic and lipophilic monomers, which has several advantages over traditional silica-based sorbents. The benefits include a wider pH range and the ability to recover a broad range of analytes, enabling a high recovery of polar analytes. SPE allows for a fast-multiplexed sample preparation step for the concentration and purification of a range of proteins. However, the drawback following SPE includes the high sample complexity of the samples, which leads to an increased analytical challenge during subsequent quantification due to the high general protein background and wide range of target protein concentrations. Additionally, for very low abundant target proteins, this dual purification concentration step might not be enough for sufficient isolation for quantification.

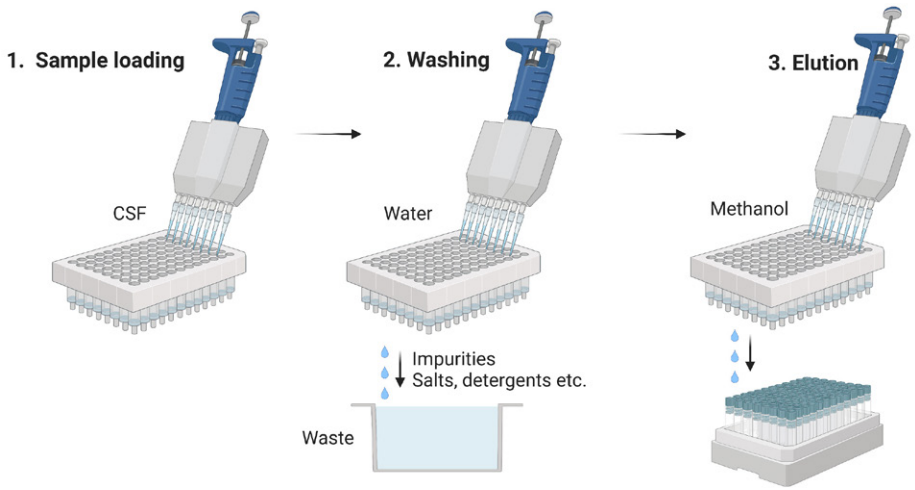

Figure 12. *Schematic of solid-phase extraction steps. Created with Biorender.com*

4.1.4 IMMUNOPRECIPITATION

IP is a common purification and enrichment method for CSF and post-mortem brain tissue to separate the target protein from the rest of the sample matrix. In MS, it is becoming increasingly prevalent as a sample preparation step since it allows for the detection of low abundant proteins. IP uses capture antibodies directed towards the proteins of interest, linked to a solid support, *e.g.*, beads for isolation. There is an abundance of different IP approaches; the one described in the following section refers to the one utilized in the thesis (Fig. 13). A direct IP approach is utilized where first, the capture antibodies are linked to magnetic beads through the bead's pre-coat with immunoglobulin G antibodies which are of the same species as the capture antibodies. The beads with the capture antibodies are then incubated with the sample, and the

target proteins bind to the beads. The magnetic beads have paramagnetic properties that can be utilized in automated magnetic-particle processing equipment, which utilizes magnetic rods to transfer the magnetic beads during the various steps. The target protein of interest can then be eluted using an elution buffer, such as formic acid, which reduces the pH causing the analyte to dissociate from the capture antibody.

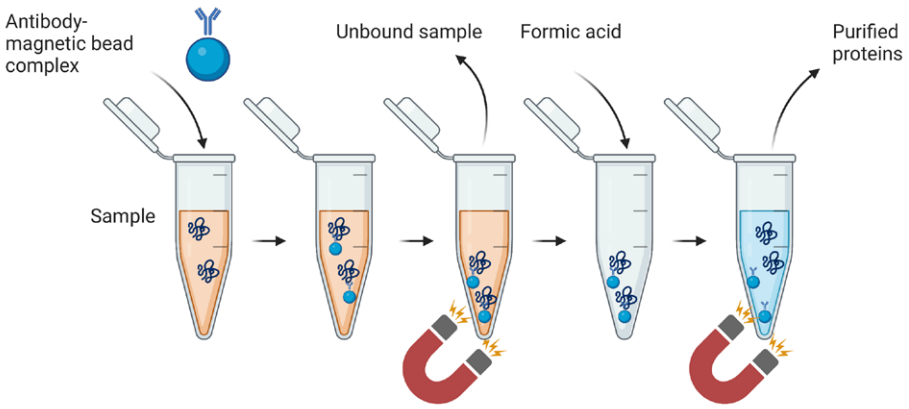

Figure 13. *Schematic illustration of immunoprecipitation. Created with Biorender.com*

In **Paper V and VI,** the described protocol above has been utilized in combination with mouse monoclonal antibody SM181 (Nordic BioSite) and mouse monoclonal antibody clone 41.1 (Synaptic systems) together with IgG-coated magnetic beads Dynabeads M-280 Sheep anti-Mouse IgG (Thermo Fisher Scientific) on the KingFisher™ Flex System (Thermo Fisher Scientific) to immunoprecipitate SNAP-25 and SYT-1, respectively. A limitation of immunoprecipitation enrichment is that the preparation relies on the availability of well-performing antibodies with high specificity. The possibility of multiplexing is also limited and costly.

4.2 LIQUID CHROMATOGRAPHY

Reverse-phase HPLC is commonly utilized in conjunction with MS for peptide characterization and quantification. It allows for separation relying on hydrophobic interactions of the analytes with the stationary phase, same as during SPE, but with a continuous flow [250]. By using a reverse-phase column (stationary hydrophobic phase) and a mobile phase composed of water and an increasing percentage of a non-polar solvent, commonly acetonitrile, the analytes will elute over time, corresponding to analyte hydrophobicity and subsequent decrease of the polarity of the mobile phase (Fig. 14). The column package material is typically porous silica beads, to which covalently attached hydrophobic ligands of carbon chains have been attached. Depending on the application, the chromatography can be run at different flows from nano to micro (L/min) and with columns with different dimensions, particle sizes, and lengths of carbon chains (C4-C18). For separation of peptides, chain lengths of 18 alkyls are preferentially used as they offer a good separation capacity, while shorter chain lengths are preferred for proteins.

To increase resolution, reducing the particle size of the column is most effective; however, increasing the length of the column is also an option, but with the drawback of increasing the run time. On the other hand, reducing the particle size while increasing the flow rate is an effective way of keeping the system's high resolution while reducing analysis time. The internal diameter of the column can be reduced to increase sensitivity while scaling injection volume and flow rate accordingly; however, this leads to an increase in run time. Additionally, all these parameters affect the pressure of the system, which leads to a give and take between the max system pressure and the aim of the analysis. A common way to relieve pressure and reduce system load is to increase the column temperature, which decreases the mobile phase's viscosity.

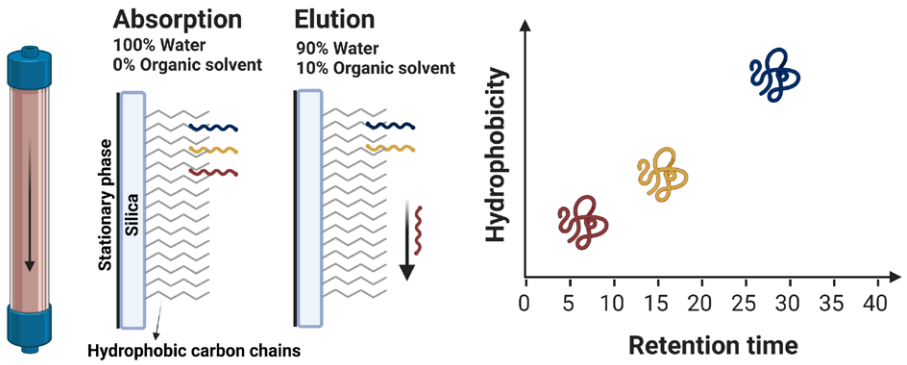

Figure 14. *Schematic of liquid chromatography separation. Created with Biorender.com*

In **all papers**, microflow HPLC equipped with a Hypersil Gold reversed-phase C18 column (dim. 100×2.1 mm, particle size 1.9 µm) was utilized with gradients optimized to reduce analysis time and increase throughput while still keeping a high resolution. This way, the separation of the analytes and interfering components can be controlled and spread out over time, which is favorable as it allows not only for matrix separation of complex samples but also works to reduce noise and increase signal [251].

4.3 MASS SPECTROMETRY

MS is a diverse tool used in proteomics for both explorative and targeted purposes. MS measures the mass-to-charge (*m/z*) ratio of a given charged molecule, allowing identification and quantification of peptides and proteins since the mass is based on its elemental composition. An MS system typically consists of three main parts; ion source, mass analyzer, and detector, of different types, some with dual functions and in different combinations. Electrospray ionization (ESI) is the most common type of ion source in proteomics. It serves to transfer and ionize relatively large and fragile analytes into the gas phase, which is necessary for the detection and introduction of the analytes into the high vacuum of the typical mass analyzer. The mass analyzer then performs the separation of the ions by their *m/z*; the two types used in this thesis are the quadrupole and orbitrap. The orbitrap also has the dual function as a detector of the ions to record output. Another common type of detector used in this thesis is the electron multiplier.

4.3.1 ELECTROSPRAY IONIZATION

ESI is a soft ionization technique, *i.e.*, it causes very little analyte fragmentation, generating multiply charged peptide ions, enabling mass analysis of large biomolecules such as peptides and proteins. Importantly, it can work online, directly coupled to the LC, converting the eluate from the LC in the liquid state to ions in the gas phase. The conversion is accomplished by emitting the liquid from a needle into a strong electric field, forming an elliptic droplet due to the equilibrium between the surface tension and the opposing electrostatic attraction. The equilibrium is then disrupted, and a Taylor cone is formed at a specific voltage threshold, causing droplets to spray from the tip. Evaporation shrinks the droplets, and when the Rayleigh limit is reached, where the surface tension is outcompeted by the repulsive forces between the ions' charges, smaller droplets brake off through Coulombic explosions. The formed ions can then enter the mass analyzer [251].

When operated at higher LC flow rates (*e.g.*, 0.3 mL/min), the evaporation is commonly aided by the assistance of heating as well as drying gas, such as nitrogen. For peptides, ESI is frequently operated in the positive ionization mode, where a trace amount of formic acid is commonly added to aid protonation into cations. In addition to settings and solvent composition, the produced charge state depends on peptide length, available sites of protonation (basic residues), and peptide sequence since some amino acids such as Arginine and Lysine are more susceptible than others. ESI sensitivity is directly dependent on sample concentration, meaning that sensitivity is considerably increased with reduced LC flow rates [252, 253]. This is primarily a consequence of the smaller drop size, leading to increased ionization efficiency and decreased charge competition between analytes.

4.3.2 QUADRUPOLE MASS FILTER

The quadrupole mass filter is composed of two pairs of opposing rods over which a combined radiofrequency (RF) potential and direct current (DC) potential of the opposing sign is applied (Fig 15). The two potentials cause combined attractive and repulsive forces where only ions within a set *m/z* ratio range have a trajectory able to pass through. This way, the mass analyzer works as an effective filter, selecting in a targeted setting the ion of choice.

4.3.3 ORBITRAP MASS ANALYZER

The orbitrap consists of an inner spindle-like electrode surrounded by an outer barrel-shaped electrode (Fig. 15). It works by trapping the ions in its electrostatic field in a circular motion around the inner electrode. Independently, the ions also oscillate axially in a frequency corresponding to its *m/z,* a motion that produces an image current on the outer electrode. Fourier transformation can then be used to calculate the *m/z* for the ions from the measured frequency, thereby converting frequency spectra into mass spectra containing both qualitative and quantitative data [251].

