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UNRAVELING PATHOGENIC PROTEINS AND PATHWAYS IN ALZHEIMER DISEASE: A FOCUS ON PROTEOMICS

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Unraveling pathogenic proteins and pathways in Alzheimer disease: A focus on proteomics

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By

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To my family.

ABSTRACT

Alzheimer disease (AD) is a multifactorial and complex neurodegenerative disorder. To date, different mechanisms, such as impairment of synaptic, mitochondrial and autophagic function, neuroinflammation and many more, are found to disrupt cellular homeostasis in AD brains. Despite the increased knowledge, it is still difficult to pinpoint which of these mechanisms is the main culprit driving the pathologic cascade, especially in the form of late-onset, sporadic AD, accounting for more than 95% of all patients.

In this thesis, we used human-based or translational approaches to investigate which pathological alterations indeed occur in AD brains. In Paper I, we investigated an amyloid precursor protein (APP)-derived band with a molecular weight of 20 kDa, most likely corresponding to the APP C-terminal fragment (CTF) called CTF-η, and showed that it is expressed at low levels in the human brain. However, we also noted that several antibodies directed to APP or other proteins also detects a presumably non-specific band of a similar size.

In Paper II, IV and V, we explored changes in the proteome of postmortem AD brains and CSF of AD patients and *App* knock-in mice. In Paper II, our aim was to identify proteins and pathways that could underlie synaptic dysfunction, a pathogenic event that happens early in disease progression. We thus explored the proteome of the outer molecular layer (OML) of the dentate gyrus using mass spectrometry (MS). This region is relatively cell-free and highly enriched in synaptic connections, and more importantly receives the main input of the hippocampus called the perforant path, which is highly affected in AD pathogenesis. Our comprehensive data analysis indicates that the OML indeed exhibits presynaptic changes, which is in line with previously published reports, whereas postsynaptic density proteins were not altered. To follow-up on the hypothesis of presynaptic impairment in AD OML, using immunofluorescence, we measured the staining densities of five presynaptic proteins in sub-regions of the hippocampus in Paper III. Similarly, we found decreased staining densities of complexin-1, syntaxin-1a, synaptotagmin-1 and synaptogyrin-1 in AD OML. However, the analysis of other hippocampal sub-regions showed no significant alterations in these presynaptic proteins, except syntaxin-1a, which showed increased staining densities in AD. Although other molecular layers of hippocampus also receive the perforant path input (as well as other important inputs), it was intriguing to find that presynaptic impairment was restricted to the OML. Together, Paper II and III point out to presynaptic failure in AD hippocampus.

To further compare our proteomic findings with the published ones, in which proteome of AD-affected brain regions (e.g. temporal and frontal cortices) was analyzed, and to identify commonalities and discrepancies between the studies, in Paper IV, we performed a meta-analysis of labeled (11753 proteins and 168 cases) and label-free (4292 proteins and 632 cases) data. We found approximately 500 significantly altered proteins that were associated with pathways such as synaptic signaling, neuron and axon development, neurogenesis, cellular respiration and catabolic process, some of which are previously reported to be involved in AD pathogenesis. Additionally, seven novel proteins were found to be consistently altered in AD.

In Paper V, we studied the CSF proteome of *App* knock-in mice and identified alterations in several blood-brain barrier and extracellular matrix proteins, for example decorin. Furthermore, in order to explore translational changes between mouse and human CSF, we compared our findings from Paper V with the CSF proteome of human patients, reflecting different stages in AD continuum (i.e normal cognition, mild cognitive impairment and AD dementia), from a recently published study. Interestingly, decorin was significantly upregulated both in the $App^{NL-F/NL-F}$ mice and in the subjects with normal cognition and Aβ-positive and tau-negative CSF levels. Additionally, this study revealed alterations in proteins that were shared in all groups and extensively associated with pathways such as cell adhesion, neurogenesis, cholesterol and lipid metabolism and acute inflammatory response.

In summary, this thesis has contributed with new knowledge on potential presynaptic failure in AD hippocampus and expanded our understanding of altered pathways that could be involved in AD pathogenesis. Future studies on this work may facilitate the development of new CSF biomarkers and therapeutic strategies for AD.

TURKISH ABSTRACT

Alzheimer hastalığı (AH) demansa sebep olan nörodejeneratif hastalıklardan biridir. Günümüzde, Alzheimer hastalarının beyinlerinde farklı mekanizmaların etkilendiği ve hücresel dengenin bozulduğu ortaya konmuştur. AH ilk olarak 100 yıl önce tanımlanmıştır. Ancak artan bilgi birikimine rağmen, özellikle hastaların yaklaşık olarak %95'den fazlasını kapsayan ve sıklıkla 65 yaş üzerinde görülen geç başlangıçlı AH'nda hangi hücresel mekanizmaların ana rol oynadığı ve patolojik süreci nasıl tetiklediği hala tam olarak aydınlatılamamıştır.

Bu doktora tezinde, AH'nda oluşan patolojik değişimleri araştırmak için AH olgularının ve demans hastalığı olmayan kontrol olgularının beyin dokuları ve ayrıca deneysel olarak AH oluşturulmuş farelerden elde ettiğimiz beyin-omurilik sıvıları kullanılmıştır. Bu tez çalışmasında farklı biyokimyasal metotların yanı sıra, proteomik yöntemi kullanılmıştır. Bu yöntem bir hastalığın oluşmasında rol oynayan binlerce proteinin, o hastalığın etkilediği biyolojik materyallerde (beyin dokusu, beyin-omurilik sıvısı vs.) aynı anda tespit edilmesini sağlamaktadır.

Makale I'de, amiloid prekürsör proteininin (APP) farklı fragmanlarını incelenmiştir. Bu proteinin bir fragmanı olan amiloid-β Alzheimer hastalarının beyinlerinde birikmektedir. Ayrıca yapılan yeni çalışmalar, beyinde bu birikimin hastalığın klinik bulgularının görülmesinden yaklaşık on ila yirmi yıl önce başladığını göstermektedir. Buna rağmen APP'nin AH gelişimindeki rolü hala tam olarak bilinmemektedir. APP'nin bazı fragmanlarının AH gelişimi açısından daha büyük önem taşıdığı tartışılmaktadır. Bu sebeple Makale I'de APP'nin yeni keşfedilen bir fragmanının düzeyi araştırılmıştır. Bu fragmanın insan beyninde düşük düzeyde ifade edildiği ve ifade düzeyinin de AH'nda farklılık göstermediği saptanmıştır.

Makale II, IV ve V'te ise, hangi proteinlerin ve mekanizmaların AH'nda etkilendiğini araştırmak amacıyla proteomik analize yoğunlaşılmıştır. Yapılan araştırmalar bu mekanizmalardan birinin sinapslardaki bozulmalar olduğunu ve bunun AH'nın erken evrelerinde ortaya çıktığını göstermiştir. Sinapslar iki nöronun birbirine çok yakın bulunduğu alanlardır ve nöronlar arasındaki iletişimi sağlarlar. İletiyi getiren nörondan (pre-sinaps) nörotransmitter denilen kimyasal maddeler salınır ve böylece iletiyi alan nöron (post-sinaps) uyarılmış olur. Nöronlar arasında uyarı beyinde sıklıkla gerçekleşir ve sinapslar hafızanın oluşumunda çok önemli bir role sahiptir. Makale II ve III'te, hafıza oluşumundan sorumlu bir

beyin bölgesinde (hipokampüs) yaptığımız çalışmalar, özellikle pre-sinapstaki proteinlerin seviyelerinde değişiklikler olduğuna işaret etmektedir. Makale IV ve V'te yapılan proteomik incelemeler yine sinaps proteinlerinin seviyelerinin AH olgularının beyinlerinde değiştiğini göstermiş ve buna ek olarak farklı hücresel işlevlerde (örneğin nöron gelişimi, hücre enerji mekanizması, kan-beyin-bariyeri işlevi gibi) rol oynayan proteinlerin seviyelerinde de önemli değişimler tespit edilmiştir.

Sonuç olarak, bu tezin literatüre en büyük katkılarından biri, AH olgularındaki sinaps değişimlerinin başlıca hipokampüsteki pre-sinapslarda gerçekleştiğinin gösterilmesidir. İleriki çalışmalarda bu değişimlerin neden pre-sinapslarda yoğunlaştığının araştırılması sinaps bozulmalarının AH'nda nasıl gerçekleştiğini aydınlatabilecektir. Ayrıca bu tez hangi hücresel mekanizmaların AH'nda etkilendiğine dair var olan bilgiye katkı sağlamıştır. Özellikle spesifik hücresel mekanizmalar üzerine yapılacak olan ileriki çalışmalar farklı tedavi yöntemlerinin geliştirilmesinde ve hastalığın teşhisinde veya seyrinde kullanılabilecek biyobelirteçlerin geliştirilmesine yardımcı olabilecektir.

LIST OF SCIENTIFIC PAPERS

I. **Hazal Haytural**[*], Jolanta L. Lundgren[*], Tansu B. Köse, Tomàs Jordà-Siquier, Marinela Kalcheva, Mohammed Seed Ahmed, Bengt Winblad, Erik Sundström, Gaël Barthet, Lars O. Tjernberg and Susanne Frykman.

   Non-specific Detection of a Major Western Blotting Band in Human Brain Homogenates by a Multitude of Amyloid Precursor Protein Antibodies.
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II. **Hazal Haytural**, Georgios Mermelekas, Ceren Emre, Saket Milind Nigam, Steven L. Carroll, Bengt Winblad, Nenad Bogdanovic, Gael Barthet, Ann-Charlotte Granholm, Lukas M. Orre, Lars O. Tjernberg, and Susanne Frykman.

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III. **Hazal Haytural**[*], Tomás Jordá-Siquer[*], Bengt Winblad, Christophe Mulle, Lars O. Tjernberg, Ann-Charlotte Granholm, Susanne Frykman, Gaël Barthet.

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IV. **Hazal Haytural**, Rui Benfeitas, Sophia Schedin-Weiss, Erika Bereczki, Melinda Rezeli, Richard Unwin, Eric B. Dammer, Eric C.B. Johnson, Nicholas T. Seyfried, Bengt Winblad, Betty Timjs, Pieter J. Visser, Susanne Frykman, Lars O. Tjernberg.

   Insights into the changes in the proteome of Alzheimer disease elucidated by a meta-analysis.
   Manuscript.

V. Richeng Jiang, Una Smailovic, **Hazal Haytural**, Robert Mihai Haret, Ganna Shevchenko, Betty Tijms, Johan Gobom, Henrik Zetterberg, Bengt Winblad, Susanne Frykman, Vesna Jelic, Jonas Bergquist, Pieter Jelle Visser, Per Nilsson.

   Autophagy-activating extracellular matrix protein decorin is increased in CSF of App knock-in mice and early stage of Alzheimer.
   Manuscript

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer disease
ADAM10	A disintegrin and metalloproteinase 10
AICD	APP intracellular domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APH-1	Anterior pharynx defective-1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	β-site APP cleaving enzyme 1
BBB	Blood-brain barrier
BCSFB	Brain-cerebrospinal fluid barrier
CA	Cornu ammonis
CAA	Cerebral amyloid angiopathy
CSF	Cerebrospinal fluid
CPLX1	Complexin-1
CPLX2	Complexin-2
CTF	C-terminal fragment
DEqMS	Differential expression quantitative mass spectrometry data
EC	Entorhinal cortex
ECM	Extracellular matrix
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
GCL	Granule cell layer
GO	Gene ontology
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase-3
GWAS	Genome-wide association study
HiRIEF	High resolution iso-electric focusing
IML	Inner molecular layer
IR	Immunoreactivity

IPA	Ingenuity pathway analysis
iTRAQ	Isobaric tags for relative and absolute quantification
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LM	Stratum lacunosum-moleculare
LMD	Laser microdissection
LUC	Stratum lucidum
LTD	Long-term depression
LTP	Long-term potentiation
MAPT	Microtubule-associated protein tau
MBP	Myelin basic protein
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NC	Normal cognition
NFT	Neurofibrillary tangle
NMDAR	N-Methyl-D-aspartic acid receptors
NSF	N-ethylmaleimide sensitive factor
OML	Outer molecular layer
PCA	Principal component analysis
PEN-2	Presenilin enhancer 2
PET	Positron emission tomography
PMI	Postmortem interval
RRM-MS	Parallel reaction monitoring-mass spectrometry
p-tau	Phosphorylated-tau
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RAD	Stratum radiatum
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP	Soluble NSF-attachment proteins
SNAP25	Synaptosomal-associated protein 25
SNARE	Soluble NSF-attachment protein receptor

STX1A   Syntaxin-1A

SYNGR1   Synaptogyrin-1

SYT1   Synaptotagmin-1

TMT   Tandem mass tag

VAMP2   Vesicle-associated membrane protein 2

1 INTRODUCTION

1.1 DEMENTIA AND ALZHEIMER DISEASE

Dementia is a clinical syndrome that is mainly characterized by progressive memory loss and cognitive impairment. These symptoms are often accompanied by behavioural changes and depression. As the disease progresses, the ability to independently perform everyday activities diminishes, which ultimately reduces life quality. Dementia is caused by neurodegeneration and observed in a variety of neurodegenerative disorders such as Alzheimer disease (AD), Lewy body dementia and vascular dementia. The main risk factor of dementia is old age, and due to increased life expectancy worldwide the prevalence of dementia is rapidly increasing. A recent report from Alzheimer's Disease International has shown that approximately 50 million people worldwide are living with dementia, of which approximately 60% are living in low- and middle-income countries (Prince et al. 2015). Due to the impact of dementia on society, World Health Organization has considered dementia as a public health priority and implemented the global action plan aiming to 1) increase dementia awareness and policies, 2) reduce risk of dementia, 3) improve diagnosis, treatment and care, and 4) support research and dementia care givers (https://www.who.int/news-room/fact-sheets/detail/dementia).

AD is a progressive neurodegenerative disorder and the most prevalent cause of dementia. In the most common form, i.e. sporadic AD, individuals usually develop late-onset AD that is seen after the age of 65. On the other hand, in familial AD, mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) or presenilin 2 (*PSEN2*) genes cause early-onset AD and a more rapid disease progression (St George-Hyslop et al. 1987; Schellenberg et al. 1992; Levy-Lahad et al. 1995). To date, more than 350 mutations have been identified in these genes (https://www.alzforum.org/mutations). For example, these mutations alter APP processing by increasing the production of the amyloid β-peptide (Aβ), especially the 42 amino acid long fragment (Aβ_{42}), which has a greater propensity to aggregate and is more toxic compared to the Aβ_{40}. Both sporadic and familial AD patients are clinically characterized by cognitive deficits affecting episodic memory, followed by executive dysfunction such as impaired decision-making, planning, recognition, and verbal fluency. As the disease progresses, a variety of other symptoms can emerge such as seizures, mood swings, confusion, behavioural changes, which diminish the ability to independently perform everyday activities, increasing the burden of responsibilities on caregivers (Winblad et al. 2016).

1.2 PATHOPHYSIOLOGY OF ALZHEIMER DISEASE

Systematic neuropathological evaluation of postmortem AD brains has shown the presence of abnormal proteinaceous deposits, i.e. amyloid plaques and neurofibrillary tangles (NFT). Amyloid plaques are composed of insoluble fibrillar form of Aβ, mainly Aβ$_{42}$ **(Figure 1A)**, and found in the extracellular space. Amyloid plaque pathology begins in the frontal and temporal cortices (phase 1), followed by neocortex (phase 2-3), lower brainstem and cerebellum (phase 4-5) (Thal et al. 2002) **(Figure 1B)**. A subset of amyloid plaques that is well documented in AD brains is the neuritic plaques **(Figure 1A)**. They display characteristic morphological differences compared to the amyloid plaques, i.e. the dense Aβ core is usually surrounded by dystrophic neurites containing abnormal phosphorylated tau aggregates, synaptic proteins, ubiquitin, lysosomal proteins and swollen glial processes. Hence, neuritic plaques cause more local synapse loss and glial activation (Serrano-Pozo et al. 2011). Emerging evidence points out that the intracellular accumulation of Aβ occurs before the presence of extracellular amyloid plaques and could be an important process in disease pathogenesis (Gouras et al. 2010).

