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CHARACTERIZATION OF PROTEINS INVOLVED IN DISEASE PROGRESSION IN ALZHEIMER DISEASE

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Characterization of proteins involved in disease progression in Alzheimer disease
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Abstract

Alzheimer disease (AD) is a neurodegenerative disease and severe neuronal loss is already taking place at the time of diagnosis. AD affects nearly 50 million people worldwide and the incidence is expected to rise significantly in the coming decades, making the disease a high priority healthcare concern. There are two main hallmarks of AD pathology, the intraneuronal tau tangles and the mostly extracellular amyloid plaques. Current treatment strategies for AD have tried targeting both tau and amyloid plaques although with limited success. However, since AD is a multifactorial disease with many risk factors, a multifaceted approach is necessary to tailor treatment. Further understanding of the pathogenesis of AD is required to enable the development of novel drugs and treatment strategies. In this thesis, we use proteomics, bioinformatics, and microscopy in an attempt to identify and characterize proteins which might influence AD progression and pathogenesis. We utilize the AD mouse model $App^{NL\text{-}F/NL\text{-}F}$, which is a knock-in mouse model that overproduces amyloid β leading to Aβ plaque pathology without artifacts associated with APP overexpression applied in previous AD mouse models. We also study AD brain and neuronal cultures obtained from the $App^{NL\text{-}F/NL\text{-}F}$ mouse.

In Paper I, we show that a combination of proteomics and bioinformatics can identify proteins involved in AD pathology in the $App^{NL\text{-}F/NL\text{-}F}$ mice. Using immunofluorescence, we discovered that huntingtin, the pathogenic protein in Huntington disease, is abundant in the hippocampus of the $App^{NL\text{-}F/NL\text{-}F}$ mouse at a presymptomatic stage. We furthermore localized the expression of huntingtin to pyramidal neuronal cells early in the mouse life span. In Paper II, we expand on our findings of huntingtin in the $App^{NL\text{-}F/NL\text{-}F}$ mouse model by studying and characterizing the expression of huntingtin in the brain of AD patients and healthy controls. We found that huntingtin was increased in the frontal cortex and the hippocampus of AD patients. Huntingtin could be found in pyramidal neurons within both the frontal cortex and the hippocampus. The accumulation pattern of huntingtin in AD did not mimic the accumulation found in Huntington disease as there was no correlation between astrocytes and huntingtin in AD brain. Furthermore, using confocal microscopy we concluded that there was no association of tau protein and huntingtin in AD brain. In paper III, we investigated the localization of the protein DDX24, a protein identified via proteomics, in AD brain and neuronal cultures derived from the $App^{NL\text{-}F/NL\text{-}F}$ mouse. DDX24 belongs to a family of proteins consisting of putative RNA helicases and is implicated in translation initiation, nuclear RNA splicing and ribosome assembly. We show that DDX24 accumulates in AD brain where it associates to areas important for memory formation. We also show that DDX24 is increased in the brain of AD patients compared to healthy controls. We found DDX24 to be increased in the brain and in the neuronal cells of the $App^{NL\text{-}F/NL\text{-}F}$ mouse. Decreasing DDX24 levels increased APP levels in neuronal cells. DDX24 levels also appeared to be regulated by amyloid load or vice versa. In paper IV, we investigate neuritogenesis and neurogenesis in AD brain and in the $App^{NL\text{-}F/NL\text{-}F}$ mouse model. We show that Ankyrin-3, a protein important for axonal development, is detectable in AD brain and in neurons derived from the $App^{NL\text{-}F/NL\text{-}F}$ mouse. We also show that a known biomarker, doublecortin, is increased in embryonic and young mice brain derived from the $App^{NL\text{-}F/NL\text{-}F}$ mouse when compared to controls.

Overall, our studies use proteomics, bioinformatics, and microscopy to describe the presence of huntingtin, DDX24, Ankyrin-3 and doublecortin in the brain of AD patients and $App^{NL\text{-}F/NL\text{-}F}$ mouse. These results can prove helpful in our future understanding of AD pathogenesis.

List of scientific papers

I. **Proteomics Time-Course Study of App Knock-In Mice Reveals Novel Presymptomatic Aβ42-Induced Pathways to Alzheimer's Disease Pathology**
Schedin-Weiss S, Nilsson P, Sandebring-Matton A, **Axenhus M**, Sekiguchi M, Saito T, Winblad B, Saido T, Tjernberg L
Journal of Alzheimer's Disease. 2020;75(1):312-335

II. **Huntingtin Levels are Elevated in Hippocampal Post-Morten Samples of Alzheimer's Disease Brain**
Axenhus M, Winblad B, Tjernberg L, Schedin-Weiss S
Current Alzheimer Research. 2020;17(9):858-867

III. **DEAD Box Helicase 24 is Increased in Alzheimer Disease Brain and Influences Presymptomatic Pathology**
Axenhus M, Doeswijk T, Nilsson P, Winblad B, Tjernberg L, Schedin-Weiss S
Manuscript

IV. **Neuritogenesis and Neurogenesis in Alzheimer Disease Brain and the *App*$^{NLF/NLF}$ Mouse model**
Axenhus M, Malik C S, Doeswijk T, Nilsson P, Winblad B, Tjernberg L, Schedin-Weiss S
Manuscript

Scientific papers not included in this thesis.

I. **Microdissected Pyramidal Cell Proteomics of Alzheimer Brain Reveals Alterations in Creatine Kinase B-Type, 14-3-3-γ, and Heath Shock Cognate 71**
Sandebring-Matton A, **Axenhus M**, Bogdanovic N, Winblad B, Schedin-Weiss S, Nilsson P, Tjernberg L
Frontiers in Aging Neuroscience. 2021;19;13:735334

Contents

List of abbreviations

AD	Alzheimer disease
AICD	APP intracellular domain
APP	Amyloid precursor protein
Asp-Glu-Ala-Asp	DEAD
Aβ	Amyloid β-peptide
CSF	Cerebrospinal fluid
CTF	C-terminal APP fragment
DEAD Box Helicase 24	DDX24
FAD	Familial Alzheimer Disease
FL-APP	Full-length Amyloid precursor protein
MCI	Mild cognitive impairment
MMSE	Mini Mental State Examination
NFT	Neurofibriallry tangles
PSEN1	Presenilin 1
PSEN2	Presenilin 2
sAPP	Soluable APP
WT	Wild type

1 Introduction

1.1 Alzheimer disease

Dementia is one of the most significant healthcare concerns facing the world today. With an aging population, the incidence of dementia is expected to increase significantly in the coming decades. The number of people suffering from dementia is currently estimated at around 50 million worldwide and expected to reach 130 million by 2050 [1]. The economic cost associated with dementia is currently over 1 trillion US dollars as measured by the World Health Organization [2]. Such a large elderly population with dementia would put an unprecedented strain on healthcare systems, highlighting a desperate need of therapeutical intervention. Alzheimer disease (AD) is the most common form of dementia and represents approximately 60% of all dementia cases [3].

The first pathological hallmarks of AD were characterized by Dr Alois Alzheimer in 1906 [4]. Dr Alzheimer noted severe neuronal loss in the cerebrum of patients suffering from personality changes and memory loss shortly before their deaths. The neuronal loss was concentrated to lesions in specific subareas of the brain. These lesions are what we today commonly refer to as amyloid β-peptide (Aβ) fibrils and abnormal accumulations of intracellular tau protein, neurofibrillary tau tangles (NFT) [5]. The disease, which would go on to be named after Dr Alzheimer, would prove to be one of the 21st century's greatest health concerns.

Research into the pathogenesis of AD has steadily increased during the last decades and the current field of AD pathology is represented by two main pathologies, Aβ plaques and NFT. It is believed that the accumulation of extracellular amyloid plaques consisting of Aβ fibrils together with neurofibrillary tangles cause neurological pathology such as neuron loss, synaptic dysfunction, neuroinflammation and brain atrophy [6]. The Aβ theory

suggests that accumulation of Aβ is the main driving factor for AD and high levels of its neurotoxic variants drive disease progression [7,8]. This Aβ centric theory is termed the "amyloid cascade hypothesis". Recent advances in anti-Aβ treatment, such as Lecanumab, an anti-Aβ antibody which inhibits Aβ accumulation and decreases cognitive decline by 27%, give credence to the idea of an anti-Aβ AD treatment strategy, prompting research into this area [9].

Risk factors that have been shown to influence AD progression such as cardiovascular status, socioeconomic factors, educational and nutritional status [10–12]. In addition, some AD cases can also be classified as early-onset, due to the autosomal dominant inheritance of specific risk genes [13–15]. Although these cases provide genetic guidance in the evaluation of AD mechanisms, they are rare and account for <1% of AD cases worldwide. The multifactorial genesis of AD requires a broad scientific approach and the presence of additional disease mechanisms, such as neuroinflammation, cannot be excluded. Thus, the characterization of further disease mechanisms is needed in order to facilitate the development of effective AD treatments.

In this thesis, we will go over the current knowledge regarding AD pathological mechanisms. We will also describe the processes used for the identification of new proteins which might be involved in AD pathogenesis and appropriate methods for the detection of proteins involved in AD pathogenesis.

The main part of this thesis will be about the identification and characterization of proteins which might be involved in AD pathogenesis. I will describe how a multimodal approach to proteomics, microscopy and animal studies can elucidate potential mechanisms driving AD progression. I will describe how we use bioinformatics to identify up-stream disease regulators in the *App*$^{NL-F/NL-F}$ mouse and how we confirm these findings in AD brain. Furthermore, I describe the proteins DDX24 and Ankyrin-3 in the context of AD using proteomics and bioinformatics.