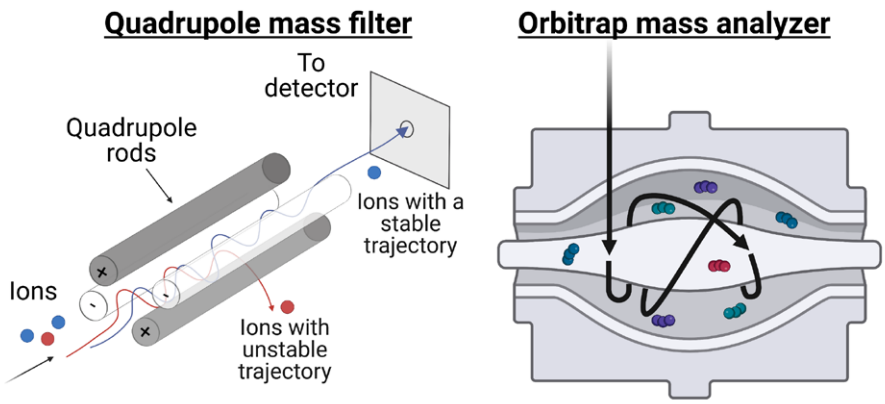

Figure 15. *Illustration of a quadrupole mass filter (left) and an orbitrap mass analyzer (right). Curtesy of Elena Camporesi. Created with Biorender.com*

4.3.4 ELECTRON MULTIPLIER

Electron multipliers act as ion detectors by a process called secondary emission, which amplifies a weak incoming particle's current into a considerably higher electron current. Secondary emission entails that when a charged particle impacts the secondary-emissive material of the multiplier, it induces the emission of a number of secondary electrons by energy transfer to the material. The secondary electrons will then be accelerated by the applied electric potential, generating further secondary emissions, resulting in an avalanche effect and a greatly amplified signal.

4.3.5 TANDEM MASS SPECTROMETRY

Tandem MS is commonly used for identifying or quantifying proteins and peptides and is also known as MS/MS (mass selection – mass separation). There are many types of experimental set-ups for tandem MS. Still, they all comprise four steps: precursor ion mass selection, fragmentation of precursor ions, fragment ion mass separation, and detection. In this thesis, two different tandem MS set-ups have been used, which both employ ESI, precursor mass selection in a quadrupole mass filter, and collision-induced fragmentation. After ionization in the ESI source, the precursor ions enter the quadrupole mass filter for narrow band mass selection. The selected precursor ions then enter the collision cell, where they experience collision-induced dissociation. Like the quadrupole, the collision cell is typically a multipole such as a quadrupole, hexapole, or octupole. By colliding with inert nitrogen gas, some of its kinetic energy converts into internal, causing the precursor ion to eventually fall apart into product ions [251].

Although fragmentation can occur in any bond, peptide amide backbone cleavage is the most useful for identification. C-terminally and N-terminally charged ions produced from peptide amide backbone cleavage are termed y- and b-ions, with a subscript indicating the number of amino acid residues of the ion [254]. For targeted tandem MS quantitation, especially the y-ions are important since the IS still carries the heavy standard label. Subsequently, the second mass analysis separates and detects the product ions, allowing for the identification and quantification of analytes with identical *m/z* since the mass determination of the product ions will increase specificity.

Depending on the type of MS instrument setups utilized for the tandem MS, two different approaches were performed; PRM or MRM (Fig. 16). In PRM, the second mass analyzer was an orbitrap, allowing for the parallel detection of all fragments, product ions, in a one mass spectrum. In MRM, on the other hand, the second mass analyzer is a second (or third if the collision cell is counted) quadrupole, allowing for the selection and detection of only one product ion at the time. Instead, for each precursor ion, multiple fast scans are performed for 3-5 product ions in a consecutive cyclic manner.

The MS/MS setup used in **Paper I** and **V** is PRM with a high-resolution instrument, *i.e.*, a Q Exactive (Thermo Fisher Scientific Inc.) for tandem mass analysis coupled to an LC system for separation. A Q Exactive consists of four main parts; an ESI source, a quadrupole mass filter/analyzer, a collision cell, and an orbitrap as the second mass analyzer and detector. This system allows for high resolution and mass accuracy, and it is thus less likely that the fragment ion signals are hidden or skewed by interfering ions. At the early stages of targeted method development, PRM is therefore highly suitable.

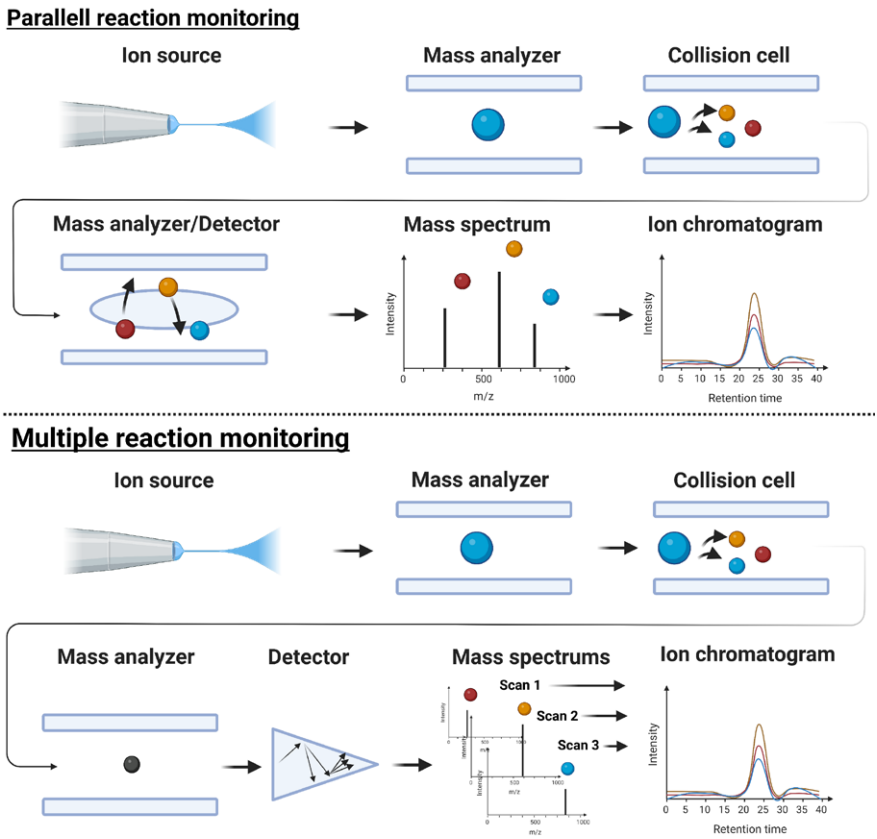

Figure 16. *Schematic representation of the principles of parallel reaction monitoring and multiple reaction monitoring. Created with Biorender.com*

In **Paper II, III, IV, and VI**, the MS setup utilized is MRM with a high-sensitivity instrument, *i.e.*, a 6495 Triple Quadrupole LC-MS system (Agilent Technologies) for selected mass analysis similarly coupled to an LC system. A triple quadrupole mass spectrometer constitutes similarly to the Q Exactive of four main parts, where the first is an ESI source. The difference, however, is that it is then comprised of a linear arrangement of quadrupoles, where the second mass analyzer is, like the first, a quadrupole for the selection of the product ions. After the second quadrupole filter, the ions are allowed to impact an electron multiplier detector. The strengths of the triple quadrupole lie in its dual use of quadrupoles as both mass analyzers allowing for selective and sensitive measurements and a broad dynamic range. These strengths are due to the quadrupole's narrow isolation and fast cycle time, as well as its ability to enable collision energy optimization for respective product ion. However, this also leads to limitations in resolving power and mass accuracy. For running larger studies, with an established targeted method, MRM setups are suitable workhorse instruments that allow for a fast and sensitive analysis [255].

4.3.6 DATA ANALYSIS – SKYLINE

For all MS data analysis, the software, Skyline (MacCoss Lab Software) was used, both for the application of initial proteomics method creation as well as quantitative data analysis [256]. The software allows for the design and exportation of method files as well as the import and analysis of output files for a range of mass spectrometers, including Agilent and Thermo Fisher Scientific instruments. For quantification of targets, it performs automatic peak picking and area under the curve calculation of all selected fragments of both endogenous peptides and IS, where the result is based on the ratio of the summed signal of the two, as well as easy peak inspection and adjustment when needed (Fig. 17).

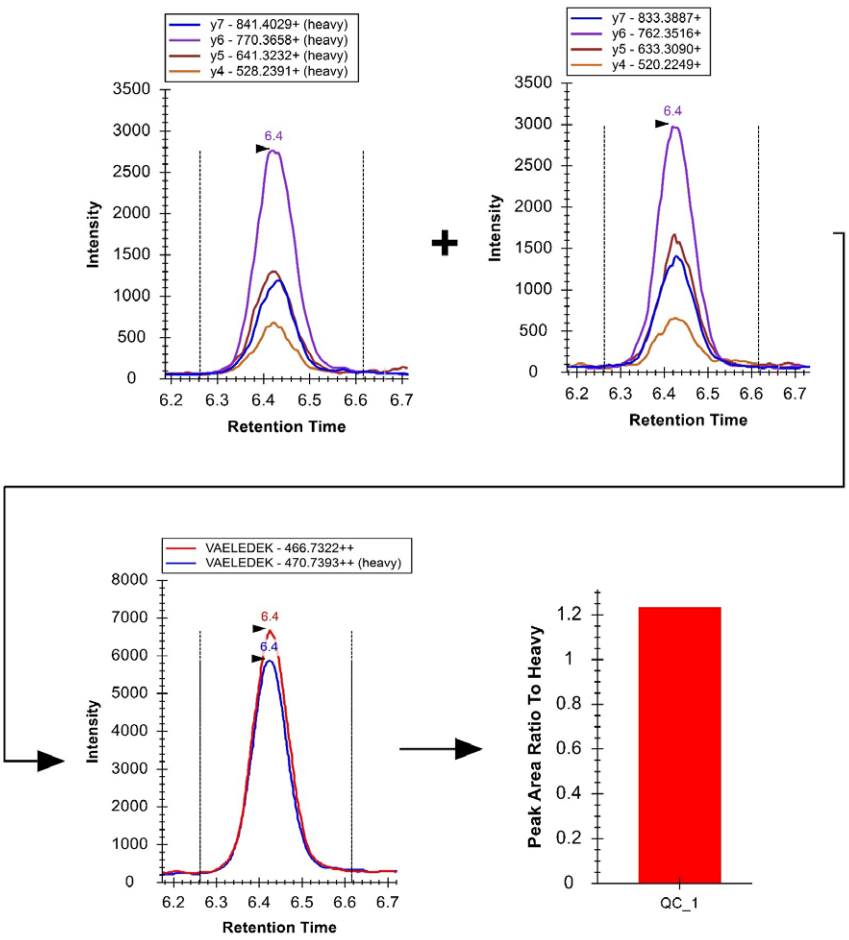

Figure 17. *Illustration of peaks, summation of fragments, and peak ratio in skyline software.*

4.4 SIMOA

Simoa is a semi-automated immunoassay technology developed by the company, Quanterix. The principle of Simoa (Fig. 18) is similar to a sandwich ELISA but at lower volume requirements, minimized variability, and increased sensitivity (200-1000-fold) [257]. Simoa utilizes paramagnetic beads conjugated with capturing antibodies which are stepwise incubated with the sample, biotinylated detection antibody, and lastly, β-D-galactopyranoside streptavidin (SBG). The beads are then transferred into a microarray, with wells that only fit one single bead, and resofurin β-D-galactopyranoside (RPG) added. Fluorescence will only be produced in those wells containing the target protein, as only beads that were able to form the immunocomplex were able to form the protein-ligand interaction of biotin to the streptavidin enzyme complex and thus contain the enzyme needed for the subsequent reaction. The number of beads is intentionally added to a higher degree than the target, which results in a Poisson distribution of analyte bound to beads and that at low analyte concentrations, only one immunocomplex will be present per bead and thus well. Digital measurement of analyte abundance can therefore be performed by automatic counting of the number of fluorescent wells in relation to the total number of wells containing beads (Fig. 18). For high abundant samples, however, quantification can also be performed in analog mode, similar to an ELISA, measuring the total fluorescent signal and thus allowing for a wide dynamic range of the technology.