NFTs are intraneuronal aggregates and mainly composed of microtubule-associated protein tau that forms paired helical filaments (Duyckaerts, Delatour, and Potier 2009) **(Figure 1C)**. In contrast to the spread of amyloid pathology (from neocortical to subcortical regions), NFT pathology starts to develop in the transentorhinal cortex and in a few brainstem nuclei such as locus coeruleus (stage I-II) (Braak and Braak 1991; Braak and Del Tredici 2012) **(Figure 1D)**. From the transentorhinal cortex, NFTs progressively spread to the hippocampal formation and some parts of the neocortex (stage III-IV), and eventually to the entire neocortex (stage V-VI). Misfolded tau aggregates seem to spread between the regions of close connectivity, such as from the entorhinal cortex (EC) to the hippocampus, indicating that tau spread likely occurs through synapses (Lace et al. 2009; de Calignon et al. 2012; Kaufman et al. 2018). Interestingly, the burden of neocortical NFT, but not amyloid plaques, has been found to correlate well with cognitive decline in AD (Nelson et al. 2012).
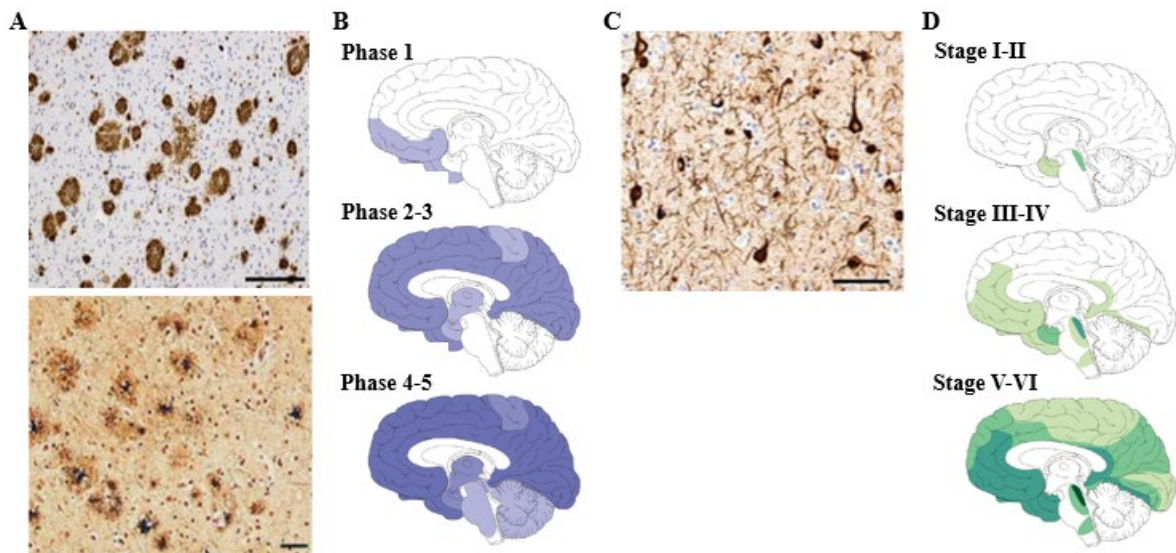
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Figure 1: The AD-related neuropathological changes and their pathological stereotypical pattern. (A) Amyloid (top) and neuritic (bottom) plaques. (B) Thal phases showing the progression of the amyloid plaque pathology. (C) NFT pathology and (D) its propagation indicated by the Braak stages. Microscopic images in A and C are adapted from (Montine et al. 2012). The schematic diagrams showing the progress of AD-related pathology in B and D are taken from (Goedert 2015).

The definitive diagnosis of AD is characterized by the postmortem neuropathological examination based on the ABC scoring system (Hyman et al. 2012): (i) amyloid plaque score, modified from Thal *et al.* (Thal et al. 2002), (ii) NFT score, modified from Braak *et al.* (Braak and Braak 1991), and (iii) CERAD neuritic plaque score (Mirra et al. 1991). Other neuropathological changes can also be observed in AD brains including synaptic and neuronal loss, atrophy, gliosis, white matter changes, cerebral amyloid angiopathy (CAA) and concomitant protein aggregates as Lewy bodies (Hyman et al. 2012).

It should also be noted that amyloid plaques and NFTs are seen in elderly individuals with no sign of dementia (Maarouf et al. 2011; Corrada, Berlau, and Kawas 2012; Perez-Nievas et al. 2013), but the extent of pathology is not as severe as in subjects with AD. Progressive loss of neurons and reduced cortical thickness are the main pathological changes that distinguish AD from normal aging (Perez-Nievas et al. 2013).

Compelling evidence from clinical and pathological findings suggests that AD pathophysiology starts many years or decades before the onset of clinical symptoms **(Figure 2)**. Based on the evaluation of current biomarkers, which will be discussed in section 1.6 (Biomarkers and treatment strategies), the pathophysiological sequence of AD is divided into three stages: preclinical or subjective cognitive impairment, mild cognitive impairment (MCI) and AD dementia (Sperling et al. 2011) **(Figure 2)**. In the preclinical stage, individuals are cognitively healthy but pathological abnormalities can be detected either in the cerebrospinal

fluid (CSF) or in the brain (Sperling et al. 2011). In MCI, the early signs of cognitive impairment, particularly problems in episodic memory, become more evident (Albert et al. 2011). In the AD stage, individuals show substantial cognitive and functional decline (McKhann et al. 2011). The degree of cognitive and functional decline varies from patient to patient and not every individual will progress to AD. Therefore, understanding the disease continuum is crucial, as it could provide a window of opportunity for potential disease-modifying therapy especially at the preclinical stage of AD while individuals are still cognitively healthy.
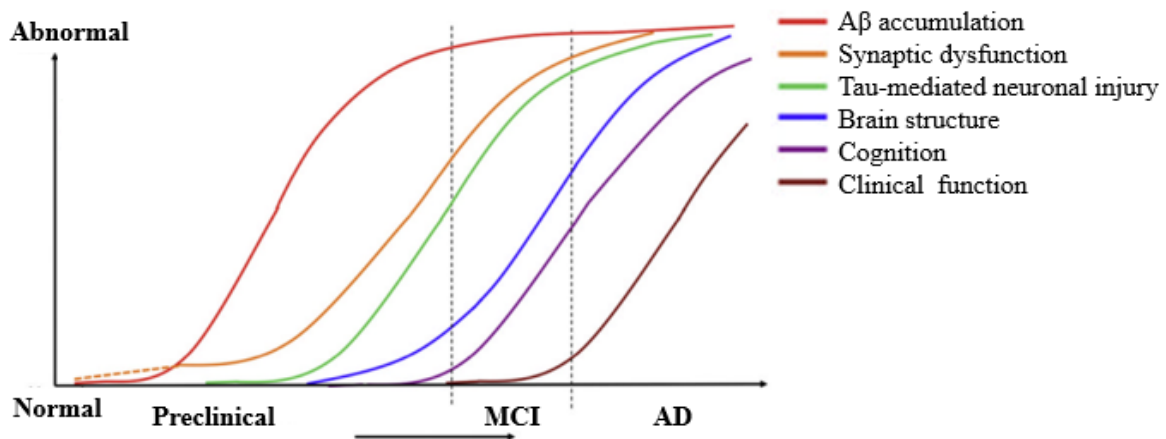

Figure 2: This hypothetical model distinguishes the pathophysiological stages of AD based on currently available diagnostic and prognostic biomarkers. This figure is taken from (Sperling, Aisen et al. 2011).

1.2.1 APP processing and Aβ

APP is a 695-770 amino acid long, type-I transmembrane protein that is expressed in a variety of tissues including brain. APP is thought to be important for neuronal function, as evidence suggests that APP is implicated in numerous cellular processes including cell adhesion, neurite outgrowth, neurogenesis, axonal transport (Nicolas and Hassan 2014). However, the exact physiological role of APP and its cleavage products still remains to be established.

The processing of APP is divided into amyloidogenic and non-amyloidogenic pathways and mediated by α-secretase, β-secretase and the γ-secretase complex, consisting of PSEN1 or PSEN2, nicastrin, anterior pharynx defective-1 (APH-1) and presenilin enhancer 2 (PEN-2) (Haass et al. 2012) **(Figure 3)**.
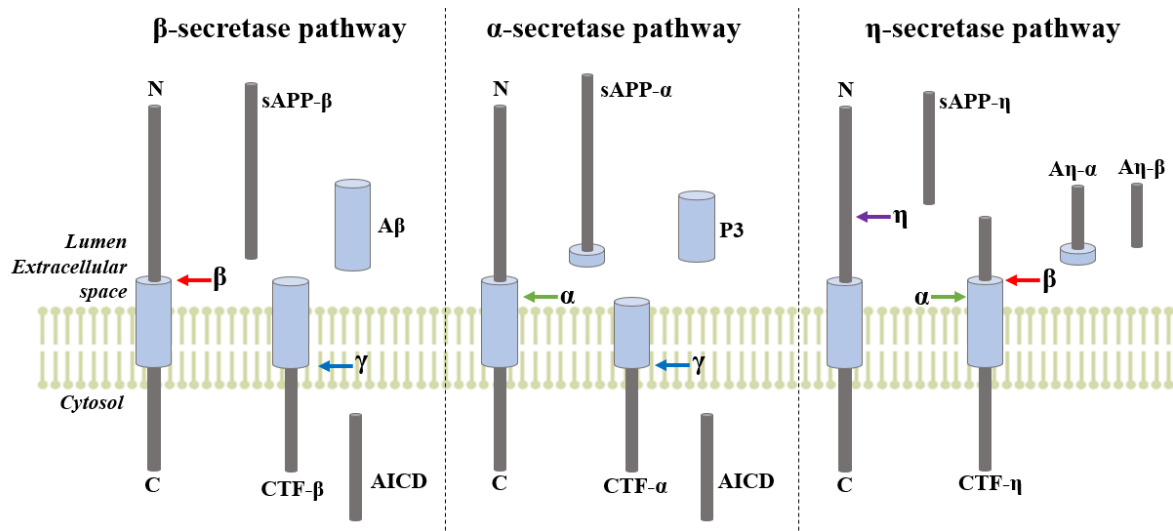
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Figure 3: *APP processing summarized by the two main pathways: amyloidogenic (β-secretase) and non-amyloidogenic (α-secretase) pathways. The sequential cleavage of APP by β- and γ-secretases gives arise to Aβ, which is released to the extracellular or intraluminal space. Alternatively, full-length APP is first cleaved by α-secretase within the sequence of Aβ, and thereby precluding the production of Aβ. In the recently identified η-secretase pathway, full-length APP is first cleaved by η-secretase, producing the soluble APP-η and the CTF-η fragments. The CTF-η is then cleaved by β- or α-secretase, giving arise to soluble Aη-β or Aη-α fragments, respectively.*

In the amyloidogenic pathway **(Figure 3, left)**, APP is sequentially cleaved by β-secretases, executed mainly by the β-site APP cleaving enzyme 1 (BACE1), and the γ-secretase complex. BACE1 cleavage generates a soluble APP-β fragment that is released into the extracellular or intraluminal space and a membrane-bound C-terminal fragment (CTF) of APP called CTF-β or C99 (Vassar et al. 1999). CTF-β is further cleaved by γ-secretase, generating Aβ and the APP intracellular domain (AICD). While Aβ is released into the extracellular space or into the lumen of vesicles such as endosomes, AICD is released into the cytosol. Depending on where exactly the γ-secretase cleavage takes place, both Aβ and AICD can vary in length. The most abundant form of Aβ is 40 amino acid long, which is followed by the longer form Aβ42. Interestingly, Aβ42 is more prone to polymerize into soluble oligomers, then to insoluble fibrils that eventually deposit into amyloid plaques. Compelling evidence points out that Aβ oligomers are the main toxic species and amyloid plaques might serve as deposits of the toxic oligomers (Mucke and Selkoe 2012). Aβ toxicity has been well documented especially at the synapses, which will be discussed in section 1.4.1 (Aβ and Tau at the synapses).

In the non-amyloidogenic pathway **(Figure 3, middle)**, APP is first cleaved within the Aβ region by α-secretases, e.g. a disintegrin and metalloproteinase 10 (ADAM10) (Allinson et al. 2003). This cleavage generates a soluble APP-α fragment that is secreted into the extracellular space and a membrane-bound truncated CTF-α (C83), lacking the amino-

terminus of the Aβ region and thereby precluding formation of Aβ (Postina et al. 2004). CTF-α is further cleaved by the γ-secretase complex, producing a short non-toxic peptide called p3 and AICD (Haass et al. 1993).

APP processing is complex, since APP has been also found to be cleaved by a number of other proteases, e.g. asparagine endopeptidase (δ-secretase) (Zhang et al. 2015), caspases (Galvan et al. 2002), and the recently identified η-secretase **(Figure 3, right)** (Wang et al. 2015; Willem et al. 2015). Moreover, in addition to the toxic role of Aβ, both CTF-β and Aη-α are shown to be neurotoxic (McPhie et al. 1997; Lauritzen et al. 2012; Willem et al. 2015). It is thus likely that other APP fragments might also be involved in AD pathophysiology.

1.2.1.1 Subcellular location of APP processing

Another important aspect of APP processing is the exact cellular location where the proteolytic cleavage of APP takes place. Evidence suggests that APP is synthesized in the endoplasmic reticulum, transported to the trans-Golgi network and then to the plasma membrane (Haass et al. 2012). At the plasma membrane, APP is cleaved by ADAM10 (Sisodia 1992) or alternatively the full-length APP is internalized and delivered to endosomes, where it can undergo BACE1-mediated processing, thereby liberating Aβ (Vassar et al. 1999). Alternatively, the internalized full-length APP can be further recycled and transported to the trans-Golgi network. BACE1 is also detected in other subcellular compartments including the trans-Golgi network (Choy, Cheng, and Schekman 2012), lysosomes (Buggia-Prevot et al. 2014), synapses (Del Prete et al. 2014; Lundgren et al. 2015; Lundgren et al. 2020). Similarly, the active form of the γ-secretase complex is reported at different subcellular sites; plasma membrane (Chyung, Raper, and Selkoe 2005), endosomal/lysosomal system (Pasternak et al. 2003), autophagosomes (Yu et al. 2005), synaptic compartments (Frykman et al. 2010; Schedin-Weiss et al. 2016), and mitochondria (Hansson et al. 2004). These studies suggest that Aβ production could take place in different subcellular compartments. Moreover, recent studies suggest that exosomes (Rajendran et al. 2006) and autophagosomes (Nilsson et al. 2013) are involved in Aβ secretion, indicating further how complex the APP processing is. However, it should be noted that many of these studies have been performed in cell lines and thus do not truly reflect the situation in neurons. Moreover, resolution obtained by traditional confocal microscopy, used for assessing co-localization, is not sufficient to truly resolve the organelles from each other.

1.2.1.2 Aβ clearance

Several mechanisms are identified to play a role in degradation of intracellular or extracellular Aβ. For example, Aβ is shown to be degraded by proteases such as neprilysin and insulin-degrading enzyme in the lysosomes, endosomes or endoplasmic reticulum/Golgi (Saido and Leissring 2012). Additionally, secreted Aβ can be taken up by microglia and astrocytes or released into the blood or the CSF via transporting Aβ across the blood-brain barrier (BBB) or the brain-CSF barrier (BCSFB) (Tarasoff-Conway et al. 2015).

As previously mentioned in section 1.1, mutations in the *APP*, *PSEN1* or *PSEN2* genes trigger more Aβ production or aggregation, thus pointing out a causal relationship between Aβ and AD pathophysiology in familial AD. However, the underlying cause of excessive Aβ accumulation remains to be elucidated in sporadic AD. APP is continuously metabolized in the central nervous system where Aβ is rapidly produced and cleared (Bateman et al. 2006). Therefore, increased Aβ production and reduced Aβ clearance over a long period of time likely contribute to the formation of Aβ deposits in the brain of sporadic AD patients (Mawuenyega et al. 2010).

1.2.2 Tau

Tau is a soluble, unfolded microtubule-associated protein that is mainly found in axons (Trinczek et al. 1995) and, at lower levels, in the dendrites (Ittner et al. 2010). In the human brain, there are six tau isoforms, encoded by microtubule-associated protein tau (*MAPT*) gene, which all contain microtubule-binding repeat domain (Goedert et al. 1989). While its physiological role is not entirely known, tau is thought to regulate microtubule stabilization and axonal transport (Trinczek et al. 1995). Under pathological conditions as in AD, tau becomes abnormally hyperphosphorylated, which causes tau to self-assemble in the somatodendritic compartment of neurons and later to aggregate into NFTs (Bancher et al. 1989). The hyperphosphorylation of tau further disrupts its interaction with microtubules, kinesin and dynein motor protein function and axonal transport, which is incompatible with neuronal function and ultimately results in neuronal death. Increased activities of kinases (e.g. glycogen synthase kinase-3 (GSK3), mitogen-activated protein kinase) and decreased activities of phosphatases (e.g. protein phosphatases PP2A and PP2B) are detected in AD brain and this imbalance causes hyperphosphorylation of tau (Iqbal et al. 2005). Additionally, different alterations including glycosylation, ubiquitylation and truncation are detected in tau (Avila et al. 2004). As with the presence of Aβ oligomers, small tau oligomers are also detected in AD brains and thought be toxic (Maeda et al. 2006; Patterson et al. 2011; Ward et

al. 2012). Tau is degraded intracellularly by different mechanisms, e.g. the autophagy-lysosomal pathway, the ubiquitin-proteasome pathway or caspases (Avila et al. 2004).

As Aβ and tau constitute the main pathological hallmarks of AD, much attention has been devoted to elucidating the mechanistic link between Aβ and tau. Based on the genetics of the familial AD, it is well established that Aβ triggers tau pathology, since mutations in *APP* gene, but not *MAPT* gene, cause familial AD of which patients develop both Aβ and tau pathologies. Although no such direct relationship has been discovered in the sporadic AD, a large body of literature suggests that Aβ and tau could have synergistic effects and therefore exert their toxic roles, or that tau could mediate Aβ toxicity - which will be discussed in section 1.4.1 (Aβ and Tau at the synapse).

