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Structural polymorphism and seeding activity of A β amyloid fibrils

Farjana Parvin

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Errata

Page vi (abstract), first paragraph, line 6, "...in neurons and glia by nsyb-Gal4 and repo-Gal4, respectively..." should be "...in neurons and glia by nsyb-Gal4 and repo-Gal4 driver **lines**, respectively..."

Page xiii (Contribution report Paper I), line 6, *manuscript* should be *paper*

Page 5, second last line, *Drosophila melanogaster* should be *Drosophila melanogaster*

Page 9, first paragraph, line 6, **PS1** should be **PS2**

Page 9, second paragraph, line 3, "...*symptom* show itself." should be "...*symptom*."

Page 11, fourth paragraph, line 5, "Everyday life activities **I like**..." should be read "Everyday life activities **like**..."

Page 19, second paragraph, line 7, *Isozyme* should be *Isomerase*

Page 44, second paragraph, line 3, "...recombinant A β ₁₋₄₂ fibrils than **A β ₁₋₄₂**." should be "...recombinant A β ₁₋₄₂ fibrils than **A β ₁₋₄₀**."

Page 44, second paragraph, line 4, "...**alkyne alkyne-conjugated** spacer..." should be "...**alkyne-conjugated** spacer..."

Paper II, page 6, second paragraph, line 13, Fig. 8E should be Fig. 8F.

Paper II, page 6, second paragraph, line 18, Fig. 8F should be Fig. 8E.

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Farjana Parvin



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Linköping 2024

Front cover: A β plaques and CAA from an 18 months old APP23 mouse model stained with two luminescent conjugated oligothiophenes, qFTAA, hFTAA, and A β specific antibody 12F4.

Back cover: A β plaques from an 18 months old APP23 mouse model stained with two luminescent conjugated oligothiophenes, qFTAA and hFTAA.

During the course of research underlying this thesis, Farjana Parvin was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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*“Above all, don’t fear difficult moments.
The best comes from them.”*

Rita Levi-Montalcini

Abstract

Alzheimer's disease (AD) is a common neurodegenerative disorder marked by fibrillar aggregates of misfolded A β peptides and tau protein in the brain. Misfolded A β peptides form extracellular senile plaques and cerebral amyloid angiopathy (CAA) in brain blood vessels. On the other hand, misfolded tau protein accumulates in intracellular tau tangles. Although the disease-causing protein shares the same primary sequence, its tertiary and quaternary fibrillar structures can exhibit polymorphism. Previous studies suggest that this structural polymorphism may be linked to distinct AD clinical phenotypes. Thus, understanding structural polymorphism is crucial to acquire insight into the disease mechanism.

In this thesis, I examined the variation in A β fibril morphology within amyloid plaques in AD mouse models carrying familial mutations in the A β PP gene. A combination of amyloid binding conformation-sensitive fluorescent dyes and A β -specific antibody staining reveals that the A β PP processing genotype influences the structure of A β fibrils within A β plaques. Plaques from APP23 mice with Swedish A β PP mutation (KM670/671NL) exhibit two distinct fibril polymorphic regions: a core and a corona. The plaque core has tightly packed A β 40 fibrils, while the corona has diffusely packed A β 40 fibrils. *App*^{NL-F} mice with the A β PP Iberian (I716F) and the Swedish mutation have tiny plaque cores of compact A β 42 fibrils.

I also examined the seeding activity of recombinant A β fibrils. The A β pathology in the brain propagates through a process called seeding, where preformed fibrils, known as seeds, promote fibril formation by bypassing the nucleation step. Previous research demonstrated that injecting brain extracts rich in A β (seeds) from transgenic mice and AD patients can induce AD pathology in transgenic mice. While research on recombinant seeds is still limited, we focused on investigating the seeding activity of pure recombinant A β fibrils of different compositions. Seeds were inoculated into APP23 mouse brains at 3 months and were analyzed after 6 months of incubation. We observed that recombinant seeds (fibrils from A β 1-42, A β 1-40, and A β 1-40+A β 1-42) accelerated plaque formation compared to non-inoculated transgenic control mice. In addition, all seeds induced profound CAA in

young APP23 mice (9 months). Interestingly, pure A β 1-42 seeds produced significantly more CAA and amyloid plaques than seeds containing A β 1-40, which is surprising given that APP23 mice produce up to five-fold more A β 1-40 than A β 1-42. I furthermore examined the seeding activity of A β 1-42 aggregates isolated from neurons and glial cells from *Drosophila melanogaster*. A β peptides were expressed in neurons and glia by nsyb-Gal4 and repo-Gal4, respectively. Seeds from neuron and glial cells were again inoculated in APP23 mice and incubated for six months. We found that both the neuronal and glial seeds were not potent in inducing seeding. However, both the seeds became potent when fibrils were first amplified *in vitro* with recombinant A β 1-42 before inoculation. These active seeds originating from neuronal expression produced more CAA and plaques than seeds from glial cells in terms of the number of aggregates per section, strongly suggesting that the amyloid fibril polymorphs are replicated into two distinct amyloid strains with different seeding efficiency.

In the last study of the thesis, we developed a multiple-ligand fluorescence microscopy approach to detect diverse pathological A β fibrils. Since A β amyloid plaques pose various fibrillar structures, using a single ligand is not enough to detect all these pathological aggregates. This study used both AD mouse models and AD patient's brain samples. It was shown that ligand binding in mice is dependent on mutation and age. Thus, combining different ligands enhances the possibility of detecting various types of A β amyloid aggregates.

In summary, this thesis provides an understanding of the diversity of structural variations of amyloid fibril aggregates in Alzheimer's disease, which will help to identify disease-relevant fibril polymorphs and provide insight for designing molecules for diagnostics and therapeutics.

Populärvetenskaplig sammanfattning

Proteiner är biomolekyler som är avgörande för att upprätthålla vår komplexa människokropp. De är cellernas byggstenar och bygger upp olika kroppsdelar som hud, hår, naglar. Bland annat transporteras de näringsämnen och andra väsentliga molekyler samt skyddar kroppen från infektioner. För att genomföra dessa kritiska biologiska funktioner behöver proteiner veckas till sina funktionella tredimensionella strukturer. Proteiner kan dock misslyckas med att vecka sig i sina funktionella strukturer av flera skäl och kan då bli skadliga för människokroppen.

Felveckade proteiner kan aggregera och klumpas samman. Vissa klumpar, så kallade amyloider, är fibrillära i sina strukturer. Amyloider orsakar flera neurodegenerativa sjukdomar genom att störa normal funktion och så småningom döda nervcellerna i kroppen. Alzheimers sjukdom är en av dessa neurodegenerativa sjukdomar. Den påverkar bland annat nervcellerna i hjärnan som ansvarar för minnet. AD är den vanligaste orsaken till demens eller minnesförlust bland äldre människor över hela världen. Tre typer av proteinaggregat finns i AD-patienters hjärnor: Amyloidplack, CAA (Cerebral Amyloid Angiopati) och tau-trassel. Felveckade A β -peptidaggregat bildar amyloidplack och CAA. Felveckade tau-proteinaggregat sammanflätas till tau-trassel. Även amyloider med samma primära aminosyrasekvens, kan ha olika tredimensionella strukturer hos fibrillerna. Detta kallas för strukturell polymorfism. Tidigare studier visade att strukturell polymorfism kan korrelera med distinkta kliniska sjukdomsbilder i Alzheimers. Därför är det mycket viktigt att studera strukturell polymorfism för att förstå sjukdomsmekanismen.

I denna avhandling har jag för detta använt musmodeller och bananflugamodeller som härmar sjukliga hjärnförändringar i Alzheimers liksom vävnadsprover från avlidna AD-patienter. Människor och möss har cirka 85% genetisk likhet. För människa och bananflugor ger samma jämförelse 60% likhet. 75% av kända sjukdomsgener i människa har en motsvarande gen i bananflugan. I det första arbetet visar jag med hjälp av konformationskänsliga amyloidbindande färgämnen att amyloidplackens struktur skiljer sig beroende på den genetiska mutationen i musmodellerna. Jag visar också att denna plackstruktur är åldersberoende inom samma

musmodell, d.v.s. placken har olika strukturer när mössen är unga och när mössen är gamla. I det andra och tredje arbetet visar jag att CAA-patologi kan induceras i en AD-musmodell genom att inokulera mössen med amyloid bildad av rekombinanta (labbtillverkade) A β -peptider och med A β -aggregat isolerade från bananflugor. I det fjärde arbetet visar vi att en kombination av nya fluorescenta amyloidmarkörer möjliggör detektion av olika polymorfa A β -aggregat i olika musmodeller av AD och i vävnadsprover från AD-patienter.

Sammantaget har mitt avhandlingsarbete bidragit till förståelsen av att A β -fibriller uppvisar stor variation rörande struktur och spridningsförmåga.

List of papers

The thesis is based on the following papers:

I. **Divergent Age-Dependent Conformational Rearrangement within A β Amyloid Deposits in APP23, APPS1, and App^{NL-F} Mice.**

Farjana Parvin¹, Samuel Haglund¹, Bettina Wegenast-Braun^{2,3}, Mathias Jucker^{2,3}, Takashi Saito^{4,5}, Takaomi C Saido⁴, K Peter R Nilsson¹, Per Nilsson⁶, Sofie Nyström^{1*}, Per Hammarström^{1*}

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II. **Efficient Seeding of Cerebral Vascular Amyloidosis by Recombinant A β 1-42 Amyloid Fibrils.**

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The manuscript is ready for submission.

III. Different brain A β -amyloidosis patterns induced by seeding from separate A β 1-42 amyloid polymorphs originating from transgenic *Drosophila*.

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Manuscript in preparation.

IV. Dual-ligand Fluorescence Microscopy Enables Chronological and Spatial Histological Assignment of Distinct Amyloid- β deposits.

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The manuscript is submitted to the Journal of Biological Chemistry.

Contribution report

I. Divergent Age-Dependent Conformational Rearrangement within A β Amyloid Deposits in APP23, APPS1, and App^{NL-F} Mice.

Farjana Parvin (FP) performed experiments, data analysis, compiled figures, and assisted in drafting the manuscript. FP is the main author of this manuscript.

II. Efficient Seeding of Cerebral Vascular Amyloidosis by Recombinant A β 1-42 Amyloid Fibrils.

FP conducted the experiments, performed data analysis, compiled the figures, and contributed to drafting the manuscript. FP is the main author of this manuscript.

III. Different brain A β -amyloidosis patterns induced by seeding from separate A β 1-42 amyloid polymorphs originating from transgenic *Drosophila*.

FP conducted the experiments, carried out data analysis, compiled the figures, and contributed to drafting the manuscript. FP is the main author of this manuscript.

IV. Dual-ligand Fluorescence Microscopy Enables Chronological and Spatial Histological Assignment of Distinct Amyloid- β deposits.

FP performed dual ligand staining and acquired data.

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List of Abbreviations

AD	Alzheimer's disease
AICD	APP intracellular domain
APOE	Apolipoprotein E
APPL	Amyloid precursor protein-like
A β	Amyloid- β
A β PP	Amyloid- β precursor protein
AFM	Atomic force microscopy
BER	Base excision repair
BTD	Bis-styryl-benzothiadiazole
CAA	Cerebral amyloid angiopathy
Cas9	CRISPR-associated protein 9
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CP	Cored plaques
CRISP	Clustered regulatory interspaced short palindromic repeats
cryo-EM	Cryogenic-electron microscopy
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
CWLP	Cotton wool-like plaque
CWP	Cotton wool plaques
DDR	DNA damage responses
diAD	Dominantly inherited AD
DP	Diffused plaque
DSB	DNA double-strand breaks
<i>E. Coli</i>	<i>Escherichia coli</i>
EOAD	Early-onset Alzheimer's disease
ERAD	Endoplasmic reticulum-associated degradation
FAD	Familial Alzheimer's disease
FDA	Food and Drug Administration
hFTAA	Hepta-formyl thiophene acetic acid
ICT	Internal charge transfer
IDP	Intrinsically disordered proteins
IF	Immunofluorescence
ISA	International Society of Amyloidosis
LCO	Luminescent conjugated oligothiophenes

LCP	Luminescent conjugated polythiophenes
LOAD	Late-onset Alzheimer's disease
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
MMSE	Mini-Mental Status Exam
MRI	Magnetic resonance imaging
ND	Neurodegenerative diseases
NER	Nucleotide excision repair
NFT	Neurofibrillary tangles
NSB	Naphthyl-stilbene
PD	Parkinson's disease
PDI	Protein Disulphide Isomerase
PET	Positron emission tomography
PSEN1	Presenilin 1
PSEN2	Presenilin 2
qFTAA	Quatro-formyl thiophene acetic acid
RNAi	RNA interference
sAD	Sporadic AD
ss-NMR	Solid-state nuclear magnetic resonance
TEM	Transmission electron microscopy
ThT	Thioflavin T
TICT	Twisted Internal charge transfer
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
WHO	World Health Organization

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Introduction

Neurodegenerative diseases (ND)

The advancement of modern medicine has elevated the overall human life expectancy of individuals. However, this prolonged life expectancy sometimes poses an enormous burden on society and the healthcare system, with the emergence of different age-related diseases, such as neurodegenerative diseases (NDs). Around 15% of the world's total population is currently affected by NDs [1]. NDs are diseases characterized by progressive loss of neuron structures and functions (**Figure 1**).