In **Paper IV,** a commercially available Simoa kit (Quanterix) for the quantification of SNAP-25 was used.

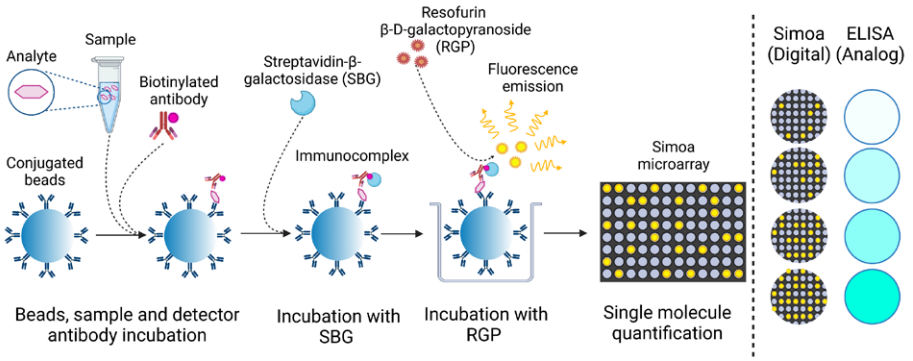

Figure 18. *Illustration of (right) the main steps of Simoa immunoassays and (left) a comparison between the quantification differences of Simoa and ELISA immunoassays. Curtesy of Juan Lantero Rodriguez. Created with Biorender.com*

4.5 STATISTICAL ANALYSIS

Statistical analyses and graphical visualization were mainly performed and created, respectively, by the use of GraphPad Prism 8.3.0 (GrahpPad Software, Inc.) or by R, version 3.6.1 (R Foundation for Statistical Computing). For specific statistical analyses, refer to the respective paper. Broadly, in order to assess group-wise comparisons, appropriate tests and adjustments were chosen based on data distribution, sample size, number of tests, and the possible inclusion of covariates such as age and sex. The receiver operating characteristic curve (ROC) contrasting groups were performed in order to provide the area under the curve (AUC) to evaluate the discriminatory ability of the biomarkers, where the Delong test was used to compare different AUC values. Exploration between the associations of the synaptic proteins and different variables, including demographics, clinical variables, or between different biomarkers, among others, was performed utilizing either correlation analysis, such as Spearman rank correlation analysis (Spearman's correlation coefficient (rho)), or using linear models, as well as linear mixed models in the cases of longitudinal data. Lastly, logistic regression models were used to determine whether the synaptic proteins were independent predictors of AD dementia (odds ratio, OR).

5 RESULTS & DISCUSSION

In **Paper I**, the aim was to set up a method for the quantification of multiple synaptic proteins, which had been indicated to be potential targets in an explorative proteomic study of AD [258]. A synaptic panel assay was established, comprising 17 synaptic proteins, including syntaxins, VAMP-2, PEBP-1, AP2B1, complexin-2, synucleins, GDI-1, neuronal pentraxins, and 14-3-3 proteins. The method was then used to quantify the proteins first in a pilot cohort of biochemically defined AD and controls, followed by validation of the results in clinically diagnosed participants. In the two cohorts, it was found that several of the proteins were altered in AD in comparison with controls. Interestingly, some proteins were higher in AD than controls while some were lower, and others unchanged, indicating that differential patterns of synaptic proteins exist in AD (Fig. 19). The method was further optimized by shortening the run time and transferring the method to another system more suitable for the continued exploration of the proteins in larger studies. In order to confirm possible disease-specific as well as common patterns among neurodegenerative diseases, the continued exploration also focused on expanding the investigation into a range of other neurodegenerative diseases as well as AD (**Paper II-VI**).

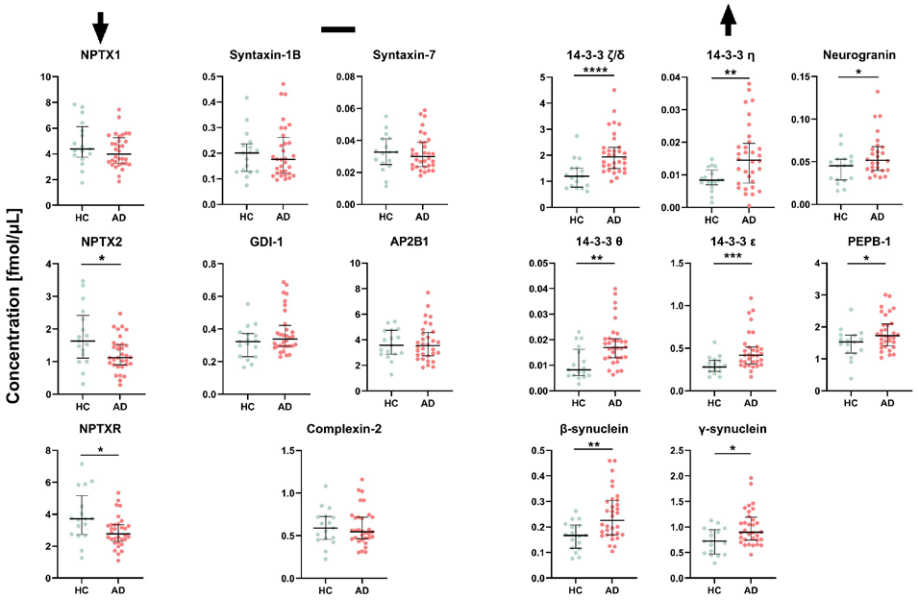

Figure 19. *CSF synaptic protein levels of the panel from Paper I in the clinical cohort that included healthy controls (HC, n=17) and Alzheimer's disease (AD, n=32) patients. The bars indicate median with interquartile range. P-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.*

In **Paper II**, the aim was to validate the findings of the previous paper in pathology-confirmed cases of AD and to explore the proteins in LBD and FTLD pathology. The study confirmed several of the previous findings of **Paper I**, as well as identifying synaptic protein changes in LBD and FTLD, including seemingly specific changes for those pathologies, AD-specific changes, and changes present across the diseases. This led to the conclusion that differential patterns of synaptic protein alterations across neurodegenerative diseases in relation to controls seem to exist. Following this, the studies were continued into the clinical spectrum of Parkinsonian disorders (**Paper III**) and genetic FTD (**Paper IV**). Also, here novel differences were discovered in synaptic proteins in both parkinsonian disorders as well as in genetic FTD with differential synaptic impairment represented by different synaptic proteins. Interestingly, multiple abnormalities were found in the symptomatic patients with *MAPT* mutations (**Paper IV**), indicating specific synaptic dysfunction in relation to the underlying proteinopathy found in each mutation of genetic FTD. Furthermore, it was demonstrated that the neuronal pentraxins have the potential as monitoring biomarkers of motor symptoms in PD (**Paper III**).

Concurrently, a novel Simoa method for the quantification of one of the most well-known potential synaptic biomarkers, SNAP-25, was developed. MS methods are often used as the gold standard for the quantification of biomarkers with high reproducibility, specificity, and sensitivity. However, new ultrasensitive semi-automated methods such as the Simoa immunoassays are interesting new alternative quantification methods in the biomarker field with low sample volume requirements that are also well suited for efficient routine use. In **Paper V**, the aim was thus to compare the novel SNAP-25 Simoa method with the in-house developed IP-MS SNAP-25 method and to evaluate their performance. The results showed that the new SNAP-25 Simoa assay can be utilized interchangeably with the IP-MS method, increasing the accessibility of biomarker quantification. Thus, demonstrating the importance of MS-based biomarker discovery, which can drive the development of new, highly accurate methods.

Lastly, in **Paper VI**, the aim was to quantify SNAP-25 together with SYT-1, another potential synaptic biomarker, and the synaptic panel method in Biofinder-2; a large-scale cohort encompassing patients in the AD continuum, in order to compare the synaptic proteins' performance and ability to predict progression and cognitive decline. The cohort also includes a range of neurodegenerative diseases for differential diagnostic comparisons. In this large AD study, higher levels were confirmed of several of the synaptic proteins that had not previously shown any differences, as well as that for many; the changes have already occurred at the preclinical stage (CU+). Several of the proteins also displayed increases across the AD continuum, with higher levels at later stages, while others increased early and seemed to stabilize.

Additionally, most were able to predict progression from prodromal to AD dementia, and six of the synaptic biomarkers, measured in samples taken at the prodromal stage, were also associated with the progression of cognitive decline.

In Table 3, an overview of all the results from the studies can be seen, demonstrating the differential patterns of synaptic protein alterations mentioned across neurodegenerative diseases in relation to controls. By grouping them together, four synaptic protein pattern alterations possibly seem to exist in neurodegenerative diseases. The first group is represented by the neuronal pentraxins, which have been found to have lower protein levels compared with controls across neurodegenerative diseases. The second group includes the 14-3-3 proteins, where foremost 14-3-3 ζ/δ displayed higher protein levels than controls, mainly in AD but possibly also in other pathologies. Thirdly, there are the synucleins together with PEBP-1 and GDI-1, which all seems to be indicative of AD-specific changes as there only were differences in AD detected across all studies. Lastly, there is a large group of proteins including, among others, the SNARE proteins (SNAP-25, syntaxins, SYT-1, and complexin-1), neurogranin, and AP2B1, of which higher levels were observed in AD while lower levels seem possibly present in other neurodegenerative diseases. Only VAMP-2 showed no difference for any neurodegenerative disease compared with controls; however, the protein was excluded from the panel after **Paper II** due to issues with the stability of the IS.