1.2.3 Other pathogenic mechanisms

1.2.3.1 Mitochondrial dysfunction

Mitochondria are the bioenergetic center of the cells and thus essential for neuronal function that requires high amount of energy such as neurotransmission. In addition to ATP production, mitochondria are involved in numerous reactions such as calcium homeostasis, apoptosis and cell signaling. Mitochondrial dysfunction is well documented in AD (Ankarcrona, Mangialasche, and Winblad 2010). For example, studies have reported a decreased number of mitochondria (Hirai et al. 2001), reduced glucose metabolism (Mosconi 2005), diminished enzymatic activity of cytochrome c oxidase, which is a component of the electron transport chain (Kish et al. 1992), reduced activity of tricarboxylic acid cycle enzymes (Bubber et al. 2005), alterations in mitochondrial proteins related to oxidative phosphorylation system (Rhein et al. 2009), imbalanced fusion/fission events (Wang et al. 2009), and increased production of reactive oxygen species, triggering oxidative stress (Eckert, Schmitt, and Götz 2011). Additionally, Aβ accumulations are found in mitochondria in AD brain as well as in the brain of transgenic mouse models of AD (Fernández-Vizarra et al. 2004; Caspersen et al. 2005; Manczak et al. 2006). Moreover, in these studies, Aβ accumulation and alterations of the mitochondrial enzymes were observed before the formation of amyloid plaques, suggesting that mitochondrial dysfunction is an early pathogenic event in AD.

Interestingly, the components of the γ-secretase complex are found in mitochondria (Hansson et al. 2004). Studies in mouse brain show that PSEN1 and PSEN2 as well as APP are

enriched at mitochondria-endoplasmic reticulum contact sites (Area-Gomez et al. 2009) and Aβ is produced at these specific sites (Schreiner et al. 2015).

1.2.3.2 Impaired autophagy

Autophagy is responsible for intracellular degradation and recycling of cellular components and therefore maintains cellular homeostasis (Nixon 2013). This process is initiated from a double membrane structure called phagophore, which is then elongated around a selected substrate such as misfolded/aggregated proteins or damaged organelles. Subsequently, the closure of the phagophore edges results in the formation of the autophagosome and its fusion with a lysosome forms a single membrane autolysosome. In the autolysosome, the autophagic content is degraded by lysosomal proteases and resulting metabolites are then released into the cytoplasm for new synthesis or as sources for energy (Nixon 2013). Several proteins are involved in this process including mTORC1, LC3B, p62 (also known as sequestosome 1), autophagy-related proteins such as Atg5, Atg7, Atg12.

A large body of literature suggests that autophagy is impaired in AD. The ultrastructural analysis of postmortem AD brain found that autophagosomes accumulate within dystrophic neurites, suggesting that the formation of mature autolysosomes is impaired in AD (Nixon et al. 2005). Similarly, this pathologic phenomenon was also observed in the brains of transgenic AD mouse (Yu et al. 2005).

1.2.3.3 Neuroinflammation

Neuroinflammation is an immune response that is characterized by the activation of immune cells such as microglia and astrocytes in the central nervous system. These cells are essential for the maintenance of brain homeostasis, as they provide neurotrophic factors and metabolic support to neurons, and play important roles in the formation of synapses and synaptic plasticity (Arranz and De Strooper 2019). Under normal conditions, soluble Aβ oligomers can be taken up by microglia or astrocytes and then degraded by proteases such as neprilysin and insulin degrading enzyme. However, in pathological conditions such as AD, excessive Aβ accumulation causes persistent activation of glial cells which release inflammatory mediators such as pro-inflammatory cytokines and chemokines, causing neuroinflammation (Heneka et al. 2015).

Until the last decade, neuroinflammation was thought to be a bystander to AD-related pathogenic changes in the brain. However, mounting evidence points towards the active involvement of neuroinflammation in AD pathogenesis, which can also be supported by the

fact that several genes, related to the innate immune system, are identified as genetic risk factors for AD by genome-wide association studies (GWAS) - which will be discussed in the next section.

1.2.4 Risk factors of Alzheimer disease

Although old age is the main risk factor for AD, over the years many other risk factors that can influence the onset and the progression of AD have been found, indicating how complex the aetiology of AD is. These factors can be categorized into two groups: non-modifiable factors, such as age and genetic risk factors, or modifiable risk factors as lifestyle choices. To date, apolipoprotein E (*APOE*) ε4 allele has been identified as the strongest and the best established genetic risk factor for sporadic AD (Corder et al. 1993). It has been shown that *APOE* ε4 allele increases AD susceptibility (Frisoni et al. 1995) in a way that one ε4 allele results in three-fold increase in risk of developing AD, while two alleles cause a 12-fold increase in risk (Farrer et al. 1997). In recent years, GWAS have led to identification of several genes that can significantly modify the risk for developing AD, such as clusterin*,* complement receptor 1, triggering receptor expressed on myeloid cells 2*,* the endocytic genes called phosphatidylinositol binding clathrin assembly protein and bridging integrator 1*,* sortilin-related receptor 1*,* and the ATP-binding cassette transporter (Lambert et al. 2009; Naj et al. 2011; Lambert et al. 2013; Guerreiro et al. 2013). These genes are involved in lipid metabolism, immune system response and endocytosis, revealing insights into the multifactorial nature of AD pathophysiology. The fact that these genes are expressed by glial cells highlights the involvement of non-neuronal cells in AD pathogenesis. However, it should also be noted that these genetic variants identified from GWAS have a small effect on AD risk.

A number of modifiable risk factors that especially affect late-life in elderly, e.g. obesity, diabetes, hypertension, depression, physical inactivity, smoking and low educational attainment, have also been identified (Deckers et al. 2015; Ngandu et al. 2015).

1.3 THE HIPPOCAMPUS AND THE PERFORANT PATH

The hippocampal formation plays a crucial role in episodic memory and consists of the cornu ammonis (CA) regions of the hippocampus, the dentate gyrus and the subiculum (Ohm 2007). The main excitatory input of the hippocampus is provided by the crucial perforant path, originating at the superficial layers of the EC, i.e. layer II and layer III **(Figure 4)**. The neurons located in the EC layer II project to the outer two-thirds of the molecular layer (in

this thesis, hereafter referred as to outer molecular layer, OML) of the dentate gyrus and to the CA3 of the hippocampus, while layer III entorhinal neurons project to the CA1 of the hippocampus. The direct perforant path fibers, arriving to CA3 and CA1, terminate at the stratum lacunosum-moleculare (LM), which is the most superficial molecular layer of each region. In the trisynaptic circuit, the dentate granule cells are innervated at the OML by the perforant path and forward the information to CA3 through mossy fibers, whose synapses are located at the stratum lucidum (LUC). The CA3 pyramidal neurons then give rise to Schaffer collaterals which in turn innervates the dendrites of CA1 pyramidal neurons located at the stratum radiatum (RAD) and stratum oriens, which is located right over the CA1 pyramidal layer. Finally, CA1 pyramidal neurons send their projections to the deep layers of the EC e.g. layer V through subiculum.
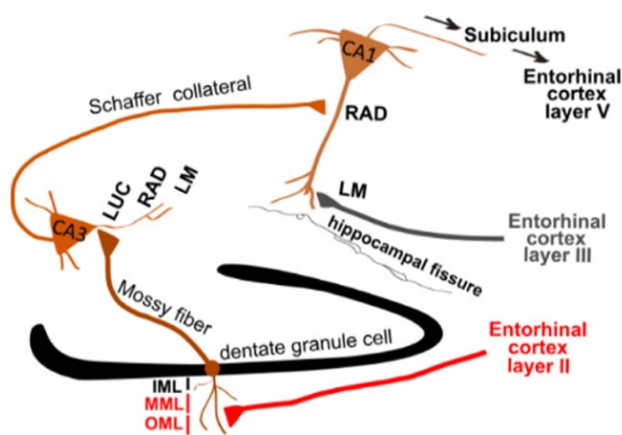

Figure 4: A schematic diagram showing the perforant path and the main hippocampal connections that are part of the trisynaptic circuit. The dentate gyrus has three molecular layers (inner, middle and outer) and extends from the dentate granule cell layer (GCL) to the hippocampal fissure, which divides the dentate gyrus and the CA1-3 regions. The molecular layers of CA3 contain LUC (right under the CA3 pyramidal layer), RAD and LM (the most superficial molecular layer). The molecular layers of CA3 (RAD and LM) further extend to the CA1 region. The perforant path, originating from EC layer II (red), terminates at the dendrites of the dentate granule cells that are located at the outer two-thirds of the molecular layer along the entire dentate gyrus.

It is important to note that the perforant path is not the only input of the hippocampus. While the dendrites of the dentate granule cells located at the OML are innervated by the perforant path fibers, the proximal dendrites of dentate granule cells, which are in the IML, receive input from the associational/commissural fibers, CA3 collaterals and other brain regions. Moreover, it is reported that the fibers from the medial septum, thalamus, locus coeruleus and amygdaloid complex also send their projections to the different sub-regions of the hippocampus, such as the molecular layers of CA3 and CA1 (Cappaert, Van Strien, and Witter 2015).

1.3.1 The perforant path and Alzheimer disease

The perforant path is crucial for memory consolidation and proposed to be vulnerable in AD pathophysiology due to:

- The presence of amyloid or neuritic plaques and NFTs both in the EC where the perforant path originates (Arnold et al. 1991; Braak and Braak 1992; Thal et al. 2000) and in the OML where the perforant path terminates (Hyman et al. 1986; Crain and Burger 1988; Thal et al. 2000);

- Drastic loss of EC neurons, particularly of layer II, reported in AD cases (Gómez-Isla et al. 1996; Kordower et al. 2001; Price et al. 2001);

- Substantial synaptic loss observed in the OML of AD and MCI cases (Scheff, Sparks, and Price 1996; Scheff et al. 2006).

Taken together, the widely acknowledged concept proposes that the loss of afferent fibers from the EC could degenerate the perforant path by triggering axonal degeneration as well as synaptic dysfunction in the dentate terminal zone of the perforant path, and thus contributes to cognitive impairment in AD. On the other hand, an alternative hypothesis, which has received insufficient attention within the field of AD research, suggests that synaptic dysfunction could precede neuronal death - a phenomenon called retrograde degeneration (Terry et al. 1991; Terry 2000), which will be discussed in section 4.3 (Paper III).

1.4 SYNAPTIC FUNCTION AND DYSFUNCTION

Synapses can be defined as the communication points between neurons and are composed of pre- and postsynaptic terminals **(Figure 5)**. At the presynaptic terminal (axon), upon depolarization synaptic vesicles that are filled with neurotransmitters fuse with the plasma membrane and release their content. At the postsynaptic terminal (dendrite), released neurotransmitters then bind to the receptors located at the postsynaptic density, which triggers signalling cascades. The neurotransmitters are then cleared from the synapse and postsynapse becomes ready for a new synaptic event. Synaptic exocytosis is tightly regulated by a highly organized machinery (Südhof 2013), involving the below-mentioned proteins that are crucial for synaptic vesicle-membrane fusion:

(i) Soluble NSF-attachment protein receptor (SNARE) proteins including vesicle-associated membrane protein-2 (VAMP2, also known as synaptobrevin-2), syntaxin-1a (STX1A) and synaptosomal-associated protein 25 (SNAP25),

(ii)   Munc18-1 or known as syntaxin-binding protein 1,

(iii)   Calcium sensor protein synaptotagmins (SYT1) and calcium ion regulator complexins (e.g. CPLX1),

(iv)   Active zone proteins such as Munc13, RIM proteins that interact with Rab3 and RIM-binding proteins.

After synaptic exocytosis, the SNARE complex is rapidly disassembled by N-ethylmaleimide sensitive factor (NSF) and soluble NSF-attachment proteins (SNAPs), and SNARE proteins become available for new membrane fusion events.
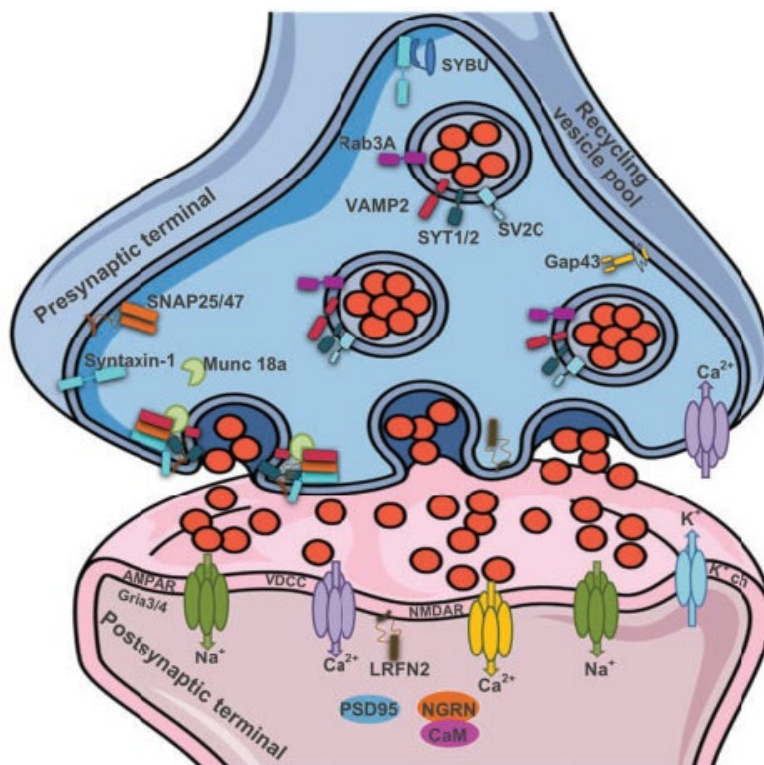

Figure 5: A schematic overview of synaptic exocytosis showing some of the important proteins of pre- and postsynaptic terminals. Upon exocytosis, neurotransmitters are released into the synaptic cleft and bound to the receptors on the postsynaptic terminal. This figure is taken from (Bereczki et al. 2018).

Synapses are plastic and synapse formation is dynamic and can be regulated. In fact cellular mechanisms of learning and formation of memories involve long-lasting changes in synaptic strength known as long-term potentiation (LTP) and long-term depression (LTD) (Cooke and Bliss 2006). To date, different cellular and molecular mechanisms are shown to induce LTP and LTD (Nabavi et al. 2014; Collingridge et al. 2010). Although these events are observed in different brain regions, they are mainly characterized at the glutamatergic synapses of the hippocampal formation, such as the synapses of CA1. Upon neurotransmitter release, glutamate binds to the postsynaptic glutamatergic receptors such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and the N-Methyl-D-aspartic acid receptors (NMDARs). For example, in CA1 synapses, high frequency stimuli directly

activate NMDARs, triggering increased activation of the AMPARs and thereby causing more depolarization of the postsynaptic cell. This can further lead to more AMPARs to be inserted in the postsynaptic terminal and consequently strengthening the synapses for further stimulation - a phenomenon known as LTP. Conversely, weaker depolarization of the postsynaptic cell causes LTD, in which AMPARs are internalized and synaptic strength is weakened.

1.4.1 Aβ and Tau at the synapses

APP has been detected both in the presynaptic active zone and in the postsynaptic density (Pliassova et al. 2016) and it could have a physiological role in enhancing the function of the glutamate receptor, NMDAR (Hoe et al. 2009). Additionally, both Aβ and secretases regulating APP processing are found at the synapses (Lundgren et al. 2020; Lundgren et al. 2015; Schedin-Weiss et al. 2016; Yu et al. 2018; Marcello et al. 2007). These studies indicate that APP processing may take place at the pre- or postsynaptic terminal, and therefore, it is very likely that Aβ, to a certain extent, may be produced at the synapse. Hence Aβ is thought to play a physiological role at the synapses. In this regards, several studies have shown that increased neuronal activity increases production and secretion of Aβ into the extracellular space (Cirrito et al. 2008; Cirrito et al. 2005; Kamenetz et al. 2003). However, more secreted Aβ has been reported to induce LTD by endocytosis of AMPARs, thereby causing decreased neuronal activity (Kamenetz et al. 2003; Hsieh et al. 2006). The synaptotoxic effects of Aβ, especially soluble oligomers, has been well reported. For example, Aβ oligomers have been found to accumulate at synapses (Tai et al. 2012; Pickett et al. 2016). Walsh and colleagues have shown that Aβ oligomers, but not monomers or amyloid fibrils, inhibit LTP (Walsh et al. 2002). Similarly, it has also been shown that solubilized amyloid plaques, which are known to be reservoirs of Aβ, inhibit LTP, enhance LTD and cause dendritic spine loss in rat hippocampus (Shankar et al. 2008). Additionally, Wei et al. has noted that overproduction of dendritic or axonal Aβ affects neighboring neurons and reduces spine density and plasticity (Wei et al. 2010). Taken together, it is plausible that over a long period of time, secreted Aβ, in response to synaptic activity, could lead to presence of oligomers and amyloid plaques in the extracellular space, while triggering synaptic dysfunction.