1.2 Neurodegeneration

1.2.1 Neurodegenerative disorders

The age-dependent decay of neuronal cells in the central nervous system is called neurodegeneration. Neurodegenerative disorders refer to those diseases in which the process of neurodegeneration is accelerated, resulting in the loss of synapses, neurons and brain functions [16]. These diseases are sometimes driven by aggregations of neurotoxic protein aggregates which are often disease specific. In AD for example, the accumulation of Aβ and neurofibrillary tau tangles (NFT) cause neurotoxic lesions and neuronal loss. Parkinson disease shows α-synuclein aggregation while Creutzfeldt-Jacob disease is caused by the accumulation of prions in the brain [17,18]. Huntington disease afflicted brains show the presence of a mutated and aggregated variant of an otherwise harmless protein called huntingtin [19]. Neurodegenerative diseases are diagnosed and treated separately although their common feature is incurability and lack of effective treatment options.

1.2.2 Dementia

Dementia is a category of diseases defined by the progressive loss of memory and cognitive functions. AD is the most common form of dementia together with vascular dementia, dementia with Lewy bodies, and frontal temporal lobe dementia [20,21]. AD is considered a primary dementia, as it is a disease caused by the specific buildup of neurotoxic proteins with memory dysfunction as a primary symptom. Some neurodegenerative diseases also cause dementia as part of their pathological process, for example the dementia observed in late-stage Huntington or Parkinson disease [22,23]. Although these dementias can be just as symptomatic as primary dementias, they are nonetheless

secondary symptoms of a disease which presents with primarily motor symptoms. These dementias are therefore referred to as secondary dementias. The onset of dementia in these diseases is also a late process and not necessarily a compulsory stage of the disease. Lastly, vascular dementia is often considered to be a third type of dementia, being the results of poor cardiovascular status rather than specific protein accumulation [24]. Lastly, there are mixed types of dementias with components from the main categories.

1.2.3 Alzheimer disease and public health

AD is a public health crisis. Current estimates of people living with AD assume that 40 million people are living with the disease. In Sweden alone the number of people living with dementia is assumed to be more than 200 000 and 100.000 with AD [25]. The incidence of AD is expected to go up as the elderly population of the world increases and the number of AD cases is expected to increase as diagnostic procedures are improved [26]. This will lead to a significant increase in AD cases during the foreseeable future. In Sweden, the socioeconomic burden of dementia is calculated to be greater than the burden of cancer, cardiovascular disease, and stroke put together [25]. The coming increase in the number of people living with dementia will therefore have a devastating effect on healthcare across the world, making the area of AD research crucial in order to prevent or at least mitigate some of the impact of the disease.

1.3 Alzheimer disease phases

1.3.1 The preclinical phase

AD is most easily understood as a gradual disease with a very long preclinical stage. The preclinical stages of AD start typically 10-20 years before the onset of symptoms and can be detected by various

biomarkers [27]. In the very early preclinical phase there is an increase in CSF-Aβ followed by a reduced ratio of Aβ 42/40 in the cerebrospinal fluid (CSF) is linked to increased accumulation of Aβ in the brain [28]. The levels of total and phosphorylated tau are increased in the CSF and serum early in the disease [29,30]. Imaging biomarkers can also be used to show different accumulation patterns of key biomarkers in the brain (**Fig. 1**) [31–34]. The onset of symptoms in AD is not indicative of an acute disease but rather the effects of multiple decades of build-up of neurotoxic elements and subsequent neuronal loss. This advanced disease stage at the time of diagnosis is one of the main reasons to why AD is considered a particular therapy resilient neurodegenerative disorder. The idea of presymptomatic diagnosis and treatment is attractive but complicated due to the difficulty in diagnosing the disease before symptoms occur.

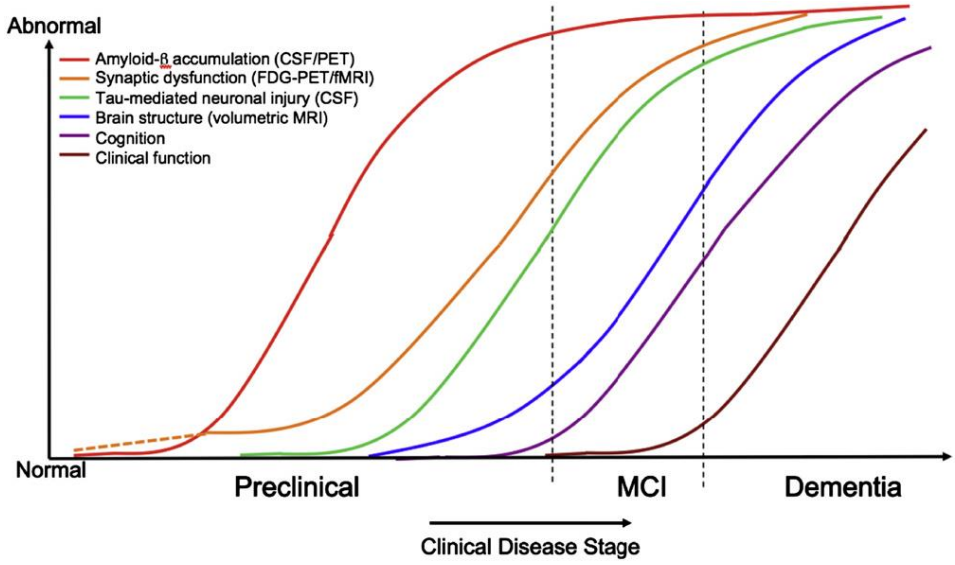

Fig. 1: Graph showing the preclinical phase of Alzheimer disease using biomarkers. Aβ is identified through cerebrospinal fluid Aβ42 assay or PET amyloid imaging, while synaptic dysfunction is detectable by FDG-PET or fMRI. Neuronal injury is identified through cerebrospinal fluid tau or phospho-tau, and brain structure is indicated by structural magnetic

resonance imaging. Clinical function is also illustrated. Figure taken with permission from publisher, Jack et al [35].

1.3.2 The clinical phase

The preclinical stage eventually develops into the clinical stage, which presents with subtle decline in episodic memory, problems with everyday tasks, and executive problems [36]. These symptoms are of more significant character than what can be expected from the normal aging process, although they are not as debilitating as fully developed AD. Rather, these symptoms are signs of the pre-AD stage of the disease, which is termed mild cognitive impairment (MCI) [37]. Patients with MCI might not necessarily progress into dementia, but they do have an increased risk of developing AD or other dementias [38].

As MCI progresses to AD, more severe symptoms start to present themselves, the patient might experience an inability to plan and solve problems. They might have more confusion, poor judgement capabilities or develop personality and behavior changes. Relatives and friends will often report that patients at this stage are behaving differently or strange. Language will deteriorate and communication becomes difficult. Patients at this stage of the disease are often dependent on others and need large amounts of help in order to perform everyday tasks [36].

Secondary psychiatric conditions might develop, such as depression and delusions [39,40]. The descriptive symptoms are many and patients vary in their presentation and progression, although the gradual decline in functionality over time is a common feature that can be expected from all AD patients.

1.3.3 Alzheimer disease diagnosis

The diagnostics of AD are extensive, requiring a team-based approach. Physicians must not only correctly diagnose the dementia disorder but must also exclude any other disorders which may mask as dementia amongst the elderly, such as depression or confusion [41]. As part of the diagnosis, the presence of neuropathology needs to be established. This includes a full patient history including caregiver history, a Mini-mental state examination (MMSE), clock test, neuropsychological evaluation, in some cases CSF and blood tests, and radiological imaging [34,42–44]. People under the age of 65 are rarely diagnosed with AD and without a genetic predisposition. At 65 years of age, 1-3% of the population is expected to have AD [45]. Above the age of 65, the AD risk increases exponentially with age. Once diagnosis has been established, an AD patient can be expected to survive for an average of 4-8 more years, although cases exist of patients living up to 20 years after diagnosis. AD patients are susceptible to comorbidity and death is usually caused by respiratory infections and ischemic heart disease [46].

1.3.4 Alzheimer disease risk factors

AD exist in a genetic or a sporadic variant. The genes associated with familial AD (FAD) account for less than 1% of the attributable risk of developing AD. The hereditary forms are caused by mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1) or 2 (PSEN2) genes, which have 100% penetrance [15]. The aggressive disease progression is often accompanied by a heavy load of Aβ plaques. Around 20 known genes are coupled to increased risk of AD and the most commonly described one of these is ApoE. ApoE exist in three isoforms, ApoE2, ApoE3, or ApoE4. ApoE regulates lipid and Aβ metabolism, processes that are suspected to be dysfunctional in the presence of the isoform ApoE4 [47].

Although the genetic component is well characterized, most AD cases are sporadic and have no as-of-yet known genetic predisposition. Rather, the development of sporadic AD seems to be a combination of risk factors that together produce the necessary pathological conditions for the underlying neuropathological processes in the brain to occur. Age is the single largest risk factor for AD as aging brings on predisposition to dementia in the form of reduction in brain volume and weight, as well as synaptic loss. Comorbidities, which are common in elderly population, also contribute to the risk of developing AD or other types of dementia. These comorbidities include, amongst others, cholesterol dysfunction, alcohol addiction, high blood pressure and psychiatric disorders [38,48]. Other common risk factors for developing AD include either external or internal neuronal stress factors such as genetic factors, traumatic brain injury, infection, vascular dysfunction, heavy metal poisoning, and others [49–51].

1.4 Alzheimer disease pathology

As mentioned, two types of neuropathological changes can be found in the AD brain. The first type consists of accumulation, either from NFTs, Aβ plaques, dystrophic neurites or other deposits. These are referred to as positive lesions. The second category of changes are referred to as negative lesions, these include changes that produce atrophy or synaptic loss [52]. Furthermore, inflammation, oxidative stress and damage to supporting neurons must also be considered when discussing AD neuropathology as oxidation causes significant neuronal damage in elderly brain tissue [53].