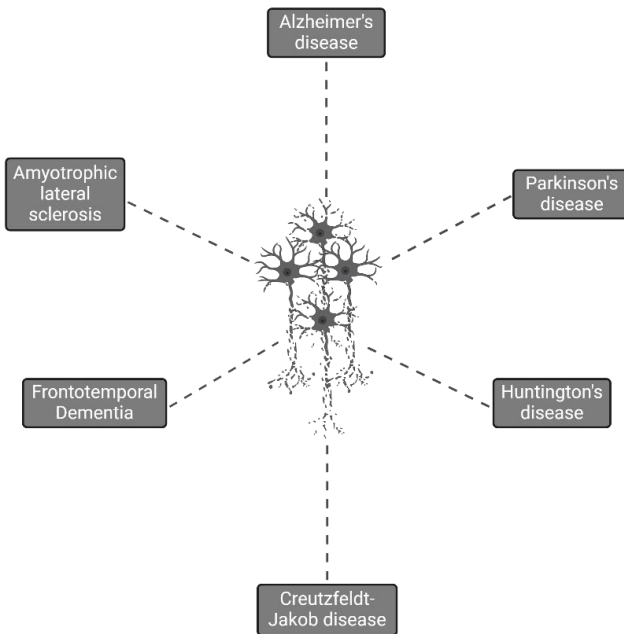


Figure 1: Example of several neurodegenerative diseases due to neuronal loss.

Aging

Multiple factors contribute to the development of NDs. Aging is the most prevalent risk factor for developing NDs. Impaired molecular mechanisms such as DNA damage, telomere dysfunction, loss of proteostasis and so on are associated with aging and are also closely correlated to the development of NDs [2] [3] [4].

The impairment of several DNA damage responses (DDRs) frequently observed in the aging process has also been identified in individuals with NDs. Increased DNA double-strand breaks (DSBs) have been reported in Alzheimer's disease patients' hippocampus compared to the non-AD brains [5]. Reduced nucleotide excision repair (NER) capacity has been testified in dermal fibroblasts from idiopathic and genetic Parkinson's disease patients [6]. Impaired BER (Base excision repair) capacities have been found in sporadic Alzheimer's disease patient's brain tissue [7].

Short telomeres have emerged as a marker associated with AD in several studies. It has been shown that AD patients have significantly shorter telomeres in the peripheral blood mononuclear cells compared to the age-matched controls. A significant correlation was found between the telomere length of the T cells and the Mini-Mental Status Exam (MMSE) of the AD group [8]. Reduced leukocyte telomere length was also found in individuals with AD compared to the control group [9]. Thomas et al. showed that AD patients have significantly shorter telomere lengths in white blood cells and buccal cells than age-matched healthy controls [10].

Reduced proteostasis is observed with aging, which also becomes an elevated factor for NDs. Proteostasis, the cellular mechanism of maintaining protein homeostasis, controls protein synthesis, folding, and degradation of misfolded proteins through a combination of pathways: the ubiquitin-proteasome system (UPS), unfolded protein response (UPR), and autophagy. Alteration of these pathways has been observed in ND patients caused by mutations in the component proteins of these pathways. Mutation in the parkin protein has been reported to cause both early onset and sporadic Parkinson's disease (PD) [11, 12]. Parkin is an E3 ubiquitin ligase, where E3 is a ligating enzyme, an element of the ubiquitin-proteasome system.

Increased levels of LC3-II protein, a marker for impaired autophagy, are observed in AD patient brains [13].

Other factors, such as genetic factors, environmental factors, and individual lifestyles, impact NDs in addition to aging. The genetic factors will be discussed in the later section for AD.

Environmental factors

With the drastic expansion of industrialization, exposure to metals has increased over time. It has been reported that welders exposed to manganese (Mn) from the welding fume have an increased risk of developing PD at an earlier age [14]. Exposure to aluminium (Al) from drinking water has been reported to pose an increased risk of developing AD in geographical areas with a higher aluminium concentration [15]. Higher concentration of aluminium has been reported in post-mortem brain tissues of AD patients [16].

Lifestyle

Evidence about an unhealthy lifestyle as a potential risk factor for NDs is also emerging. Moderate physical exercise, a balanced diet, quality sleep and mindfulness induce the anti-inflammatory state in a healthy individual by elevating the level of anti-inflammatory factors like antioxidants, anti-inflammatory cytokines like CD200-CD200R, etc. On the other hand, an unhealthy lifestyle, for example, excessive stress, chronic alcohol consumption, sleep deprivation, and high-fat diet induce the inflammatory state by increasing the pro-inflammatory cytokines such as IL-1 β , TNF- α and so on [17].

Aim of the thesis

Misfolded proteins can form fibrillar protein aggregates termed as amyloid which cause several neurodegenerative diseases such as Alzheimer's disease (AD). In AD, senile plaques and cerebral amyloid angiopathy (CAA) are considered as pathological hallmarks, which are accumulations of amyloid fibrils consisting of misfolded A β peptides. Amyloid fibrils can be diverse in their structures albeit the disease-causing misfolded protein has same primary amino acid sequence. This phenomenon is termed as amyloid fibril polymorphism. Protein structure and function is intimately connected. Spreading of the AD pathology appears to occur through a seeding mechanism, *i.e.* already formed aggregates acts as seed to initiate new fibril formation.

The specific aims of this thesis are to:

1. Further develop staining methods and analyses as a scientific and diagnostic tool to identify and discriminate amyloid aggregates
2. Investigate A β fibril polymorphism within different AD mouse models
3. Monitor the seeding activity of A β amyloid fibrils of diverse compositions and polymorphs of recombinant fibrils and isolates from of *Drosophila melanogaster* using the APP23 mouse model

Background

Alzheimer's disease (AD)

Alzheimer's disease is a fatal neurodegenerative disease that causes cognitive impairment among elderly people mainly, affecting their memory. The disease was first described in 1906 by the German psychiatrist Alois Alzheimer. Alzheimer had a long-term follow-up study of his 51-year-old female patient, Auguste D., who had been admitted to the Frankfurt psychiatric hospital for memory impairment and behavioral changes. Her condition deteriorated drastically over time, so she had to be admitted to the hospital until her death. Upon the death of the patient, Alzheimer had the opportunity to do an autopsy of the brain. Alzheimer observed severe brain atrophy. Alzheimer and his colleagues also observed depositions of peculiar substances in the brain with histopathological staining [18]. Nowadays, this deposition of peculiar substances is known as senile plaques and neurofibrillary tangles (NFTs), which are widely accepted pathological hallmarks of AD. Aside from plaques and tangles, cerebral amyloid angiopathy (CAA), aggregates found in the blood vessels are also considered pathological hallmarks of AD nowadays. All these pathological hallmarks are fibrillar aggregates of different misfolded proteins, generally termed as amyloid (**Figure 2**). Senile plaques or also called plaques interchangeably. Plaques and CAA are fibrillar aggregates of misfolded A β peptides. Meanwhile, the NFTs are composed of misfolded tau proteins.

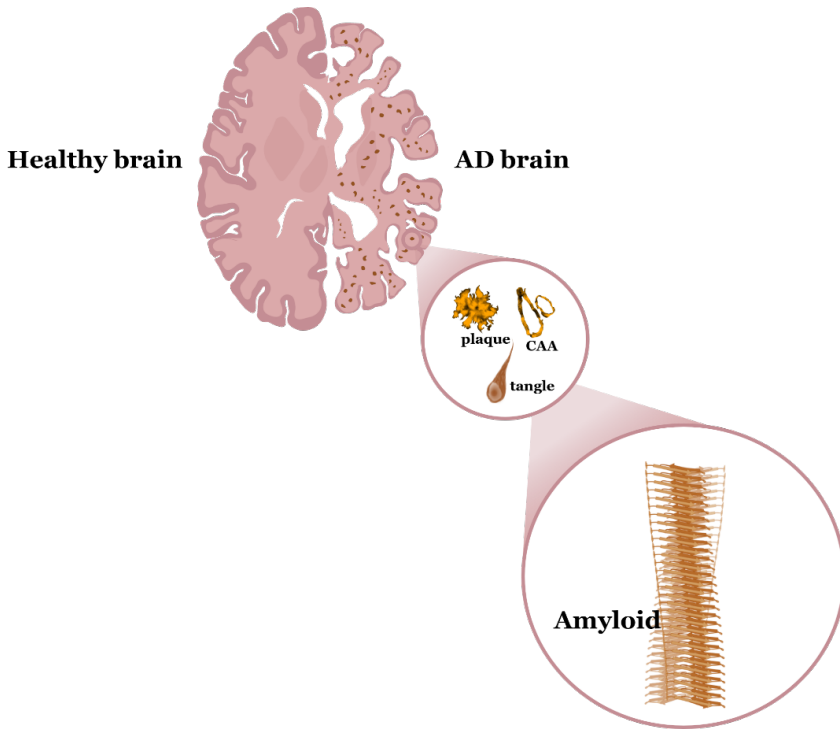


Figure 2: Illustration of AD-affected brain. The AD-affected brain is atrophied significantly compared to the healthy brain, which is full of amyloids: plaques, tangles, and CAA.

Since the first discovery of AD, it has become a global health crisis. According to the World Health Organization (WHO), an estimated 55 million people are living with dementia, among which 60-70% of cases are AD [19]. 6.9 million Americans aged 65 or older are estimated to have AD [20]. In Europe, approximately 7 million people are living with AD [21]. Each year, approximately 20,000 people are diagnosed with AD in Sweden [22]. This vast population affected by AD necessitates more research and initiatives to reduce the burden from the individual level to healthcare providers.

AD is classified into two categories, based on the onset of the disease: early-onset AD (EOAD) and late-onset AD (LOAD). EOAD affects approximately 5% of the total AD population and the disease onset is before 65 years of age. EOAD is familial, i.e., hereditary and referred as familial Alzheimer's disease (FAD). Mutations in three genes, amyloid precursor protein (APP), presenilin-1 (PS1) and presenilin-2 (PS2) are involved in causing the EOAD. The disease onset of LOAD is after 65 years and it is considered to be sporadic (sAD) [23].

While symptoms of AD might not appear until later in life, changes in AD biomarker levels begin about 20 years before manifestation of the first cognitive symptom shows itself. **Figure 3** illustrates the temporal progression of biological alterations, as evidenced by biomarker changes. CSF A β 42 precedes all the biomarkers, followed by amyloid PET and CSF tau. MRI and FDG biomarkers later indicate cognitive impairment in terms of brain dysfunction. While the high-risk group of people with genetic risk factors is already affected by the disease, the low-risk group can retain their normal cognitive activity, albeit preserving changes in biomarkers for AD pathology.

According to the European intersocietal recommendations for biomarker-based diagnosis of neurodegenerative disorders, published in *Lancet Neurology* in 2024, AD is biologically defined by the presence of A β plaques and tau protein tangles in the brain. These are key neuropathological features that can be detected through specific biomarkers such as CSF analysis and PET imaging (**Figure 3**). The recommendation emphasizes the importance of using biomarkers to aid in the diagnosis of AD, particularly in the early stages [24].

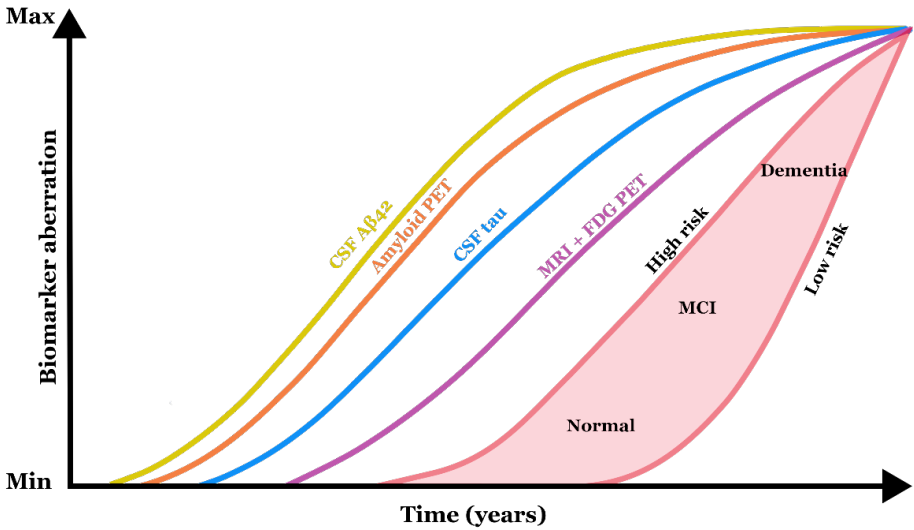


Figure 3: Temporal change in biomarkers over the years of AD pathology. CSF = Cerebrospinal fluid, MRI = Magnetic resonance imaging, FDG = Fluorodeoxyglucose, PET = Positron emission tomography, MCI = Mild cognitive impairment. Redrawn from [25].

The AD continuum, from measurable brain changes with no clinical symptoms to severe symptoms, spans three different stages: preclinical AD, mild cognitive impairment (MCI) and AD dementia. AD dementia is further subdivided into three groups (**Figure 4**).



Figure 4: Alzheimer's disease continuum showing different stages of the disease. The duration of each stage of the continuum is not equal and varies from person to person. Modified from [20].

Preclinical AD: The preclinical AD is asymptomatic, i.e., there are no visible symptoms of cognitive impairment. Individual's everyday life remains unhindered. However, changes in the brain are observed by PET imaging technique, for example, increased accumulation of A β and tau, decreased level of glucose metabolism [26, 27].

Mild cognitive impairment (MCI) due to AD: Mild cognitive impairment is referred to as the marginal stage between normal aging and the early form of dementia. Individuals affected with MCI experience very mild symptoms of memory problems or other cognitive declines. Different AD biomarker changes in the brain are also observed along with the subtle symptoms. However, individuals can maintain their everyday activities without major obstacles. This stage is also often stated as prodromal AD. About 15% of the MCI-affected individuals aged over 65 years, develop some sort of dementia within the next two years [28]. Within five years, approximately 33% of these individuals develop AD dementia [29].

Mild AD dementia: Individuals start to experience noticeable cognitive decline in many of their everyday life activities. They require more time to accomplish their daily tasks and face challenges in paying bills and making financial decisions. At this stage, individuals can often manage many of their everyday tasks by themselves; however, in certain areas, assistance might be needed to make their lives easy and properly functional.

Moderate AD dementia: At this stage, individuals suffer from further memory problems. They have difficulty recognizing their familiar persons. They have difficulties with their language skill, too. They undergo certain personality and behavioral changes such as becoming suspicious, aggressive, and withdrawing from social activities. Everyday life activities like bathing and dressing become difficult without assistance.