Tau also plays a role in synaptic function, since it regulates microtubule stabilization and axonal transport (Trinczek et al. 1995). By isolating synaptic terminals from postmortem human brain tissue, tau as well as hyperphosphorylated-tau were detected both at the pre- and the postsynaptic terminals (Tai et al. 2012). Additionally, under normal conditions, tau was

also detected in the dendritic spines, where it interacts with postsynaptic density protein the Fyn kinase, which phosphorylates one of the subunits of the NMDAR to facilitate NMDAR-PSD95 interaction (Ittner et al. 2010). Moreover, the reduced interaction between tau and the Fyn kinase caused to reduce Aβ toxicity, suggesting that tau mediates Aβ toxicity in the dendritic spines. Interestingly, a mechanistic link between neuronal activity and extracellular tau was reported in which increased neuronal activity leads to an increase in the levels of extracellular tau. These findings support the notion that spread of tau pathology occurs via trans-synaptic connections and is regulated by synaptic activity (Pooler et al. 2013; Yamada et al. 2014).

1.4.2 Synaptic changes in Alzheimer disease

A large body of literature, using electron microscopy or densitometrical analysis of synaptic protein-immunoreactivity (IR), has noted that synaptic dysfunction occurs in AD brain and correlates strongly with the cognitive deficits. Synaptic loss are detected in different regions of AD brains including the OML (Scheff, Sparks, and Price 1996; Scheff et al. 2006), the IML (Scheff and Price 1998), the CA1-RAD (Scheff et al. 2007), the inferior temporal gyrus (Scheff et al. 2011), the frontal cortex (DeKosky and Scheff 1990), and the cingulate gyrus (Scheff et al. 2015). In fact, in these studies, synaptic loss was reported to be the best correlate of cognitive decline in AD.

Decreased protein or mRNA expression of synaptic markers, e.g., SNAP25, synaptophysin, VAMP2, SYTs, Rab3a, PSD95 and GAP43 (known as neuromodulin), are also reported in AD brains (Masliah et al. 2001; Reddy et al. 2005; Counts et al. 2014; Scheff et al. 2015; Bereczki et al. 2016). Similarly, decreased levels of synaptic markers are found to be correlated well with cognitive decline. Recently, a meta-analysis was performed to analyse the overall changes in the levels of 57 synaptic proteins, which were originally measured in postmortem human brain by immunodetection methods (de Wilde et al. 2016). Using random-effects-modeling, the standard mean difference between AD and control groups was found to be much larger for presynaptic proteins, and thus the authors conclude that presynaptic proteins are affected more than postsynaptic proteins (de Wilde et al. 2016). However, it has been previously reported that not all presynaptic proteins are equally affected in AD (Honer 2003).

1.5 THE ANALYSIS OF THE PROTEOME IN ALZHEIMER DISEASE

Mass spectrometry (MS)-based proteomics is a powerful technique that allows a simultaneous identification and quantification of proteins in biological samples such as brain tissue. There is a growing interest in applying this technique in the field of AD for a better understanding of disease pathogenesis and to identify potential biomarkers reflecting different stages of the disease.

A bottom-up approach, in other words from peptide to protein, is commonly used for generating MS data. In this approach, proteins are first digested to small peptides, which are then analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and finally, the generated mass spectra of the peptides are used as a fingerprint to depict the relevant protein by comparing the generated spectra against the theoretical one from the databases. Earlier studies used two-dimensional gel electrophoresis in combination with LC-MS/MS to explore AD-related changes especially in plasma and blood samples (Hye et al. 2006; Liao et al. 2007). Recent advances in the proteomics field provides a high mass accuracy, thereby resulting in a more reliable quantification of the proteins, and increases proteome coverage by detection and quantification of even low abundant proteins. Two main approaches of MS analysis are commonly used; label-free (Zhu, Smith, and Huang 2010) or stable isotope labelling. In the label-free MS, each biological sample is individually analyzed by LC-MS/MS and the chromatographic peak of the precursor ion (MS1) is used for relative quantification. Labelled MS can be done by e.g. tandem mass tags (TMTs) or by isobaric tags for relative and absolute quantification (iTRAQ) (Thompson et al. 2003; Wiese et al. 2007). A key advantage of isobaric labeling is that it allows for multiplexing so that different biological samples are simultaneously analysed by LC-MS/MS, thus reducing the inter-run variability. A given peptide, independent of which isobaric tag it is labeled with, has the same mass due to the chemical structure of tags. Therefore, the same peptides (labeled with different tags) elute from the column at the same time and thus have the same retention time. Following fragmentation, the reporter ions are released and spectra from the second MS (MS2) are then used for relative quantification. Both approaches are extensively used for the proteomic analysis of CSF and brain tissue from AD cases and controls (Andreev et al. 2012; Donovan et al. 2012; Musunuri et al. 2014; Seyfried et al. 2017; Bereczki et al. 2018; Johnson et al. 2018; Mendonça et al. 2019; Xu et al. 2019; Wang, Dey, et al. 2020) as well as AD mouse models (Schedin-Weiss et al. 2020; Wang, Dey, et al. 2020; Sebastian Monasor et al. 2020).

Thanks to increased sensitivity of LC-MS systems and novel dissection techniques, it is possible to perform remarkably detailed studies on specific regions and even on selected neurons or structures. For example, as hippocampus plays an important role in memory and is heavily affected by AD-related changes, the proteome of specific hippocampal sub-regions has been carried out in AD compared to control: CA1 pyramidal neurons (Hashimoto et al. 2012), CA4 (Ho Kim et al. 2015), and CA1 and subiculum (Hondius et al. 2016). Protein content within amyloid or neuritic plaques have also been analyzed in AD brain tissue using LC-MS/MS. While the amyloid plaque core only contain Aβ (Söderberg et al. 2006), many other synaptic, cytoskeletal, chaperone proteins are also detected within the plaques (Liao et al. 2004; Nijholt, Stingl, and Luider 2015; Drummond et al. 2017).

Another MS-based approach used for biomarker validation or studying posttranslational modifications is called targeted proteomic analysis, applied by parallel reaction monitoring (PRM)-MS (Rauniyar 2015). The main difference between this and the above-mentioned MS approaches is that a peptide standard (e.g. synthetic peptide) of known concentration is injected to the LC-MS for absolute protein quantification. In the field of AD, PRM-MS is often used to quantify the levels of disease-relevant proteins in order to search for potential CSF biomarkers (Brinkmalm et al. 2018; Duits et al. 2018; Andersson et al. 2019; Sjödin et al. 2019; Sathe et al. 2019).

1.6 BIOMARKERS FOR DIAGNOSIS AND PROGRESSION

As mentioned earlier, dementia is observed in different neurological disorders. Converging evidence from clinical, pathological and genetic findings suggests that there is some overlap between different dementia disorders, which makes it difficult to accurately diagnose individuals at an early stage of the disease. Hence, the development of better diagnostic and prognostic biomarkers will enable screening for early detection and monitoring of disease progression. In this regard, the recent ATN criteria, which stands for Aβ deposition, abnormal tau and neurodegeneration (Jack et al. 2018), allows to clinically diagnose AD patients using *in vivo* biomarkers. Current Aβ and tau biomarkers include CSF measures of Aβ$_{42}$ and phosphorylated-tau (p-tau) (Blennow et al. 2010), as well as detection of amyloid and tau pathologies in the brains of AD patients using positron emission tomography (PET) (Nordberg et al. 2013; Palmqvist et al. 2014; Mattsson et al. 2015; Marquie et al. 2015; Mattsson et al. 2017). The levels of Aβ$_{42}$ are decreased in the CSF while the amyloid burden is increased in the brains of AD patients, reflecting the deposition of Aβ in the brain (Fagan et al. 2006). On the other hand, both the levels of p- and total-tau are increased in the CSF of

patients. Biomarkers of neurodegeneration include CSF measure of total-tau (Blennow et al. 2010), glucose hypometabolism detected by fluorodeoxyglucose PET imaging (Landau et al. 2011) and brain atrophy detected by structural magnetic resonance imaging (MRI) in the brains of AD patients (Frisoni et al. 2010). It should be noted that measures of neurodegeneration are most likely not specific for AD but rather non-specific indicators of neuronal damage that could result from different causes.

To diagnose AD patients more accurately, the development of new biomarkers is of utmost importance and has taken increasing attention in the field of AD research. Several studies have shown increased levels of synaptic proteins in AD CSF, for example the neuronal calcium sensor protein called visinin-like protein 1 (Lee et al. 2008; Tarawneh et al. 2011), the postsynaptic protein neurogranin, the presynaptic proteins SNAP25 and SYT1, and the pre-/postsynaptic protein GAP43 (Thorsell et al. 2010; Kvartsberg et al. 2015; Öhrfelt et al. 2016; Tible et al. 2020). Notably, Tible and colleagues have shown that the change in the CSF levels of GAP43, neurogranin, SNAP25 and SYT1 were able to distinguish AD and MCI due to AD groups from other MCI and non-AD dementia groups, whose CSF levels were very similar to the control group (Tible et al. 2020). Moreover, GAP43, which is associated with nerve growth, was the only synaptic protein that showed significant increase in AD compared to the MCI due to AD group. These studies suggest that the alterations in the CSF levels of synaptic proteins could be used for monitoring the rate of synaptic dysfunction, neuronal injury as well as disease progression. Additionally, ongoing research attempting to identify CSF biomarkers that could reflect neuroinflammation has shown increased levels of YKL-40 (also known as chitinase 3-like 1) protein in AD patients (Wang, Gao, et al. 2020; Nordengen et al. 2019) as well as in subjects with amnestic MCI (Alcolea et al. 2015).

It seems that a combined analysis of at least two biomarkers would allow to more accurately diagnose AD patients. Although the above-mentioned biomarkers, especially CSF Aβ and tau levels and PET imaging, are nowadays used in research for patient stratification, it will be challenging to utilize them in the memory clinics, due to expensive cost and logistical problems such as accessibility to the instruments. Alternatively, using less invasive and cost-effective approaches would be beneficial for screening purposes, hence there is a growing interest in identifying blood-based biomarkers (Hampel et al. 2018). Recently, Janelidze and colleagues reported increased plasma p-tau181 levels in individuals with preclinical (normal cognition, NC), MCI and AD dementia compared to the healthy controls (Janelidze et al. 2020). Moreover, plasma p-tau levels were not increased in individuals with non-AD dementia such as Parkinson disease with dementia or dementia with Lewy bodies, indicating

that p-tau181 could hold a great potential as an AD-specific plasma biomarker (Janelidze et al. 2020).

1.7 TREATMENT STRATEGIES

Despite the extensive research and increased knowledge on AD, there is still no treatment available that could halt disease progression. The current treatment strategies are able to delay the cognitive decline in AD patients and include acetylcholinesterase inhibitors (donepezil, galantamine and rivastigmine) and the NMDAR antagonist (memantine) (Winblad et al. 2016). The acetylcholinesterase inhibitors are often used in patients with mild-to-moderate AD and inhibit acetylcholinesterase, which catalyse the breakdown of acetylcholine, and thereby increasing the level and the duration of action of acetylcholine in the nervous system. In turn, memantine is available for patients with moderate-to-severe AD and blocks the prolonged calcium ion influx into the postsynaptic terminal, which is the main basis of neuronal excitotoxicity. In addition to the approved drugs available in the market, there is an ongoing research targeting Aβ and tau in order to reduce production of Aβ (by inhibiting BACE1), increase clearance of Aβ (by active or passive immunotherapy), reduce the abnormal hyperphosphorylation of tau (by inhibiting GSK3) or its fibrillation/deposition into NFTs (by active or passive immunotherapy) (Winblad et al. 2016).

It is important to keep in mind that AD is a complex multifactorial neurodegenerative disorder. To date, many clinical trials against single targets have failed and therefore multi-target therapies, addressing different pathogenic aspects of AD (Zagórska and Jaromin 2020), will be the key in future therapeutic approaches. Better understanding of AD continuum and implications of different sets of biomarkers could enable determining the window of opportunity for potential disease-modifying treatments as well as identifying subsets of patients that could potentially receive different treatment strategies.

2 AIMS OF THE THESIS

The main aim of this thesis was to investigate proteins that could be involved in AD pathophysiology. We investigated a recently identified fragment of APP as well as focused on identifying novel proteins by performing unbiased proteomics using postmortem human brain tissue and mouse CSF.

More specific aims were:

- To investigate whether the abundantly expressed 20 kDa band, detected in human brain tissue by western blotting, is indeed an APP-CTF and could be of importance in AD pathogenesis (**Paper I**).

- To study the proteome of a vulnerable, synapse-rich region of the hippocampus, which receives the crucial perforant path input, in order to identify proteins and pathways that could be involved in synaptic impairment in AD (**Paper II**).

- To assess the detailed hippocampal expression pattern of five presynaptic protein hits, which were identified in Paper II, in AD brain (**Paper III**).

- To get insights into the proteins and pathways that could be crucial for AD pathophysiology by performing a meta-analysis of the proteomic studies (**Paper IV**).

- To explore the translational changes in the CSF proteome of *App* knock-in mice *versus* human subjects with NC, MCI and AD dementia stages (**Paper V**).

3 METHODOLOGY

3.1 ETHICAL CONSIDERATIONS

In this thesis, we conducted research using postmortem human brain tissue (Paper I-III, Paper V) as well as brain tissue and CSF from laboratory animals (Paper I and V). In Paper IV, we used published MS data or unpublished work from our research group (manuscript in preparation) for the purpose of performing a meta-analysis (2015/1803-31/2 including amendment 2020-01322). The use of human brain material in this thesis was conformed to the Declaration of Helsinki and approved by the regional ethical review board of Stockholm (2015/18/03-31/2, 2007/1477-3 and 2013/1301-31/2) and obtained Institutional Review Board approvals by the VU Medical Center, Amsterdam, the Netherlands and the Medical University of South Carolina, USA. All donors or their next-of-kin gave informed consent. The laboratory animals used in this study were handled according to the Karolinska Institutet guidelines, Swedish national guidelines and current European Law (Directive 2010/63/EU). The breeding and the collection of CSF and tissue from laboratory animals were approved by different ethical committees in Sweden (rat brain (S21-14), mouse brain (ID 156) and *App* knock-in mice CSF (ID 407)). Additionally, commercially available brain lysates from guinea pig and macaque was purchased from Novus Biologicals who ensure that the animals have been handled according to the ethical legislation in the United States. All research performed abroad were performed in alignment with the ethical legislation of their respective countries.

3.2 POSTMORTEM HUMAN BRAIN TISSUES

Postmortem tissue is an end-stage material, but it could still provide valuable information on relevant changes occurring in the brain during disease pathogenesis. However, it is important to have a well characterized cohort in order to minimize the variability between the cases. In this thesis, while selecting AD and control cases, we tried to control this variability as much as possible by considering age, gender, postmortem interval (PMI) and AD-related pathology which are mainly assessed by Braak stages and Thal/CERAD stages. Despite including samples from different brain banks **(Table 1)**, we made sure that all AD cases were clinically and pathologically diagnosed, and all control cases showed little or no pathological alterations beyond normal age-appropriate changes including a few plaques and tangles.

Studies	Sample size	Brain region	Material	Brain Bank
Paper I	10 AD cases (Braak IV-VI) 10 control	Prefrontal cortex	Frozen	Brains for Dementia Research, London, UK
	1 control	Mixed cortex	Frozen	Brain Bank at the Karolinska Institutet, Stockholm, Sweden
	4 human fetuses, post-conception age 7–11 weeks	Cortex	Frozen	Developmental Tissue Bank at Karolinska Institutet, Sweden
Paper II	5 AD cases (Braak IV) 5 controls	Hippocampus	Frozen	Netherlands Brain Bank, Amsterdam, the Netherlands
	5 AD cases (Braak IV-VI) 7 controls	Hippocampus	Formalin-fixed paraffin-embedded (FFPE)	Carroll A. Campbell Jr. Neuropathology Laboratory Brain Bank at the Medical University of South Carolina, USA
Paper III	8 AD cases (Braak V-VI) 7 controls	Hippocampus	FFPE	Netherlands Brain Bank, Amsterdam, the Netherlands
Paper V	3 AD cases (Braak VI) 3 controls	Hippocampus, Temporal cortex	FFPE	Brain Bank at the Karolinska Institutet, Stockholm, Sweden

Table 1: The details of the postmortem human brain tissues included in this thesis.

3.3 LABORATORY ANIMALS

In Paper I, we used brain tissue from rat, mouse, guinea pig and macaque for comparison between the species. Brain tissues were collected from the male Wistar rats (Charles River) and female C57BL/6 mice, while brain lysates from guinea pig and macaque was purchased from Novus Biologicals. In Paper V, we collected both CSF and brain tissue from wild-type, App^{NL-F} and App^{NL-G-F} mice (n = 4 per group). The details of the knock-in mouse models of AD will be explained in the next section. Additionally, embryos were collected from wild-type C57BL/6 mice E16-E18 in order to prepare primary cultures of hippocampus and cortex.