Table 3. *Simplified overview of changes observed for the neurodegenerative diseases compared against controls across the studies included in the thesis for all synaptic proteins quantified.*

		Clinical										Pathology			Genetics
		AD continuum			Parkinsonian disorders										
	Protein	CU+	MCI+	AD	FTD	DLB	PD	MSA	PSP	CBD	Vad	AD	FTLD	LBD	FTD
Panel I	*NPTXR*			↓	↓	↓	↓	↓	↓	↓		↓	↓	↓	↓ All
	NPTX1			↓		↓	↓	↓	↓	↓	↓	↓	↓		↓ All
	NPTX2		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓ All
	14-3-3 ζ/δ	↑	↑	↑↑								↑↑	↑	↑	↑ MAPT
	14-3-3 ε	↑	↑	↑											
	14-3-3 η			↑											↑ MAPT
	14-3-3 θ	↑	↑	↑											
	β-synuclein	↑	↑	↑↑								↑			↑ MAPT
	γ-synuclein	↑	↑	↑↑											↑ MAPT
	PEBP-1	↑	↑	↑↑											↑ MAPT
	GDI-1	↑	↑	↑↑											↑ MAPT
	Neurogranin	↑	↑	↑↑				↓	↓				↓	↓	↑ MAPT
	Complexin-2	↑	↑	↑		↓		↓	↓						↑ MAPT
	AP2B1	↑		↑		↓		↓	↓				↓	↓	↑ MAPT
	Syntaxin-1B												↓		↑ MAPT
	Syntaxin-7			↑									↓		↑ MAPT
Panel II	*SNAP-25*	↑	↑	↑↑				↓*	↓*						
	SYT-1	↑	↑	↑		↓		↓*	↓*						

Notes: *Only observed in a combined group of MSA and PSP.

The reason for the difference in the synaptic markers causing the patterns remains elusive. Overall, it could be speculated from the current results that it seems that there are underlying AD pathology mechanisms that cause increases in most synaptic proteins, while one or several mechanisms possibly in common to neurodegenerative diseases, with the exception of AD, lead to lower levels of synaptic proteins. Different sensitivities, functionalities, and cell localization or regional distributions might then cause the synaptic proteins to be more or less affected by said mechanisms or in a temporal manner, thus leading to the appearance of different synaptic protein patterns. By looking at the synaptic proteins studied, no clear indications of differences in either function or localizations are apparent between the groups of proteins with different patterns. Drawing any conclusion regarding so is especially hard in light of the fact that much is left to learn about these proteins, particularly in regard to function.

However, as increases of some of these synaptic proteins have been found to be higher already in early AD, *i.e.*, Aβ-positive persons with no evidence of tau pathology, this has been attributed to be due to amyloid-β triggered synaptic dysfunction prior to tau accumulation and neurodegeneration [259]. A recently published hypothesis [260] on the temporal relation of synaptic dysfunction with Aβ and tau pathology stipulates the following. In early AD disease initialization, localized synaptic dysfunction occurs due to soluble Aβ. Attracted microglia phagocytose the damaged synapses, advancing removal of particular synapses but reducing more extensive damage to the axon, thus resulting in minimal effect on the network where compensation allows for maintained cognition. Once plaque load increases and multiple plaques are found in the proximity of individual axons, tau hyperphosphorylation occurs at multiple sites. This is the starting point of neurodegeneration, where tau dissociates from the microtubules causing dysfunctional axon removal, including all its synapses. The synapse is a dynamic structure that undergoes ongoing formation and removal in the adult brain. In *vivo* studies of mouse models found that synapse formation continues at a normal pace after plaque formation but in the presence of enhanced removal [93]. Increased synaptic protein levels in AD, especially at early stages, could thus be indicative of widespread synaptic dysfunction and removal in the presence of reformation. On the other hand, at later stages, lower synaptic protein levels when neurodegeneration occurs might be indicative of the permanent greater synaptic loss, which follows with no possibility of continued formation.

In the following sections, the results for each protein are described in more detail in the context of the four synaptic protein patterns noted as well as in regard to the literature.

5.1 NEURONAL PENTRAXINS

Lower CSF concentrations of the neuronal pentraxins (NPTX1, NPTX2, and NPTXR) were found in all neurodegenerative diseases explored in this thesis compared to controls (Fig. 20). Of the three pentraxins, NPTX2 seems to outperform the others with consistently better results compared to the other two protein family members, and as the proteins correlate (rho>0.71, **Paper II**) with each other, much focus is on NPTX2 in the current section.

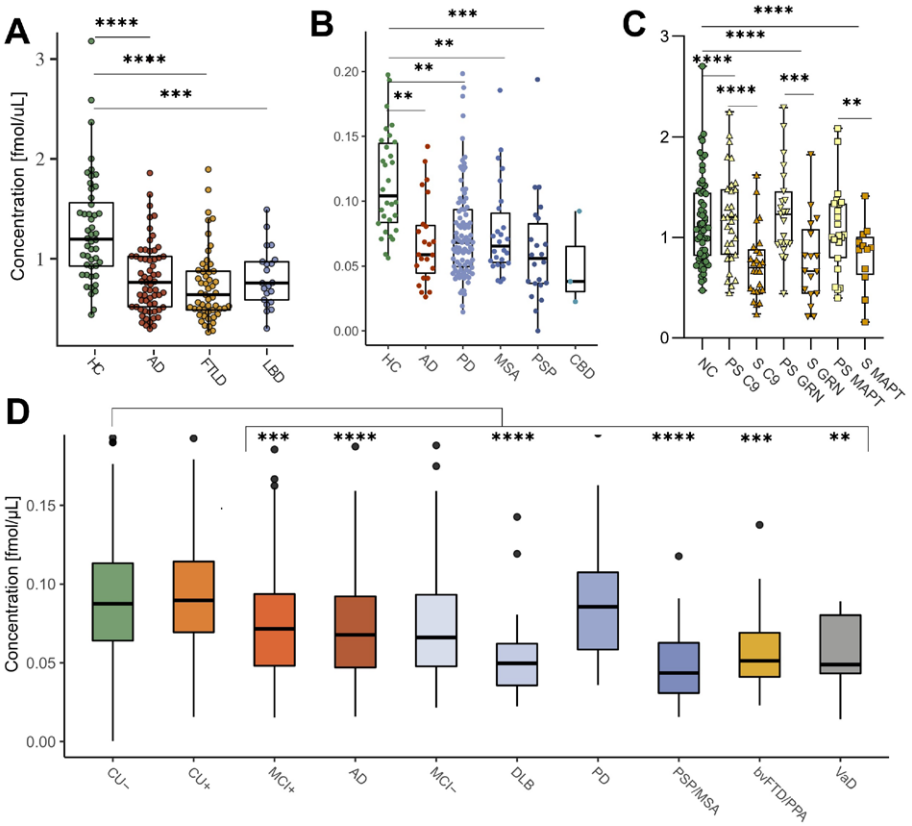

Figure 20. *Neuronal pentraxin-2 levels. (A) The pathologically confirmed cohort consisting of; healthy controls (HC), Alzheimer's disease (AD), Lewy body spectrum of disorders (LBD), and frontotemporal lobar degeneration (FTLD). (B) The Umeå parkinsonian cohort consisting of; HC, Parkinson's disease (PD), corticobasal degeneration (CBD), progressive nuclear palsy (PSP), multiple system atrophy (MSA), and AD. (C) The GENFI cohort consisting of; non-carriers (NC) and presymptomatic (PS) as well as symptomatic (S) MAPT, C9orf72, and GRN mutation carriers. (D) Biofinder-2 consisting of; cognitively unimpaired (CU) controls and patients with mild cognitive impairment (MCI) divided by Aβ status, as well as a spectrum of other neurodegenerative diseases, including AD. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.*

One of the most important findings of this thesis is that the pentraxins seem to have potential as monitoring biomarkers of general cognition observed across neurodegenerative diseases and other functions such as motor function in parkinsonian disorders. They do, however, not seem to be useful as differential diagnostic biomarkers among neurodegenerative diseases since they show changed protein levels among neurodegenerative diseases regardless of clinical diagnosis (**Paper III, Paper VI**, Fig 20B & 20C), underlying pathology (**Paper II**, Fig 20A) as well as genetic subtype of FTD (**Paper IV**, Fig 20C).

NPTX2 was repeatably found to be the synaptic protein to have the highest association with cognitive measures (*e.g.*, MMSE) in AD dementia. Similarly, in **Paper III** in PD, lower CSF NPTX2 was found to be associated with lower MMSE scores (rho=0.21, p=0.048), *i.e.*, worse cognition, as well as cognitive decline over time (β-estimate=0.32, p=0.021). Interestingly, when looking at motor scores, higher levels of the pentraxins were found to be associated (rho=0.36-0.39, p<0.01) with higher scores in tremor, *i.e.*, less severe tremor at baseline, while lower levels were found to be associated (β-estimate=-0.025 to -0.038, p<0.05, Fig. 21) with faster PIGD progression. Thus, lower levels of NPTX2 were associated with more rapid advancement in both motor- and cognitive aspects of PD. Since a tremor-dominant PD phenotype is associated with lower dementia risk and a more benign prognosis [261], this indicates that NPTX2 might be useful for predicting subtype severity in PD cases. Further confirmed by the correlation of lower CSF NPTX2 levels with early denervation in the caudate nucleus in PD (rho=0.25, p-value=0.029) but not in the putamen (rho=0.10, p-value=0.39) – since impaired function of the caudate nucleus is associated with an increased risk of developing dementia [262, 263].

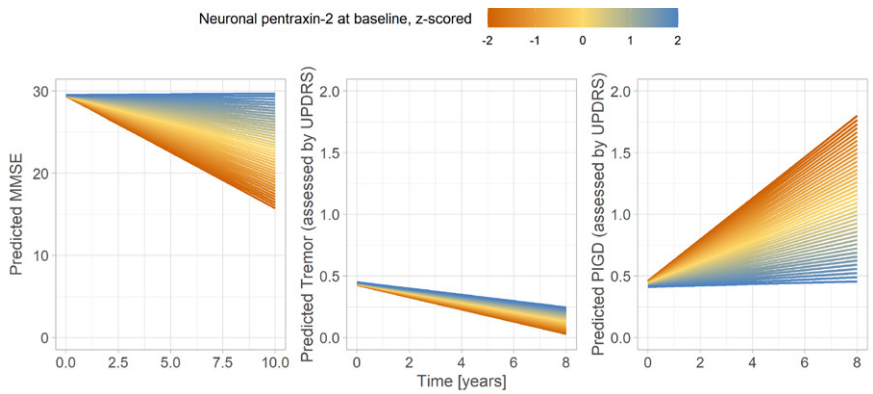

Figure 21. *Estimated marginal means plots for neuronal pentraxin-2 from mixed effect model analyses of the associations with the longitudinal performance of cognition (mini-mental state exam (MMSE)) and motor (Tremor and postural imbalance and gait difficulty (PIGD)) scores in Parkinson's disease patients. The linear mixed models were adjusted for age, sex, and baseline values.*

Furthermore, in **Paper VI,** lower levels were observed of NPTX2 also at early stages of AD, but only at prodromal AD and not preclinical (Fig. 20D). It was again found that CSF NPTX2 seems to be generally lower across neurodegenerative diseases, including FTD, DLB, PSP/MSA, and VaD, compared with controls. NPTX2 was found to be the synaptic protein studied to best separate non-AD from controls (AUC=0.74), but not even the top five at separating AD from controls (AUC=0.67). As previously mentioned, also in this paper, for AD dementia, it was confirmed that there is a particularly strong association between baseline pentraxin concentrations and MMSE (NPTX2, β-estimate=1.09, p-value=0.002). Interestingly, it was shown that the pentraxins concentrations are particularly associated with more rapid cognitive decline in AD dementia (NPTX2, β-estimate=0.49, p-value=0.0043), something not found for the other synaptic proteins. Unlike some of the other synaptic proteins, NPTX2 did not predict cognitive decline in prodromal AD (MCI+) subjects or have an association with baseline cognition in those patients. However, when investigating progression to AD dementia diagnosis, lower NPTX2 levels were predictive, increasing the probability of conversion to dementia by 33% (OR=0.67, p-value=0.044). Thus, according to the results of **Paper VI**, in the AD continuum, NPTX2 seems to be more affected in later stages of AD, especially in relation to cognitive decline compared with other synaptic biomarkers but is still predictive of progression at prodromal stages.