1.4.1 Amyloid precursor protein

Aβ is derived from APP which is a type I transmembrane protein found throughout the body. It is ubiquitously expressed in the brain and encoded by the APP gene found on chromosome 21 [54]. There are about 25 mutations in the APP gene associated to AD [47]. There is only a single known protective APP mutation named APPA673T, more commonly referred to as the Islandic mutation. Common in Icelandic and Scandinavian populations, the Icelandic mutation is associated with protection against amyloid pathology and AD. APP belongs to a family of proteins which also includes amyloid precursor-like proteins 1 and 2 [55]. The APP family can be found throughout the body, including the central nervous system [56]. APP contains multiple domains, which are cleaved and processed during maturation allowing for APP and its fragments to be involved in numerous processes within the body [57]. APP have known roles within neurodevelopment, cell division, synaptic functionality, and cell signaling [58]. There are also studies suggesting that APP have regulatory effects that could influence memory function and synaptic density [59]. APP is mainly involved in regulation of the growth of neurites, vesicular transport, and cell interactions. The constitutional APP family is important for basic synaptic functionality including memory and learning [60]. APP is also an essential protein for brain development, and thus cannot be easily targeted for genetic silencing in embryonic models [61]. The deletion of APP in mice models have unilaterally produced pathological results including lowered spine density, disrupt learning processes, motor dysfunction, and induced autism-like behavioral phenotypes [62,63]. Removing two or more of the APP family members induce shorted life spans in animal models [64].

Even though APP is a key player in AD pathogenesis, the targeting and manipulation of APP has met with limited success in clinical drug development [65]. APP is however a useful tool in the development of

AD animal models. The integration of humanized APP into mice in order to produce a stable animal model for AD pathogenesis has been a very successful endeavor during the last years, which has resulted in highly stable animal models that are useful for studying Aβ pathology and metabolism [66].

1.4.2 The amyloid β-peptide, Aβ

When discussing Aβ in the context of AD we are mainly referred to peptides of 40-43 amino acids that constitute the Aβ plaques found in the AD brain. Aβ is derived from the proteolytic processing of APP by β- and γ-secretase. Human APP is a transmembrane protein with the C-terminus facing the cytosolic side and the N-terminus facing the extracellular/luminal side [54]. The processing of APP is usually described in the form of two pathways, the amyloidogenic pathway and the non-amyloidogenic pathway.

1.4.3 The amyloidogenic pathway

The amyloidogenic pathway starts with full-length APP (FL-APP) undergoing cleavage by β-secretase. The resulting β-cleaved fragments of APP are a soluble (sAPP) fragment and a membrane-bound C-terminal fragment (CTF). CTF goes on to be processed by γ-secretase, resulting in Aβ and a residual APP fragment named APP intracellular domain (AICD) [54,67]. γ-Secretase can generate Aβ of different lengths by processing at various sites along the protein sequence. The most common form contains 40 amino acids, Aβ40, and is less neurotoxic and aggregation-prone than its longer cousins Aβ42 and Aβ43. Aβ42 tends to aggregate into oligomers and form fibrils, which make up the main components of Aβ plaques in AD brain [68]. The amyloid cascade

hypothesis is a mainstay theory of AD pathology that state that accumulation of Aβ oligomers is a crucial step in the disease that disrupt normal neuronal function, induce dysfunction and cell death [69].

Although the amyloid cascade is the prevalent theory behind amyloid pathology in the human brain, the underlying mechanisms are slightly different between familial and sporadic cases. In familial cases, mutations affecting the amyloidogenic pathway lead to increased ratio of Aβ42/Aβ40 or increased total levels of Aβ42 [70]. In sporadic cases it is less clear what triggers the disease development, although Aβ clearance, e.g. autophagy, and enzymatic degradation could be important factors. No Aβ fragments are generated in the non-amyloidogenic pathway, due to FL-APP being processed via α-secretase instead of β-secretase, generating CTF, which cleaves at either amino acid 15 or 16 in the Aβ sequence. CTF can then be cleaved by γ-secretase to produce a short peptide called p3 and AICD [71].

1.4.4 The non-amyloidogenic pathway

The non-amyloidogenic pathway involves the cleavage of APP by the α-secretase enzyme. This cleavage produces a soluble fragment known as sAPP, which has been shown to have neuroprotective effects [72]. The remaining fragment, known as C83, is then cleaved by γ-secretase, which results in the production of the p3 peptide. The p3 peptide is not as soluble as Aβ and likely more prone to aggregation but not found in the brain [73].

While the non-amyloidogenic pathway has been identified as a significant therapeutic target for AD, there are currently no drugs that target this pathway. However, research in this area is ongoing and it has been suggested that the

use of α-secretase stimulators could promote the processing of APP via non-amyloidogenic pathway and provide neuroprotection against AD [74].

In conclusion, while the amyloidogenic pathway is the primary focus of research on AD, it is important to remember that there is another pathway that does not involve amyloid formation.

1.4.5 Tau protein

Tau is an intracellular protein that regulates cytoskeleton function. It is coded by the MAPT gene in human and consists of six isoforms. During normal conditions, tau stabilizes microtubules and regulates axonal transport while functioning as a scaffolding protein [75].

In the amyloid cascade hypothesis, the presence of tau pathology is considered to be downstream of Aβ pathology. Post-translational modifications cause the release of tau proteins from microtubules and the formation of insoluble aggregates [76]. Although both Aβ and tau are present in the AD brain, little is known about their interactions with each other and the role of tau in Aβ pathology is not elucidated.

During AD, hyperphosphorylation of tau is induced, resulting in aggregations of tau proteins. Phosphorylation of tau is an essential part of AD pathology as levels of phosphorylated tau are significantly higher in AD brain compared to normal brain [77]. Non-phosphorylated tau tends to be similar in levels between AD and controls, implicating phosphorylated tau in the development of AD pathology [78].

The presence of NFT interferes with normal synaptic function. Synaptic dysfunction is an important aspect of AD, since receptors, organelles

and proteins, which rely on functional microtubules for transport, are unable to make it to the synapse [79]. NFT are ubiquitous in AD but can also be found in other diseases, which are collectively named tauopathies [80]. Little is understood about the mechanism of tau tangle formation and why tauopathy develops in the brain. Regardless of the cause, the impact of tau tangles on the pathology in AD is well established. Furthermore, Aβ toxicity appears to be mediated by tau while Aβ increases tau pathology in mouse models injected with Aβ [81].

1.5 Other diseases with tauopathy

As discussed, tau, in particular phosphorylated tau, is an important part of AD pathology although tau is also detected in other neurodegenerative diseases, termed tauopathies. Niemann-Pick type C disease, a rare inherited lysosomal lipid storage disorder, is also characterized by the accumulation of tau pathology, although not of phosphorylated tau [82]. Other diseases include progressive supranuclear palsy, chronic traumatic encephalopathy and subacute sclerosing panencephalitis [83–85].

1.6 Alzheimer disease clinical trials

1.6.1 Treatment strategies

The pathogenesis of AD makes drug development complicated and very expensive. Although, due to the debilitating nature of the disease and the huge cost involved in the care for dementia patients, research continues.

One obvious intervention against AD may seem to be to inhibit the γ-secretase function and reduce amyloid load in the brain. However, γ-secretase is ubiquitous and have over 100 substrates and is thus important for vital physiological functions, making total blockage of the enzyme problematic [86]. Thus, clinical trials with γ-secretase inhibitors have been associated with considerable side effects and, in the end, failure. Recent drug development has tried to avoid this shortcoming by focusing on lowering Aβ42/40 ratios and leaving γ-secretase active [87].

Clinical trials concerning AD treatment have ranged from lifestyle interventions to vaccines against toxic Aβ and tau protein build-up. Currently, only two drugs, the monoclonal antibodies Lecanemab and Aducanumab, have proven significant effects on lowering Aβ plaque pathology whereas only Lececanemab and Donanemab have shown a positive effect on cognition [9,88]. Increasing nerve growth in AD is considered a novel approach and has recently gained some headway as a potential treatment although the clinical implications are far away [89,90].

1.6.2 Alzheimer disease treatment

Except for therapies such as Lecanumab and Aducanumab, two types of drug classes can be used to treat AD symptoms, cholinesterase enzyme inhibitors and an NMDA receptor antagonist [91,92].

The blockage of acetylcholinesterase by inhibitors results in increased acetylcholine levels at the synaptic cleft, improving cognitive function [93]. NMDA receptor antagonists prevents overactivation of glutamate receptors and allow for normal neuronal function by preventing cell death and synaptic dysfunction [92]. Neither of these drugs cure or

reduce the onset of the disease and are only used to treat symptoms. Commonly used AD medications include Donepezil, Galantamine, Rivastigmine and Memantine.

The current compounds in clinical trials often target only one protein or peptide, such as Aβ, tau or BACE1. Difficulties with defining homogenous patient groups, starting too late during disease development and low blood-brain-barrier penetration are causes that are often cited for the reason of clinical trial failure [94]. However, the most likely explanation for the failures of AD treatment trails can be found in the origin of the disease. Old patients with multiple pathological processes are unlikely to benefit significantly from effecting a single pathological pathway and multimodal intervention is most likely needed.

Treatments for AD has focused on the two most prominent pathological elements of the disease, namely the tau protein and the Aβ plaque. The current state of research into AD is more and more focused on the presymptomatic stage of the disease. The idea of prevention and hindrance has gained popularity during the latest decades of failed attempts of reversing the neuronal injury induced during AD. The search for disease mechanism modulators is a new area which has recently become popular within AD research.