3.3.1 Mouse models of Alzheimer disease

To date, different animal modes of AD have been generated in order to study different aspects of AD pathophysiology (Sasaguri et al. 2017). The identification of mutations e.g. *APP* and *PSEN1* genes in familial AD or *MAPT* gene in frontotemporal dementia, have led to generation of transgenic models of AD. Recently, two new models called App^{NL-F} and App^{NL-G-F} were generated using a knock-in strategy in which APP is manipulated in a way that

humanized Aβ sequence, containing mutations of familial AD, is inserted (Saito et al. 2014). In App^{NL-F} mice, the Swedish (KM670/671NL) and the Beyreuther/Iberian (I716F) mutations were incorporated into Aβ sequence, while a third mutation, the Arctic, was introduced in App^{NL-G-F} mice. In contrast to the APP transgenic mice models, both App^{NL-F} and App^{NL-G-F} mice express APP at endogenous levels while exhibiting profound Aβ pathology. The pathology occurs as a result of increased Aβ production and $Aβ_{42}:Aβ_{40}$ ratio as well as increased oligomerization of Aβ, due to the presence of familial mutations. Although the Aβ pathology is similar between these models, App^{NL-F} mice display milder Aβ pathology starting at nine months of age, whereas in the more aggressive model App^{NL-G-F}, the Aβ pathology starts already at two months of age. Indications of inflammation including astrocytosis and gliosis as well as synaptic alterations are observed in these mice. In addition, App^{NL-F} and App^{NL-G-F} mice start to exhibit cognitive impairments at 18 and 6 months of age respectively.

3.4 IMMUNODETECTION TECHNIQUES

3.4.1 Western Blotting

In Paper I and V, western blotting was carried out using the LI-COR system in order to investigate APP and its fragments (Paper I) and markers of autophagy (Paper V). Western blot is commonly used to detect and quantify proteins from a biological sample. Briefly, the denatured proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular weight and transferred to nitrocellulose or PVDF membranes, which were blocked and then incubated with primary antibodies. Subsequently, membranes were washed and incubated with fluorescently labeled secondary antibodies (LI-COR). Digital fluorescent visualization of signals was detected using the Odyssey CLx Imaging System (LI-COR). Additionally, in some experiments of Paper I, such as the comparison between AD and control cases, membranes were stained with total protein stain using REVERT^{TM} Total Protein Stain (LI-COR) and the signal was immediately detected at the 700 nm channel. Quantitation of protein of interests was done using Image Studio Lite v5.2 (LI-COR).

3.4.2 Immunoprecipitation

In Paper I, we performed immunoprecipitation experiments to concentrate a protein of interest from a complex biological sample using an antibody. Briefly, brain lysates were first precleared using magnetic beads in order to remove any potential non-specific binding. Subsequently, direct (antibody-bead complex) or indirect (antibody-antigen complex)

immunoprecipitation was performed. The input (lysate before and after pre-absorption steps), the unbound sample and the immunoprecipitated sample were collected and subjected to western blotting as described above.

3.4.3 Immunohistochemistry/Immunofluorescence

In this thesis, we also did immunostaining in order to investigate staining densities of presynaptic proteins in postmortem human brain (Paper II and III), to visualize the presence of the autophagic markers both in postmortem human brain and in mouse brain as well as to detect decorin and its colocalization with the markers of interneurons in mouse brain (Paper V). Briefly, using immunohistochemistry (Paper II and V), paraffin-embedded sections were deparaffinized, rehydrated, and following the antigen retrieval step, sections were blocked and incubated with primary antibodies. Subsequently, sections were washed, incubated with secondary antibodies and after DAB staining, sections were coverslipped. Images were acquired by the Nikon Eclipse E800 light microscope using NIS Elements software. Alternatively, in Paper III and V, we used immunofluorescence method, which was similar to the above-described immunohistochemistry. In Paper III following incubation with primary antibodies, the sections were incubated with TrueBlack Lipofuscin Autofluorescence Quencher to reduce autofluorescence. Afterwards, the sections were incubated with IgG conjugated secondary antibodies, followed by DAPI staining. Slides were scanned using the semi-automated Nanozoomer 2.0HT slide scanner (Hamamatsu) and images were acquired using the NDP.view2 software (Hamamatsu). Both in Paper II and III, semi-quantitative densitometrical analysis was done by measuring the mean pixel intensities of region of interest on ImageJ Fiji. In Paper V, to detect decorin, following rehydration steps, the sections were incubated with chondroitinase ABC, which digests O-linked chondroitin sulfate-like glycosaminoglycan (Snow et al. 1992). While the remaining steps were followed accordingly, the signals were amplified with TSA Fluorescein System. Images were acquired by the Nikon fluorescence microscope and quantified by ImageJ Fiji.

3.5 LASER MICRODISSECTION

Under microscopic visualization, LMD is used to isolate specific cells, regions or structures from a complex tissue, thereby providing a more homogenous sample for molecular analyses. In Paper II, LMD was employed to specifically microdissect the OML in order to study the changes in the proteome **(Figure 6)**. Consecutive frozen hippocampal sections (20 μm thick) were cut in the cryostat and mounted on polyethylene naphthalate membrane coated slides, which facilitates the laser cutting during LMD. Using toluidine blue staining, the nuclei of all

cell types are stained enabling us to identify the molecular layer of the dentate gyrus that is located right underneath the granule cell layer (GCL). Approximately 0.6 mm^3 of microdissected OML was collected per case.
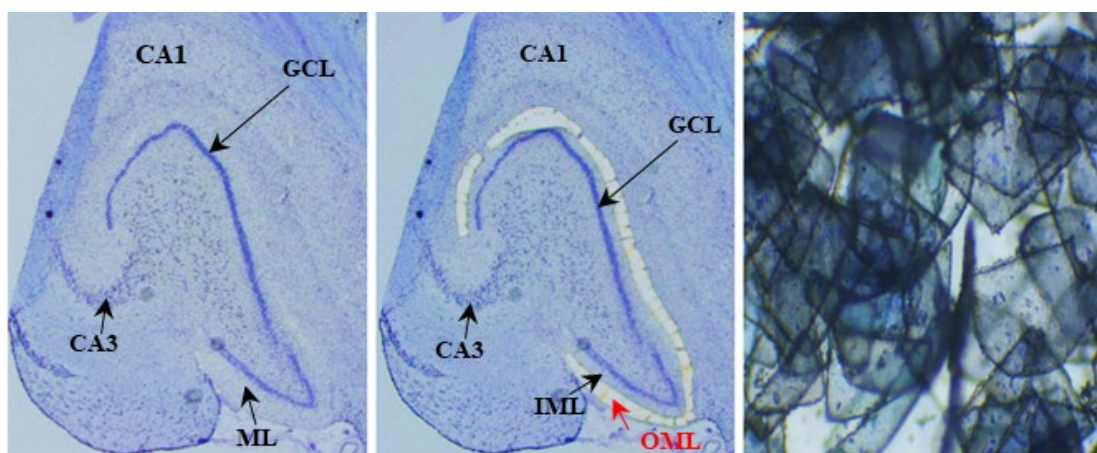

Figure 6: The process of LMD. A 20 μm thick hippocampal section form a control brain was stained with toluidine blue. The entire molecular layer (ML), which is located between the granule cell layer (GCL) and the hippocampal fissure, is seen (left). The OML is successfully microdissected (middle) and the microdissected tissues are collected inside a tube cap (right).

3.6 MASS SPECTROMETRY-BASED PROTEOMICS

MS is a sensitive analytical method that measures mass-to-charge ratio of charged molecules and is commonly used for identification and quantification of proteins in a complex biological sample. The bottom-up approach is often used where peptides are identified through pattern matching between the generated MS spectra and the theoretical MS spectra, coming from the protein database. Briefly, proteins are extracted from a lysate and reduced, alkylated and digested by trypsin, which cleaves after lysine and arginine residues, to generate peptides with properties suitable for analysis. Following sample clean-up steps (e.g. removal of salts), the resulting peptides are then injected to the LC-MS. As it stands from the name, in the LC, the peptides are separated on a column based on their hydrophobicity. Once the peptides are eluted from the column, they are ionized by the electrospray. The precursor ions are detected at the first MS (MS1). Following fragmentation for example by collision induced dissociation, peptide bonds are broken and smaller fragment ions are generated. At the second MS (MS2), the spectra of fragment ions are detected, providing a more resolved sequence of peptide fragments.

In this thesis, MS-based proteomic approaches were applied for different purposes. While in Paper I, our aim was to investigate whether the 20 kDa APP band (detected by APP antibodies using SDS-PAGE) is a true APP fragment, in Paper II and V, relative

quantification was performed. More specifically, in Paper II, peptides from each case were labeled with a different TMT (TMT10plex 126–131Da) and pooled together for LC-MS/MS analysis **(Figure 7)**. To increase the proteome coverage, the pooled sample was pre-fractionated into 72 fractions using high resolution iso-electric focusing (HiRIEF) (Branca et al. 2014). Finally, each fraction was analyzed by the LC-MS/MS.
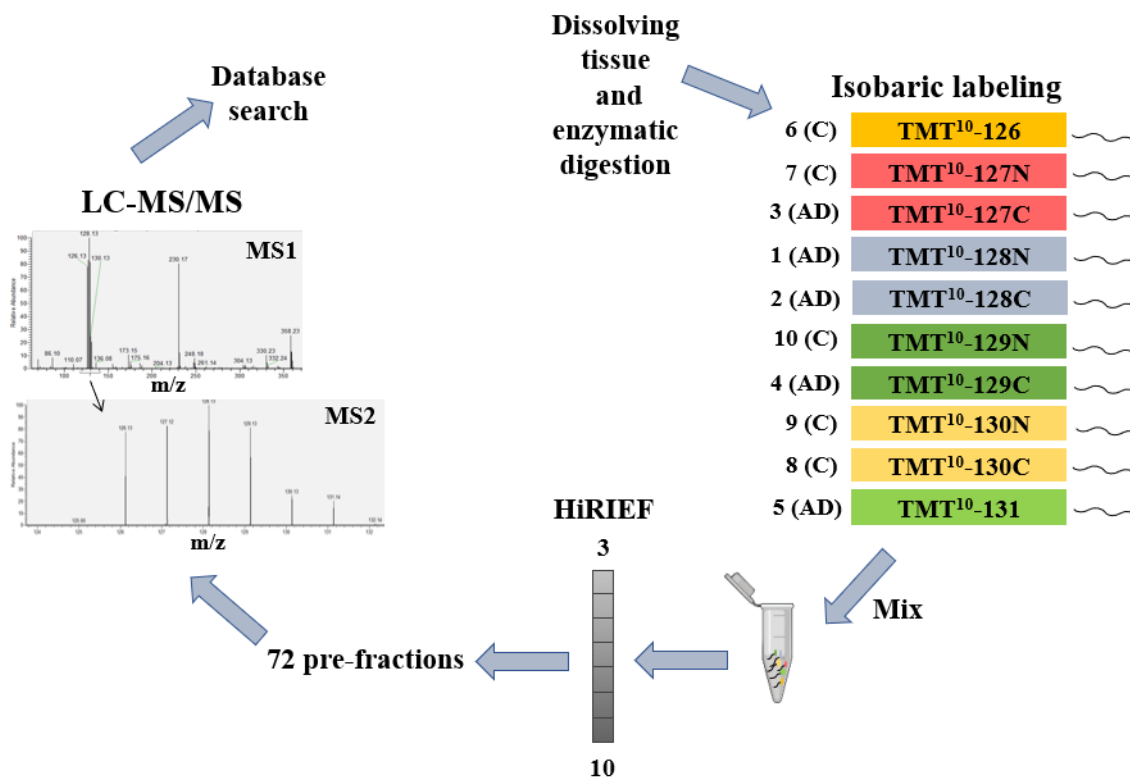

Figure 7: The microdissected tissues were dissolved and digested by trypsin. The resulting peptides from 10 cases were labeled by different TMTs and pooled. The peptide mix was then pre-fractionated into 72 fractions by HiRIEF and each fraction was analysed by LC-MS/MS.

In Paper V, since small volume of CSF (~ 10 µl) is collected from living mice, we instead used label-free MS approach in order to explore the CSF proteome from *App* knock-in mice and wild-type mice.

3.7   DATA ANALYSIS

In this thesis, demographic characteristics (i.e. age, gender, PMI, brain pH, Braak and amyloid stages) and biochemical data were analyzed in GraphPad PRISM 7.0. Depending on whether the data follows a normal distribution or not, which was checked by Kolmogorov-Smirnov test, either two-tailed Student's t-test or Mann-Whitney test was used for assessing the statistical significance. Only in Paper V, we had more than two groups, and therefore, one-way ANOVA followed by Dunnett's multiple comparisons test was performed for the analysis of the biochemical data, if not stated otherwise in the manuscript.

For the analysis of the MS data, in Paper II, differential expression analysis of quantitative mass spectrometry data (DEqMS) package in R was used to detect the significant alterations in average protein expression between AD and control groups. Briefly, DEqMS works on top of limma package and considers the number of peptide spectra matches per peptide, detected by LC-MS/MS analysis, while calculating t-statistics (Zhu et al. 2020). Benjamini-Hochberg method was then used for multiple hypothesis testing (Benjamini and Hochberg 2000) and a cut-off level of 10% false discovery rate (FDR) was applied. In Paper IV, random-effects-model was used to perform meta-analysis of MS data which will be explained in section 3.7. Proteins with FDR < 20% were considered as statistically significant. In Paper V, a two-tailed Student's t-test was performed to identify proteins that were significantly altered in *App* knock-in mice compared to wild-type mice using Qlucore. A p-value < 0.05 was considered as statistically significant. The human CSF data that was used in Paper V was recently published (Tijms et al. 2020). Multivariate data analysis was performed to find the biggest variation in our data using an unsupervised principal component analysis (PCA) in SIMCA or Qlucore. Volcano plots of all proteins were generated in R or GraphPad Prism 8. Heatmaps of differentially expressed proteins were generated using Morpheus (Broad Institute, https://software.broad_institute.org/morpheus). Venn diagrams were generated by the Interactive Venn tool (Heberle et al. 2015) or Venny 2.1 (Oliveros 2007-2015).

3.7.1 Bioinformatic analyses

Thanks to advances in the field of LC-MS, it has become possible to identify and quantify thousands of proteins in a complex biological sample. Therefore, knowing that a given protein is up- or downregulated is not very insightful when many proteins together influence a pathway. This is the main reason why there is a growing interest in performing functional enrichment analyses which allows us to put single protein alterations in a biological context. For this purpose, we used the following tools: Gene set enrichment analysis (GSEA, Broad Institute) (Subramanian et al. 2005), GOrilla tool (Eden et al. 2009), DAVID Bioinformatics Resources (Huang da, Sherman, and Lempicki 2009), and Gene ontology enrichment analysis by Panther (Ashburner et al. 2000; Mi et al. 2019).

Although the display of results is different between these analyses, they all are based on the molecular signature database that contains gene sets derived from gene ontology (GO) annotations. Therefore, the same pathways are expected to be overrepresented for certain set of proteins, independent of which tool is used. The input is a list of proteins with valid protein identifiers and proteins are often unranked, meaning that they are not sorted by e.g. fold

change prior to the analysis, except in GSEA. However, to identify which processes are truly altered and to minimize false interpretations, it is preferable to upload the list of significantly altered proteins. Moreover, up- and downregulated proteins are often analyzed separately to get a better understanding of the biological changes. While statistical methods to compute p-value are different between different tools, the main outcome of enrichment analyses is the same and include: enriched GO biological process, enrichment score, number of genes/proteins that are associated with a given GO term from the uploaded data and the GO database, p-value and FDR-value. In all tools, Benjamini-Hochberg method is used for multiple hypothesis testing (Benjamini and Hochberg 2000) and biological processes with FDR < 5% are considered as statistically significant.

In summary, functional enrichment analyses can tell us which processes are affected in a pathological condition. Moreover, by visualizing whether the affected processes are linked to each other, it can allow us to select candidate processes and proteins for further studies.

GSEA uses a running-sum statistic method, therefore all identified proteins are ranked from the most upregulated to the most downregulated, regardless of their p-values **(Figure 8)**.
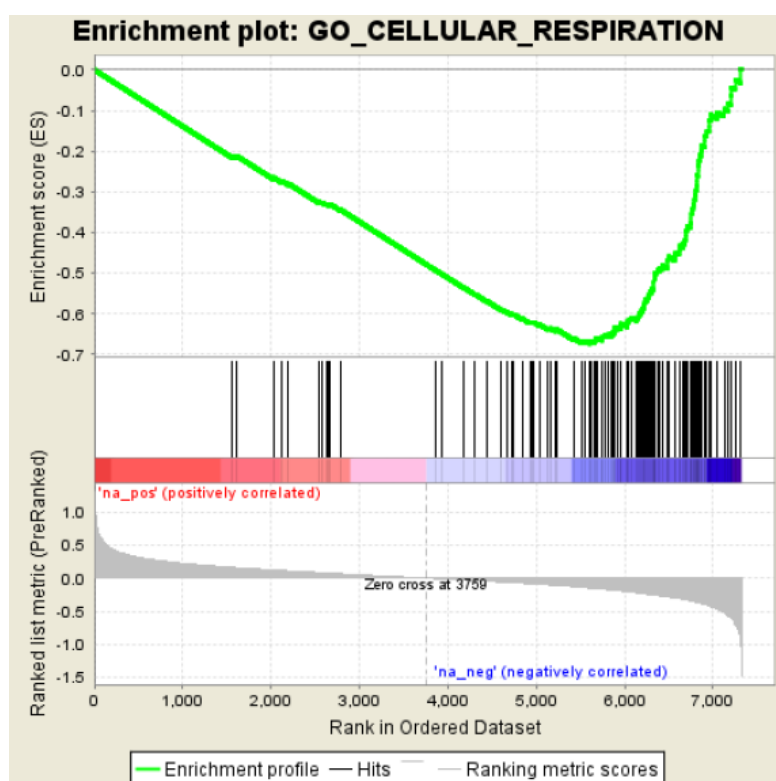

Figure 8: *An example of a biological process called cellular respiration. All 7322 identified proteins were subjected to GSEA software. Proteins related to this process are indicated as lines, shown at the middle part of the graph. Upregulated proteins are shown to the left and downregulated proteins to the right. As most proteins are downregulated, this pathway is found to be negatively enriched.*

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In GOrilla tool, either a single list of proteins or two lists of proteins (target and background lists) can be uploaded. For example, in the latter approach, a target list consists of differentially expressed proteins (with a preferred cut-off for p-value or FDR-value) while a background list contains all protein identifications **(Figure 9)**. The main difference, when subjecting two unranked lists, is that the enrichment score takes into account the number of all protein identifications, which is provided as the background list.