Severe AD dementia: In this stage, individuals require full-time assistance from the caregiver. Their language skill declines to single words or sometimes complete loss of verbal communication. Due to damage in the brain associated with mobility and

swallowing, individuals become completely bedridden and incapable of feeding themselves, leading to malnutrition. Individuals affected with severe AD are vulnerable to acute lung infections, such as aspiration pneumonia. Aspiration pneumonia is caused by inhaling oral or gastric materials like food particles and saliva into the lungs, leading to infection. It is one of the major causes of death among people suffering from AD dementia. Due to a lack of mobility, individuals pose a high risk of skin infections and sepsis, which might lead to organ failure and ultimate death [20].

AD demands immense resources, both for patient care and research. In the United States alone, the total cost of caring for individuals with AD is estimated at a staggering \$360 billion for 2024. [20]. Moreover, the research dedicated to AD clinical trials from January 1, 1995, to June 21, 2021, consumed approximately \$42.5 billion.[30]. Despite these substantial investments, a permanent cure for AD remains elusive. Since tacrine, the first drug for AD gained FDA (Food and Drug Administration) approval in 1993, the success rate for phase II and III clinical trials of Alzheimer's treatments has been a dismal 2% until 2022 [31]. Developing a new drug, from preclinical trials to FDA approval, typically takes about 13 years. As of January 1, 2024, 127 drugs were undergoing evaluation in 164 clinical trials aimed at treating AD [32]. However, only 11 drugs have received FDA approval since 1993. **Table 1** lists all the FDA-approved drugs until July 2024, grouped by drug category.

Early-stage AD drugs were primarily symptomatic. These drugs aimed to improve cognitive functions and address neuropsychiatric issues without altering the core biological mechanism of AD. Subsequently, drug development research shifted focus towards targeting and modifying the underlying disease mechanism *i.e.* aggregated A β protein to slow the disease progression.

Table 1: List of FDA-approved drugs for AD [30, 33, 34]. The commercial names of the drugs are in parenthesis. AD = Alzheimer’s disease, MCI = mild cognitive impairment. * Withdrawn from the market immediately after being available due to adverse side effects on the liver [35]. ** Manufacturer Biogen will discontinue commercialization and clinical trials to readjust resources for their AD franchise [36].

Drug category	Drug name	Molecule type	Aimed for	Year of approval
Symptomatic	Tacrine	Cholinesterase inhibitor	Mild to moderate AD	1993*
Symptomatic	Donepezil (Aricept)	Cholinesterase inhibitor	All stages of AD	1996
Symptomatic	Rivastigmine (Exelon)	Cholinesterase inhibitor	Mild to moderate AD	2000
Symptomatic	Galantamine (Razadyne)	Cholinesterase inhibitor	Mild to moderate AD	2001
Symptomatic	Memantine (Namenda)	Glutamate regulator	Moderate to severe AD	2003
Symptomatic	Memantine & donepezil (Namzaric)	Cholinesterase inhibitor + glutamate regulator	Moderate to severe AD	2014
Symptomatic	Suvorexant (Belsomra)	Orexin receptor antagonist	Moderate to severe AD	2020
Symptomatic	Brexpiprazole (Rexulti)	Atypical antipsychotics	Moderate to severe AD	2023
Disease-modifying	Aducanumab (Aduhelm)	Monoclonal antibody	MCI due to AD to mild AD	2021**
Disease-modifying	Lecanemab (Leqembi)	Monoclonal antibody	MCI due to AD to mild AD	2023
Disease-modifying	Donanemab (Kinsula)	Monoclonal antibody	MCI due to AD to mild AD	2024

Proteins, protein folding, and misfolding

Proteins are fundamental macromolecules indispensable for all living organisms. They exhibit a diverse array of functions, including structural support of tissues and organs, integral roles in immune responses and metabolic pathways, and the transport of essential molecules such as nutrients and neurotransmitters. The basic building blocks of these proteins are amino acids. Proteins comprise one or more polypeptide chains, made of 20 common amino acids (**Figure 5**).

Amino acid is an organic molecule composed of four key components attached to a central carbon atom, often referred to as alpha (α) carbon (**Figure 6**). The four key components are:

1. Amino group (NH_2): A nitrogen atom connected to two hydrogen atoms.
2. Carboxyl group (COOH): A carbon atom double-bonded to an oxygen atom and single-bonded to a hydroxyl group.
3. Hydrogen atom (H): A single hydrogen atom.
4. Side chain (R group): A variable group that varies among 20 common amino acids.

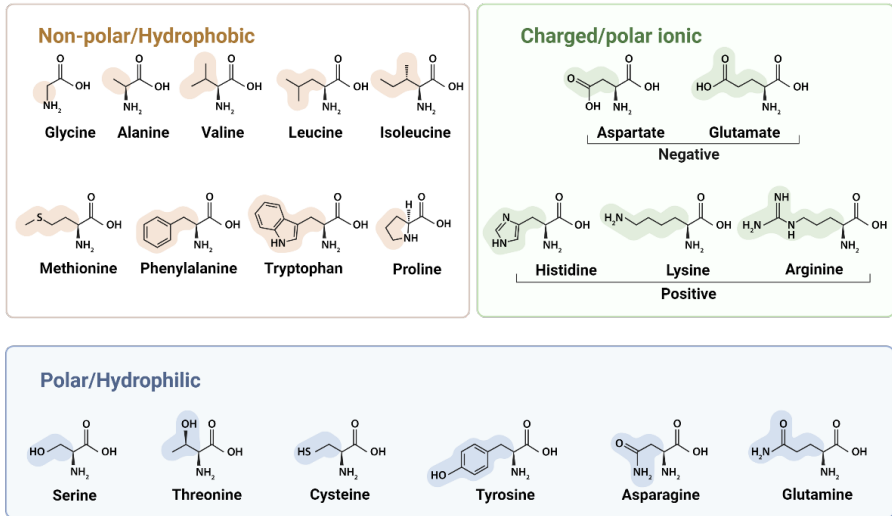


Figure 5: 20 common amino acids in three different groups based on the properties of their side chains. Glycine is the only amino acid devoid of sidechain.

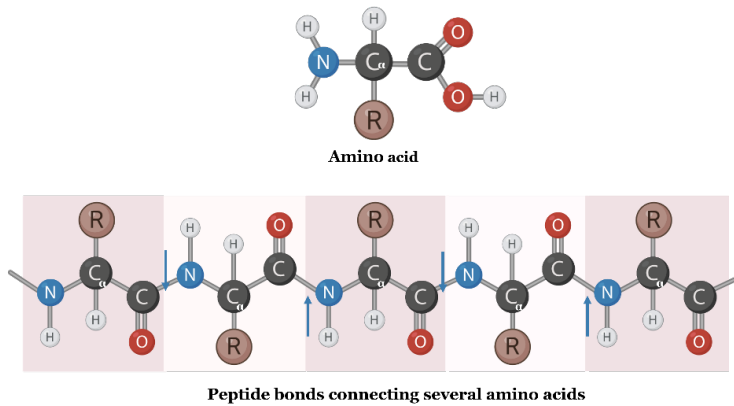


Figure 6: Top panel - general structure of an amino acid. Bottom panel – a polypeptide chain comprising several amino acids connected through peptide bonds. Peptide bonds are marked with blue arrows.

Amino acids link together to form a polypeptide chain through peptide bonds. Peptide bonds are formed between a carboxyl group of one amino acid and the amino group of another, releasing a water molecule in the process. Amino acids making the polypeptide chain through peptide bonds are called residues since they are no longer in the free form of amino acids. This polypeptide chain builds up the primary structure of a protein. The R group, or side chain, distinguishes one amino acid from another. Its properties determine the unique characteristics and functions of the amino acid within a protein. Based on the properties of R groups, amino acids can be divided into three groups: nonpolar/hydrophobic, polar/hydrophilic and charged/polar ionic amino acids.

1. Nonpolar/hydrophobic: Repel water and tend to cluster within the protein core of soluble proteins or on the surfaces of membrane transitioning protein segments.
2. Polar/hydrophilic: Interact with water and often found on the exterior of soluble proteins.
3. Charged/polar ionic: Have a positive or negative charge at physiological pH.

Apart from the 20 common amino acids synthesizing most proteins, two rare amino acids, selenocysteine and pyrrolysine, are incorporated into proteins through distinct biosynthetic pathways. Selenocysteine is predominantly localized within the catalytic sites of a limited subset of selenoproteins, ubiquitously distributed across the domains of Archaea, Bacteria, and Eukarya [37]. However, pyrrolysine is exclusively incorporated into proteins of certain methanogenic Archaea and Bacteria species [38].

Proteins need to fold to perform their function because their specific three-dimensional structure is essential for their biological activity. The precise folding creates active sites, binding sites, and interaction surfaces necessary for the protein to interact with other molecules accurately and efficiently. Proper folding stabilizes the protein, enables enzyme catalytic activity, and facilitates regulatory mechanisms.

Without correct folding, proteins can lose function, fail to interact correctly with other molecules, and even cause diseases through misfolding and aggregation.

A protein transitions from its linear primary structure to a complex three-dimensional conformation through a folding process involving the formation of secondary and tertiary structures. In some cases, multiple polypeptide chains assemble to form quaternary structures, ultimately resulting in a fully functional molecule (**Figure 7**).

The secondary structure arises due to the hydrogen bonding within the polypeptide chain. Commonly occurring secondary structures are the α -helices, β -sheets and turns. α -helices are spiral arrangements of the residues in the polypeptide chain. Each turn of the spiral has 3.6 residues. These structures are stabilized by repeated hydrogen bonds formed between residue i and $i+4$ in the next turn. β -sheets are folded sheet-like structures composed of two or more β -strands, arranged in parallel or antiparallel manner. β -sheets are stabilized by hydrogen bonds formed between the backbones of adjacent β -strands. Another element of secondary structure is turn. Turns connect the other secondary structures α -helices and β -strands and help the polypeptide chains to change directions to adopt a globular conformation. Based on the number of residues, turns can be divided into different groups: α -turn, β -turn, γ -turn, and loop. α -turn comprises five residues and is stabilized by a hydrogen bond between residue i and $i+4$; β -turn comprises four residues and is stabilized by a bond between residue i and $i+3$; γ -turn comprises three residues and is stabilized by a hydrogen bond between residue i and $i+2$. Loops have more residues than the other types of turns and without any fixed hydrogen bond among the residues [39].

The tertiary structure describes the overall three-dimensional shape of a polypeptide chain. It results from the interaction between amino acid side chains (R), including hydrogen bonding, ionic bonding, disulfide bridges, hydrophobic interactions and Van der Waals forces. This structure determines the protein's function.

The quaternary structure occurs only in proteins with multiple polypeptide chains (subunits) coming together. It describes how these subunits arrange and interact with each other. Hemoglobin is an example of a protein with a quaternary structure [40].

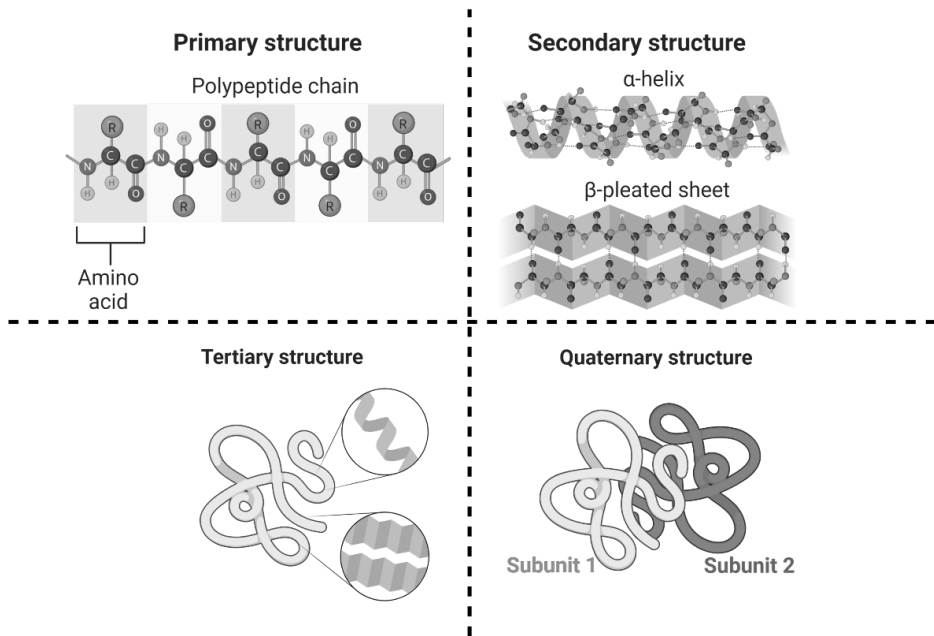


Figure 7: Schematic representation of different structures adopted by a protein during protein folding.

A well-maintained proteostasis is essential for the cells in our body to perform all biological functions. Proteostasis refers to the cellular process of maintaining protein homeostasis. In simpler terms, it is the delicate balance between protein synthesis, folding, and degradation within a cell. A well-established proteostasis is achieved by different pathways activated by the cell to aid newly synthesized proteins in folding correctly and degrading misfolded or unfolded proteins (**Figure 8**). Proteins are produced on ribosomes based on the genetic information stored in

cellular DNA. Some proteins begin to fold co-translationally while being synthesized on the ribosome. A major portion of the proteins fold in the cytoplasm post-translationally, while others fold within organelles like mitochondria and the endoplasmic reticulum (ER) [41]. The folding of mammalian secretory and membrane proteins occurs predominantly in the ER. In mammals, approximately 34% of proteins are secretory and destined for ER for folding before reaching their final destination of the secretory pathways by the Golgi apparatus [42].