1.7 Mouse models for Alzheimer disease

Due to the advanced disease stage at symptom presentation, the multifactorial pathogenesis and the widespread epidemiology of AD, the study of the disease presents many challenges. Animal models have become indispensable in the recent decades as a tool to understand

the pathological mechanisms of the disease [95]. They have also been used to evaluate therapeutical strategies and studying the disease progression in controlled environments. The earliest mouse models employed transgenic methods which overexpressed genes identified from familial cases and that were implicated in AD pathogenesis. Transgenic AD mouse models rely on the integration of AD pathology associated gene variants which produce a disease phenotype [96]. Examples of these include the 5xFAD, Tg2576 and the Tg-Arc/Swe mouse models [97]. The basis for this rational was that the overexpression of underlying disease genes such as APP and tau might enable study and prevention of the underlying disease lesions. AD animal models, while relatively easy to produce, have several drawbacks. AD mouse models employing transgenic APP overexpression have an overproduction not only of Aβ but also of by-products which naturally occur during the amyloid metabolism, in particular APP artefacts [98]. The use of the transgenic AD models is still popular within AD research, due to the relative availability of transgenic mouse models, but mouse models with more exact gene modification are gaining popularity. There are also inherent limitations to the use of mouse model system such as differences in anatomy and life span.

New generations of mouse models for AD can be created by knock-in techniques, where the overexpression observed in transgenic mouse models can be avoided. Thus, an increase in Aβ load can be obtained without artifacts from the overexpression of APP. These mouse models typically lack the tau pathology observed in human brain and do not produce NFT. One such mouse model for AD is the $App^{NL-F/NL-F}$ mouse, which is an *App* knock-in mouse model with APP that contains a humanized Aβ sequence and the Swedish and Beyreuther mutations, leading to increased Aβ42 levels, making it suitable for *in vivo* studies of amyloid pathogenesis [99]. Developed in Japan, this mouse model has

been the basis of thousands of studies during the last years and have accelerated the scientific understanding of Aβ-related pathology.

1.8 Novel Alzheimer disease mechanisms

1.8.1 Regulating Alzheimer disease progression

The widespread failure of clinical trials and experimental drugs to reduce the disease burden in AD patients with advanced or clinical stages of the disease have moved drug therapy research back into the preclinical stage. The preclinical stage of AD is being vigorously studied to identify a possible mitigating protein or mechanism which can be efficiently targeted as a potential treatment before the onset of symptoms. The identification and characterization of proteins which influence disease progression in AD is an active field of research. Using newly developed methods, such as transcriptomes and artificial intelligence, complex protein analysis which were previously unavailable can be performed [100,101]. Identifying proteins that influence AD pathogenesis at the preclinical stage is the first step to describe mechanisms that can be targeted to delay disease onset.

The underlying pathological processes in AD are mainly related to neuronal dysfunction. Interest have therefore been aimed at preventing neuronal decay. Although the current treatment strategies of clearing out toxic protein products have shown promise, presymptomatic targeting of pathological elements is essential in preventing neuronal loss early in the disease progression.

1.8.2 Identification of proteins involved in Alzheimer disease pathogenesis

The identification of proteins involved in AD pathogenesis requires a multimodal approach utilizing bioinformatics and biomolecular testing. Simply elevated proteins levels are rarely correlated to disease severity and a combination of techniques is required to elucidate any potential mechanisms by which proteins might exert their influence on pathogenesis. A recent proteomics study of $App^{NL-F/NL-F}$ mice has shown that protein levels are altered even before the appearance of amyloid plaques and cognitive decline [102]. Particularly, proteins involved in synaptic function, neuronal development and neuronal growth are affected. Thus, it is speculated that Aβ42 induces alterations to neurogenesis, neuritogenesis and synaptogenesis prior to the development of Aβ plaques or cognitive decline.

1.8.3 Aβ as mediator of Alzheimer disease pathology

Aβ is a key player in AD pathology but the molecular details of its role in the initiating events that eventually lead to clinical AD are unknown. Several mechanisms of Aβ mediated toxicity have been suggested. Extracellular oligomeric Aβ42 is suspected of mediating vulnerability in AD brain and cause impairment in mice models [103]. Other studies suggest that intracellular Aβ42 is neurotoxic [104]. Recent data using super-resolution microscopy have enabled detailed studies of the subcellular location of Aβ42 and its substrate in hippocampal neurons. Interestingly, both CTF, γ- secretase and Aβ42 were found to be enriched in small vesicles in the presynaptic side of the synapse [105]. Thus, it is highly probable that the presynapse is a site of Aβ production and may reflect that Aβ42 have a physiological function at this site. In contrast, larger vesicles, such as late endosomes, lysosomes and

autophagosomes contained Aβ in the soma region, where it was also shown to aggregate. Thus, Aβ42 is clearly present in different pools in neurons, some of which may be neurotoxic and some of which may be physiologically relevant. These findings make it highly important to study the relationship of APP processing and Aβ with other pathways and determine how they are regulated in different conditions. Such studies may enable more specific Aβ42 targeting approaches than those that have been in clinical trial up to now.

1.8.4 Huntingtin and Alzheimer disease

Our recent efforts aimed at finding proteins that regulate AD pathology have so far resulted in two such protein candidates, namely Huntingtin and DEAD Box Helicase 24 (DDX24). Huntingtin, which is the pathogenic protein of Huntington disease, is increased in AD brain and accumulates in a pattern that does not correlate with the disease pattern of Huntington disease [106]. Huntingtin is a protein of a predicated mass of around 350 kDa. Its physiological function is not well understood but it is implicated in several physiological processes including axonal transport and fetal development [107]. Huntingtin knock-out mice do not survive adolescence, indicating a necessary developmental function [108]. Huntingtin is also strongly associated with vesicles and appears to have a functional role in cytoskeletal stability and transportation of mitochondria [109]. Mutations in the HTT gene can lead to PolyQ expansions within the protein. These accumulations are neurotoxic and result in the development of specific neurodegenerative pathways. Such pathological huntingtin is associated with its namesake, Huntington disease [110], a disease which causes degeneration of dopamine-producing neurons in the Substantia Nigra, resulting in significant neurological symptoms and eventually death. The disease is inherited in an autosomal dominant fashion, and therefore, family clusters of the

disease are common [111]. Treatment strategies targeting huntingtin have so far not led to a successful treatment. Significant side effects and insufficient clinical effects have been the main obstacles to developing an effective treatment for Huntington disease [112].

Using proteomics, bioinformatics, and microscopy we were able to identify the presence of huntingtin in both the AD brain and in the brains of AD mice. Similar results have previously only been described in a small observational study. The idea of cross-seeding between AD and Huntington disease has gained ground in the recent years with studies having found increased huntingtin levels in AD mouse models as well as increased number of intermediate huntingtin CAG-repeat alleles in AD patients [113,114]. However, there are currently no studies that clearly connect the different pathological processes which occur in Huntington disease and AD.

1.8.5 DDX24 and Alzheimer disease

DDX24 is a fairly uncharacterized protein with an undefinable function. DDX24 is an enzyme belonging to the DEAD box family of proteins. Its main characteristics is the presence of the conserved motif Asp-Glu-Ala-Asp (DEAD). The DEAD Box family are a class of putative RNA helicases and mediate their function through the alteration of RNA processing [115]. DDX24 has a limited structural similarity with other human DEAD box proteins, but is similar in structure to the mouse variant of DDX24 [116]. The current literature suggests a role for DDX24 in RNA-mediated immune signaling and tissue growth [117,118]. Oncology focused studies have shown that DDX24 might regulate certain processes through the interaction with p53 [119,120]. The implication of DDX24 as a potential regulator of RNA processing lends credence to the

hypothesis that it might play a role in disease progression. Although current data on the role of DDX24 is lacking, we make significant effort in this thesis to elucidate the potential mechanisms and characteristics of DDX24 in AD brain.

1.8.6 Neurogenesis in Alzheimer disease

The birth of new neuronal cells, neurogenesis, and the development of neurites, neuritogenesis, are important processes in the brain. Neurogenesis is the process by which neuronal progenitor cells develop into mature neurons and occurs consistently throughout the mammalian kingdom [121]. Some activities and circumstances can enhance neurogenesis in adults, such as running or mental stimulation [122–124]. Neurodegenerative disease such as AD are speculated to be associated with reduced adult hippocampal neurogenesis although the exact mechanism for impaired neurogenesis in AD is not known [125–128].

1.8.7 Neuritogenesis in Alzheimer disease

Neuritogenesis, the formation of new neuronal processes is the first step in the formation of neuronal morphology [129,130]. Neurite formation and growth has been extensively studied and described [129,131,132]. Theoretically, the influence of these processes could help in alleviating some of the disease processes in AD. Neuroinflammation, common in AD, has been described as a potential mediator of adult hippocampal neurogenesis [125,133,134]. Furthermore, stress and age also contribute to reduced neuronal production [135–137].

1.8.8 Ankyrin-3 and Alzheimer disease

Ankyrin-3 (ANK-3) is a protein encoded by the *ANK3* gene and belongs to the ankyrin family of proteins. The main function of ankyrins is to mediate attachments between integral membrane proteins and cytoskeletal components [138]. This linkage is essential to the integrity of the plasma membrane and the functionality of ion metabolism and processing across the plasma membrane. The ankyrin family consists of three genes – *ANK1, ANK2,* and *ANK3* which all produce different proteins through alternative splicing. ANK-3 can be found at neuromuscular junction, the initial axonal segment and the nodes of Ranvier [139]. It is believed that ANK-3 influences action potential propagation though binding to neurofascin and voltage-gated sodium channels [140,141]. ANK-3 is also important for the axon initial segment where it promotes assembly and maturation [142]. The lack of ANK-3 in neurons tends to cause dysfunction at clusters of voltage-gated sodium channels and disturb action potential firing. Although largely unstudied in the context of neurodegeneration, there are suggestions that ANK-3 plays a role in some neurological disorders. *ANK3* has also been shown to be associated with cortical thickness and cognitive performance in patients with psychotic episodes [143,144]. A small study on the genetic variation of ANK-3 in patients with bipolar disorder suggests that ANK-3 influences brain atrophy and might be associated with age-related neurodegeneration [145]. Active vaccination with ANK-3 antibodies in mice models for AD have shown positive results [146].