It is particularly interesting to note that unlike the other synaptic proteins studied, the neuronal pentraxins were the only proteins to have lower CSF concentrations in AD instead of higher. The neuronal pentraxins seem thus to be exempt from whatever AD-specific mechanism induces higher synaptic protein CSF levels than normal. It has earlier been suggested that the decreases seen for NPTX2 are connected with a reduced protein expression, as indicated by lower mRNA as well as protein levels in AD brain tissue [131], possibly due to reduced neuronal activity, which is a known regulator of its expression as an induced early gene [123]. However, the same article found no changes in NPTX1 mRNA expression, while for the studies included in this thesis, the CSF NPTX1 protein levels were also found to be lower in AD. An additional fact that doesn't align with this theory is the reported upregulated mRNA expression in PD for NPTX2 [125], again compared with the presence of lower levels of CSF protein NPTX2 in PD patients found in the studies included herein. In PD, lower NPTX2 levels could be explained by LB inclusion pathology [125]. However, this has not been reported for other pathologies to our knowledge and does thus not explain all the changes observed for CSF NPTX2 levels.

NPTX2 has been found to prominently and specifically accumulate at excitatory synapses on parvalbumin-expressing interneurons in the presence of perineuronal nets [264]. Changes in NPTX levels have thus been suggested to infer specific disruption of pyramidal neuron-parvalbumin circuits [131], circuits which are important for brain

rhythmicity and homeostasis of excitability [265]. In light of the discordance with NPTX mRNA levels, specific vulnerability and degeneration of parvalbumin interneurons might thus be the source of the lower NPTX levels in the CSF. Additionally, since NPTX1 and NPTX2 function depend on their secretion into the synaptic cleft from the presynaptic compartment, lower levels might be due to alterations in said release, following synaptic dysfunction of vesicle exocytosis. This also aligns with the fact that higher levels of SNARE proteins are detected at the early stages of AD seemingly preceding the lower levels of NPTXs found only at later stages of the AD continuum.

Similarly, NPTXR needs to be cleaved and released from the postsynaptic membrane to exert its function. In a mouse model of AD, higher levels of membrane-bound NPTXR were found with more advanced Aβ pathology, supporting impaired secretion of NPTXR at later AD stages as well [266]. However, NPTXR cleavage is mediated by TACE (Tumor necrosis factor-α converting enzyme), whose enzymatic activity is reported to be upregulated in AD [267], not downregulated. All in all, further studies are warranted to discern the exact mechanistic pathways which underlie the decreased levels of NPTX in CSF of neurodegenerative disease.

Neuronal pentraxin-2 is a potential synaptic monitoring biomarker of decline in cognition and motor functions across neurodegenerative diseases.

To summarize, the neuronal pentraxins seem to be potential monitoring biomarkers of the decline of cognitive and motor functions across neurodegenerative diseases. However, they seem to change later in the AD continuum than other synaptic biomarkers and possibly be more affected at later stages of AD. The protein family should be continued to be further studied in other neurodegenerative diseases and to confirm the changes noted in the studies included in this thesis. They might also be interesting targets in mechanistic studies of synaptic dysfunction pathways of neurodegenerative diseases.

5.2 14-3-3 PROTEINS

Out of the four 14-3-3 proteins (ζ/δ, θ, η, and ε) included in the synaptic panel, a better performance was continually seen from the 14-3-3 ζ/δ protein. Results for 14-3-3 η protein were only included in **Paper I** due to issues with repeatability. Furthermore, it was found that the 14-3-3 proteins correlated with each other (**Paper II**, rho>0.61), where the differences in their results possibly can be attributed to analytical variability. The following section thus focuses on the results observed for 14-3-3 ζ/δ. Across the studies included in this thesis, higher protein concentrations of 14-3-3 ζ/δ were found in AD compared with both controls and other neurodegenerative diseases (**Paper II, III & VI,** Fig. 22).

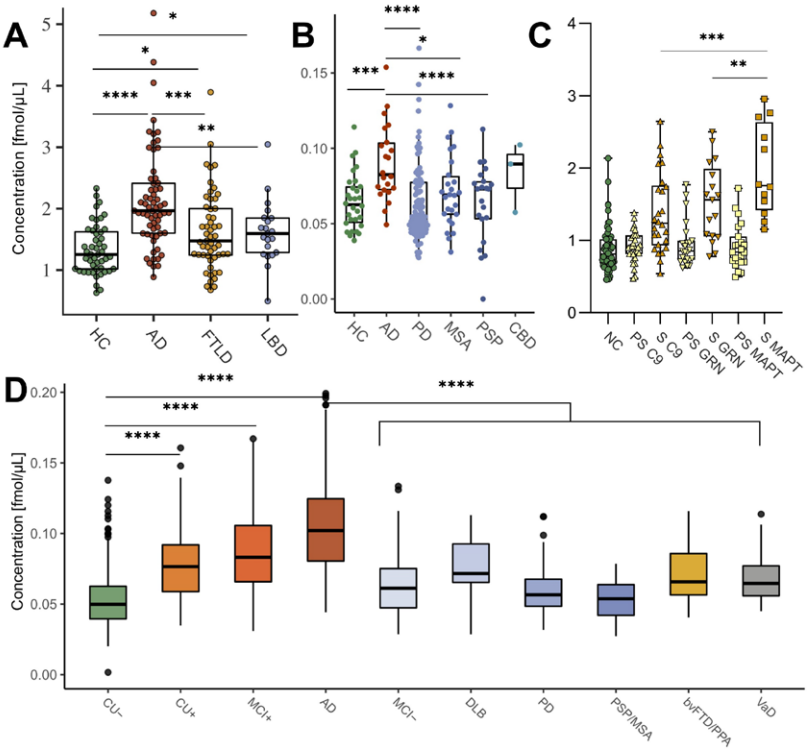

Figure 22. *14-3-3 ζ/δ levels. (A) The pathologically confirmed cohort consisting of; healthy controls (HC), Alzheimer's disease (AD), Lewy body spectrum of disorders (LBD), and frontotemporal lobar degeneration (FTLD). (B) The Umeå parkinsonian cohort consisting of; HC, Parkinson's disease (PD), corticobasal degeneration (CBD), progressive nuclear palsy (PSP), multiple system atrophy (MSA), and AD. (C) The GENFI cohort consisting of; non-carriers (NC) and presymptomatic (PS) as well as symptomatic (S) MAPT, C9orf72, and GRN mutation carriers. (D) Biofinder-2 consisting of; cognitively unimpaired (CU) controls and patients with mild cognitive impairment (MCI) divided by Aβ status, as well as a spectrum of other neurodegenerative diseases, including AD. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.*

In **Paper VI,** it was found that 14-3-3 ζ/δ outperforms the other synaptic biomarkers in the separation of AD from controls (AUC=0.92). In addition, stepwise higher levels across the AD continuum were found, with moderately well separation already at the preclinical stages of AD (CU+, AUC=0.79), compared with controls, similar to neurogranin and SNAP-25. 14-3-3 ζ/δ was also one of the strongest predictors of AD conversion in prodromal cases, increasing the risk by 132% (OR=2.32, p-value≤0.0001). Additionally, at those early AD stages (MCI+), higher 14-3-3 ζ/δ levels were also associated with more rapid cognitive decline (β-estimate=-0.36, p-value=0.013), something not observed in Aβ-negative subjects (MCI-).

Even though no indication of changes in 14-3-3 CSF levels was found in other neurodegenerative diseases in the clinical cohorts, changes in pathologically confirmed cases of non-AD pathology were observed. In **Paper II** (Fig. 22A), higher protein concentrations were observed in pathologically confirmed cases of FTLD and DLB compared with controls. This is of note since similar findings were not observed in the corresponding clinical diagnosis groups with this kind of underlying pathology. This can be explained by the complexity present in the underlying pathology and the neurodegenerative syndromes, with a high presence of overlap as well as the presence of co-morbidity. This increase might thus partly be attributed to AD co-pathology, at least in the LBD cases, which were observed in 50% of the cases. For FTLD, for which only one patient presented with AD-comorbidity according to pathology, the results are more surprising. In addition, in the genetic study of FTD, higher levels of 14-3-3 ζ/δ were found to be present in symptomatic MAPT carriers (**Paper IV,** Fig 22C). Since 14-3-3 ζ/δ has been associated with tau and its phosphorylation [149, 155-157], it might thus be of interest to investigate 14-3-3 ζ/δ concerning the FTLD subtype to explore if there is an association between the higher levels found in pathology confirmed cases of FTLD and genetic FTD MAPT carriers with the presence of tau pathology.

On the other hand, 14-3-3 proteins are established biomarkers of CJD [268]. Early methods to confirm 14-3-3 positivity in CJD included sodium dodecyl sulfate (SDS)-PAGE immunoblotting which does not differentiate between the 14-3-3 family members [269]. Today, ELISA and Western blot utilize protein-specific antibodies, commonly targeting 14-3-3 β or 14-3-3 γ [268], but 14-3-3 pan-specific antibodies can also be used, which capture all 14-3-3 proteins [269]. Less work, however, seems to have been done on 14-3-3 ζ/δ, possibly due to the fact that early studies demonstrated only the presence of β, γ, ε, and η in the CSF of CJD [269, 270]. In CJD, the higher levels of 14-3-3 proteins in the CSF have been explained to be due to a general passive protein leakage present caused by neurodegeneration [271]. A theory corroborated by the finding that higher levels of the proteins are also present in other conditions characterized by extensive neuronal loss, including multiple sclerosis, stroke, and

meningoencephalitis [272, 273]. The higher levels for 14-3-3 ζ/δ seen in AD, FLTD, and LBD in **Paper II** could thus be explained by the same reasoning. Furthermore, in addition to the higher levels for 14-3-3 ζ/δ in non-AD pathology in **Paper II**, when the non-AD diseases were combined into one group in **Paper VI** and compared against controls, 14-3-3 ζ/δ showed a moderately well separation (AUC=0.68), indicating possible higher levels in the non-AD diseases here as well. As hypothesized in **Paper II,** 14-3-3 ζ/δ might thus be a general marker of neurodegeneration across neurodegenerative diseases, which is also particularly higher in AD, according to the studies included in this thesis work. Similarly, for 14-3-3 γ, a study has reported higher levels in AD compared with controls and FTD as well as higher levels in FTD compared with controls, finding the protein to perform comparably to NfL and total tau as a marker of neurodegeneration [154]. However, it should be noted that direct mechanistic involvement of the different 14-3-3 protein family members in some diseases cannot be excluded due to the multiple reported links with pathology, such as interactions with both tau [149, 155-157] and α-synuclein [158]. Furthermore, in this thesis work, CJD cases were not included in any study, and for the disease studied, primarily, changes were only observed for AD. Thus, no firm conclusions can be drawn on 14-3-3 ζ/δ and its role as a biomarker in other diseases than AD.

14-3-3 ζ/δ is a potential biomarker of AD able to predict progression and possibly monitor cognitive decline. The protein might be a general marker of neurodegeneration across neurodegenerative diseases.