Most of the mammalian proteins are translocated into ER co-translationally (nascent polypeptide chain remains attached to the ribosome) through the translocon, a protein complex. Post-translational translocation is also observed in mammals; however, this is more observed in yeast cells [43]. After entering the ER lumen, the nascent polypeptide chain undergoes post-translational modification, such as N-linked glycosylation and disulfide bond formation, catalyzed by Protein Disulfide Isozyme (PDI), which is crucial for protein folding. The ER lumen has a high concentration of different chaperone families, like HSP70s and HSP90s, which facilitates proper protein folding. Properly folded proteins are transported to the Golgi apparatus through transport vesicles and undergo further glycosylation. Later, these proteins are shipped to their next destination, like cytosol or the extracellular space, by the Golgi apparatus.

The ER also initiates the disposal of the unfolded proteins by transporting them to the cytosol through the ER-associated degradation (ERAD) system. In the cytosol, proteins are degraded by two different pathways: ubiquitin-proteasome system (UPS) or by lysosome-mediated autophagy. In the UPS system, misfolded proteins are tagged with ubiquitin, a small protein, in a multi-step process involving ubiquitin-activating, conjugating, and ligating enzymes. The ubiquitinated proteins are then recognized and degraded by the proteasome, a large protein complex, into short peptides, which can be further processed or recycled.

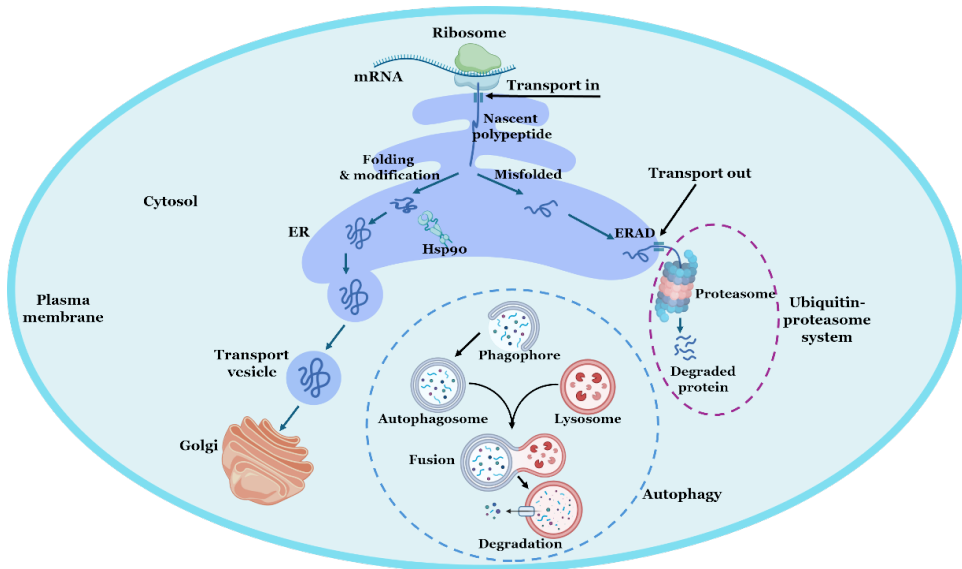


Figure 8: Protein quality control in the cell. Folded proteins in the ER are shipped to the Golgi apparatus for further secretion. Misfolded proteins are degraded by the Ubiquitin-proteasome system (UPS), encircled with a purple line and autophagy encircled with a blue line. Other organelles except for ER and Golgi of the cell are not highlighted.

The lysosomal degradation pathway or autophagy can be subdivided into three classes: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy is the most common form initiates with the formation of a phagophore, a cup-like structure originating from the ER or other membranes. This structure elongates and closes to form an autophagosome, engulfing cytoplasmic material including organelles, protein aggregates, and pathogens. Autophagosomes mature through a series of modifications and ultimately fuse with lysosomes, creating autolysosomes. Within this acidic compartment, lysosomal hydrolases degrade the enclosed contents into reusable building blocks, such as amino acids and fatty acids, which are released into the cytosol to support cellular metabolism and survival. Microautophagy directly involves the invagination of the lysosomal

membrane to form small vesicles containing cytoplasmic material, leading to their degradation within the lysosome. This process is less selective compared to macroautophagy and CMA. CMA is a highly selective process where specific proteins containing a KFERQ-like motif are recognized by chaperone proteins and directly delivered to the lysosomal membrane for translocation into the lysosomal lumen [44]. CMA is primarily involved in the degradation of soluble cytosolic proteins [45].

Despite the cell's robust mechanisms for maintaining proteostasis, failure of these systems can lead to the accumulation of misfolded protein aggregates and the development of several NDs. Aging is a primary factor contributing to proteostasis impairment, including compromised autophagy as previously discussed [2, 3, 13]. Certain proteins are inherently susceptible to misfolding and aggregation due to their amino acid sequences and are commonly implicated in NDs. These proteins lack stable three-dimensional structures and are termed intrinsically disordered proteins (IDPs) [46].

Proteins can form different types of aggregates. One form of these aggregates is amyloid, which is associated with NDs; hence, this thesis discusses only amyloid aggregates.

Amyloid fibrils

Amyloids are defined as fibrillar aggregates of misfolded proteins. These fibrils consist of multiple twisted protofilaments composed of repetitive β -strands oriented perpendicular to the fibre axis, forming a cross- β sheet structure. X-ray diffraction patterns reveal characteristic spacings of 4.8 Å between strands and 10-12 Å between sheets. Amyloid fibrils typically measure 70-120 Å in diameter and have an undetermined length, appearing as straight, unbranched structures under electron microscopy (EM) [47]. Clinically, amyloids are identified as pathological deposits that exhibit apple-green birefringence when stained with Congo red under polarized light, a gold standard for amyloid detection [48]. To date, the

International Society of Amyloidosis (ISA) Nomenclature Committee has classified 42 human proteins as amyloidogenic [49].

In vitro fibril formation kinetics shows a sigmoidal growth profile, which can be monitored by Thioflavin T (ThT), an amyloid-binding fluorescent dye. The kinetic profile has three different phases: lag phase/nucleation phase, growth phase/elongation phase and stationary phase (**Figure 9**). In the lag phase/nucleation phase, the protein monomer undergoes a conformational change, which is amyloidogenic and β -sheet rich structure. These amyloidogenic monomers grow further into oligomers that act as seeds/nuclei for further growth of larger aggregate species. In the lag phase, a large number of nuclei are formed [50]. Hence, this phase is also called the nucleation phase.

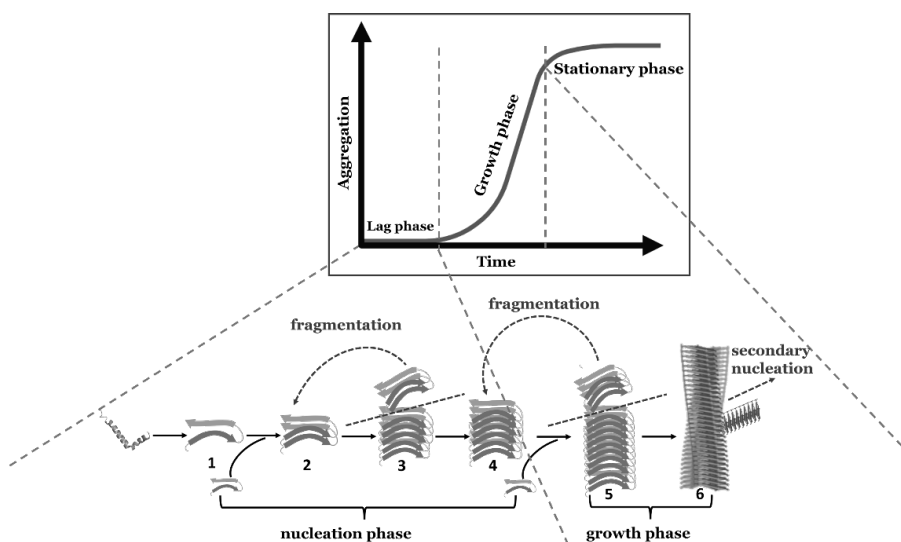


Figure 9: Sigmoidal kinetics of *in-vitro* fibril formation monitored by ThT fluorescence. Sharp ThT fluorescence is observed during the growth phase when enormous numbers of fibrils are formed. The numbers represent different species participating in the fibrillation process. 1 - misfolded monomer, 2 - dimer, 3 - oligomer, 4 - protofibril, 5 - protofilament, and 6 - mature fibril.

In the growth phase/elongation phase, previously formed nuclei grow further into prefibrillar structures such as protofibrils and protofilament by elongation. In the elongation process, monomers are added to the ends of the already-formed nuclei. At the same time, the fragmentation of the preformed aggregate species happens, generating more fibril ends to act as new nuclei. The growth of these new nuclei by elongation further generates prefibrillar structures. Secondary nucleation, i.e., the growth of new fibrils on the surface of existing fibrils, also takes place[51].

The stationary phase of fibril formation kinetics is where mature fibrils are formed. In this phase, an equilibrium is reached between the association and dissociation of monomers to the fibril ends. Different species are formed in all phases of fibril formation; however, certain species dominate at certain phases [50].

Proteins in Alzheimer's disease

Amyloid- β precursor protein

The Amyloid- β precursor protein or A β PP is the gene encoding the pathological A β peptide responsible for forming extracellular senile plaques. The sequential cleavage of A β PP by β - and γ -secretases generates A β , highlighting the critical role of A β PP processing in AD pathogenesis. The gene is located on chromosome 21 [52]. Alternative splicing of this gene gives rise to three different major isoforms: A β PP695, A β PP751 and A β PP770[53]. Where A β PP751 and A β PP770 have ubiquitous expression, A β PP695 is predominantly expressed in neurons [54-57].

A β PP is a complex protein with multiple domains, including a large extracellular domain, a transmembrane region, and a short intracellular domain. While the precise physiological function of APP remains elusive, it is thought to be involved in various cellular processes, such as neuronal plasticity, cell adhesion, and cell migration [58-60].

A β PP undergoes proteolytic processing either through the non-amyloidogenic or amyloidogenic pathways (**Figure 10**). In the non-amyloidogenic pathway, the soluble extracellular fragment sA β PP α is released by α -secretase cleavage between

Lys16 and Leu17 of the A β domain. This cleavage leaves a membrane-bound fraction α -CTF or C83 [61]. The membrane-bound α -CTF is later cleaved by a multi-meric protein complex γ -secretase releasing p3 fragment and membrane-bound intracellular C-terminal APP intracellular domain (AICD) [62].

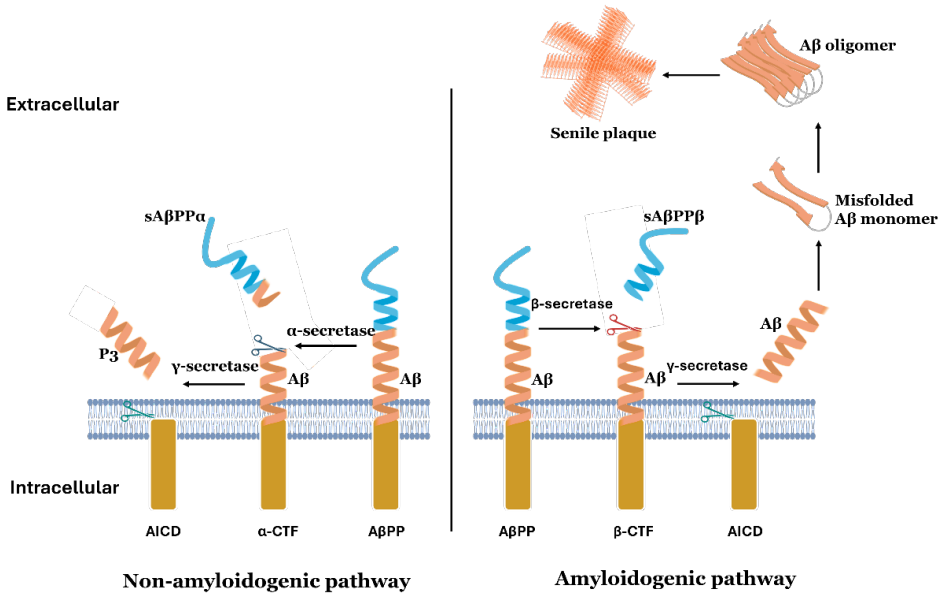


Figure 10: Non-amyloidogenic and amyloidogenic pathways. The non-amyloidogenic pathway is initiated by α -secretase while β -secretase initiates the amyloidogenic pathway.

The amyloidogenic pathway is initiated by the β -secretase BACE1 at the N-terminus of the A β domain at residue Asp1, generating a soluble sA β PP β fragment and leaving membrane-bound β -CTF or CTF99 fragment [63]. A multimeric protein complex, γ -secretase cleaves β -CTF within the transmembrane domain, releasing A β and the AICD. The core components of gamma-secretase are presenilins (PS1 and PS2). These proteins are believed to house the catalytic site of the enzyme [64].

Other essential components include nicastrin, APH-1, and PEN-2 [65]. The cleavage of C99 by γ -secretase generates various A β isoforms, differing in length from 37 to 43 amino acids. The most abundant isoforms are A β 40 and A β 42, with the latter being considered more amyloidogenic and neurotoxic [61].

While mostly A β peptide is described as a pathological agent due to its association with the senile plaques, several studies indicate that A β peptide also has a physiological role. A β has antimicrobial properties, helping to defend the brain against infections [66, 67]. At low concentrations, it can enhance synaptic plasticity [68]. It also aids neuronal growth and survival [69, 70]. A β might have a protective role in some forms of cancers by suppressing tumor growth [71-73].

Mutations associated with the genes responsible for A β processing pathways are prevalent for EOAD or FAD. To date, 34 pathogenic mutations in the APP gene, 221 pathogenic mutations in PS1, and 19 pathogenic mutations in PS2 have been identified [74]. PS1 mutations affect A β production by increasing the A β 1-42/A β 1-40 ratio [75]. Mutations in the APP gene are associated with both CAA and FAD. Pathogenic mutations commonly interfere with the secretory pathway of APP, causing an increase in A β generation and/or a change in the proportion of various A β peptides [76].