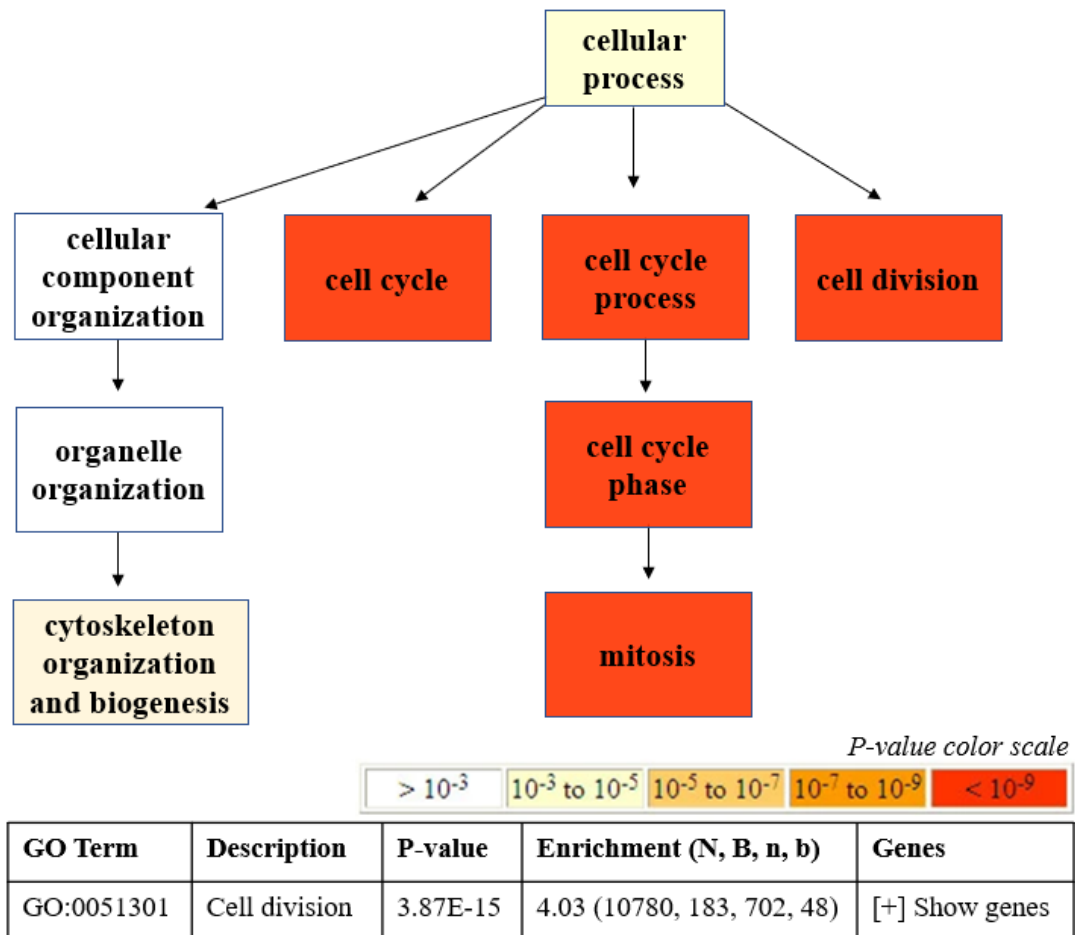| GO Term	Description	P-value	Enrichment (N, B, n, b)	Genes
GO:0051301	Cell division	3.87E-15	4.03 (10780, 183, 702, 48)	[+] Show genes

Figure 9: GOrilla makes a summary chart where all identified biological processes, which appeared to be affected by the uploaded proteins, are shown. Additionally, this chart visualizes the hierarchy of a given GO term and whether different processes are linked to each other. The enrichment score is defined by (b/n) / (B/N). N is the total number of all identified proteins in the data; B is the total number of proteins associated with a given GO term; n is the total number of proteins from the target list (with a determined cut-off); and b is the number of proteins in the target list that are associated with a given GO term. This figure is modified from (Eden et al. 2009).

Alternatively, DAVID or Panther can be used for functional enrichment analysis. The results are displayed in a table format in both tools and GO terms can be sorted by the statistical significance or the hierarchy **(Table 2)**.

GO Term	Count	%	P-value	Genes	Fold Enrichment	FDR-value
GO:0006099 Tricarboxylic acid cycle*	13	3.9	8.11E-14	P31040…	23.2	1.45E-10
GO:0006120 Mitochondrial electron transport, NADH to ubiquinone*	14	4.2	5.47E-12	P19404...	14.8	4.88E-09
GO:0044281 Small molecule metabolic process	126	37.8	6.58E-37	P78417...	3.3	3.04E-33
GO:0044710 Single-organism metabolic process	165	49.5	1.39E-25	A0A0A…	2.1	2.14E-22

*Table 2: Example of functional enrichment analysis performed by DAVID. Count column is the number of proteins that are associated with a given GO term, and individual proteins are listed under the column genes. By clicking on the GO term, more information about a given pathway can be obtained. *DAVID summarizes the list of processes that are at the very bottom in a hierarchy under "Direct" category.*

Additionally, Ingenuity Pathway Analysis (IPA) can be used for biological interpretation of the data. While a valid protein identifier is sufficient to perform the above-mentioned analyses, here in IPA, log2 fold change is also provided. Common analysis types in IPA include biological functions and upstream regulators **(Table 3, Figure 10)**.

Diseases and Functions	Predicted activation state	Activation z-score	p-value	Molecules	# Molecules
Transport of metal ion (GO:0030001)	**Decreased**	-2.619	4.44E-04	↓AKT3...	27
Secretory pathway	**Decreased**	-2.570	2.44E-11	↑ANXA1... ↓CADPS2…	29
Upstream regulator	**Predicted activation state**	**Activation z-score**	**p-value of overlap**	**Target molecules in dataset**	**# Molecules**
Nuclear factor erythroid 2-related factor 2 (NFE2L2)	**Inhibited**	-2.525	0.0045	↑GFAP… ↓CHGB…	11
Vascular endothelial growth factor A (VEGFA)	**Activated**	2.186	0.0163	↑ICAM1…	5

Table 3: Examples of diseases and functions analysis (top) and upstream regulator analysis (bottom). The column called 'molecules' or 'target molecules in dataset' contain the proteins that are associated with a given pathway or upstream regulator. Based on the log2 fold change of proteins

from the data, downregulated proteins are indicated in green while upregulated ones are shown in red. IPA makes a prediction about the activation state of a function or upstream regulator by calculating a z-score, which are considered significant if z-score >2 or z-score <-2.
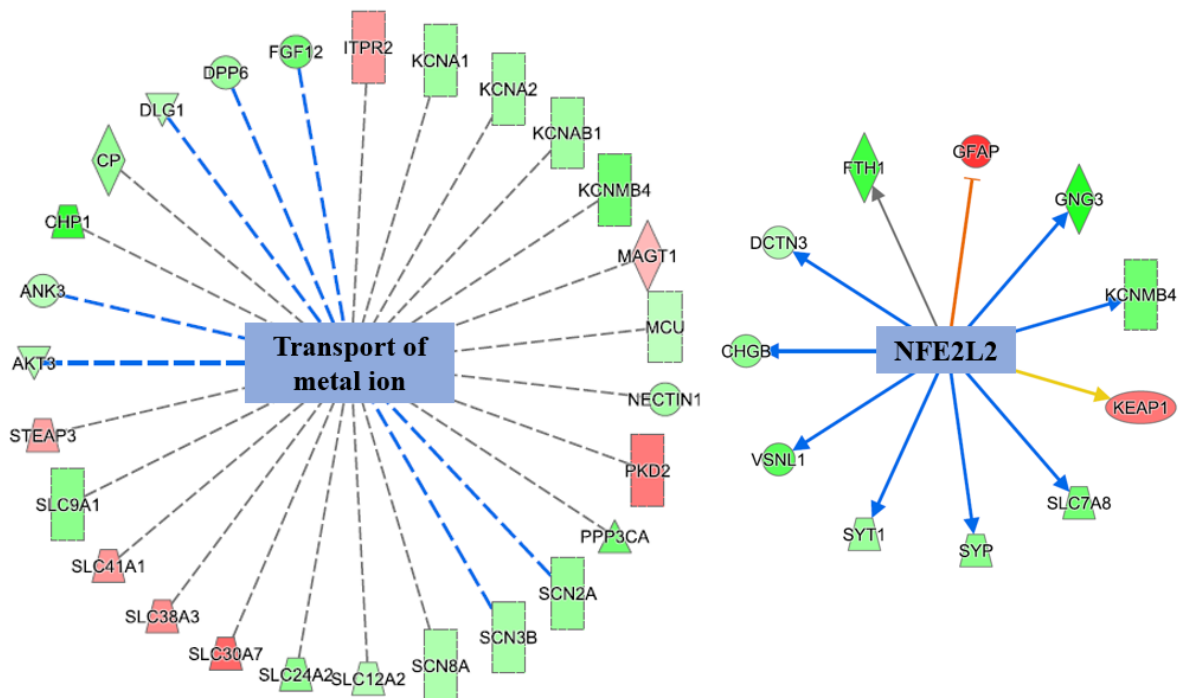

Figure 10: *Examples of diseases and functions analysis (left) and upstream regulator analysis (right). IPA can also predict the relationship between individual proteins and associated functions or upstream regulators, based on the literature findings (IPA knowledgebase). In a function (transport of metal ion) or in an upstream regulator (NFE2L2), blue arrows indicate an inhibition, red arrows indicate an activation, yellow is contradictory findings between the data and literature, and grey indicates that the effect is not predicted.*

3.8 META-ANALYSIS BY RANDOM-EFFECTS-MODEL

A meta-analysis of proteomic studies could verify proteins that are previously known to be altered during disease pathogenesis as well as result in differences in the nature of proteome by identifying novel proteins that could be important for disease pathogenesis. Additionally, it could allow us to detect discrepancies between the datasets. We used random-effects-modeling (Harrer, Cuijpers, and Ebert 2019) that assumes a distribution of effect sizes, i.e. mean differences between disease and control groups, under the influence of different biasing effects. In Paper IV, we followed the guideline called "Doing Meta-analysis in R" and performed meta-analysis through the package meta (version 4.13). The metacont function included the following parameters:

meta-analysis.results = metacont(

        final_dataset, *input MS dataset*

        N_AD, *number of cases in AD group*

Mean_AD, *mean values in AD group per proteins*

SD_AD, *standard deviation in AD group per proteins*

N_C, Mean_C, SD_C, *for control groups*

studlab = paste(reference), *reference*

comb.fixed = FALSE, *whether to use a fixed-effects-model*

comb.random = TRUE, *whether to use a random-effects-model*

method.tau = "SJ", *the selected estimator for between-study variance*

hakn = TRUE, *whether to use the Hartung-Knapp method*

prediction = TRUE, *whether to print a prediction interval*

sm = "MD" *the summary measure which is the mean difference*

)

The random-effects-modeling approach gives an overall mean difference for each protein by taking into account the mean and standard deviation within each group (e.g. AD and control) as well as the sample size per group, thus accounting for group differences even for proteins that were not initially quantified in all datasets or for those showing opposite directional changes between datasets.

4 RESULTS AND DISCUSSION

4.1 PAPER I. NON-SPECIFIC DETECTION OF A MAJOR WESTERN BLOTTING BAND IN HUMAN BRAIN HOMOGENATES BY A MULTITUDE OF AMYLOID PRECURSOR PROTEIN ANTIBODIES

It has been shown that fragments of APP, not just Aβ, can exert neurotoxic effects (McPhie et al. 1997; Lauritzen et al. 2012; Willem et al. 2015). Therefore, investigation of APP and its fragments is crucial in order to better understand the role of APP in AD pathogenesis.

Using six different APP antibodies, which are directed against the C-terminal part of APP sequence (Y188, C1/6.1, A8717), the Aβ sequence (6E10, 7N22), and the N-terminal part of the CTF-η sequence (9478), we consistently detected a band migrating around 20 kDa in human brain **(Figure 11)**. Notably, the signal intensity of 20 kDa band was considerably different between the studied APP antibodies. Besides, the 20 kDa band, detected by APP antibodies, was overlapped very well with a major band that became visible by total protein staining. In addition to the human brain, we found that the 20 kDa band was present in brain homogenates from guinea pig and macaque, but not in mouse or rat brain homogenates.
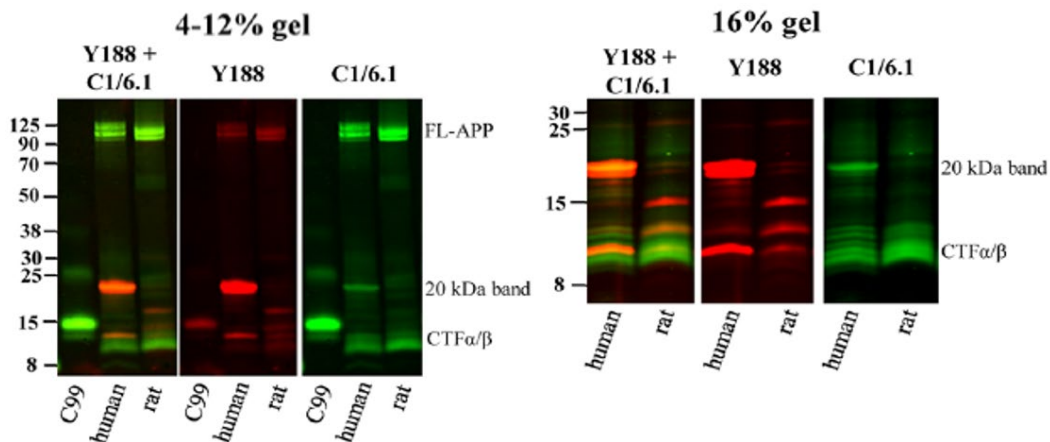

Figure 11: *The APP antibodies, Y188 and C1/6.1, showing the presence of 20 kDa band in human brain homogenate. Using 16% SDS-PAGE gel, the 20 kDa band appeared as a double band. Especially using the C1/6.1 antibody, several APP-CTFs including CTF-α and -β were detected both in human and rat brain homogenates.*

An APP-CTF called CTF-η, migrating around 25 kDa on an SDS-PAGE gel, was recently discovered (Wang et al. 2015; Willem et al. 2015). Although we do not have any conclusive evidence, the 20 kDa band corresponds to the expected weight of the APP CTF-η (Willem et al. 2015). Furthermore, when using 16% SDS-PAGE gels, we observed that the 20 kDa band appeared to be a double band, in agreement with two previous reports in which an APP-band of similar size (~25 kDa) was detected in human cell-lines (Wang et al. 2015) and in human

CSF (García-Ayllón et al. 2017). Increased levels of the CTF-η and its fragment Aη are detected in the CSF of AD subjects and in the brain of AD transgenic mice (Willem et al. 2015; García-Ayllón et al. 2017) but we did not observe any change in the levels of the 20 kDa band in AD brain compared to control, using both the Y188 and the C1/6.1 antibodies. Additionally, we noticed that the levels of the 20 kDa band varied considerably between cases and showed a correlation with the intensity of the major band detected by total protein stain.

Based on these observations, we questioned whether the studied antibodies could cross-react or bind non-specifically to constituents of this total protein band other than APP. Thus, different APP fragments were separated on SDS-PAGE and two gel pieces, corresponding to the size of the 20 kDa band and the full-length APP (~100 kDa), were cut out and in-gel digestion was done for the LC-MS/MS analysis. Several APP-derived peptides were detected in the gel preparation from the full-length APP, while only one APP-derived peptide was found in the 20 kDa band. It is probable that the same peptide was detected in both preparations, since the APP-derived peptide in the 20 kDa showed a similar spectrum and the same retention time as one of the peptides identified in the full-length APP band. This could further suggest that the 20 kDa band derive from APP but it is probably expressed at low levels. We next tried to immunoprecipitate this band using the Y188 and the A8717 antibodies. While the full-length APP and the lower molecular weight CTFs were efficiently immunoprecipitated, the attempts to immunoprecipitate the 20 kDa band was not successful. This is in agreement with the MS analysis and suggests that the true levels of the 20 kDa APP band is quite low in human brain. Alternatively, the immunoprecipitation experiments might not have worked because (i) conformation of the 20 kDa band might be different than the other APP CTFs and therefore the epitope for antibody binding site might be hidden, (ii) the tissue was homogenized using a mild lysis buffer which could result in a detection of low signal intensity, and (iii) we used a total brain lysate instead of using soluble and insoluble fractions, which could have improved the detection of this band since it contains the transmembrane site of the APP sequence.