1.8.9 Doublecortin and Alzheimer disease

Doublecortin (DCX) is a known neurogenesis marker and has been

extensively used for the staining of neurogenesis related processes in mammals [147–149] . DCX is a microtubule associated protein and essential for the migration of neurons throughout the nervous system [150]. DCX is nearly exclusively expressed in developing neurons and has previously been described for the use of neurogenesis quantification in AD and AD animal models [151–154].

However, there are conflicting reports about the correct methodology for staining DCX in AD brain, which has resulted in contradictory studies [155,156] . Nevertheless, the use of DCX as neurogenesis marker is well established and its incongruent results as a marker for neurogenesis in AD prompt further studies.

2 Research aims

Significant efforts have been made in elucidating the pathogenesis in AD and strides have been made. However, the complete image of cellular and molecular pathogenesis is not fully understood. Due to the prodromal phase of the disease starting before the detection of these hallmarks, the contribution of other disease-related proteins needs to be evaluated. By using a multimodal approach and combining the knowledge and experience from several different fields of research, we aimed to try and identify and characterize proteins that have an effect on the presymptomatic disease progression in AD. We hypothesize that this combination of techniques will help us discover new disease mechanisms related to Aβ pathology.

In this project we therefore ask the following questions:
Which pathways can be identified by unbiased proteomics approaches in AD brain and AD mouse models? Which proteins and pathways are induced by Aβ42 at different stages of disease development in animal models of AD? How are these alterations related to the disease development?

The specific aims were to:

1. Characterize early regulators of AD pathology identified by unbiased proteomics of a) an APP knock-in mouse model of AD b) postmortem human AD and control brain

2. Visualize the effects of novel early regulators of AD pathology in postmortem human AD and control brain

3. Examine neurogenesis and neuritogenesis in AD brain and in an APP knock-in mouse model of AD

3 Materials and methods

3.1 Wild type mice

Wild type (WT) mice from the C57BL/6 strain were obtained from the Comparative Medicine Biomedicum (KM-B) in Solna campus and were used for all studies described in this thesis.

3.2 App knock-in mice

We used *App*$^{NL-F/NL-F}$ mice, which are generated on C57BL/6 background (Fig. 2). Both females and males were used indiscriminately for analysis and culturing of neurons and brain sectioning. The mice were kept on a 12:12 day and night cycle and with access to food and water *ad libitum*. The animal experiments were conducted according to the guidelines of Karolinska Institutet. Animals were obtained from KMB in Solna Campus.

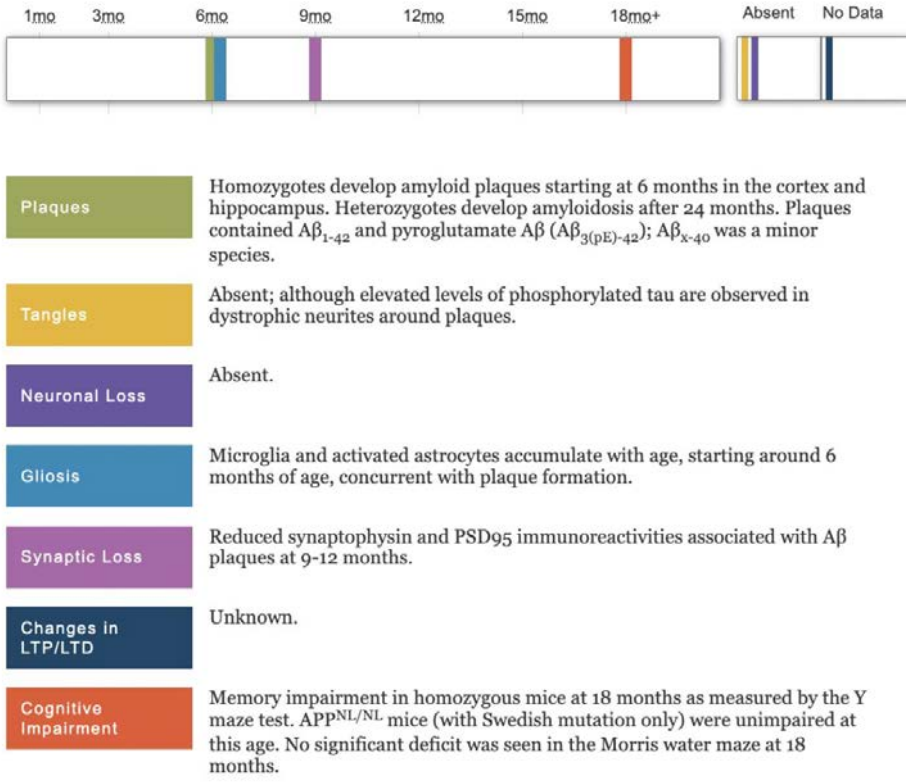

Fig 2: Phenotype characterization of the *App*$^{NL-F/NL-F}$ mice. Image taken with permission from https://www.alzforum.org/research-models/app-nl-f-knock.

3.3 Primary cultured neurons

App$^{NL-F/NL-F}$ mice and WT mice were used to develop primary cultures of hippocampal and cortical neurons. Embryo brains from 16-17 days old mice were collected and dissected. Brains were kept in Hibernate-E medium between transfers from the animal facility and the research lab. Brains were dissected under transmitted light microscope and the cortex and hippocampus were isolated. Cortex and hippocampus were kept in cold HBSS buffer and transferred to a 15 ml Falcon tube and warmed using neurobasal medium with 2% B27 and 1% L-glutamine. Pipetting was used to dissociate brain tissue, approximately 10-12 times. Cells were counted using manual

methods and then seeded onto culture plates coated with poly-D-lysine at least 24 hours before plating. For standard 9 mm glass plates, approximately 150 000 cells were seeded as supporting cells and 7500 cells were seeded in the main well, contact between different types of neuronal cells were always avoided. Supporting cells always consisted of cortical cells to support hippocampal or cortical cells in the center of the well plate. Cells were incubated in 37°C and 5% CO_2 for 7, 14 or 21 days in vitro before being fixed using 4% formaldehyde in neurobasal medium for 10 min. Fixed samples were stored at 4°C. Samples were imaged within 14 days of fixation.

3.4 Sample preparation and labeling for confocal microscopy

Fixed neurons were permeabilized with 0.45%(W/V) CHAPSO for 10 min before being blocked with 10% normal goat serum for 10 min. Primary antibody incubation was performed overnight (ON) in 4°C. After incubation, samples were washed with PBS x 3 and incubated with secondary antibodies for 3 hours (h) at RT. After secondary antibody incubation, samples were washed with phosphate buffered saline (PBS) 3 x 5 min before being rinsed with water and post-fixed for 10 min using a solution of 3% formaldehyde and 0.1 % glutaraldehyde in PBS at room temperature (RT). Lastly, samples were mounted using ProLong gold antifade reagent. We used fluorescently labelled secondary antibodies with different emission spectrums. For dual labelling, we used mainly Alexa Fluor 488 and Abberior Star 635P and for triple labelling we used Alexa Flour 488, Alexa Flour 555 and Abberrior star 635P.

3.5 Sample preparation for LC/MS

In this thesis we analyzed brain tissue from 6-, 9-, and 18-month-old *App*$^{NL-F/NL-F}$ and WT mice (three from each age and strain) by an LC-MS/MS proteomics

approach. The mice were anesthetized with pentobarbital, perfused by cardiac perfusion with PBS, brains were isolated, hippocampal and cortical regions were dissected, snap frozen, and homogenized using Multi-Beads Shocker (Yasui Kikai) at 2500 rpm for 20 s in 8 M urea, 400 mM ammonium bicarbonate. The protein concentration was determined by BCA Protein assay (Pierce). 50 μg protein was digested by trypsin (1:50 trypsin:protein ratio) in 100 mM ammonium bicarbonate, 2 mM $CaCl_2$ and 0.2% RapiGest (Waters Corporation, WI, USA) for 12 h at 37°C. The reaction was acidified by addition of 1.5 μl 37% HCl, in order to hydrolyze RapiGest, and centrifuged for 10 min at 15,000 rpm at 4°C to separate hydrophobic debris from hydrolyzed RapiGest and lipids. An internal standard was made for each time point and brain region by pooling 25 μg of homogenate from the six individual samples, drying them by speedvac and digesting them with trypsin in the presence of 97% ^{18}O-labeled H_2O (by reconstituting ammonium bicarbonate, $CaCl_2$, RapiGest, and trypsin in $H_2{}^{18}O$). A total amount of 10 μg of labeled internal standard was mixed with 10 μg of tryptic peptides of each sample and purified by ZipTipC_{18} chromatography (Millipore, MA, USA) according to the manufacturer's instructions, dried and dissolved in 0.1% formic acid by sonication two times for 5 min. For further fractionation, the peptides were subjected to SCX ion exchange chromatography using handmade stage SCX pipette tips containing four 3M Empore AnionExchange SCX discs (Varian, 1214–5012) placed on top of each other and inserted into a 200 μL micropipette tip. Peptides in a quantity of 20 μg were bound to the matrix, washed with 0.1% formic acid in 45% acetonitrile. Four fractions were sequentially eluted by centrifugation of the tips inserted to Eppendorf microcentrifuge tubes at 2500 rpm for 60 s with 1) 60 mM ammonium formate, 2) 100 mM ammonium formate, 3) 150 mM ammonium formate, and 4) 5% NH_3, 20% MeOH, all containing 0.1% formic acid and 15% acetonitrile. The elution conditions were chosen so that approximately equal amounts of peptides (5 μg) were eluted in each fraction as determined from the respective chromatogram. The eluted fractions were dried in a speedvac, dissolved in 0.2% formic acid and purified by ZipTipC_{18}.