95% of people affected with Down syndrome (DS) have an extra copy of chromosome 21, a condition known as Trisomy 21 [77]. People with Trisomy 21 have overexpression of A β PP, thus increasing the risk of developing AD [78, 79]. In many cases, AD develops at an earlier age in individuals with Down syndrome compared to those without. AD is one of the leading causes of death for people suffering from DS [80] and was around 30% of the Swedish DS population from the year 1969-2003 [81].

Microtubule-associated protein tau (MAPT)

Microtubule microtubule-associated protein tau gene is located on chromosome 17 and encodes the tau protein that plays a central role in the formation of NFTs [82].

Tau is predominantly expressed in neurons in the CNS (central nervous system) and localized mainly on the axons in adults [83]. However, the presence of lower levels of tau has also been reported in different glial cells like astrocytes and oligodendrocytes [84, 85]. Its primary function is to stabilize microtubules, which are essential for maintaining neuronal structure and function [86]. Tau also regulates microtubule dynamics and axonal transport [87, 88]. In humans, tau has 6 different isoforms produced by alternative splicing of exons 2, 3, and 10 (**Figure 11**).

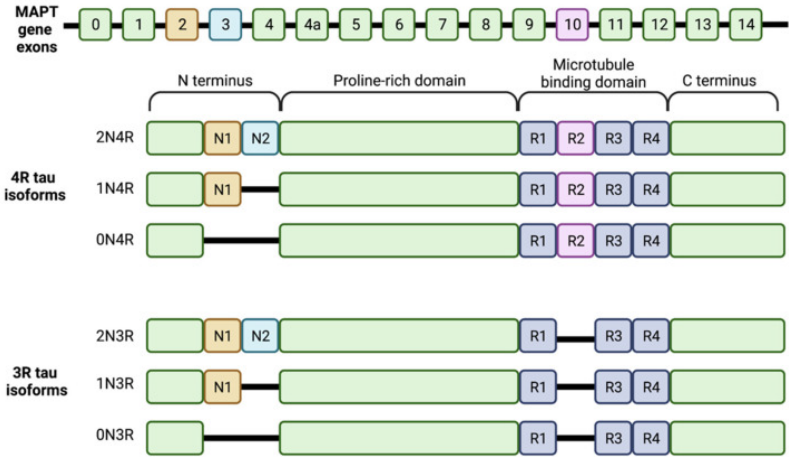


Figure 11: Six isoforms of human tau due to alternative splicing of exons. The image is taken from [89].

Hyperphosphorylated tau protein detaches from microtubules, leading to its self-assembly into insoluble NFTs, which are associated with AD and several other tauopathies [90]. The longest isoform of tau 2N4R has 85 potential sites for phosphorylation, primarily at serine and threonine residues [91].

Amyloid fibril polymorphism

Despite amyloid fibrils being similar in overall appearance characterized by highly ordered cross- β sheet structures perpendicular to the fibril axis, they can form different types of fibrils with distinct structural features and biological properties (Figure 12). This structural variation is even evident in fibrils derived from the same polypeptide chain. This feature is known as the structural polymorphism of amyloids [92]. Amyloid polymorphism is observed most often for fibrils formed *in vitro*. Figure 12 shows an example of different polymorphic structures for A β 1-42 fibrils formed *in vitro* [93-95]. The misfolded A β 1-42 monomers are arranged differently in the protofilament. Then, the protofilaments are also rearranged differently when they form the fibrils.

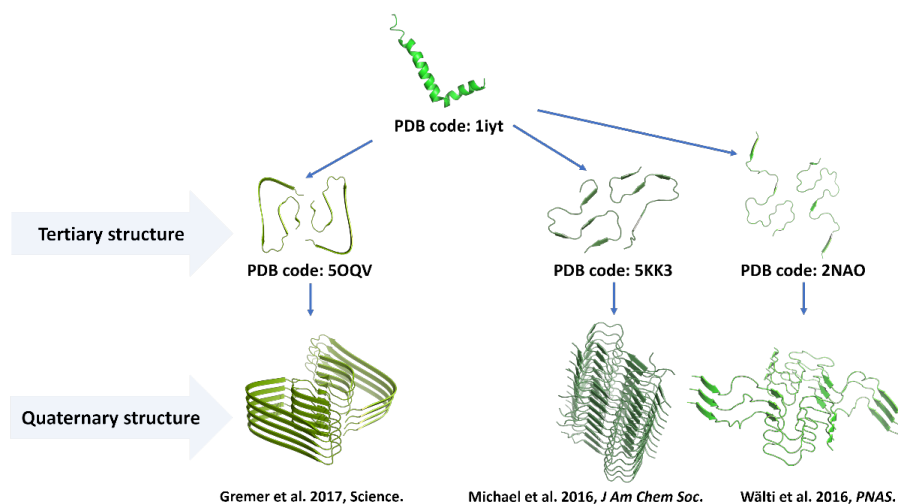


Figure 12: The variation in structural conformation of A β 1-42 fibrils generated *in vitro*. Structures were created using PyMOL software based on the provided PDB codes. The A β 1-42 fold and assembly of Gremer et al. 2017 fibrils are completely different from the Michael et al. 2016 and Wälti et al. 2016 fibrils, which are quite similar in their respective core.

Modifications to the *in vitro* fibrillation environment, including pH, buffer, salt concentration, and temperature, can generate diverse fibril polymorphs [95, 96]. For example, Petkova et al. demonstrated that almost identical experimental conditions can produce two distinct polymorphic fibrils except for differences in agitation state. A β 40 fibrils formed at 24°C and pH 7.4 exhibit a predominant "striated ribbon" morphology when the A β 40 solution is gently agitated during growth, whereas fibrils grown under identical conditions without agitation primarily display a "twisted" morphology [96].

This polymorphic morphology of the amyloid fibrils can be recognized by different techniques: solid state nuclear magnetic resonance (ss-NMR) [97], atomic force microscopy (AFM) [98], transmission electron microscopy (TEM) [99], cryogenic-electron microscopy (cryo-EM) [100] and fluorescence spectroscopy with the aid of conformation-sensitive luminescent conjugated oligo- and polythiophenes (LCOs and LCPs) dyes [92, 101, 102].

Amyloid polymorphism is not exclusively observed *in vitro* but is also evident in *in vivo* studies. Prusiner and colleagues found that synthetic A β fibrils can induce amyloid deposition in mice, and the resulting plaques vary depending on the fibril structure [103]. Jonson et al. demonstrated cell type-specific expression of A β 1-42 in transgenic *Drosophila* generated fibrils with distinct morphology detected by LCOs [104].

Lu et al. reported the presence of two distinct amyloid fibril structures composed of A β 40 fibrils in two Alzheimer's disease patients with different clinical histories. Patient I, a female who died at the age of 72, was diagnosed with Lewy body dementia and primary progressive aphasia. Fibrils isolated from this patient exhibited a single, predominant A β 40 fibril morphology with a constant diameter of 7 ± 1 nm, lacking any self-association into thicker bundles. Conversely, patient II, a female diagnosed with probable Alzheimer's disease died at the age of 80. Fibrils isolated from her brain tissue displayed a periodic twist as observed by transmission electron microscopy. These fibrils were composed of A β 40 [105].

Qiang et al. investigated the amyloid-beta ($A\beta$) fibril structures in different AD clinical subtypes. They found that while $A\beta_{40}$ fibrils from typical AD (t-AD) and posterior cortical atrophy (PCA-AD) have a dominant structure, those from rapidly progressive AD (r-AD) exhibit greater structural diversity. This suggests that factors beyond fibril structure may influence AD progression and that the increased structural variability of $A\beta_{40}$ fibrils in r-AD might contribute to increased neurotoxicity [106]. Yang et al. reported two different folds of $A\beta_{42}$ within fibrils derived from human AD brain material using cryo-EM [107]. The *in vivo* derived fibril polymorphs from human AD differed from those reported *in vitro* but showed similarities to fibrils formed in certain AD mouse models with $A\beta$ amyloidosis.

Rasmussen et al. analyzed LCO-stained postmortem brain tissues from different clinical subtypes of FAD and sAD. They observed cloud-like diversity in $A\beta$ plaques within each patient, reflecting the variation of $A\beta$ fibrils within a single sample. However, the structures of $A\beta$ plaques differed significantly between different AD subtypes. The distribution of this conformational cloud was biased towards specific types of mature or immature fibrils, depending on the existing mutations in the PS1 and APP genes in FAD cases. Interestingly, LCO staining features from FAD-derived fibrils could be templated by seeding into APP23 mice [108].

These *in vivo* studies suggest a strong correlation between the structural polymorphism of $A\beta$ fibrils and the diverse clinical phenotypes observed in different AD subtypes. This diversity in the amyloid structures makes PET imaging quite challenging because some structures may remain undetected by conventional PET ligands [109]. Therefore, accurate diagnosis requires a comprehensive approach capable of capturing the structural heterogeneity of amyloid deposits. Combinations of ligands or other biological molecules, such as a mixture of monoclonal antibodies targeting different epitopes of $A\beta$ fibrils, might resolve this problem.

Like *in vitro*, several factors influence amyloid fibril morphology *in vivo*, such as post-translational modification [110-112], disease mutation [113, 114], interacting molecules [115, 116] and so on.

Animal models of Alzheimer's disease

Animal models have been significantly used in AD research for understanding the pathophysiology of the disease, developing potential treatments, and exploring the efficacy and safety of novel therapeutic strategies. Since AD has a multifactorial complex disease mechanism, it is challenging to recapitulate the disease pathology *in vitro* or in cell culture. Unlike humans, animal models have shorter lifespans, allowing researchers to observe their entire lives and multiple generations. This is crucial for studying how diseases evolve and interact with the complex biological systems of living organisms. However, unnecessary use of animals should be avoided, and animal care should be prioritized during the experimental procedures to reduce their suffering.

The most widely used animal models in AD research are rodents [117]. To date, 227 rodent models have been developed for AD research. Among them, 17 models were generated in rats and 210 models were generated in mice [118]. Rodent models are primarily limited by their focus on FAD [119, 120]. Additionally, rodents require genetic modification and human FAD mutations to replicate AD pathology [117, 121].

Although rodent models cover some aspects of AD-related cognitive impairment, neuronal loss (observed only at old age and region-specific), and synaptic loss to some extent, they lack widespread neuronal loss at different brain regions, severe brain atrophy [120]. Animals that develop AD pathology spontaneously or naturally would be suitable for studying sAD disease pathology. Nonhuman primates and dogs are examples of sAD animal models since they develop plaques and CAA, in some cases NFT spontaneously without any genetic modification [122-124].

In addition to rodents and other higher animal models, lower animal models such as zebrafish and invertebrate animal models such as *Drosophila melanogaster* (fruit fly) and *C. elegans* are also used in AD research [125]. Though these lower-order model organisms are anatomically different from humans, the ease of genetic manipulations becomes the primary advantage of these models. Numerous

transgenic lines expressing human A β PP, and tau have been developed for these organisms [126-129]. Additionally, invertebrate models offer practical advantages such as easy handling, low cost, and short lifespans. These benefits make transgenic invertebrate models suitable for high-throughput genetic and drug screening[120].

In this thesis, mouse and *Drosophila melanogaster* have been used as model organisms. The next section will include a brief background of these two model systems.

Mouse (*Mus musculus*)

The first generation of mouse models were transgenic mouse models that overexpress the human A β PP along with the mutations of FAD. These mouse models are referred to as APP mouse models. Some mouse models also include mutations in PS1 and PS2, components of γ -secretase. These mutations lead to increased production and accumulation of A β , particularly more aggregation-prone A β 42 peptide [130]. These mouse models do not show any tau pathology. Therefore, transgenic mouse lines incorporating mutations of tau pathology were also generated [121].

Since this thesis focuses on the structural polymorphism and seeding activity of A β amyloid fibrils, this section will only discuss the APP mouse models used for this purpose.

The FAD mutations of AD for A β pathology span across the A β coding region including mutations in the upstream and downstream of A β coding region. The mouse A β peptide sequence differs from human by three amino acids at positions 5, 10, and 13 [131]. This sequence needs to be humanized to produce A β pathology. Figure 12 represents a schematic diagram showing different FAD mutations included in the A β PP for generating different APP mouse models (**Figure 13**).

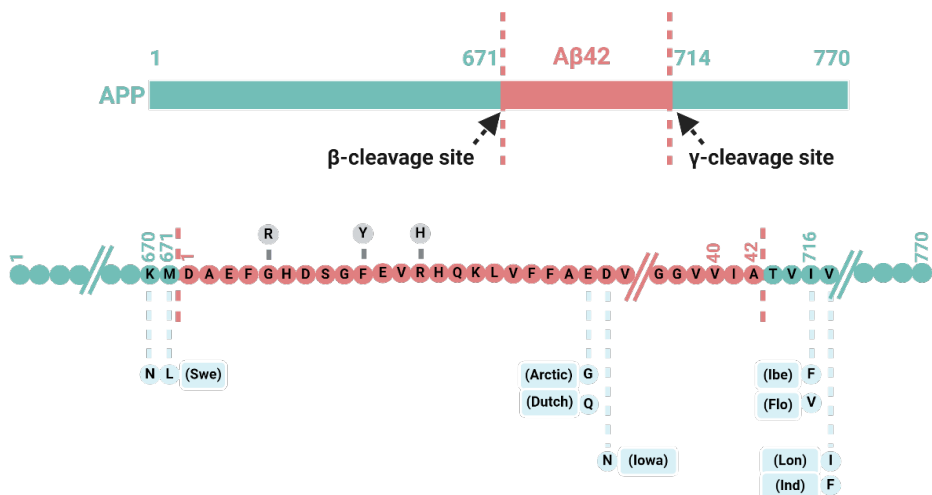


Figure 13: Illustration of the APP gene modification incorporating different FAD mutations in different APP mouse models. The Aβ peptide domain is highlighted in red. The amino acid residues at positions 5, 10 and 13 differ in mouse and human Aβ peptide sequences. The residues in human Aβ peptide sequences are shown in grey circles, which are required to be incorporated in the respective positions, replacing the mouse residues to create these mouse models.