To summarize, for the first time, it is shown that 14-3-3 ζ/δ is thus seemingly an early AD biomarker able to both differentiate from other neurodegenerative diseases and support the diagnosis of AD already at the preclinical stage. 14-3-3 ζ/δ also seems to have the potential to be able to predict conversion as well as cognitive decline but less so than SNAP-25 and possibly monitor such cognitive changes. Additionally, 14-3-3 ζ/δ might have potential as a possible biomarker in other neurodegenerative diseases and should be further studied in this context to find its potential role across neurodegenerative diseases. The studies included in this thesis have contributed to the study of 14-3-3 proteins outside of their use as biomarkers of CJD.

5.3 SYNUCLEINS, PEBP-1, & GDI-1

The group of synaptic proteins which represent the third possible pattern includes the synucleins together with PEBP-1 and GDI-1. The increased levels of β-synuclein in AD compared with controls found in the first study (**Paper I**, Fig. 19) were confirmed across the studies and found to be AD-specific both in pathologically confirmed cases (**Paper II,** Fig. 23A) as well in clinical cohorts (**Paper III & VI**, Fig 23B & 23D) with a wide range of neurodegenerative diseases.

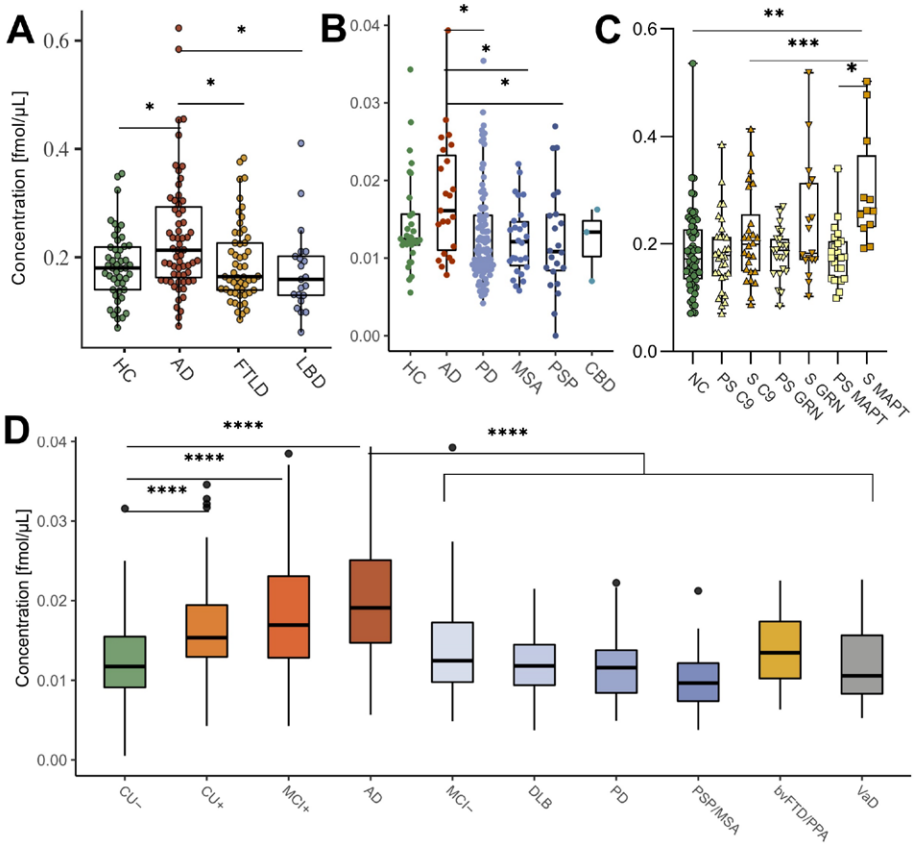

Figure 23. *β-synuclein levels. (A) The pathologically confirmed cohort consisting of; healthy controls (HC), Alzheimer's disease (AD), Lewy body spectrum of disorders (LBD), and frontotemporal lobar degeneration (FTLD). (B) The Umeå parkinsonian cohort consisting of; HC, Parkinson's disease (PD), corticobasal degeneration (CBD), progressive nuclear palsy (PSP), multiple system atrophy (MSA), and AD. (C) The GENFI cohort consisting of; non-carriers (NC) and presymptomatic (PS) as well as symptomatic (S) MAPT, C9orf72, and GRN mutation carriers. (D) Biofinder-2 consisting of; cognitively unimpaired (CU) controls and patients with mild cognitive impairment (MCI) divided by Aβ status, as well as a spectrum of other neurodegenerative diseases, including AD. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.*

Even though less well-performing, similar patterns of protein change was observed across studies for γ-synuclein, PEBP-1, and GDI-1. For all three proteins, higher levels were observed in pathologically confirmed AD compared with FTLD (**Paper II**) and in AD compared with both controls and other neurodegenerative diseases (**Paper VI**). The proteins, however, showed no differences in **Paper III** for any of the groups. These proteins, the synucleins, PEBP-1, and GDI-1, seem thus, according to the results presented herein, to be potentially specifically higher in AD and unchanged in other diseases.

Of the two synucleins studied in this thesis, β-synuclein and γ-synuclein, β-synuclein consistently showed more promising results than the other. However, the synucleins seemed to reflect the same changes across the different diseases and correlated strongly with each other (**Paper II**, rho=0.82). The lower performance of the γ protein could be possibly attributed to its more widespread neuronal localization and peripheral expression [274]. A fact that can also explain the lower performance of GDI-1 and PEBP-1, which similarly have low region and tissue specificity. However, even if these don't show potential as biomarkers, the study of the proteins might contribute to mechanistic insight. GDI-1 is a protein that modulates several rab family members, and thus potentially, its changed levels are indicative of other pathological mechanisms in AD, such as trafficking of Aβ [182]. Similarly, further studies might explore PEPB-1s potential role in cholinergic dysfunction [179]. However, since cholinergic dysfunction is a central part of other neurodegenerative diseases such as PD, where no differences were found, possibly the changes observed in AD are due to another function of the protein. Another explanation could also be that it reflects AD-specific cholinergic dysfunction pathways. The proteins' possible pathological involvement and function in AD neurodegeneration should thus be further studied to answer these questions and give insight.

β-synuclein, on the other hand, does seem to have potential as a biomarker of AD. In fact, of all synaptic proteins, β-synuclein was found to be one of the best to identify AD (**Paper VI**) compared with both controls (AUC=0.81) and non-AD (AUC=0.82), comparable to neurogranin and only surpassed by 14-3-3 ζ/δ and SNAP-25. Furthermore, it was also found that β-synuclein seems to have higher levels already at the preclinical stage with good separation from controls (Fig. 23D, AUC=0.72) and seems to increase step-wise across the AD continuum. Higher levels at the prodromal stage were also indicative of a more rapid cognitive decline (β-estimate=-0.40, p-value=0.0036) as well as a 104% higher risk of progression to AD dementia (OR=2.04, p-value≤0.0001).

The AD-specificity for β-synuclein has also been corroborated by other studies [170, 171], and the reason for its specificity has been contributed to the protein being enriched in the hippocampus [275], an area specifically vulnerable to AD neurodegeneration [276]. Only in the genetic study of FTD (**Paper IV**) were increased levels found outside of AD, this time in symptomatic *MAPT* carriers compared with non-carriers as well as asymptomatic *MAPT* carriers. Even though β-synuclein was outperformed by 14-3-3 ζ/δ and SNAP-25 in **Paper VI**, the protein is an interesting potential synaptic AD-specific biomarker. Especially in light that it is highly brain-specific, which has led to the investigation of it as a prospective blood biomarker. In 2020, it became one of the first synaptic biomarkers to be found to be able to differentiate AD patients from controls in serum [173].

β-synuclein is a potential differential AD-specific blood biomarker able to predict progression and possibly monitor cognitive decline.

To summarize, the synucleins, PEBP-1, and GDI-1, seem to indicate AD-specific changes and should be further studied to discover their potential roles in AD pathology. β-synuclein is a promising biomarker of AD with potential for quantification in blood, and the studies included in this thesis contribute to the field, expanding the knowledge of the protein and its potential.

5.4 SNARES, NEUROGRANIN, & AP2B1

The last group of proteins includes the SNARE proteins (syntaxins, complexin-2, SYT-1, and SNAP-25), neurogranin, and AP2B1. Of these proteins that are part of the panel (the syntaxins, complexin-2, neurogranin, and AP2B1), only neurogranin displayed higher protein levels in AD in comparison to controls in **Paper I** (Fig. 19). In **Paper II** (Fig. 24A), complexin-2 again showed no change, but it was found that the syntaxins and AP2B1 displayed lower levels in FTLD compared with controls, and also in LBD for AP2B1. It was thus hypothesized at the time that lower levels of the syntaxins and AP2B1 were indicative of non-AD-specific changes. In the same study, neurogranin displayed specifically higher levels in AD compared with both FTLD and LBD, as well as lower levels in FLTD and LBD compared with controls. Furthermore, in **Paper III** (Fig 24B), lower levels were found of AP2B1, neurogranin, as well as complexin-2 in PSP and MSA. In the genetic study of FTD (**Paper IV**, Fig. 24C), higher levels were observed in symptomatic *MAPT* carriers compared with noncarriers, and with the other genetic groups, only AP2B1 showed lower levels in symptomatic mutation carriers of *GRN* compared with asymptomatic.

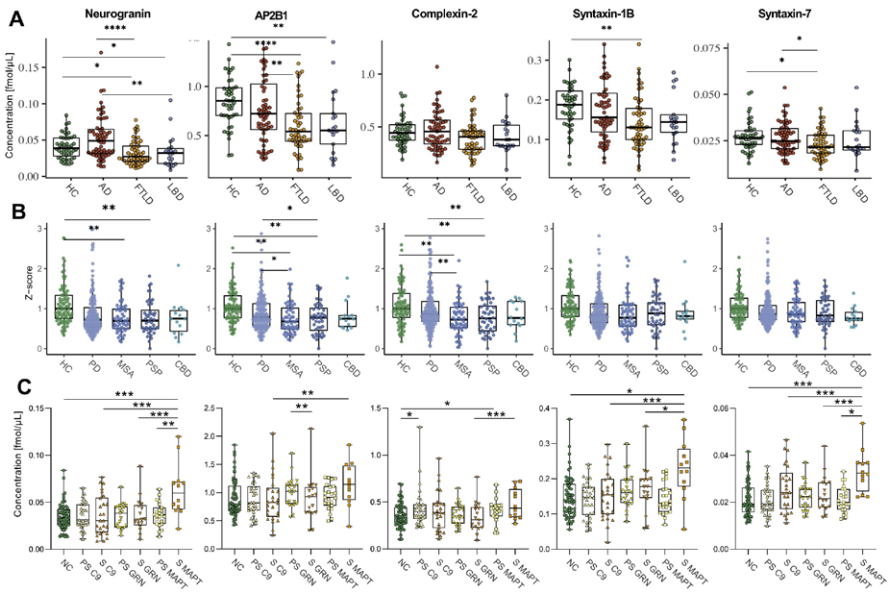

Figure 24. *Neurogranin, AP2B1, the syntaxins, and complexin-2 levels A) The pathologically confirmed cohort consisting of; healthy controls (HC), Alzheimer's disease (AD), Lewy body spectrum of disorders (LBD), and frontotemporal lobar degeneration (FTLD). (B) The combined parkinsonian cohort (Umeå plus Sahlgrenska) consisting of; HC, Parkinson's disease (PD), corticobasal degeneration (CBD), progressive nuclear palsy (PSP), multiple system atrophy (MSA), and AD. (C) The GENFI cohort consisting of; non-carriers (NC) and presymptomatic (PS) as well as symptomatic (S) MAPT, C9orf72, and GRN mutation carriers. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.*