We also used human hippocampal or cortical homogenates in aliquots corresponding to 50 μg protein (determined by the BCA protein assay, Pierce) from control and AD cases (Table 1). Samples were first digested by trypsin and internal standards for the hippocampal and cortical samples were prepared by tryptic digestion of a mixture of all the samples from each brain region in the presence of 97% ^{18}O-labeled H_2O. Each sample was mixed with the internal standard at a 1:1 ratio, fractionated into four fractions by using ion-exchange pipette tips and subjected to LC-MS/MS analysis on a Q Exactive instrument (ThermoScientific)

Table 1. Patient information for AD and control samples used for proteomics

Variable		**Non-demented controls (n=5)**	**AD (n=5)**
Age of death		79 ± 6	81 ± 5
Number (%) of females		3 (60%)	3 (60%)
PMD (hours)		6.8 ± 2.0	6.3 ± 1.1
Braak scores	0	1	0
	I/II	4	0
	III/IV	0	1
	V/VI	0	4

3.6 **LC-MS/MS analyses**

Samples destined for LC-MS/MS analysis were dissolved in 2% acetonitrile/0.2% TFA and injected to the LC-MS/MS in a volume of 9 μl (Q Exactive, ThermoScientific). Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile with 0.1% formic acid. The peptides were eluted by a 120 min long gradient; 0–30% B from 0–100 min and 30–65% B from 100–120 min at a flow rate of 300 nl/min. The analytical column was a

NANO-HPLC capillary C18 column, 0.075×150 mm (Nikkyo Technos) and the trap column was an Acclaim® PepMap100 pre-column, 100 μm×2 cm (Thermo Fisher Scientific). Peptide selection was set at m/z 350–1800. The combined resulting mass spectra data from the four fractions were subjected to Proteome Discoverer (Ver.1.4) with the MASCOT search engine (Ver. 2.5.1) against SwissProt database 2015_04 with search criteria; ^{18}O incomplete labeling (Heavy+Medium)/Light. The median ratio was set to 1 for each analysis.

3.7 **Statistical analyses of proteins identified by LS-MS/MS**

Proteins identified by LC/MS analyses were uploaded to Qlucore Omics Explorer version 3.3. Principal component analysis (PCA) was performed after filtering with a threshold of 1.05-fold change upon performing a two-group comparison between the $App^{NL-F/NL-F}$ mice and the WT mice and human samples. Heat maps were generated for each time point for the cortical and hippocampal proteomes after performing a two-group comparison and filtering with threshold values of both a 1.5-fold change and a p-value < 0.05. UniProtKB was used to explore the functions of the proteins with altered levels.

3.8 **Ingenuity pathway analyses (IPA)**

T-test was performed with two-tailed two sample unequal variance and the resulting file containing protein identification number, fold change in protein levels, and p-values were uploaded to IPA. Set cutoffs: Experimental p-value of $p < 0.1$ and experimental fold change 1.1 times up- or downregulated.

3.9 **Brain Tissue Collection**

Brain samples containing hippocampus and frontal cortex from AD (n=15) and control patients (n=15) were obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam (open access: www.brainbank.nl) and the Karolinska Brain Bank (KBB), Karolinska Institute,

Department of Neurobiology, Care Sciences and Society (open access: www.ki.se/en/nvs/the-brainbank-at-karolinska-institutet). All AD subjects met the criteria for definitive AD according to the Consortium to Establish a Registry for AD. The control subjects had no known psychiatric or neurological disorders. Detailed patient information about the sex distribution, age at death, neuropathological diagnosis, brain weight, post-mortem processing intervals (PMI), Braak staging, and brain bank origin of the donors were available (Table 2). Samples were paraffinized in whole tissue sections, cut and mounted on glass slides.

Table 2. Patient information for AD and control samples used for immunohistochemistry

Clinical Diagnosis	**Braak**	**AD (n=15)**	**Control (n=15)**
Age of death (years), mean, SD (range)		82 ± 2.2 (72-89)	83 ± 6.3 (76-93)
Number of females		8	8
Brain weight (g), mean, SD (range)		985 ± 100.8 (843-1135)	1119 ± 122 (1001-1361)
Post-mortem interval (hours), mean, SD (range)		4.3 ± 0.7 (3-5)	5.5 ± 1.3 (5-7.4)
Distribution of Braak scores	0	0	11
	I/II	0	4
	III/IV	0	0

	V/VI	15	0
Brain bank	NBB KBB	9 6	9 6

3.10 Immunohistochemistry

Immunohistochemistry (IHC) was used to validate the presence of specific proteins in AD and mouse brain. Brain sections from AD patients obtained from NBB and KBB together with brain sections of *App*$^{NL-F/NL-F}$ and WT mice were used for immunolabeling. We cut 4 μm thick sections from paraffin-embedded brain tissue derived from 6-, 12-, and 25-month-old *App*$^{NL-F/NL-F}$ and *App*$^{wt/wt}$ mice and mounted these on slides. Human brain sections were purchased paraffinized and mounted on glass slides. The sections were deparaffinized in Xylene baths and hydrated using a series of solutions of decreasing concentrations of ethanol. Antigen retrieval was performed in citrate buffer, at 110°C for 20 min.

Immunostaining was performed using either standard IHC protocol with DAB/Chromogen visualization or via the use of specialized fluorescence amplification kits.

For amplification, the NEL701001KT TSA amplification kit (Perkin Elmer, MA, USA) was used by adapting the protocol from the manufacturer. Sections were incubated overnight with an antibody for huntingtin EPR5526 (Abcam, CAM, UK), DDX24 (Sigma-Aldrich, Cambridge, UK) ANK-3 (Synaptic Systems, Göttingen, GER) or DCX (Sigma-Aldrich, Cambridge, UK) in TNB blocking buffer. After washing steps, the samples were incubated with secondary biotinylated

antibody, in TNB blocking buffer, for 2h at RT. Samples were incubated with chromogen-DAB solution for 5 min at RT before being rinsed with water. Samples were mounted using water soluble mounting medium (ThermoFisher, MA, USA) and covered with cover glass. All samples were stored dry and dark when not in use.

3.11 Preparation of samples for confocal microscopy

The cellular localizations of proteins of interest were studied using confocal microscopy. Paraffinized human brain tissue slides were deparaffinized and treated for antigen retrieval, as previously described. Samples were washed 3 x 5 min in PBS-T and incubated with primary antibodies anti-huntingtin EPR5526 diluted 1:100 and either Milli-Mark Pan Neuronal Marker (MAB2300, SigmaAldrich) diluted 1:100, anti-phospho tau (AT8, ThermoFisher) diluted 1:200 or anti-GFAP (G3893, Sigma Aldrich, MO, USA) diluted 1:400 in PBS ON at 4°C. For negative control samples, the primary antibody was omitted. After washing for 3 x 5 min with PBS-T, a secondary incubation step was performed at RT for 2h in RT with anti-rabbit secondary antibodies conjugated with Alexa Flour 647 or Abberior Star 635P and anti-mouse secondary antibodies conjugated with Alexa Fluor 488 or Alexa Flour 555 (Sigma Aldrich, MO, USA) using dilutions of 1:500 in PBS. After a washing step of 3 x 5 min, mounting was performed using ProLong gold antifade reagent P36930 (Life Technologies, CAL, USA), and slides were covered with coverslips with a refractive index of 1.5. Samples were stored dark and at 4°C when not in use.

3.12 Image capture-Confocal and brightfield microscopy

Image capture of fixed samples was done using a Zeiss LSM 900 with Airyscan 2 using the ZEN version 3.6 software (Zeiss, STK, SWE). Images were captured using 20X and 40X objectives with an image size of 1024 x 1024 pixels.

Excitation lasers with wavelengths of 488, 561 and 640 nm were used. The settings, including laser power intensities, pinhole and detector gain, were chosen to optimize the dynamic range to show no fluorescence signal in the negative control and limited or no saturation in the strongest signal localizations. The pinhole size was set to one airy unit.

Images of immunolabeled sections were captured using a 20X and 40X objectives and a Nikon Camera DS-Qi2 (Nikon, TKY, JPN) with capture software NIS-elementsD (Nikon, TKY, JPN). Same settings were used for the capture of each image with 2-s exposure time and 1.6 digital gain. Exposure was kept to a minimum to limit photobleaching.

3.13 Data analysis-Images

Images of IHC staining were analyzed using quantification in ImageJ imaging software (NIH, UK). Positive signal was detected based on mean signal intensity in each sample area. Regional signal was defined as the whole signal detectable in the anatomically identified area. Neuronal signal was defined as the average signal of 40 neurons per subarea. The signal intensity was displayed in an interval of 0-250. The signal in neuronal cultures was measured by drawing a mask around the neuron and calculating the mean signal intensity per channel. Statistical analysis of signal intensity data obtained via ImageJ was performed using Graphpad Prism software (Graphpad Software, CA, USA). Unpaired *t*-tests were used to determine significance between signal intensity, p-value < 0.05. Data is displayed as Mean+/- SD.

3.14 Antibodies

All primary and secondary antibodies are shown in Table 3:

Table 3: Primary antibodies used in this thesis.