APP23 mouse model: The APP23 mouse is a widely used transgenic model for AD research. It expresses the Swedish double mutation (KM670/671NL) in the APP751 isoform. Expressing the Swedish double mutation under the mouse Thy1 promoter element gives 7-fold overexpression of AβPP, which results in the overproduction of total Aβ40 and Aβ42 [130]. Aβ plaques begin to form in these mice around six months of age and plaques grow in size and number as they get older [132]. These mice generate profound CAA starting around 12 months of age, which increases with age [133]. Significant neuronal loss occurs in the CA1 region of the hippocampus, reaching up to 25% in mice with high plaque burden [134, 135]. Microglia

activation is observed near dense amyloid plaques [136]. These mice show age-dependent cognitive decline starting at 3 months of age [137].

APPPS1 mouse model: The APPPS1 mouse model was generated by co-expressing two FAD mutations, the Swedish double mutation (KM670/671NL) and presenilin-1 (PS1) with the L166P mutation under mouse Thy1 promoter element. A β plaques start to appear at 6 weeks of age and increase in size and number with age [138]. 7-month-old mice show spatial learning and memory impairment [139]. Neuron loss has been reported in the dentate gyrus in 17 months old mice [140]. Loss of dendritic spines around plaques has been reported to start about 4 weeks after plaque formation and persist for several months [141]. 3-fold increased microglia were observed surrounding plaques in 8-month-old mice [138].

First-generation APP mouse models have been essential in advancing our understanding of Alzheimer's disease, particularly in elucidating the role of A β pathology. These mice models, overexpressing A β PP have enabled the development and testing of a wide range of anti-A β antibodies [142-144]. As a result, significant progress has been made in discovering compounds that can lower A β accumulation in the brain, marking a major milestone in Alzheimer's research. Despite their benefits, these models also have certain limitations. The expression of A β PP under different promoters leads to variation in the expression level and regional distribution of exogenous A β PP among various mouse models [145]. In addition, overexpression of A β PP does not only lead to overproduction of A β but also other different A β PP fragments. Overproduction of non-A β A β PP fragments leads to unusual interactions with cellular proteins and causes unusual pathophysiological activity [146, 147]. Thus, some phenotypes observed in these mouse models are artifacts due to the ectopic expression of A β PP.

To eliminate these artifacts, single knock-in mouse models *App*^{NL-F} and *App*^{NL-G-F} were generated under mouse endogenous A β PP promoter to have the physiological expression of A β PP in the appropriate cell types and brain regions. In this thesis, *App*^{NL-F} was used, so a brief description of only this model is included.

App^{NL-F} model: This mouse model is generated by incorporating the Swedish double mutation (KM670/671NL) and Iberian mutation (I716F). Introducing the Iberian mutation produces elevated levels of A β ₄₂ and a high A β ₄₂/A β ₄₀ ratio without overexpressing A β PP [148]. A β plaques appear at 6 months of age in homozygote mice. Heterozygote mice develop plaques after 24 months. Microglia accumulation and astrocyte activation surrounding A β plaques, an indication of neuroinflammation is also observed in this mouse model. This mouse model also shows synaptic dysfunction. Memory impairment was observed in homozygote mice at 18 months [148]. A side-by-side comparison of the used mouse models in this thesis can be found in Table 2.

Table 2: A systematic overview of Alzheimer's disease mouse models utilized in the research. Table reused from [149].

Mouse models/strains	APP23	APP/PS1	<i>App^{NL-F}</i>
Generation	First	First	Second
Genetic modification	Transgenic	Transgenic	Knock-in (KI)
Promoter	mouse Thy1	mouse Thy1	mouse endogenous APP
Transgene	huA β PP ⁷⁵¹ (Swe)	huA β PP ⁶⁹⁵ (Swe); huPSEN1(L166P)	huA β PP ⁷⁵¹ (Swe)
Mutations	A β PP ^{KM670/671NL}	A β PP ^{KM670/671NL} ; PS1 ^{L166P}	A β PP ^{KM670/671NL,I716F}
APP expression level	7-fold overexpression	3-fold overexpression	1-fold expression
Ratio of A β amyloid (A β ₄₂ /A β ₄₀)	0.2-0.42*	2.8-4.3*	11-8600**
Availability	The Jackson Laboratory #stock 030504	Mathias Jucker & The Jackson Laboratory	Takaomi Saido
Reference	Sturchler-Pierrat, C., et al., Proc Natl Acad, 1997 PMID: 9371838	Radde, R., et al., EMBO Rep. 2006 PMID: 16906128	Saito, T., et al., Nat Neurosci. 2014 PMID: 24728269

*6 – 25 months for APP23
6 – 18 months for APPPS1

**6 – 21 months for *App^{NL-F}*

Ye, L., et al., EMBO Rep., 2017. PMID: 28701326

Heilbronner, G., et al. EMBO Rep., 2013. PMID: 23999102

Fruit fly (*Drosophila melanogaster*)

Despite fruit flies (*Drosophila melanogaster*) having less anatomical similarity to humans than rodents, many essential cellular processes like cell cycle regulation [150, 151], metabolic pathways [152], signalling pathways [153-155], cell death [156, 157] are highly conserved between the two species. Approximately 75% of human disease-causing genes have homologs in *Drosophila* [158, 159]. This model organism offers powerful genetic tools, including the GAL4/UAS system [160], RNA interference (RNAi) [161] and CRISPR-Cas9 [162] for controlled gene expression. In addition, the *Drosophila* genetic system utilizes balancer chromosomes, which avoid recombination in female flies and prevent the loss of mutant alleles due to crossing over. Balancer chromosomes are rearranged chromosomes with multiple inversions and distinct visible markers. These markers can be used to identify genes that are physically linked to a mutation of interest [163]. The life cycle of *Drosophila* is short, making it convenient to raise large populations for genetic, biochemical, and molecular analyses. The life cycle is approximately 10 days from fertilized egg to adult fly, with a maximum lifespan ranging from 60 to 80 days, depending on culture conditions. In controlled laboratory environments, *Drosophila* is typically cultured at 25°C, although 18°C is often employed for maintaining stock populations [164]. Given these advantages, *Drosophila* has been widely used over the past two decades to study neurodegenerative diseases, including Alzheimer's disease [127, 165], Parkinson's disease [166], Huntington's disease [167], amyotrophic lateral sclerosis [168] and others.

The ortholog of the human APP gene in *Drosophila* is *Appl* (Amyloid precursor protein-like), which encodes the protein APPL [169]. APPL expression is exclusively localized to the nervous system. *Drosophila* also possesses homologs of β -secretase and γ -secretase: dBACE [170] and Psn [171]. Despite the orthologous relationship between A β PP and APPL, a significant divergence is apparent in the A β region, characterized by limited sequence similarity [172]. Therefore, there is no endogenous production of A β in *Drosophila*. However, *Drosophila* β -secretase and γ -secretase cleavage of APPL produce a 64-residue A β -like fragment, which

aggregates and shows neurotoxic effects in the brain and eye. This aggregate is also Thioflavin-S positive [127, 170].

Over the years, two types of primary transgenic *Drosophila* models have been employed to investigate the various aspects of Alzheimer's disease. One type utilizes the secretase activity by coexpressing human A β PP, BACE1, and endogenous *Drosophila* Psn, thereby generating human A β aggregates in *Drosophila*. Transgenic flies expressing human A β PP, BACE1, and *Drosophila* Psn with pathogenic FAD mutations exhibited pronounced amyloid pathology, confirming the toxic effects of A β 42 in physiologically relevant *Drosophila* models of AD [173]. Another more straightforward approach for modeling AD neurotoxicity involves generating flies that produce a secreted form of the human A β 42 peptide, thus mimicking the extracellular accumulation of the primary pathogenic factor in this condition. In this method, different signal peptides were fused to the N-terminal of the human A β [127]. Utilizing this direct method, Luheshi et al. investigated the specific peptide properties and aggregated species accountable for toxic phenotype. Systematic substitute of single amino acids revealed that the sequence-specific propensity of the A β 42 peptide to aggregate into protofibrillar structures is the primary determinant of its pathological effects in living organisms [174]. Another study demonstrated that a single amino acid substitution can be sufficient to induce toxicity in A β 40 (E3R) and to abolish the toxicity of A β 42-E22G (I31E). These mutations modify the relative levels of prefibrillar soluble aggregates, which are hypothesized to be the most toxic forms [175].

Drosophila has also been utilized as a model system to evaluate different pharmacological agents or biomolecules for their potential to suppress A β 42 toxicity. For instance, Caesar et al. demonstrated that the administration of curcumin to A β 42 flies resulted in a significant enhancement of lifespan, locomotor function, and

notable acceleration of A β 42 fibril formation [176]. *Drosophila* models have also been developed to study tau toxicity, although these are not discussed in this thesis.

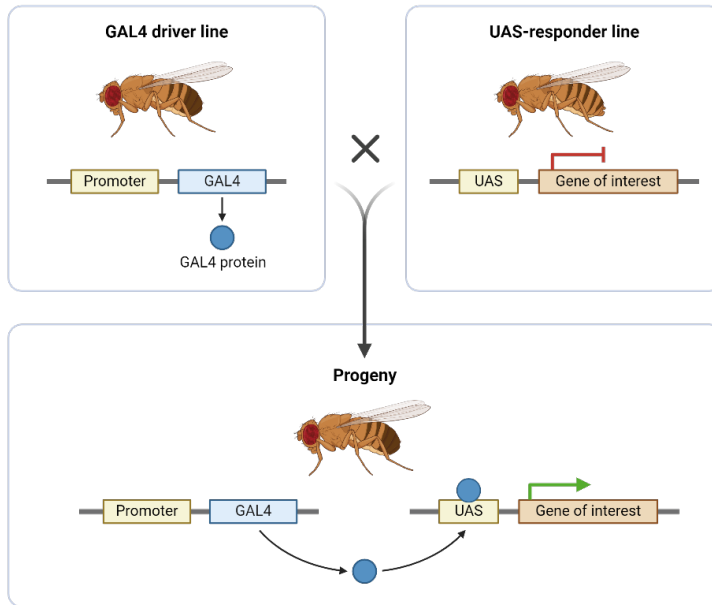


Figure 14: Illustration of the GAL4/UAS system. Crossing the driver line and responder line results in targeted gene expression in the offspring.

In this thesis, we have used the GAL4/UAS system to express the human A β peptide (**Figure 14**). The GAL4/UAS system is a versatile and widely used genetic tool for targeted gene expression in *Drosophila* research, developed by Andrea Brand and Norbert Perrimon in 1993 [160]. The system comprises two key components: the GAL4 protein and the Upstream Activating Sequence (UAS). GAL4, a yeast protein, functions as a transcriptional activator that specifically binds to UAS sequences located upstream of a gene of interest. In the GAL4/UAS system, two distinct transgenic fly lines are generated: one line carrying the GAL4 gene under the control of a tissue-specific promoter, and the other line carrying the UAS sequence linked to the target gene. When these two lines are crossed, the resulting offspring express GAL4 in the specific tissues dictated by the promoter. The tissue-specific

expression of GAL4 subsequently activates transcription of the gene downstream of the UAS sequence, enabling targeted gene expression [177]. Notably, the GAL4/UAS system is temperature sensitive, with GAL4 expression being enhanced at a higher temperature, 29°C compared to a lower temperature [178].

Fly lines used in this thesis:

To achieve the targeted expression of A β 1-42, we employed two driver lines: nsyb-Gal4 and repo-Gal4. The nsyb-Gal4 driver is a pan-neuronal, expressing the gene of interest in all neurons of the CNS [179]. Conversely, the pan-glial driver repo-GAL4 expresses the gene of interest in all glial cells of the CNS [180]. As the reporter line, we utilized a previously optimized UAS-A β 1-42 transgenic line designed for high-level expression. The A β 1-42 sequence was integrated into a pUASattB vector, which was landed at the 65B locus on chromosome 3. For further details regarding this transgenic line, please refer to the cited article [179].

Methods

A β peptide production and purification

In this study, recombinant amyloid- β (A β) protein was produced in *Escherichia coli* (*E. coli*) using a plasmid-based expression system [181]. The gene of interest was inserted downstream of the lac operator and the T7 promoter, allowing for IPTG-induced gene expression. The A β construct was transformed into *E. coli* cells and protein expression was induced by IPTG.

After cell lysis, A β protein was purified using a combination of ion exchange and size exclusion chromatography. Ion exchange chromatography involves binding A β to a stationary phase with charged molecules, either cation exchange or anion exchange, depending on the charge of A β . A β was then eluted from the column by increasing the ionic strength of the buffer or changing the pH, disrupting its interaction with the stationary phase.

Size exclusion chromatography was used as a final purification step to ensure the removal of any remaining contaminants and to obtain a homogeneous A β protein preparation. This technique separates molecules based on their size, with larger molecules eluting first and smaller molecules eluting later.

By using a size exclusion column with appropriate pore sizes, A β protein could be effectively separated from other molecules in the lysate. More details, about the A β ₁₋₄₀ and A β ₁₋₄₂ protein expression and purification steps, can be found in this article [182].

***In vitro* fibrillation**

The *in vitro* fibrillation process of A β -peptide was initiated as soon as possible after the peptide elutes from the size exclusion chromatography since A β -peptide is highly aggregation-prone. The eluted peptide solution was kept on ice during the preparation process of fibrillation. The peptide concentration was determined at

absorption at 280 nm by spectrophotometer. All the fibrillation processes were performed in 96 well plate, in non-shaking condition at 37°C (**Figure 15**)

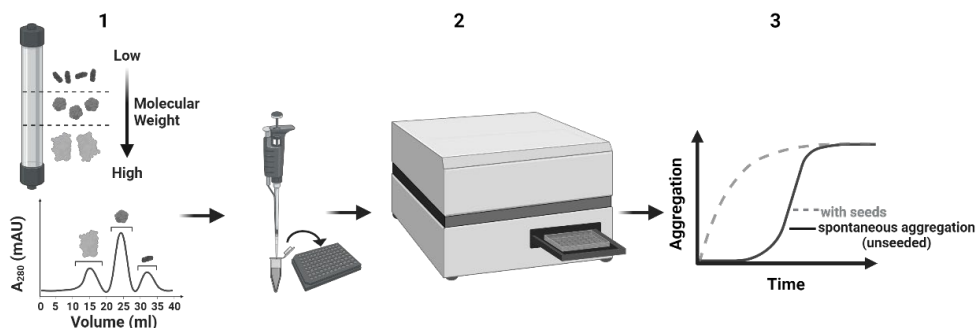


Figure 15: Schematic representation of protein purification (1), *in vitro* fibrillation (2), and seeding experiment (3).