In **Paper IV**, it was found that the two methods for the quantification of SNAP-25, the Simoa and the IP-MS method, correlated strongly (rho>0.88; p<0.0001, Fig. 25). Furthermore, for both methods, higher CSF SNAP-25 protein concentrations in CI Aβ+ in comparisons with CI Aβ- (Simoa, $p \leq 0.01$; IP-MS, $p \leq 0.05$) and CU Aβ- (Simoa, $p \leq 0.01$; IP-MS, $p \leq 0.05$) was observed. The results thus indicated no difference between the two methods' clinical performance as they were equally able to identify Aβ pathology, *i.e.*, AD pathophysiology. It was furthermore explored if the Simoa SNAP-25 assay could be used in plasma since studies have indicated SNAP-25 to be present, although, so far, only measured in neuronally derived exosomes [277]. However, SNAP-25 was not able to be quantified in plasma with any of the methods, and it was thus concluded that both assays lacked the analytical sensitivity required. Furthermore, it was observed that one of the SNAP-25 peptides (*e.g.*, [Total] Ac-AEDADMRNELEEMQR) seemed to perform less well than the other in comparison with the Simoa method. This is supported by an earlier study which suggests that the longer soluble forms of SNAP-25, which seem to be captured better by the more N-terminally located peptide (*e.g.*, [Long] MLQLVEESK) as well as the Simoa, which targets N-terminally acetylated amino acid 2-47 of SNAP-25, results in the best differential diagnostic performance for SNAP-25 when comparing AD and controls [114].

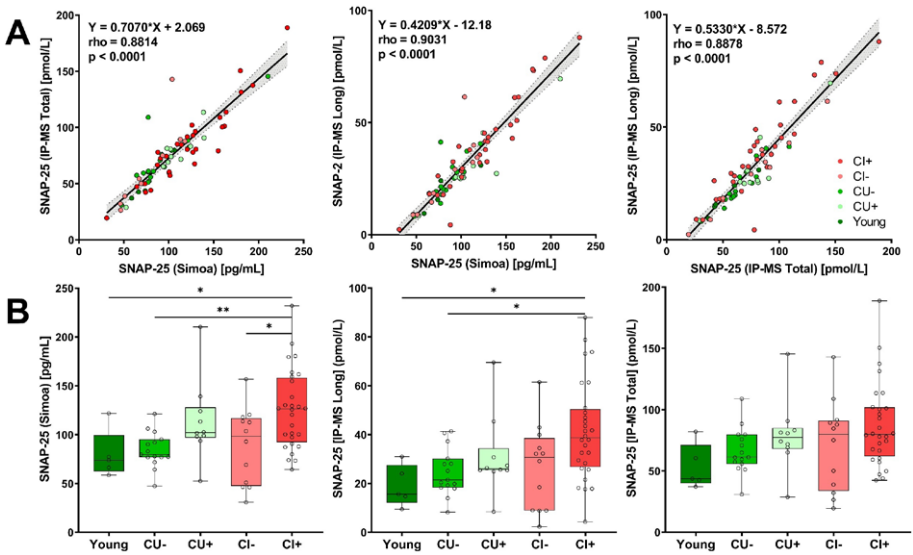

Figure 25. *SNAP-25 levels in the TRIAD cohort consisting of young (n=5), cognitively unimpaired Aβ negative (CU-, n=15), cognitively unimpaired Aβ positive (CU+, n=10), cognitively impaired Aβ negative (CI-, n=12), and cognitively impaired Aβ positive (CI+, n=28) (A) Correlations between SNAP-25 [Simoa] and the two SNAP-25 forms, SNAP-25 [Long] and SNAP-25 [Total], quantified with MS. (B) Group comparisons for the three SNAP-25 measurements. P-values: * $p \leq 0.05$ and ** $p \leq 0.01$.*

Lastly, in the study of **Paper VI** (Fig. 26), higher levels of syntaxin-7, complexin-2, AP2B1, and neurogranin were found in AD compared with controls. Similarly, SNAP-25 and SYT-1, which were also quantified in this study, also showed higher protein levels in AD compared with controls. Additionally, decreased levels were observed in the atypical parkinsonian disorders, both in the combined group of MSA and PSP for neurogranin, complexin-2, AP2B1, and SNAP-25, as well as in the DLB group for SYT-1, complexin-2, and AP2B1.

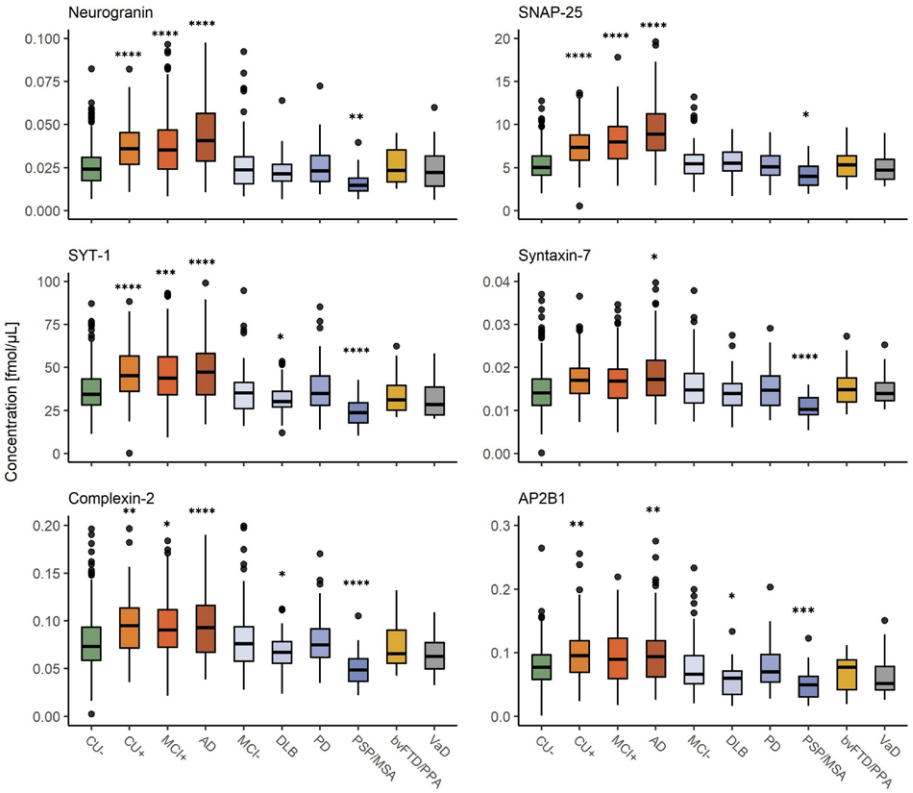

Figure 26. *Protein levels of neurogranin, SNAP-25, SYT-1, syntaxin-7, complexin-2, and AP2B1 in Biofinder-2 consisting of; cognitively unimpaired (CU) controls and patients with mild cognitive impairment (MCI) divided by Aβ status, as well as a spectrum of other neurodegenerative diseases, including AD. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001, with significance shown compared with controls (CU-) only.*

Our studies thus indicate that the SNARE proteins (SNAP-25, syntaxins, SYT-1, and complexin-1), neurogranin, and AP2B1, have AD-specific higher levels as well as possible lower levels in other neurodegenerative diseases. Of these synaptic proteins, only SNAP-25 and neurogranin are particularly well explored, but also, for them, some of the reported findings are novel. However, all changes were not confirmed across studies or corroborated with the literature, and some results should thus be interpreted

with caution. Notably, the higher levels in symptomatic *MAPT* carriers for these proteins misalign with the other observed non-AD changes.

Neurogranin and SNAP-25 are the most studied synaptic proteins with known higher levels in the CSF of AD patients [114, 130, 196, 197, 207, 208]. AD-specific higher levels were found in all studies included in this thesis for neurogranin compared with other diseases, which is corroborated by others [199, 200]. In the last study, higher levels were found for both neurogranin and SNAP-25 in AD compared with controls, increased already at preclinical stages and across the continuum (**Paper VI,** Fig. 26), a fact also recently corroborated [209]. Of all synaptic proteins studied, they turned out to be two of the best synaptic proteins in identifying AD at late stages (Neurogranin; AUC=0.80, SNAP-25; AUC=0.87) and at preclinical stages (Neurogranin; AUC=0.80, SNAP-25; AUC=0.87) compared with controls. They also showed a good performance in the separation of AD from other diseases (Neurogranin; AUC=0.84, SNAP-25; AUC=0.88). At the early stages, neurogranin and SNAP-25 performed as well as 14-3-3 ζ/δ. However, at later stages, SNAP-25 generally performed better than the other synaptic proteins, including neurogranin, only outperformed by 14-3-3 ζ/δ at separating AD from controls. Furthermore, it was also found that higher levels of both proteins at the prodromal stage was also indicative of a more rapid cognitive decline (Neurogranin; β-estimate=-0.28, p-value=0.049, SNAP-25; β-estimate=-0.45, p-value=0.0024) as well as higher risk of progression to AD dementia (Neurogranin; OR=1.72, p-value≤0.0001, SNAP-25; OR=2.65, p-value≤0.0001). In fact, in this study, SNAP-25 was the synaptic protein best able to predict both cognitive decline and progression. Interestingly, since SNAP-25 and neurogranin display so similar performance even with their different localizations, this possibly indicates that whatever AD-specific mechanisms are causing their changes, it seems to affect both the pre- and post-synapse equally. Or at least no evidence of the contrary was found.

SNAP-25 and neurogranin are potential differential AD-specific biomarkers able to predict conversion and monitor cognitive decline.

To summarize, SNAP-25 and neurogranin are thus seemingly early AD-specific biomarkers able to both differentiate from other neurodegenerative diseases and support the diagnosis of AD already at the preclinical stage. The two proteins also seem to have the potential to be able to predict conversion as well as cognitive decline and possibly monitor such cognitive changes.

For the syntaxins, complexin-2, SYT-1, and AP2B1, only in the last large AD study were changed levels found (**Paper VI,** Fig. 26), but also there were they all outperformed by other proteins in the separation of AD. On the other hand, the changes observed for these proteins as well as neurogranin and SNAP-25 in the non-AD neurodegenerative diseases were mostly novel and certainly interesting for further study. However, these changes were, in most cases, not particularly promising. Furthermore, due to the novelty, most of the changes cannot be corroborated, and even opposite findings have been found for some of the proteins.