Antigen	Host Species	Manufacturer	Catalog	Dilution
Phospho-tau	Mouse	Thermofischer	G3893	1:200/
GFAP	Mouse	Sigma-Aldrich	G3893	1:400
Huntingtin	Rabbit	Abcam	EPR5526	1:100/1:500
Neuronal Marker	Mouse	Sigma-Aldrich	MAB2300	1:100
DDX24	Rabbit	Sigma-Aldrich	HPA002554	1:100
ANK-3	Guniea Pig	Synaptic Systems	386 004	1:200
APP	Mouse	BioLegend	C1/6.1	1:200

Table 3: Secondary antibodies used in this thesis

Antigen	Host Species	Manufacturer	Catalog	Dilution
Alexa Flour 647	Rabbit	Invitrogen	A-21246	1:500
Alexa Flour 647	Mouse	Invitrogen	A-21236	1:500
Abberior Star 635P	Rabbit	Invitrogen	2-0012-007-2	1:500
Alexa Flour 488	Mouse	Invitrogen	A-11029	1:500
Alexa Flour 555	Rabbit	Invitrogen	A-21244	1:500
Alexa Flour 555	Mouse	Invitrogen	A-21424	1:500
Alexa Flour Phalloidin	N/A	Invitrogen	A12379	1:200

3.15 Ethical considerations

Neuronal cell cultures and brain sections were obtained from live mice and mice embryos. Brain sections from healthy controls and AD patients were also used in this project. We utilized brain homogenate from mice and human samples. No experiments were performed on live animals during this project. All experiments were performed in accordance with local ethical guidelines at Karolinska Institutet and subjected to ethical review according to national guidelines before initiation.

4 Results and discussion

The identification and characterization of proteins involved in AD regulation is difficult due to the multifactorial origin of the disease. Although the Aβ hypothesis and the success of Aβ lowering treatment have lent credence to the approach of lowering Aβ, other treatments are needed. To this end, we focused on identifying proteins involved in the presymptomatic pathogenesis of AD.

4.1 Paper I. Proteomics Time-Course Study of Knock-In Mice Reveals Novel Presymptomatic Aβ42 Induced Pathways to Alzheimer's Disease Pathology

The following questions were answered in this study: What proteins are alternated at an early age in the *App*$^{NL-F/NL-F}$ mouse model? Which proteins are potential regulators of disease pathology in different areas of the brain during the life span of the *App*$^{NL-F/NL-F}$ mouse model? Is huntingtin different in the brain of *App*$^{NL-F/NL-F}$ mice compared to WT mice? Is there a time dependent accumulation of huntingtin in the brain of *App*$^{NL-F/NL-F}$ mice. Where is huntingtin located on a subcellular level in neurons from the *App*$^{NL-F/NL-F}$ mouse?

Identification of protein changes in mouse models for neurodegenerative disorders has been extensively described in the literature [100,157]. No study in AD mouse models have yielded clinically translatable results [158,159]. We utilized a multimodal approach in which we combined proteomics, bioinformatics, and microscopy in order to identify proteins of interest that might have a regulatory role in disease progression in a highly relevant animal model for AD called *App*$^{NL-F/NL-F}$. Because it is a knock-in model, the *App*$^{NL-F/NL-F}$ produce high levels of Aβ42 but do not cause artefacts due to overexpression of APP, which is a common problem with transgenic mouse models. Overproduction of APP can interfere with both disease progression and protein

levels in animal models. This makes the *App*$^{NL-F/NL-F}$ mouse model particularly suited for studying Aβ-driven pathology in AD, in particular Aβ42-driven pathology.

We performed proteomics on brain homogenate from hippocampus and cortex of *App*$^{NL-F/NL-F}$ mice at various time points and subsequently subjected the data to IPA. Huntingtin was noted as a disease regulator in the hippocampus of 3-,6- and 9-months old *App*$^{NL-F/NL-F}$ mice using IPA. Furthermore, huntingtin was detected as a potential regulator in the cortex of 6-, 9- and 18-months old *App*$^{NL-F/NL-F}$ mice. At 6-months of age the *App*$^{NL-F/NL-F}$ mice had significantly increased levels of huntingtin. This accumulation was retained in CA1 at 12-months of age in the *App*$^{NL-F/NL-F}$ mouse. At 25 months of age, there was no significant difference in huntingtin levels between any brain areas of the *App*$^{NL-F/NL-F}$ and WT mice. Confocal microscopy of the pyramidal neurons in CA1 of 12-months old *App*$^{NL-F/NL-F}$ mice displayed elevated huntingtin levels in the soma and apical dendrites with a clear absence of huntingtin in the nucleus.

Normal huntingtin regulates a myriad of functions in the soma of neuronal cells including axonal transport, nuclear import and apoptotic processes. Increased intracellular levels of huntingtin has been suggested to act as a neuroprotective element [160,161]. Our findings of accumulations of huntingtin early in the life span of the mouse might therefore be evidence of a compensatory mechanism in response to cellular stress. The *App*$^{NL-F/NL-F}$ mice do not develop amyloid plaques until 9 months of age and symptoms do not appear until at least 18 months. The presence of neuropathology in the early life span of the mice could explain the observed increase in huntingtin levels. The absence of increased huntingtin in the brain of 25- months old *App*$^{NL-F/NL-F}$ mice might be due to the advanced stage of the mouse model and the subsequent neuronal loss which inevitably occurs. These findings indicate that huntingtin is upregulated early in the life span of the mouse, even before the accumulation of Aβ.

We found several proteins with a known pathological function within AD such as MAPT, PSEN1 and MTOR. However, the discovery of huntingtin as a top novel regulator raises the question about the implication of huntingtin in the development of AD. Another study performed by a different lab using a different mouse model has also shown implication of huntingtin in AD in similarity to our results [113]. Studies have found extra alleles of huntingtin in the brains of AD patients and studies in other mouse models have shown increased huntingtin levels in similarity to our findings [162,163].

Our results have a potential for clinical approach as techniques for increasing and decreasing huntingtin levels are already described [164,165]. It is possible that huntingtin has a positive regulatory effect on diseased neurons as healthy normal length huntingtin has been showed to be neuroprotective [160]. Our results corroborate this theory as we studied normal length huntingtin rather than the extended mutant version found in Huntington disease. Further studies into the effects of huntingtin on AD is warranted and future researchers should focus on elucidating mechanistic relationships between huntingtin and known AD disease modulators such as Aβ and tau. There are several available techniques which could be used to change the protein levels of huntingtin intracellularly such as siRNA treatment, transduction and genomic expansion.

Taken together, our findings indicate that huntingtin might play a role in the early regulation of AD and efforts should be made to further elucidate its role in the pathogenesis of AD.

4.2 Paper II. Huntingtin Levels are Elevated in Hippocampal Post-Mortem Samples of Alzheimer Disease Brain

Having discovered that huntingtin was elevated in an AD mouse model brain, both presymptomatically and before the formation of amyloid plaques, we wanted to examine if we could discover increased levels of huntingtin in the brain tissue of AD patients. Therefore, in this paper we sought to answer the questions: Is huntingtin increased in the brain of AD patients? What areas of the brain of AD patients is mostly affected by huntingtin accumulation? Is the accumulation of huntingtin in the AD brain similar to the accumulation of huntingtin in the Huntington disease brain?

We studied brains from AD and healthy controls using two different brain banks. We discovered that huntingtin accumulates in dystrophic neurites, the soma of pyramidal neurons, CA1, CA3, in the pila of the hippocampus and in pyramidal neurons within layer III-V of AD patients which did not occur in control cases. Huntingtin in human brain was found in the nucleus whereas in the AD mice model we only noticed huntingtin in the soma of neuronal cells.

The presence of huntingtin in the frontal cortex and CA1/CA3 are particularly interesting findings as these areas are important areas for memory formation and cognition. CA1 and CA3 are closely linked via Schaffer collaterals, raising the question regarding translocation of huntingtin via neuronal connections, similar to the suggested neuronal spread of oligomers [166]. We also found accumulation of huntingtin in the frontal cortex of AD patients compared to healthy controls, although these levels were lower than those seen in the hippocampus. There was some variance between brains acquired from different brain banks, which we believe is due to variance in slide quality and tissue processing. All of our samples were AD brains with high amyloid load and Braak staging, our results therefore have to be interpreted in the context of advanced AD.

In Huntington disease, huntingtin is produced and secreted by astrocytes. We therefore investigated whether this was the case in AD and therefore co-

stained astrocytes using GFAP and huntingtin. We utilized confocal microscopy to investigate whether there were any unique patterns to the accumulation of huntingtin in AD brain.

We found that the accumulation of huntingtin in AD brains did not colocalize with GFAP staining, showing that AD huntingtin accumulation has a distinctive pattern compared to the huntingtin accumulation observed in Huntington disease. Neither did we find any significant colocalization between huntingtin and tau tangles, a key AD pathological marker. Huntingtin was significantly elevated early in the life span of our AD model as described in paper I. The quantification of huntingtin in the early stages of AD could therefore be an area of future interest. Theoretically, PET tracer markers for huntingtin could be used to quantity huntingtin levels in MCI and SCI patients.

There are many neurodegenerative disorders that show concurrent proteinopathies. Neurodegeneration often occurs due to the influence of several different mechanisms. Huntingtin has not been extensively studied in post mortem brains of AD patients, to our knowledge only a single study has examined the presence of non-mutated huntingtin in the brain of AD patients and that study was relatively small and provided mostly an overview of protein accumulation rather than specific findings [167]. Our study is the first one to extensively detail the presence of huntingtin in AD brain and its cellular localization in relation to tau tangles and astrocytes. Our study is also the first to our knowledge that describes low amounts of huntingtin in astrocytes in AD.

4.3 Paper III. DEAD Box Helicase 24 is Increased in Alzheimer Disease Brain and Influences Presymptomatic Pathology

Following up on paper I, this study intended to examine protein levels that might be influential to disease progression amongst AD patients using

hippocampal or cortical homogenates. The questions this study sought to answer were: What proteins are increased or decreased in AD hippocampus and cortex? Which proteins act as a disease regulator in AD brain? What is the anatomical distribution of DDX24 in human and mouse AD brain?