Seeding

The lag time of fibril formation can be shortened or, in some cases, diminished completely by the addition of preformed fibrils called seeds. These seeds bypass the nucleation step and accelerate the fibril formation. This phenomenon is termed as seeding (**Figure 15**). To validate the seeding activity *in vitro*, a ThT fibril formation kinetic assay was used. All seeding experiments were performed in a Tecan infinite M1000 PRO plate reader using a 96-well plate (Corning 96 Flat black) under non-shaking conditions. Experiments were performed at 37 °C. To monitor the aggregation kinetics, the amyloid-specific fluorescent dye ThT was used. 1% seeds from different seed types were used for seed validation experiments.

Basic principles of fluorescence

Fluorescence

Fluorescence is a type of luminescence characterized by the relaxation of a molecule from an excited electronic state to its ground state, accompanied by the emission of a photon. This process differs from phosphorescence, which involves a slower relaxation due to spin restrictions.

In fluorescence, fluorophores undergo electronic excitation from the ground singlet state (S_0) to a higher singlet state (S_1 or higher). Internal conversion quickly relaxes the molecule to the first singlet state, followed by vibrational relaxation to the lowest vibrational level. This energy loss results in the Stokes shift, where the emitted photon has a longer wavelength than the excitation photon.

The fluorescence lifetime is the average duration a fluorophore resides in its excited state before emitting a photon. It is influenced by factors such as the molecule's environment and interactions. The quantum yield represents the efficiency of fluorescence, with higher quantum yields indicating brighter molecules.

The dipole moment of a molecule can change upon excitation, affecting its interaction with the environment. In polar environments, this can lead to energy losses and a red-shifted emission spectrum.

Some molecules, known as charge transfer molecules, can undergo internal charge transfer (ICT) when excited. This involves the transfer of an electron from a donor group to an acceptor group, resulting in a separation of charge within the excited molecule. In some molecules, the donor and acceptor groups are connected through a single bond and undergo a rotation or twist to facilitate the charge transfer. This process is referred to as twisted internal charge transfer (TICT). The emissive properties of the ICT and TICT state can vary significantly depending on the molecular structure and environmental factors.

Jablonski Diagram

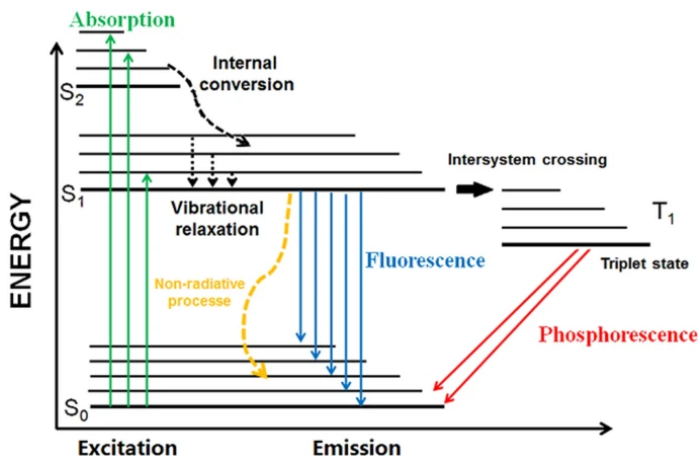


Figure 16: Jablonski diagram representing the fluorescence and phosphorescence. The image is taken from [183].

The Jablonski diagram is a visual representation of the energy levels and transitions involved in fluorescence (**Figure 16**). It illustrates the ground state (S_0), excited singlet states (S_1 , S_2 , etc.), and triplet states (T_1 , T_2 , etc.). The diagram shows the absorption of a photon, leading to excitation to a higher singlet state. Internal conversion and vibrational relaxation quickly bring the molecule to the lowest vibrational level of the first singlet state. From here, fluorescence occurs, emitting a photon and returning the molecule to the ground state [184].

Immunofluorescence

Immunofluorescence (IF) is a powerful technique employed in biomedical research to visualize the localization of specific proteins or antigens within tissues. It involves using antibodies that bind specifically to the target antigen, followed by the detection of these antibodies using labelled secondary antibodies or other detection methods.

In this thesis, flash-frozen mouse brain and paraffin-embedded human brain sections were used for IF. Paraffin-embedded tissue sections were deparaffinized to remove the paraffin wax and rehydrated to restore tissue integrity. Antigen retrieval was performed by incubating the tissue sections in boiled citric acid for epitope exposure of the hidden antigens due to the fixation process. Non-specific binding of antibodies to the tissue sections was prevented by incubating in a blocking solution, goat serum. Following the blocking step, tissue sections were incubated with primary antibodies overnight at 4 °C. A secondary antibody labeled with a detectable marker, *i.e.* a fluorophore, is applied to bind to the primary antibody. After the completion of the staining procedures, the tissue sections were mounted with mounting media and covered with a cover slip. The stained sections were later visualized by fluorescence microscopy.

Amyloid ligand staining

Amyloid ligand staining is a valuable technique used to visualize and characterize amyloid fibrils. Various fluorescent dyes and ligands have been developed to specifically bind to amyloid aggregates, providing valuable information about their structure, distribution, and dynamics (**Figure 17**). For this thesis, ThT (Thioflavin T) and LCOs (Luminescence Conjugated Oligothiophenes), BTD-3, and NSB were used extensively.

ThT

ThT is a benzothiazole dye exhibiting a fluorescence enhancement upon binding to amyloid fibrils (**Figure 17**). The rotational restriction induced by binding to amyloid fibrils enhances ThT's fluorescence intensity. ThT is commonly used to monitor the kinetics of amyloid fibril formation *in vitro* and to detect amyloid plaques in brain tissue. ThT can also bind to other types of protein aggregates, potentially leading to false-positive results. Additionally, ThT staining can be affected by factors such as pH and ionic strength [185].

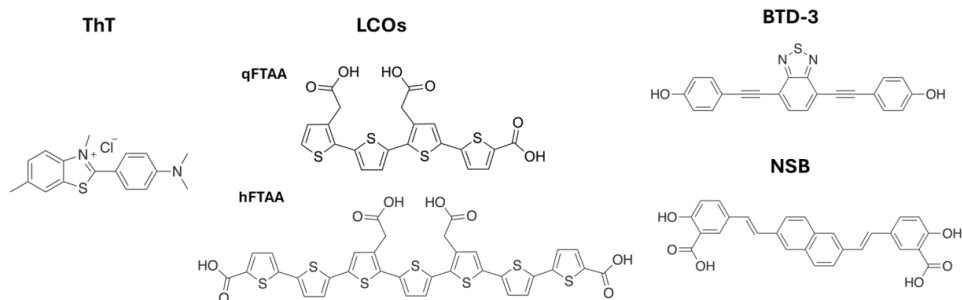


Figure 17: Structure of amyloid binding ligands fluorescent ligand. Chemical structures courtesy: Marcus Bäck, Linköping University.

Luminescence Conjugated Oligothiophenes (LCOs): LCOs are thiophene-based fluorescent ligands that show distinct spectral properties upon binding to amyloid fibrils. Based on the flexibility and length of the thiophene backbones the ligands could give a wide range of emission spectra in the visible spectrum [186]. Combinations of LCOs can offer increased contrast and enhance differences between amyloid structures.

BTD-3

BTD-3 is an analogue of the bis-styryl-benzothiadiazole probe with a hydroxyl group (-OH). This hydroxyl analogue shows a higher binding affinity towards recombinant A β 1-42 fibrils than A β 1-42. In BTD-3 the styryl group is replaced by alkyne alkyne-conjugated spacer (\equiv), i.e. a triple bond (**Figure 17**) [187]. The central moiety of this dye is benzothiadiazole (BTD). BTD derivatives have become popular fluorescence probes because of their photophysical properties including large Stokes shift, high quantum yield, high molar extinction coefficient, good signal-to-noise ratio, bright emission, and high storage stability [188].

NSB

NSB is an analogue of X-34 (Congo red derivative) [189]. NSB was synthesized to replace the single central benzene group with a naphthyl group (**Figure 17**). This ligand shows high binding affinity towards recombinant A β 1-42 fibrils compared

to X-34 and ThT. NSB shows an emission maximum at 505 nm in PBS, which is blue-shifted around 448 to 446nm upon binding to A β 1-42 fibrils, with a 79-fold increase in intensity compared to the free probe in PBS. It also has a higher quantum yield compared to X-34 and ThT when bound to A β 1-42 fibrils [190].

Fluorescence microscopy

Fluorescence microscopy is a powerful technique for visualizing biological structures and processes based on their fluorescence properties. It involves the excitation of fluorophores within a sample using specific wavelengths of light, followed by the detection of the emitted fluorescence signal.

Hyperspectral imaging microscopy

Hyperspectral imaging microscopy is an advanced technique that captures images across a broad spectrum of wavelengths, delivering spectral data for each pixel. In contrast to traditional imaging methods, which capture images at only one or a few specific wavelengths, hyperspectral imaging gathers information across many wavelengths, rendering full spectral information over the image (**Figure 18**).

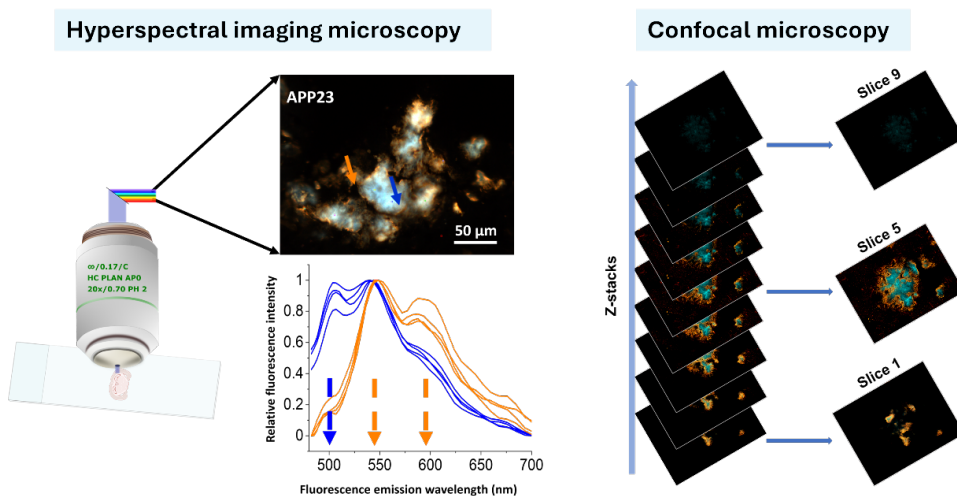


Figure 18: Illustration of two different types of fluorescence microscope. Image reused from [149].

This technique provides powerful insights into ND research, by exhibiting distinct spectral signatures for amyloid structures and fibril polymorphs [191].

Confocal microscopy

Confocal microscopy allows for the acquisition of high-resolution images from the sample's different optical sections (**Figure 18**). In light microscopy, the sample is illuminated uniformly. Therefore, thicker samples can suffer from blurriness due to out-of-focus light and scattering. Fluorescence microscopy shares this issue due to the excitation of dye molecules in all planes. Confocal microscopy overcomes this by selectively rejecting out-of-focus light, enabling high-resolution imaging of thick tissues. This is achieved by a tiny light point, a so-called pinhole, which blocks the out-of-focus planes, focusing the laser beam of the microscope onto a specific point, which is also the detection point, resulting in a sharp image. The laser beam is then moved to a different depth within the sample, and the process is repeated to acquire a series of images at different focal planes [192].

MSD immunoassay

MSD (Meso Scale Discovery) is a highly sensitive sandwich immunoassay that allows the detection and quantification of analytes such as protein in biological samples (serum, plasma, and tissue homogenates). This immunoassay utilizes the Electrochemiluminescence (ECL) from the detection antibody conjugated with an electrochemiluminescent tag. In the MSD assay, a microplate with a high-binding carbon electrode is used (**Figure 19**). The plate is coated with a capture antibody specific to a target protein. After the sample is added to the plate, the target protein binds to the capture antibody. To detect the protein, a Sulfo-Tag labeled detection antibody is applied. Electricity is then applied to the plate electrode by an MSD instrument, resulting in a chemical reaction catalyzed by the ruthenium-containing Sulfo-Tag, which emits light. The light intensity is proportional to the amount of target protein in the sample.

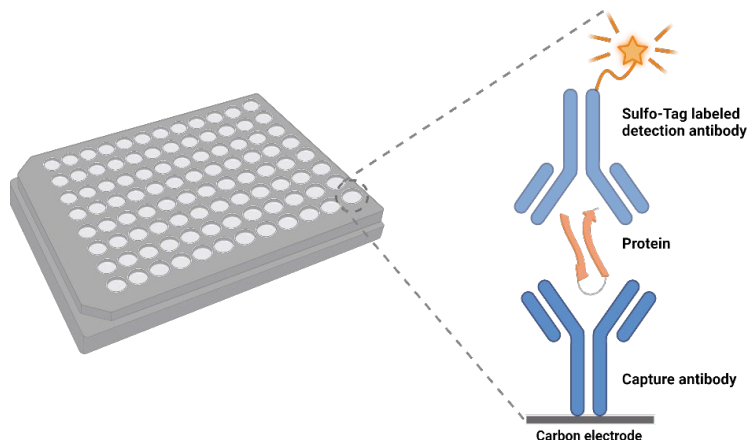


Figure 19: A schematic presentation of the MSD immunoassay.

The advantage of this immunoassay is it offers high sensitivity, enabling detection of proteins in low concentration. It can also quantify protein over a wide range of concentrations.