The LC-MS/MS analysis of the 20 kDa band identified myelin basic protein (MBP) with the highest score, indicating that the peptide fingerprint of MBP (detected MS spectra with respect to the theoretical spectra) had the best coverage among all detected proteins. Since MBP is an abundant protein in the brain and the prepared brain homogenates contain white matter as we did not dissect the tissue beforehand, we further investigated whether the APP antibodies might potentially react to MBP. Using western blotting, we found several MBP

isoforms between 14–21 kDa, of which one perfectly overlapped with the 20 kDa APP band detected by the C1/6.1 antibody in AD brain. As this overlap does not indicate that the APP antibodies could interact with the MBP, we did immunodepletion experiments. While the 15 kDa MBP band was efficiently immunoprecipitated in the human brain homogenate and the levels of MBP staining was depleted depending on the concentration of the MBP antibody, the 20 kDa band was not specifically immunodepleted or immunoprecipitated using both the MBP and APP antibodies. This could indicate that the detection of 20 kDa band by MBP antibody could also be non-specific. Additionally, we observed that several other antibodies detected a band migrating at 20 kDa in human brain homogenates, suggesting that this band is to some extent is unspecific.

Taken together, our findings suggest that the 20 kDa band is a true APP fragment, since the same fragment was detected by six different APP antibodies and an APP-derived peptide was identified by the LC-MS/MS analysis. However, this fragment is most likely expressed at low levels in human brain.

4.2 PAPER II. THE PROTEOME OF THE DENTATE TERMINAL ZONE OF THE PERFORANT PATH INDICATES PRESYNAPTIC IMPAIRMENT IN ALZHEIMER DISEASE

As explained in section 1.3, compelling evidence suggests that the perforant path, which provides the main input of the hippocampus, is vulnerable to AD-related changes. Since synaptic changes are early pathogenic features of AD and observed at the OML, where the perforant path fibers terminate, in this study, we investigated the proteome of this vulnerable, synapse-rich region in order to identify proteins and pathways that could be involved in synaptic dysfunction in AD.

The OML was specifically cut out from postmortem human brain tissue using LMD. By employing HiRIEF (Branca et al. 2014), the proteome coverage was greatly increased and 7322 proteins were quantified in the microdissected OML in all 10 cases with no missing values. Using DEqMS algorithm (Zhu et al. 2020), 724 proteins were found to be significantly altered in AD ($p < 0.01$ and FDR < 10%), consisting of 382 downregulated and 342 upregulated proteins. To interpret our findings in biological context, we performed functional enrichment analyses on all proteins by GSEA and on differentially expressed ones by GOrilla tools. Biological processes including cellular respiration, oxidative phosphorylation and electron transport chain were negatively enriched in AD OML. As we included all proteins regardless of their p-values in the GSEA, it is important to note that

many of those mitochondrial proteins were not significantly altered, but they could still have an impact on mitochondrial function. Given that synaptic signalling requires high amount of energy, altered mitochondrial bioenergetics could impair synaptic homeostasis in AD (Devine and Kittler 2018). The analysis of the significantly decreased proteins, done by GOrilla, revealed the involvement of biological processes related to neurotransmission in AD OML **(Figure 12A)**. In turn, upregulated proteins were associated with processes related to gene expression such as RNA processing and ribonucleoprotein complex biogenesis in AD OML. Furthermore, 724 differentially expressed proteins were subjected to IPA. Using diseases and functions tool, the exocytosis pathway was predicted to be significantly decreased in AD **(Figure 12B)**. Several proteins such as STX1A and SNAP25 (indicated by blue arrows in **Figure 12B**) were found to be associated with decreased exocytosis.
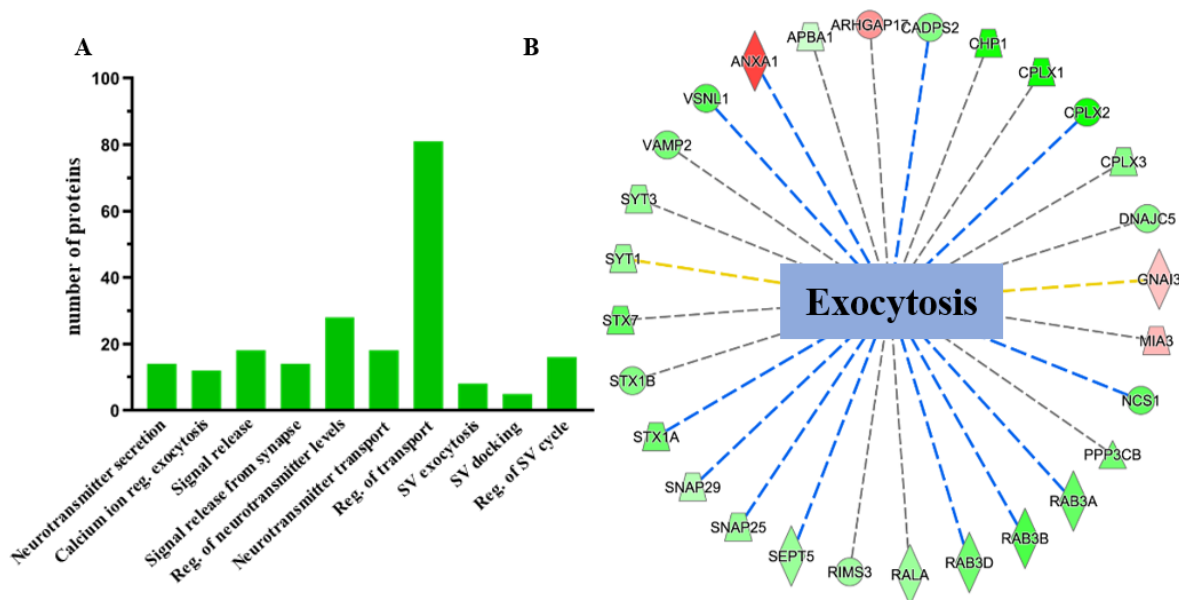

Figure 12: *(A) Functional enrichment analysis of significantly downregulated proteins (n = 382), using GOrilla tool, showed that biological processes related to neurotransmission were significantly altered in AD OML. (B) Similarly, exocytosis was predicted to be significantly decreased in AD OML by IPA of differentially expressed proteins (n = 724). Blue arrows indicate that proteins lead to an inhibition of the pathway. Yellow arrows indicate that the association with proteins and pathway is inconsistent with the literature findings, and grey arrows suggest that the effect is not predicted even though proteins are involved in this pathway.*

Based on the fold change between AD and control, biological function and availability of commercial antibodies, we selected three presynaptic proteins that have not been previously studied in AD, i.e. synaptogyrin-1 (SYNGR1), CPLX1 and complexin-2 (CPLX2), for immunohistochemical assessment. Using a different group of AD and control cases, we confirmed that the staining densities of all selected proteins were reduced in AD OML.

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Our proteomics findings are in line with the literature and indicate that the OML indeed exhibits synaptic changes during AD pathogenesis. More importantly, presynaptic proteins were found to be significantly altered in AD OML while the postsynaptic density proteins (e.g. AMPARs, NMDARs, drebrin and PSD95) were not. This supports the notion that presynaptic changes could be more important than the postsynaptic changes in this region during disease pathogenesis.

4.3 PAPER III. SPECIFIC PRESYNAPTIC LOSS IN THE OUTER MOLECULAR OF THE DENTATE GYRUS IN ALZHEIMER DISEASE

To follow-up on our proteomics findings from Paper II and further explore the synaptic impairment within the hippocampal formation, we investigated the expression of five presynaptic proteins using immunofluorescence. The selected presynaptic proteins play important roles in neurotransmission and were: the cytosolic protein CPLX1, the cell surface SNARE protein STX1A, the synaptic vesicle proteins SYNGR1 and SYT1, and the vesicular SNARE protein called VAMP2. The presynaptic protein IR was measured in the following regions: (i) molecular layers of the dentate gyrus: OML and IML; (ii) molecular layers of the CA3: LUC, RAD and LM; (iii) molecular layers of the CA1: RAD and LM; and neuronal layers: CA4, CA3 and CA1.

The staining densities of CPLX1, STX1A, SYNGR1 and SYT1 were significantly decreased in AD OML, supporting our proteomic findings (Haytural et al. 2020). However, VAMP2 showed a non-significant decreased tendency in AD OML. In the adjacent molecular layer, IML, the densities of CPLX1, STX1A, SYNGR1 and SYT1 were not altered in AD, while VAMP2 showed a non-significant decreased tendency. Several studies have reported reduced staining densities of synaptic proteins in AD OML (Hamos, DeGennaro, and Drachman 1989; Masliah et al. 1994; Robinson et al. 2014). Although the semi-quantitative densitometric analysis of synaptic proteins was not reported specifically for IML in these studies, the change in synaptic protein-IR was reported as a ratio of OML/IML, suggesting that the levels were not altered in AD IML. As previously explained in the section 1.3, these two regions receive distinct excitatory inputs, thus it is not surprising that the extent of synaptic changes might be different between these regions.

We furthermore explored presynaptic protein-IR in other molecular and neuronal layers of the hippocampus and surprisingly observed no profound alterations in the staining of CPLX1, SYT1, SYNGR1 and VAMP2 in AD. Interestingly, we detected a significant increase in the STX1A staining in CA4 neuronal layer and a non-significant trend towards increased levels

in CA3 neuronal layer, CA3-LUC, CA3-RAD, CA1 neuronal layer and CA1-RAD in AD compared to control. The STX1A staining could be increased in these regions, possibly via increased branching or sprouting, in order to compensate for the reduced input that dentate granule cells receive. Taken together, our findings indicate that the reduction in the staining of these presynaptic proteins, assessed by semi-quantitative densitometric analyses, were highly specific to the OML in AD, despite that AD cases had severe pathology (Braak stages V-VI). Moreover, the fact that other terminal zones of the perforant path, i.e. CA3-LM and CA1-LM, were unaffected in AD could emphasize the importance of the perforant path terminating at the OML.

In the hypothesis of synaptic loss, both pre- and postsynaptic sites are expected to be affected. However, no alterations in the staining densities of postsynaptic density protein SHANK2 and the dendritic marker MAP2 was detected in AD OML, supporting our previous proteomic findings (Haytural et al. 2020). Moreover, the density of granule cells, whose dendrites are in the OML, showed no difference between AD and control. These observations suggest that postsynaptic compartment is intact in the OML and the deficit specifically arise from the presynaptic compartment, for example as a result of the loss of EC layer II neurons, which has been well documented in the field of AD research (Gómez-Isla et al. 1996; Kordower et al. 2001). Although we were not able to investigate this cause-consequence relationship, since the brain sections in our cohorts were too posterior, we could investigate whether there was an axonal degeneration in AD OML. We showed that the staining densities of MBP (for myelinated axons), SMI-312 (for phosphorylated neurofilaments M and H) and tau were not altered in AD OML. This supports the notion that the presynaptic impairment observed in the OML does not directly result from loss of projecting EC fibers. Alternatively, a retrograde degeneration could be involved in AD pathogenesis (Terry 2000). In this theory, synaptic dysfunction and degeneration precede axonal degeneration and neuronal death, which has been supported by the fact that synaptic loss correlates well with cognitive decline, in fact much better than the neuronal loss (Terry et al. 1991). Importantly, this hypothesis could also explain why all presynaptic proteins were not altered to the same degree in AD brain compared to control (Haytural et al. 2020).

Increasing evidence suggests that Aβ and tau play a role in synaptic dysfunction, explained in section 1.4.1. Since both amyloid plaques and NFTs are present in AD OML (Hyman et al. 1986; Crain and Burger 1988; Thal et al. 2000), it is plausible that these pathological changes could also cause synaptic impairment in this region. Therefore, lastly, we investigated whether the burden of amyloid plaque and NFT pathology, could have an effect on this

specific presynaptic impairment. We found no correlation between the amyloid load, detected by Aβ antibody, and the reduced staining densities of the studied proteins in OML. Similarly, there was no difference in terms of the area covered by neurites, detected by p-tau antibody AT8, between the OML and the other molecular layers of the hippocampus, suggesting that the observed presynaptic alterations did not result from the burden of the p-tau positive neurites.

In summary, using a different group of AD and control cases, we showed that the OML exhibits presynaptic changes while the postsynaptic compartment seems to be rather intact, which corroborates our findings from Paper II. Furthermore, investigation of other molecular layers of the hippocampus strongly suggests that this presynaptic failure specifically affects the perforant path extending from EC layer II to the OML.

4.4 PAPER IV. INSIGHTS INTO THE CHANGES IN THE PROTEOME OF ALZHEIMER DISEASE ELUCIDATED BY A META-ANALYSIS

There has been a growing interest for carrying out proteomic studies in order to shed light on the pathogenic processes that are involved in AD. In this study, we performed a meta-analysis using the random-effects-model, which was previously described in section 3.8.

After literature search done in PubMed, we selected 20 datasets from nine published studies (and one unpublished work from our research group) based on the following inclusion criteria: (i) sample size of at least five cases per group, (ii) minimum number of 1000 quantified proteins, (iii) studies in which quantified protein intensities were reported for each individual biological sample, and (iv) studies in which the proteomic analysis was carried out using frontal and/or temporal cortices or cingulate gyrus. The selected datasets were further grouped into different categories: brain region (frontal = 10 datasets, temporal = 8 datasets, and cingulate gyrus = 2 datasets), and MS approaches (labeled = 12 datasets, and label-free = 8 datasets). In each dataset, the relative protein intensities were log2 transformed, median-centered and scaled (i.e. median = 0 and standard deviation = 1) so that they would be comparable for further statistical analyses. To visualize the largest variability between samples and to determine whether there were any systematic patterns depending on MS techniques or brain regions, PCA was performed using the log2 protein intensities from the concatenated data consisting of 512 proteins that were quantified in all 895 samples (566 AD and 329 control cases) in all datasets. We detected no clear separation between the two groups (AD *versus* control), the brain regions (cingulate gyrus, frontal and temporal cortices) or the datasets. However, two main clusters, corresponding to labeled and label-free MS

approaches, were observed **(Figure 13)** and therefore we decided to perform the meta-analysis of labeled and label-free datasets separately.
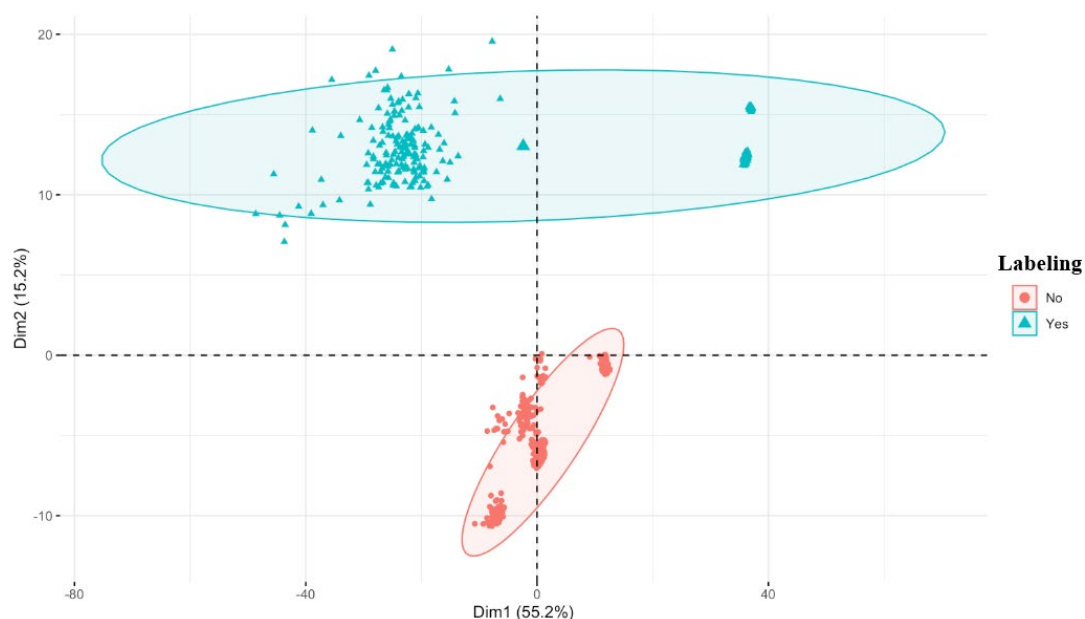

Figure 13: *PCA of 512 proteins that were quantified in all 895 samples and in all 20 datasets showed a clear separation between the labeled and the label-free data. Ellipses indicate the 95% confidence interval of samples in each of the groups.*