In fact, for the few studies published on the syntaxins, reports of decreased levels of syntaxin-1B in preclinical AD [218] and unchanged levels in AD dementia and FTLD pathology [218-220] are found. Similarly, SYT-1 and SNAP-25 have previously been found to be specifically increased in relation to AD pathology [151, 230] with no changes in other neurodegenerative diseases. Notably, however, Tible *et al.* found lower levels of SYT-1 in a combined group of "other dementias" composed of DLB, FLTD, and VaD, which corroborate the lower SYT-1 levels observed in **Paper VI** for DLB and the combined group of PSP and MSA. The observed changes presented herein for the syntaxins, complexin-2, SNAP-25, and SYT-1 are, however, interesting since both the syntaxins and complexin-2 are almost not at all explored as biomarkers and for SYT-1 and SNAP-25 less in other neurodegenerative diseases outside of AD. The studies included in this thesis thus contribute to the study of these synaptic proteins in the CSF in relation to neurodegenerative diseases. For example, lower levels of complexin-2 were found in two of the studies (**Paper III & VI,** Fig. 24B & 26) in MSA and PSP, something never before reported.

Contrarily, previous studies for AP2B1 have mostly found differences in PD and DLB [141, 193], to which genetic and functional links also exist [191, 192], and reported unchanged levels in AD [193]. These observed changes also corroborate the findings of lower levels of AP2B1 in DLB in **Paper VI (**Fig. 26) and LBD pathology in **Paper II** (Fig. 24A). However, in the two clinical cohorts of **Paper III** and **VI,** lower levels could not be confirmed in PD (Fig. 24B & 26). Novel findings for AP2B1, on the other hand, were the finding of lower levels in the other atypical parkinsonian diseases of PSP and MSA (**Paper III & VI**). Thus, endocytic impairment, an implicated feature of many neurodegenerative diseases, not the least in AD [278], might be reflected by changed AP2B1 CSF levels across neurodegenerative diseases. The results of **Paper II** suggested that endocytic impairment may be a more prominent feature of FTLD and LBD pathology than of AD pathology. However, later finding changed levels in AD but in the opposite direction might indicate that endocytic impairment is also present but potentially through different mechanisms. AP2B1 seems thus to be a potential biomarker in several neurodegenerative diseases, but less so in AD. In AD, other biomarkers also outperform AP2B1 performance and future studies should thus focus

on AP2B1 as a biomarker of non-AD diseases including; MSA, PSP, and DLB, and generally across neurodegenerative diseases to mechanistically discern different pathological mechanisms between AD and other neurodegenerative diseases.

Additionally, while studies of neurogranin have mostly focused on its use as a biomarker for AD, several studies on other neurodegenerative diseases exist. Lower levels have been reported in both FTD and VaD [279] as well as parkinsonian disorders [280], corroborating the findings presented herein. Neurogranin is thus a potential synaptic biomarker of both AD and possibly other neurodegenerative diseases. Furthermore, even though lower levels were found of both neurogranin, the syntaxins, and AP2B1 in FTLD pathology (**Paper II,** Fig. 24A), lower levels were only found in the genetic cases of FTD for AP2B1 and again only for symptomatic *GRN* mutation carriers (**Paper IV**, Fig. 24C). In Biofinder-2, no changes were confirmed for these proteins in the clinical group of FTD. This can potentially be explained by the low number of patients included in both the genetic FTD and Biofinder-2 study or by the complexity found in the underlying pathology in FTD. This is potentially corroborated by the genetic study of FTD, where the observed higher levels in symptomatic *MAPT* cases misalign with the noted pattern of lower levels for these synaptic proteins in non-AD diseases, indicating specific synaptic dysfunction in relation to the underlying proteinopathy. However, in the genetic FTD study, the groups were very small, and there was also a large age difference between presymptomatic and symptomatic carriers, which might have occluded potential differences.

Notably, the study of synaptic proteins at large so far has been focused on AD, and much work is left on other neurodegenerative diseases. Specifically, the studies included in this thesis show that there might be changes in other neurodegenerative diseases which might be overlooked when focusing on AD and grouping it into “other dementias”. To summarize, even though the results do not show great promise of these synaptic proteins as potential biomarkers of disease outside of AD, they should be studied further to confirm possible changes in the studies included in this thesis and in order to gain possible mechanistic insight into the differential involvement of SNARE proteins and synaptic transport processes into pathological mechanisms present in different neurodegenerative diseases.

6 CONCLUSION & FUTURE PERSPECTIVE

Over the last 20 years, several potential biomarkers of synaptic pathology have been suggested and studied, both to find biomarkers of the underlying pathology and to try to grasp pathological mechanisms. However, for a long time, the progress in the field was slow, and it was not until 2022 that neurogranin, as the first synaptic biomarker, was implemented in the clinic (Sweden). Nonetheless, to this day, no synaptic biomarker is used widely, and much research is still ongoing to solve several key questions. Early contradicting results between studies and biomarkers, including high overlap between groups, lead to skepticism of the clinical utility and use of synaptic biomarkers. Recent progress in the development of techniques with greater specificity and sensitivity has allowed for better measurements as well as the quantification of low abundant proteins, increasing the number of possible markers. With the increased number of potential biomarkers, a key question remains if they all reflect the same changes in AD but also in different diseases and, of course, which one is the one with the best performance.

In this thesis, by using an unbiased selection approach of explorative proteomics to find new potential synaptic biomarkers to set up methods for, in addition to the usage of already developed methods for the exploration of well-known suggested biomarkers (*e.g.*, SNAP-25 and SYT-1), both new and known synaptic biomarkers were able to be compared. This approach allowed for the study of synaptic proteins with different functions and localizations, exploring their potential and to the find of general and specific pathological patterns across neurodegenerative diseases. It is relevant to highlight the positive aspect of using mass spectrometric panels in this area of study, which allows for reduced variability, time consumption, and costs when the aim is to compare multiple targets. This methodological approach is relatively novel in the study of synaptic biomarkers. Additionally, for several of these biomarkers, some of the studies included in this thesis were also the first to explore them in the CSF.

One of the main findings of this thesis is that out of potential synaptic biomarkers, almost all of them showed specifically higher concentrations in the AD continuum, indicating that higher levels of synaptic proteins are possibly a specific feature of AD and thus a biomarker result of specific synaptic dysfunction and degeneration mechanisms in AD compared with other neurodegenerative diseases. It was found that potential biomarkers of this phenomenon are SNAP-25, neurogranin, and β-synuclein. The possible exemption to this seems to be 14-3-3 ζ/δ which higher levels might be a general biomarker of synaptic degeneration mechanisms across neurodegenerative diseases. Mechanisms that still are particularly affected in AD pathology. Together SNAP-25, neurogranin, β-synuclein, and 14-3-3 ζ/δ show promise to be potential AD-

specific diagnostic biomarkers, able to differentiate AD from other neurodegenerative diseases. Importantly they are early biomarkers since they change already at the preclinical AD stage, a phase that might last up to 20 years. However, due to the presence of the already established AD core biomarkers, which have high specificity and sensitivity in AD diagnostics, the addition of synaptic biomarkers for this purpose is most likely of low clinical utility. Instead, it is most important to highlight the synaptic proteins' potential use as stage and monitoring biomarkers of progression and cognitive decline already at the early stages of AD, something which is in great need in the field both in the clinic and especially as endpoints in clinical trials when developing therapeutics.

Another exemption and main finding of this thesis is the presence of lower levels of the neuronal pentraxins across neurodegenerative diseases, indicative of synaptic dysfunction and degeneration mechanisms equally affected across diseases. It was shown that the neuronal pentraxins are promising possible prognostic and monitoring biomarkers of cognitive and motor symptoms across neurodegenerative diseases. As much of the field focuses on AD, fewer studies are performed on other conditions even though the need for biomarkers is equally dire for the same reasons, *i.e.*, to predict progression and for use as endpoints when developing therapeutics. Furthermore, several neurodegenerative diseases are not explored in the current studies, such as CJD, ALS, and MS. The continued study of these diseases is of value to give further insight into synaptic pathology patterns and confirm some of the presented results, such as AD-specificity. It is also important to continue exploring biomarkers such as neurogranin and SNAP-25 in non-AD to confirm the indications of lower levels observed for those biomarkers and investigate if the lower levels are specifically affected or signs of a general non-AD pattern. Furthermore, psychiatric diseases, which, similarly to neurodegenerative diseases, are affected by synaptic dysfunction, have also been pointed out to be diseases where synaptic biomarkers can be of use. In addition to the vital question of exploring synaptic pathology mechanisms in those diseases, several psychiatric disease symptoms are also frequent in neurodegenerative diseases such as depression, and biomarkers allowing for differential diagnosis are key [281].

Altogether, even though some of the presented biomarkers in this thesis show great promise and considerable progress in the study of synaptic biomarkers has been made in the last couple of years, there is still a long way to go from implementing synaptic biomarkers in the clinic worldwide. Currently, in the field of biomarkers, much work is focusing on the long-sought goal of a blood test for AD, mainly focusing on different phospho-forms of tau, including p-tau$_{181}$ and p-tau$_{231}$. In primary care, such a test would be revolutionary for AD diagnostics, reducing costs and removing the need for specialized centers. Primarily to facilitate worldwide implementation since current

CSF biomarkers are hampered by their invasiveness, especially in countries outside of Europe where spinal taps are less frequent. Similarly, if and when the time of implementation for synaptic biomarkers comes, blood would be a favorable matrix above CSF. In the thesis, the critical question of whether synaptic proteins can be quantified in blood was attempted to be addressed. However, even though SNAP-25 could not be quantified in blood in the studies, a central finding of the field in recent years is that β-synuclein is and is thus the first potential synaptic blood biomarker. Nonetheless, in the thesis, the performance of a new Simoa assay of SNAP-25 quantification was verified. The increase of available assays on different analytical platforms such as Simoa is favorable and promising for future implementation in primary care since they are well suited for routine clinical work and contribute to higher accessibility of biomarker quantification.

An important next step for studies of synaptic biomarkers would be longitudinal CSF studies, which would make it possible to establish trajectories and track how the biomarkers change over time in a patient. These studies would give important insight into how the biomarker patterns arise and provide temporal information about when they change and in which order. The information would be another vital piece of the puzzle on the complex mechanics of synaptic dysfunction in neurodegenerative diseases. Additionally, some of the synaptic biomarker work included in this thesis is ongoing. The next steps include the continued exploration of the results from **Paper II**, where further characterization of the underlying pathology is in progress. The characterization primarily includes quantification of AD comorbidity present in FLTD and LBD, as well as the precise identification of FTLD subtypes, which will allow us to explore how the biomarkers are affected by either factor. Personally, I would also like to see more mechanistic studies in the field on how synaptic proteins get released into the CSF and why some proteins show higher or lower protein levels as a consequence of synaptic dysfunction. The knowledge from those studies might be able to pinpoint key interactions of the concordant and discordant pathological pathways that seem to be present in neurodegenerative diseases. Those studies might give vital insight into possible therapeutic targets for slowing or hindering cognitive decline as a treatment for said diseases.

In summary, the synapse is a complex and dynamic structure with central involvement in neurodegenerative disease through a number of pathways. Thus, it should not come as a surprise that the synaptic dysfunction and degeneration mechanisms in said diseases are highly complex and, as a result, reflected by the synaptic proteins in the CSF. A better understanding of the mechanistic pathways of synaptic dysfunction across and between diseases may thus contribute to improving diagnostics and potentially also to the development of new therapeutic strategies targeting said pathways. Additionally, the results indicate that several of these potential synaptic

biomarkers show promise as possible complements to other CSF and imaging markers as diagnostic, prognostic, stage, or monitoring biomarkers of cognitive decline and synaptic pathology. Lastly, current fast developments generally in the field of biomarkers show great promise for the continued study of synaptic proteins in neurodegenerative diseases.

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