Ethical considerations

Mouse experiment

The experiments on the APP23 mice were conducted at Linköping University under ethical permit #10925-2020 #13028-2021. Mice with strain number 030504 were purchased from Jackson Laboratory for breeding and housed at Linköping University animal facility. Standard conditions were applied during the housing of mice: 12-hour light/dark cycle, room temperature $22\pm 2^{\circ}\text{C}$, humidity $55\pm 10\%$. Enrichment materials were provided in each cage, and trained technicians checked the cages daily for food and water. During the seed inoculation at 3 months, mice were provided analgesics 30 minutes prior to the procedure. For details about the procedures, please see Paper II. Mice were anesthetized by isoflurane prior to inoculation. Mice were closely observed for 24 hours after the inoculation procedure. Then,

mice were evaluated once every two weeks by a standard test routine procedure for neurological assessment, introduced by world-leading expert Adriano Aguzzi. Any mouse exceeding the cut-off for health assessment scoring used at the animal facility was euthanized. After 6 months of incubation, mice were killed at the age of 9 months to collect their brains. Mice were first anesthetized by isoflurane and later killed by carbon dioxide (CO₂). Then, the brains were collected by opening the skulls and frozen in dry ice for LCO histological studies.

Human brain sample

The human brain tissue experiment was approved under ethical permit #2020-01197 at Linköping University. Formalin-fixed paraffin-embedded human brain tissue samples were obtained from the Netherlands Brain Bank (NBB). NBB obtained written informed consent for the brain autopsy and use of clinical information, and the privacy of the patients was ensured during the research.

Drosophila melanogaster

Ethical permission is not required to perform experiments on *Drosophila melanogaster*. For details on experimental procedures, please see Paper III.

Figure compilation

All the figures in this thesis were compiled in BioRender.com except Figure 11, Figure 16, and Figure 18. Figures 11,16 and 18 are taken from articles with CC BY 4.0, which allows sharing, copying, and redistribution if credit to the original creation is given.

Summary of the papers

Paper I.

Divergent Age-Dependent Conformational Rearrangement within A β Amyloid Deposits in APP23, APPPS1, and App^{NL-F} Mice.

This study investigates A β fibril structures in various AD mouse models using LCOs (qFTAA and hFTAA) and A β -specific antibodies (4G8 and 12F4). LCOs and hyper-spectral microscopy provide valuable tools for studying A β fibril structures *in situ*. These methods enable the analysis of nearly intact amyloid structures in their natural environment, providing crucial insights into A β aggregation and its relationship to AD.

Different A β fibril polymorphs were observed in APP23, APPPS1, and App^{NL-F} mice models, influenced by A β PP genotype. Additionally, we observed that A β fibril structures evolved over time in APP23 and APPPS1 mice, but not in App^{NL-F} mice. This indicates the dynamic nature of A β aggregation and its potential relevance to disease progression.

In APP23 mice, we identified a profound core-corona structure with distinct A β 40 fibril polymorphs in each region. In contrast, App^{NL-F} mice exhibited smaller plaques with distinct A β 42 fibril polymorphs. This suggests that the spatial arrangement of A β fibrils within plaques may be influenced by the relative abundance of A β 40 and A β 42.

In summary, our study provides novel insights into the structural diversity of A β fibrils within plaques in AD mouse models. We demonstrate that A β fibril polymorphism is influenced by genetic factors, age, and A β isoform composition. Our findings highlight the importance of understanding A β fibril structures for developing targeted therapeutic strategies. By elucidating the structural heterogeneity of A β fibrils, researchers can gain insights into the mechanisms underlying AD pathogenesis and identify potential therapeutic targets.

Paper II.

Efficient Seeding of Cerebral Vascular Amyloidosis by Recombinant A β 1-42 Amyloid Fibrils.

Our study investigated the ability of different recombinant A β fibrils to seed A β -amyloid plaques and cerebral amyloid angiopathy (CAA) in APP23 mice. We generated fibrils from A β 1-40, A β 1-42, and A β 1-40+A β 1-42 and inoculated them into young APP23 mice.

We found that all three types of fibrils could effectively induce A β -amyloid plaques and CAA in APP23 mice. A β 1-42 fibrils were particularly potent in seeding CAA, suggesting that their unique structure or properties may contribute to their increased efficacy.

We also used LCOs, qFTAA, and hFTAA to analyze the fibril structure of amyloid deposits in seeded mice. We compared the fluorescence emission ratios of these LCOs in different types of plaques and found that the core of plaques in seeded mice had lower qFTAA binding than in old mice. However, some plaques in A β 1-42 and A β 1-40+A β 1-42 seeded mice showed higher qFTAA/hFTAA ratios than expected for their age. This suggests that accelerated plaque formation may be associated with a certain level of plaque maturation.

To further investigate the fibril structure of amyloid deposits in seeded mice, we used the fluorescent amyloid probes NSB and BTD3 from our in-house produced library of ligands. These ligands bound differently to A β 1-40, A β 1-42, and A β 1-40+A β 1-42 fibrils, suggesting distinct structural characteristics. The emission spectra of NSB and BTD3 were blue-shifted for A β 1-40 fibril seeds compared to A β 1-42 and A β 1-40+A β 1-42 seeds. In seeded mice, NSB and BTD3 staining revealed different spectral profiles in CAA and amyloid plaques, suggesting that the fibril structure of these deposits may be influenced by the type of seed used.

Overall, the study provides evidence for the *in vivo* seeding of A β -amyloidosis by recombinant A β fibrils in a mouse model and highlights the importance of understanding the role of A β fibril structure and composition in disease progression.

Paper III.

Different brain A β -amyloidosis patterns induced by seeding from separate A β 1-42 polymorphs originating from transgenic *Drosophila*.

In this study, we investigated the influence of A β 1-42 fibril structural polymorphs, derived from different *Drosophila* transgenic flies expressing A β 1-42 in neurons or glial cells, on seeding activity *in vitro* and *in vivo* in APP23 mice. Despite the different cell types expressing A β 1-42 in *Drosophila*, purified A β 1-42 fibril seeds from the fly brains were initially inactive in inducing A β -amyloidosis in APP23 mice. However, after amplifying these seeds *in vitro* with recombinant monomeric A β 1-42, they became highly active, even surpassing the seeding efficiency of pure recombinant A β 1-42 seeds. This suggests the formation of novel A β 1-42 amyloid strains.

Furthermore, LCO staining of amyloid plaques in the seeded APP23 mice revealed distinct maturation profiles for seeds originating from *Drosophila* neurons and glial cells. Seeds from neurons exhibited a significantly higher maturation profile of plaque cores and corona compared to seeds derived from glial cells. These findings demonstrate the high evolvability of A β -amyloid polymorphs and highlight the potential impact of cell-type-specific A β 1-42 fibril formation on the propagation of A β -amyloidosis.

Overall, this study provides insights into the diverse nature of A β 1-42 fibril polymorphs and their potential roles in the pathogenesis of Alzheimer's disease. The findings suggest that targeting specific A β 1-42 amyloid strains could be a promising therapeutic approach for this neurodegenerative disorder.

We have shown that the structural polymorphs of A β 1-42 fibrils derived from *Drosophila* can influence seeding activity *in vitro* and *in vivo*. We have shown that these polymorphs can evolve during amplification and potentially contribute to the progression of Alzheimer's disease. Our findings suggest the need for further investigation into amyloid strain-specific role in disease.

Note: *In paper III, a smaller number of sections were analyzed compared to paper II, meaning that a comparison of absolute numbers between the two papers cannot be made. However, within paper III, the comparisons are made on the same number of sections.*

Paper IV.

Dual-ligand Fluorescence Microscopy Enables Chronological and Spatial Histological Assignment of Distinct Amyloid- β deposits.

The study presents a novel approach for the histological assignment of distinct amyloid- β ($A\beta$) deposits in Alzheimer's disease (AD) patients using new dual-ligand fluorescence microscopy in addition to qFTAA and hFTAA protocols used in paper I-III. By combining five fluorescent thiophene-based ligands with distinct binding modes and photophysical properties, we developed various dual-staining protocols to differentiate $A\beta$ aggregates in brain tissue sections from transgenic mouse models and AD patients. We hypothesized that by using multiple ligands with varying binding affinities and specificities, we could distinguish between distinct $A\beta$ aggregates based on their structural and compositional differences.

We applied dual-ligand protocols to brain tissue sections from various AD models, including transgenic mice expressing different $A\beta$ mutations and human AD patients with disease-associated mutations. We observed distinct staining patterns for different $A\beta$ deposits, such as cored plaques (CP), diffused plaque (DP), cotton wool plaques (CWP), and cotton wool-like plaques (CWLP). These findings suggest that the dual-ligand approach can effectively differentiate between various $A\beta$ aggregates based on their unique characteristics.

One of the significant findings of the study was the ability to identify age-dependent and region-specific $A\beta$ deposits. In transgenic mouse models, we observed changes in $A\beta$ deposit morphology and distribution over time, indicating the dynamic nature of $A\beta$ aggregation. Additionally, it was found that different brain regions exhibited distinct $A\beta$ deposit patterns, suggesting that the composition and distribution of $A\beta$ aggregates can vary depending on the brain region.

The study also provides insights into the differences between sporadic AD (sAD) and dominantly inherited AD (diAD). By analyzing brain tissue sections from patients with different AD subtypes, we identified unique ligand staining patterns for $A\beta$ deposits in each group. These findings suggest that the composition and

structure of A β aggregates may differ between sAD and diAD, potentially contributing to the distinct clinical manifestations of these two disease subtypes.

Furthermore, the study demonstrates the potential of dual-ligand fluorescence microscopy for clinical applications. By accurately characterizing A β deposits, these protocols may help to identify patients who are most likely to benefit from specific treatments and monitor the progression of the disease.

In conclusion, the study presents a valuable approach for the histological assignment of distinct A β deposits in AD. The dual-ligand fluorescence microscopy technique offers a powerful tool for characterizing A β aggregates, understanding their heterogeneity, and exploring their role in AD pathogenesis. The findings of this study have significant implications for the development of novel diagnostic and therapeutic strategies for AD.

Concluding remarks

This thesis presents a comprehensive investigation into the structural polymorphism of amyloid- β ($A\beta$) fibrils associated with Alzheimer's disease (AD) and its implications for disease pathogenesis.

Through a series of experiments, we have demonstrated that $A\beta$ fibrils exhibit diverse structural variations in different AD mouse models. Genetic factors, age, and $A\beta$ isoform composition can influence this structural variation. Our findings highlight the importance of understanding $A\beta$ fibril structures for developing targeted therapeutic strategies.

We have shown that recombinant $A\beta$ fibrils and fibrils originally isolated from transgenic *Drosophila* are potent in inducing $A\beta$ -amyloid pathology in APP23 mice and the pathology spreads through different brain regions like prion propagation. These fibrils are different in their structural properties, i.e. polymorphic. These polymorphs exhibit varying seeding activities, suggesting that they play a crucial role in the propagation of $A\beta$ -amyloidosis.

Furthermore, we have demonstrated that $A\beta$ fibril polymorphs can evolve over time, potentially contributing to the development of novel $A\beta_{1-42}$ strains *in vivo*. This finding emphasizes the vibrant nature of $A\beta$ aggregation and the need for therapeutic strategies that can address evolving $A\beta$ species.

By further developing dual-ligand fluorescence microscopy approaches, we have demonstrated the feasibility of distinguishing between distinct $A\beta$ deposits in brain tissue sections from AD patients. This method offers a valuable tool for characterizing $A\beta$ aggregates and understanding their heterogeneity.

Our findings have significant implications for the development of novel diagnostic and therapeutic strategies for AD. By targeting specific $A\beta$ fibril polymorphs, we may be able to develop more effective treatments that can prevent or slow down disease progression. Additionally, the dual-ligand fluorescence microscopy

approach can be used as a diagnostic tool to identify patients who are most likely to benefit from specific therapies.

In conclusion, this thesis provides a comprehensive understanding of the structural polymorphism of A β fibrils and its implications for AD pathogenesis. Our findings may offer valuable insights into the development of novel therapeutic approaches for this devastating disease.

Future perspectives

Based on the findings presented in this thesis, future research could more deeply investigate the prion-like nature of amyloid-beta aggregates in AD, with a particular focus on the critical role of A β fibril polymorphism.

Fibrils/seeds produced under different chemically controlled experimental conditions, such as in different buffers, and compositions, can be tested on mice to examine the templated propagation of the seeds. Furthermore, strain-specific neurotoxic properties of fibrils can be examined. For example, to investigate if A β fibril polymorphs produce toxic effects in the host mice. It can be analyzed by investigating different neurological markers of neurodegenerative disease, such as astrogliosis, microglia activation, and neuronal cell death. Determining the molecular structure of this toxic strain will help in developing biomolecules for therapeutics.

In this thesis, we showed that spontaneous aggregation in the APP23 mice generates age-dependent polymorphs. Further research could explore the potential for novel A β polymorphs to emerge in seeded APP23 mice with extended incubation periods beyond six months. Additionally, longitudinal *in vivo* studies tracking the evolution of these polymorphs could provide valuable insights into the pathophysiological stages of Alzheimer's disease. It would also be interesting to study the outcome of A β -targeting therapies and how this influences the distribution of amyloid polymorphs.

Deciphering the molecular determinant of strain evaluation of the A β fibril polymorphisms can help in designing strain-specific therapeutic agents. Therapeutic approaches should focus not only on existing A β species but also on the potential for new, more pathogenic variants to emerge. This may involve developing treatments that can target a broader range of A β structures or inhibit the evolution of these polymorphs.

The study of changes in gene expression in the seeded mice will also help to understand the interplay between factors responsible for different A β polymorphic strains. For that purpose, the RiboTag system, which is the background breed of

our current APP23 mice, could be employed. RiboTag system comprises two components: RiboTag Cre reporter line and Cre driver lines. Crossing the RiboTag mice with the Cre driver mice allows cell type-specific gene expression. For example, the Cx3CR1-Cre-ERT2 driver line is used for gene expression in microglia.

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Papers

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