The labeled data contained 11753 unique proteins and was considerably larger than the label-free data, which consisted of 4292 unique proteins. The increased proteome coverage that is observed in the labeled data can be explained by the employment of the extensive pre-fractionation methods prior to LC-MS/MS analysis. Using the random-effects-model, the mean difference between AD and control groups were computed for each protein. In the meta-analysis of the labeled data, comprising 88 AD and 80 control cases, 509 proteins (232 down- and 277 upregulated) were significantly altered in AD after adjusting for multiple hypothesis testing (FDR < 20%). In turn, in the meta-analysis of the label-free data, consisting of 424 AD and 208 control cases, 505 proteins (259 down- and 246 upregulated) were significantly altered in AD (FDR < 20%). While 3604 unique proteins were found to be shared between the two meta-analyses, corresponding to 30% of proteins in the labeled data and 84% of the proteins detected in the label-free data, only 12 proteins were significantly altered in AD in both meta-analyses - indicating that there was a considerable difference between these analyses **(Figure 14)**. Among those 12 proteins, cytosolic arginine sensor for mTORC1 subunit 2 (*CASTOR2*), integrin alpha-6 (*ITGA6*), plectin (*PLEC*), ribosomal protein S6 kinase alpha-2 (*RPS6KA2*), sodium- and chloride-dependent GABA transporter 3 (*SLC6A11*), structural maintenance of chromosomes protein 3 (*SMC3*) and synaptotagmin-11 (*SYT11*) showed consistent direction of changes across the datasets.
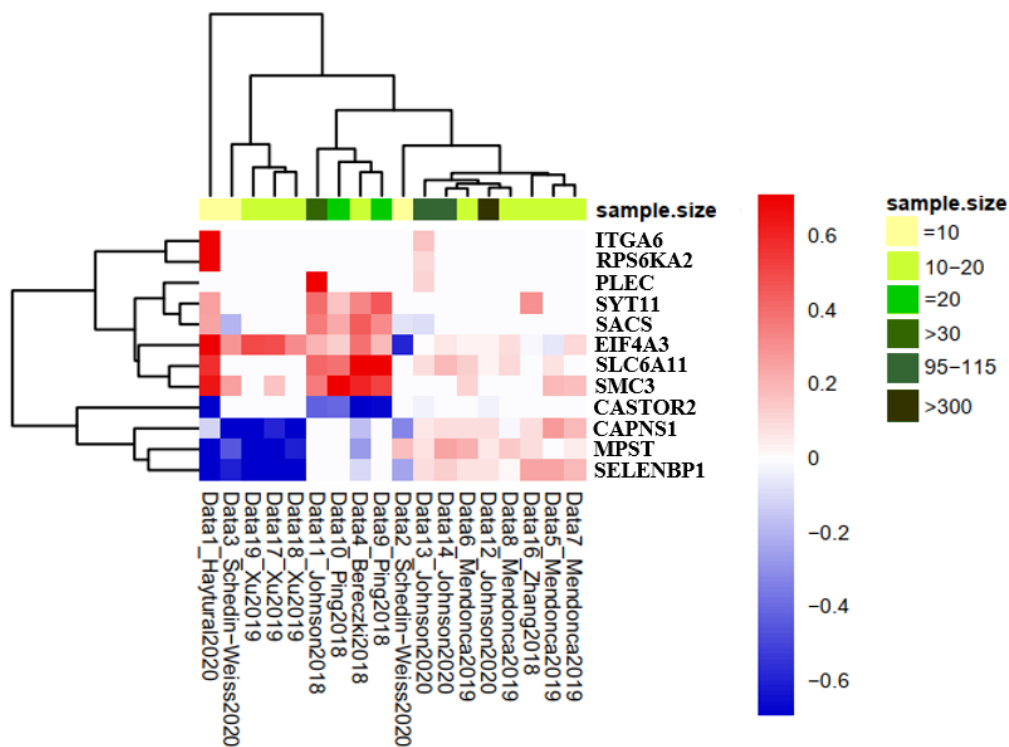
42

Figure 14: Heatmap of 12 significantly altered proteins, which were identified in both meta-analyses.

To get a better understanding of the pathways that are involved in AD, we performed functional enrichment analyses and uploaded the lists of significantly down- and upregulated proteins (FDR < 20%) to DAVID. The downregulated proteins from the labeled data were mainly associated with biological processes related to neuron and axon development, neurogenesis and synaptic signaling, while the upregulated ones were associated with generic processes such as localization and transport. In turn, the downregulated proteins from the label-free data revealed processes involved in mitochondria and energy metabolism, and the upregulated proteins were associated with pathways such as small molecule metabolic process and catabolic process. The functional enrichment analyses revealed that 19 pathways were commonly enriched in both meta-analyses and were, to a large degree, associated with neuron and axon development as well as synaptic signaling. Notably, when investigating individual proteins belonging to the synaptic signaling ontology, only a few proteins were commonly detected in both meta-analyses and these were: brain-specific angiogenesis inhibitor 1-associated protein 2, serine/threonine-protein phosphatase 2B catalytic subunit beta isoform, SNAP25 and septin-5. This points out that even though the precise proteins affected were different in the two meta-analyses, common biological processes could still be altered, and such processes could play crucial roles in AD pathophysiology.

In conclusion, we found proteins that were dysregulated in the same direction between the studies. However, depending on whether the labeled or label-free MS approaches were used,

different proteins appeared to be altered and only seven were commonly and consistently altered in both MS approaches. This could suggest that proteomic studies should be treated with care. Additionally, as reported here, the same biological processes may be detected, despite that individual proteins may differ between the studies.

4.5 PAPER V. EXTRACELLULAR MATRIX PROTEIN DECORIN IS INCREASED IN CSF OF APP KNOCK IN MICE AND EARLY STAGE OF ALZHEIMER'S DISEASE

Examination of postmortem human brain shows that autophagy is impaired in AD (Nixon et al. 2005) and investigation of autophagy status in the *App* knock-in mice is of great interest. In this study, we found increased levels of p62 and LC3-II, markers of autophagy, in $App^{NL\text{-}G\text{-}F}$ mice brain but not in $App^{NL\text{-}F}$ mice brain. As explained in section 3.3.1, $App^{NL\text{-}G\text{-}F}$ mice is a more aggressive model due to the presence of the Arctic mutation, and therefore, it is not surprising that the markers of autophagy were only altered in $App^{NL\text{-}G\text{-}F}$ mice. To explore the CSF proteome of *App* knock-in mice models as well as to investigate whether any proteins related to autophagy could be altered in CSF, we performed label-free MS. We quantified 246 proteins in all samples without any missing values. Compared to the wild-type mice, 38 proteins were significantly altered in $App^{NL\text{-}F}$ mice while 36 were significantly altered in $App^{NL\text{-}G\text{-}F}$ mice (p-value < 0.05). Among those proteins, only 13 were commonly altered in both *App* knock-in models. Notably, we detected six autophagy-related proteins including the extracellular matrix (ECM) proteins decorin and lumican, cathepsin B, cathepsin D, cathepsin S and alpha-mannosidase, which were significantly altered in either $App^{NL\text{-}F}$ or $App^{NL\text{-}G\text{-}F}$ mice.

We further investigated the translational changes in the CSF proteome between the *App* knock-in mice and human subjects across AD continuum (Tijms et al. 2020). Briefly, the human cohort consisted of three AD stages: preclinical AD (NC), prodromal AD (MCI) and mild to moderate AD-type dementia, based on the cognitive performance tests. Additionally, the cohort was also categorized into two groups based on the presence of markers of AD-related pathology: subjects with abnormal CSF $A\beta_{42}$ and t-tau (a+t+) and subjects with abnormal CSF $A\beta_{42}$ and normal CSF t-tau (a+t-). In NC, MCI and AD groups, 1777, 1845 and 1690 proteins were quantified respectively. Since relatively a smaller number of proteins were quantified in the mice CSF proteome compared to the human proteome, to be able to have a better overview of potential changes in the CSF, we did two different comparisons in which either all quantified proteins or only significant changes (p-value < 0.05) were

included in the analyses. Moreover, as *App* knock-in models do not show tau pathology and more common changes were detected between the mouse and human (a+t-) CSF proteome comparisons, we stratified to focus on the human cohorts with a+t- status. We divided the MS data into up- and downregulated proteins in order to get better insights into the protein changes. The comparisons done by Venn diagrams showed that 33 proteins were commonly upregulated and 76 were downregulated both in NC and App^{NL-F} mice. In MCI and App^{NL-F} mice, 46 proteins were commonly upregulated and 84 were downregulated. In AD and App^{NL-F} mice 33 proteins were commonly upregulated and 76 were downregulated. When only significant alterations (p-value < 0.05) were considered, interestingly, the ECM protein decorin was found to be significantly upregulated both in App^{NL-F} mice and NC subjects. In turn, comparing the CSF proteome of App^{NL-G-F} mice with the human cohorts revealed that 38 proteins were commonly upregulated and 84 were downregulated both in NC and App^{NL-G-F} mice. In MCI and App^{NL-G-F} mice, 50 proteins were commonly upregulated and 90 were downregulated. In AD and App^{NL-G-F} mice 37 proteins were commonly upregulated and 86 were downregulated. While decorin was again commonly upregulated across all comparisons, it was not significantly altered in App^{NL-G-F} mice. In the comparisons between *App* knock-in mice and human cohorts, we found several other significantly and commonly altered proteins, including contactin-1, dickkopf-3, fibronectin 1, neurotrimin, SPARC-like protein 1, ECM protein 1, limbic system-associated membrane protein and C-type natriuretic peptide, which are associated with the BBB. Interestingly, the dysfunction of BBB and BCSFB is reported in aging and AD (Montagne et al. 2015; Lendahl, Nilsson, and Betsholtz 2019). Hence, the detection of BBB-related proteins in the CSF could indicate that there might be changes in the BBB composition in both AD models as well as in human patients with a+t- status.

As similar number of proteins were commonly up- and downregulated between *App* knock-in mice and human cohorts, we further assessed how many of those changes could be observed across all groups. This comparison allowed us to detect changes that occur throughout the course of AD both in human and mouse as well as pinpoint specific alterations in one of the *App* knock-in mouse models, thereby enabling us to further explore the differences between the two mouse models. Among those changes, 12 were commonly upregulated in all groups while 54 were commonly downregulated. Furthermore, functional enrichment analyses of upregulated proteins identified processes such as acute inflammatory response, cholesterol and lipid metabolism, while downregulated proteins were associated with processes including cell adhesion, neurogenesis and positive regulation of amyloid fibril formation.

Among the above-mentioned protein hits, we selected decorin for further biochemical characterization, since it has previously been shown to activate autophagy in endothelial cells. Decorin is an ECM proteoglycan, therefore using a modified immunohistochemistry method, we show that decorin was expressed both in somata and neurites of CA2 pyramidal neurons and the parvalbumin-positive interneurons of the hippocampus. While the distribution of decorin-positive neurons did not show any difference between the *App* knock-in *versus* wild-type mice, we found that decorin-positive neurite length of parvalbumin-positive interneurons was significantly decreased in *App*$^{NL-G-F}$ mice. Using western blotting, no difference in the levels of decorin was detected in the membrane fraction of hippocampus between the groups. However, since the entire hippocampus was used for this biochemical analysis, it might not truly reflect to the decorin levels of the parvalbumin-positive interneurons and CA2 pyramidal neurons. These findings suggest that the localization of decorin might be altered in the hippocampus of especially *App*$^{NL-G-F}$ mice. Decorin has previously been shown to activate autophagy (Neill, Sharpe et al. 2017). Using primary neuronal cultures derived from wild-type mice that were treated with decorin, we investigated the effect of decorin on autophagy and found a significant reduction in LC3-II levels. Moreover, when bafilomycin A1, inhibitor of lysosomal proteolysis, was introduced, decorin treatment did not result in any change in the levels of LC3-II, suggesting that decorin most likely increases the autophagosomal-lysosomal degradation.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis provides insights into different mechanisms that are affected in AD pathophysiology, by investigating the recently identified fragment of APP (Paper I) as well as exploring alterations in the proteome of postmortem AD brain and CSF of *App* knock-in mouse (Paper II, IV and V).

In Paper I, we raise a precaution about a non-specific detection of the 20 kDa band, most likely corresponding to the recently identified APP-fragment called CTF-η. Our findings suggest that this fragment derives from APP, evident by the LC-MS/MS analysis, but probably expressed at low levels in human brain.

Another focus of this thesis was to investigate synaptic dysfunction in AD brain in an unbiased manner. We therefore studied a synapse-rich, vulnerable region of the hippocampus, i.e. the OML, that receives the crucial perforant path input. In Paper II, we showed that the combination of LMD with MS (especially with the usage of pre-fractionation prior to LC-MS/MS analysis) is a powerful technique to investigate the proteome of a specific region, as 7322 proteins were quantified in all samples. Our findings suggest that this region indeed exhibited a presynaptic impairment in AD, since many presynaptic proteins, but not postsynaptic proteins, were significantly altered. Five presynaptic proteins (CPLX1, STX1A, SYT1, SYNGR1 and VAMP2) were then selected for immunostaining reported in Paper III. We found that the staining densities of CPLX1, STX1A, SYT1 and SYNGR1 were significantly reduced in AD OML, supporting our proteomics results. The detailed immunohistochemical investigation of hippocampal sub-regions (six other molecular layers) indicates a specific presynaptic impairment of the OML, thereby highlighting the importance of the perforant path (from EC layer II to dentate gyrus) in AD pathogenesis. Since synaptic dysfunction is an early pathogenic event in disease pathogenesis and the maintenance of functional synapses is important for memory and learning, it is plausible that therapeutic approaches aiming to prevent synaptic dysfunction could slow or halt cognitive deficits. Moreover, cascades involved in synapse dysfunction can hold the key to the onset of AD, being the earliest events known to the disease. Together, Paper II and III highlight the importance of a presynaptic failure in AD and suggest that future interventional strategies should be targeted to presynaptic proteins. However, our findings point towards the notion that not all presynaptic proteins were altered to the same degree, suggesting that specific presynaptic pathways could be more vulnerable than others.

To systematically identify proteins and pathways that are commonly altered in AD brain, in Paper IV, we performed a meta-analysis of the MS data, which were generated by either labeled (including our own data from Paper II) or label-free MS approaches. Interestingly, most of the alterations in the proteome (proteins and associated pathways) appeared to be different between the two meta-analyses. For example, functional enrichment analyses found that pathways such as neuron development, neurogenesis were enriched in the labeled data, while pathways related to mitochondria and energy metabolism were enriched in the label-free data. Mounting evidence points towards the involvement of these pathways in AD pathogenesis. Hence, our future plans include a systematic investigation of the proteins that were associated with these pathways. Although we found substantial differences between the two meta-analyses, several pathways such as synaptic signalling were commonly enriched in both. In Paper II, in order to identify proteins related to synaptic signalling that could be altered in AD, our focus was on a highly specific, cell-free region, which is enriched in synapses and nerve fibers. Not surprisingly, we detected substantial alterations in synaptic signalling pathways in the OML of the hippocampus. However, the fact that synaptic signalling pathway was commonly altered in both meta-analyses regardless of the studied brain region (frontal and temporal cortices or cingulate gyrus) emphasizes the importance and involvement of synaptic impairment in AD brain. We are currently investigating the proteins belonging to the synaptic signalling pathway. Despite all methodological differences between the selected studies in Paper IV, seven novel proteins were significantly altered in both meta-analyses and more importantly showed consistent fold changes across the proteomic studies. Future studies should focus on better understanding their role in AD.

The investigation of CSF proteome of *App* knock-in mice, in Paper V, revealed alterations in several BBB-associated proteins such as decorin, suggesting that BBB composition might be affected in *App* knock-in mice, which needs to be clarified. This could further illuminate how well the AD models can mimic the other components of AD pathology, for example CAA, which is often observed in AD brains. In this study, we also explored the translational changes in the CSF proteomes between *App* knock-in mice and human subjects (i.e. NC, MCI and AD stages) and reported commonly up/downregulated proteins. Importantly decorin was significantly upregulated both in App^{NL-F} mice and NC subjects. Additional biochemical analysis showed that decorin is exclusively expressed in different neuronal subgroups within the hippocampus, with a role to be discovered in *App* knock-in mice.

The comparison of CSF proteomes between human and mouse also allowed us to detect proteins that are altered throughout the entire course of AD, in other words from NC to MCI

and to AD stages as well as from the milder mice model $App^{NL\text{-}F}$ to the more aggressive model $App^{NL\text{-}G\text{-}F}$. The functional enrichment analyses show the involvement of pathways such as cholesterol and lipid metabolism, acute inflammatory response, cell adhesion and neurogenesis. The detection of translational changes between mouse and human CSF proteome is indeed exciting and promising, as it illustrates that the *App* knock-in mice indeed recapitulates some aspects of the AD pathogenesis. It is interesting that neurogenesis pathway was altered in the CSF proteomics and complements our findings from the meta-analysis. An immediate perspective to the present work would be to compare the results of Paper IV and V in order to explore which of the proteomic changes in AD brain are reflected in AD CSF and how they could translate to the CSF of AD mouse model.

In summary, this thesis adds substantial new knowledge on proteins and pathways involved in AD pathogenesis from a boarder (analysis of bulk tissue and CSF) to specific (EC-dentate gyrus connection) perspective. Future studies of the reported pathways could elucidate the involvement of specific proteins in AD. In order to better understand the presynaptic failure that is restricted to the OML in AD, several questions need to be addressed. Why are certain presynaptic proteins more affected than others? Which mechanisms might be driving the presynaptic impairment? Is the downregulation of specific presynaptic proteins the cause or the consequence of synaptic impairment? Could the EC neuronal loss be the direct cause of the observed presynaptic failure?

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