Using a similar approach to proteomic analysis as described in paper I, we identified 266 significantly altered proteins in the hippocampus and 223 proteins in the cortex of AD patients when compared to healthy controls. DDX24 in AD hippocampus was the most significantly elevated protein. Ranking signaling pathways and identifying networks which were the most affected also revealed DDX24 as a prominent protein in the highest ranked network. We interpreted these results to indicate that DDX24 might influence AD pathogenesis.

IHC of AD and control brains revealed DDX24 positive pyramidal neurons within CA1 and CA3 of AD patients but no or very little DDX24 in non-neuronal cells, such as astrocytes or microglia. Both AD and controls showed small dot-shaped nuclear inclusions of DDX24, suggesting an enrichment of DDX24 in the nucleolus. There was also diffuse cytosolic staining of DDX24 throughout the neuronal cells.

Following-up on our findings of intracellular DDX24 accumulations, we examined neuronal cells for signs of colocalization of DDX24 and hyperphosphorylated tau. Using confocal microscopy, we observed that DDX24 and tau did not colocalize to any significant extent.

Next, we characterized the expression of DDX24 in the hippocampus of the *App*$^{NL-F/NL-F}$ mice. Staining hippocampal sections from 6-, 12- and 25-months old mice revealed that DDX24 was increased in the hippocampus of 6 months old *App*$^{NL-F/NL-F}$ mice. DDX24 was mostly located to the soma and nucleolus of pyramidal neuronal cells, mimicking the findings in advanced AD brain. There

was no change in DDX24 levels in *App*$^{NL-F/NL-F}$ compared to WT in mice older than 12 months. The absence of DDX24 differences in older mice might indicate that changes in DDX24 levels is mostly expressed before the onset of amyloid accumulation and the onset of symptoms. Since the *App*$^{NL-F/NL-F}$ mice do not express significant levels of tau tangles at 6 months, we assume that DDX24 expression levels is a consequence of either the increased amyloid load of the *App*$^{NL-F/NL-F}$ mice or the increased cellular stress which occurs in these mice at 6 months of age.

Next, we examined DDX24 and APP in WT neuronal cultures. Silencing DDX24 expression using siRNA caused decreased levels of both DDX24 and APP. Lower levels of DDX24 expression also correlated to decreased levels of Aβ42 in both cell culture medium and cell lysate. siRNA treated cultures displayed lower spine density but not decreased number of living neuronal cells, indicating that DDX24 might be needed for maintaining spine density.

Although silencing of DDX24 resulted in lower Aβ levels, addition of Aβ to cell cultures resulted in lower DDX24 levels. We speculate that these results are indicative of a negative feedback loop between Aβ42 and DDX24.

Imaging DDX24 in primary neuronal cultures from WT and *App*$^{NL-F/NL-F}$ mice showed that DDX24 levels in the soma and nucleus were time dependent. *App*$^{NL-F/NL-F}$ mice neurons incubated 7 DIV displayed significantly higher levels of DDX24 in the soma, nucleus and nucleolus than the older cultures did. We found no evidence of increased DDX24 expression over time in the neurites of *App*$^{NL-F/NL-F}$ or WT mice neurons. We believe that these results suggest that DDX24 production and/or nuclear transport might be increased in *App*$^{NL-F/NL-F}$ at a very early stage in the disease progression.

Although inconclusive, our results warrant further studies into the mechanistic relationships between DDX24 and Aβ42.

4.4 Paper IV. Neuritogenesis and Neurogenesis in Alzheimer disease Brain and in the *App*$^{NL-F/NL-F}$ Mouse Model

In this paper we wanted to further expand on our previous results from paper I and III which implicated certain neuronal process as dysregulated in AD. In particular, we found indications that neurogenesis and neuritogenesis might be influenced in AD. We therefore aimed to answer the questions: Are neurogenesis and neuritogenesis altered in AD and an AD mouse model as measured by protein markers? What is the distribution of neuritogenesis- and neurogenesis-markers in AD and in and AD mouse model? What is the influence of AD pathogenesis on neuritogenesis and neurogenesis?

To enable the study of neuritogenesis in AD and an AD mouse model, we utilized ANK-3 as a marker for neuritogenesis. Using IHC of AD brain we discovered that ANK-3 is heavily elevated in the AD brain. ANK-3 was significantly increased in the AD brain compared to controls in areas such as CA3, CA1, the dentate gyrus, and in corpora amylacea. ANK-3 was increased also in the frontal cortex layers III-V of AD patients,

We used DCX as a marker for neurogenesis in AD and stained postmortem human brain sections. We found no evidence of DCX positivity in AD brain, neither in the hippocampus or its subareas nor the frontal cortex, indicating that neurogenesis does not take place in advanced AD or is difficult to detect using standard methods.

Having found evidence of increased neuritogenesis in AD in the form of ANK-3 positivity, we wanted to see if these results were translatable to the AD mouse model, *App*$^{NLF/NLF}$. We therefore stained a time series of *App*$^{NL-F/NL-F}$ mouse brain, using 6-, 12- and 25-months old mice. We found that ANK-3 is increased in the *App*$^{NL-F/NL-F}$ mice at 6 months but not in older mice. Studying subareas of the AD mouse model brain we found that ANK-3 was accumulated in a similar pattern

to what we found in the AD brain with CA3, dentate gyrus and frontal cortex displaying high levels of ANK-3. DCX positivity was detected in 6- and 12 months old *App*$^{NL-F/NL-F}$ mice, indicating increased neurogenesis at these time points before and after the onset of amyloid plaque deposition, respectively.

With our *App*$^{NL-F/NL-F}$ results indicating that neuritogenesis and neurogenesis are induced might be taking place at an early stage in the disease progression, we decided to utilize embryonic brain samples of the *App*$^{NL-F/NL-F}$ mice to detect neuro- or neuritogenesis related changes at the embryonic stage. We found that ANK-3 was not increased at the embryonic stage, but DCX was increased in certain regions which correlated to the cortical layers. The knowledge of synaptogenesis in the *App*$^{NL-F/NL-F}$ mouse model at an early stage is lacking and our results suggest increased neurogenesis but not neuritogenesis at this stage of the mouse life span.

Lastly, having discovered evidence of neurogenesis in the *App*$^{NL-F/NL-F}$ mice at the embryonic stage, we studied the cellular levels of ANK-3 and DCX in primary neurons derived from AD mouse model. We found that ANK-3 was heavily increased in cultures at 14 and 21 DIV, compared to controls, with ANK-3 located mostly to the soma. We found that DCX was increased in the neuronal cultures at 7 and 21 DIV compared to controls.

Taken together the results from paper IV suggest that neurogenesis and neuritogenesis are affected in AD with DCX staining of *App*$^{NL-F/NL-F}$ showing increased neurogenesis at the cellular, embryonic stage as well as through the life span of the mice. Our findings regarding neuritogenesis show that AD brain have evidence of increased neuritogenesis in advanced AD brain and the AD mouse model showed increased ANK-3 on a cellular level as well at 6 months of age. The most likely explanation for increased a neuronal process intended to promote neuronal growth and proliferation in the AD mouse model would be a compensatory mechanism in response to neurodegeneration.

5 Concluding remarks

AD is fast becoming a global health crisis and there is an urgent need for additional and effective treatment strategies to combat an ever-growing elderly population. Without a cure, an aging world population could spell disaster for healthcare systems around the world within the coming decades. The characterization of proteins involved in AD pathogenesis can help to increase our understanding of AD. Studying the disease associated protein changes in AD brain and in the brain of the *App*$^{NL-F/NL-F}$ mouse will further our knowledge of this terrible disease.

The project described in this thesis aimed at identification and characterization of specific proteins that might influence AD pathogenesis before the onset of symptoms. The results in this thesis have increased our understanding of AD pathogenesis and describe several proteins as of yet unstudied in the context of AD pathogenesis. We also suggest potential mechanisms by which these proteins could influence the disease. Taken together, our main conclusions were:

⇒ Huntingtin is elevated in AD brain and in an AD mouse model. Accumulation of huntingtin in AD brain appears to be present in areas important to memory formation in the brain. Huntingtin accumulation in AD does not mimic huntingtin accumulation in Huntington disease.

⇒ The accumulation of huntingtin in the AD brain might precede amyloid deposition as demonstrated by the findings of increased huntingtin in our AD mouse model before the onset of amyloidosis or cognitive impairment.

⇒ DDX24 accumulation can be detected in AD brain and in an AD mouse model. DDX24 appears to accumulate in neuron-rich areas such as the hippocampus and frontal cortex.

⇒ DDX24 is increased before the onset of amyloidosis in the AD mouse model.

⇒ DDX24 is influenced by Aβ and affects APP metabolism.

⇒ Neurogenesis and neuritogenesis changes are detectable in AD brain and in the early life span of an AD mouse model prompting further research into these processes and their implications for AD pathogenesis.

6 Points of perspective

Although, we describe several novel proteins in the context of AD in this thesis, the identification and characterization of proteins involved in disease progression is an ongoing process. There are several questions that have been raised due to the implications of our results and the answers to these might help us further our understanding of the disease mechanisms which prelude symptoms in AD.

⇒ The molecular mechanisms by which huntingtin influences neuronal health are unknown and motivates further research on the effects of overexpression and silencing of huntingtin in AD models.

⇒ We confirmed the presence of HTT in AD brain and mouse tissue, however, further sampling should be used to determine its metabolism in AD.

⇒ We examined the effects of down- but not upregulation of DDX24 on the processing of APP and found that this increased APP expression. Further studies should focus on other potential mechanisms by which DDX24 might influence AD progression, such as protein overexpression.

⇒ Neurogenesis and neuritogenesis should be further studied in the context of AD. Future studies should focus on trying to implement strategies to further elucidate the effects of AD pathological elements on these processes. This could be accomplished by the study of brain tissue containing lower Braak staging and amyloid load or the use of other animal models such as rats. Related processes such as synaptogenesis and pruning could also be of interest.

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