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**Investigating Amyloid β toxicity in
*Drosophila melanogaster***

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Cover: A β aggregates in glial cells of a *Drosophila* Alzheimer's disease model stained with two luminescent conjugated oligothiophenes, q-FTAA and h-FTAA.

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

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Till mina solstrålar, Lucas and Linnea

Abstract

In this thesis *Drosophila melanogaster* (the fruit fly) has been used as a model organism to study the aggregation and toxic properties of the human amyloid β (A β) peptide involved in the onset of Alzheimer's disease (AD). AD is one of many misfolding diseases where the important event of a protein to adopt its' specific three-dimensional structure has failed, leading to aggregation and formation of characteristic amyloid fibrils. AD has a complex pathology and probably reflects a variety of related molecular and cellular abnormalities, however, the most apparent common denominator so far is abnormal Amyloid- β precursor protein (APP) processing, resulting in a pool of various A β -peptides. In AD, the A β peptide misfolds, aggregates and forms amyloid plaques in the brain of patients, resulting in progressive neurodegeneration that eventually leads to death.

By expressing the human A β protein in the fly, we have studied the mechanisms and toxicity of the aggregation in detail and how different cell types in the fly are affected. We have also used this model to investigate the effect of potential drugs that can have a positive impact on disease progression. In the first and second work in this thesis, we have, in a systematic way, proved that the length of the A β -peptide is essential for its toxicity and propensity to aggregate. If the peptide expressed ends at amino acid 42 it is extremely toxic to the fly nervous system. However, this toxicity can be completely abolished by expressing a variant that is shorter than 42 amino acids (1-37 to 1-41 aa), or be significantly reduced by expressing a longer variant (1-43 aa). Toxicity can be partly mitigated in trans by co-expressing the 1-42 variant with a 1-38 variant. This supports the theory that the disease progression could be inhibited if the formation of A β 1-42 is decreased. In the third work we demonstrate that amyloid aggregates can be found in various cell types of *Drosophila*, however, the toxicity seem to be selective to neurons. Our results indicate that the aggregates of glial expressing flies have a more mature structure, which appear to be less toxic. This also suggests that glial cells might spread A β aggregates without being harmed. The last work in this thesis investigates how curcumin (turmeric) can affect A β aggregation and toxicity. Curcumin appears to shift the equilibrium between the less stable aggregates and mature fibers toward the final stage resulting in an improved lifespan for treated flies.

In summary, this thesis demonstrates that the toxicity of A β in *Drosophila* is highly dependent on the A β variant expressed, the structure of the protein aggregates and which cell type that expresses the protein. We have also shed light on the potential of using *Drosophila* when it comes to examining possible therapeutic substances as a tool for drug discovery.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom är den tredje vanligaste dödsorsaken i västvärlden och drabbar fler och fler människor i takt med att den förväntade livslängden ökar. Genom att använda bananflugan som modell har vi studerat mekanismerna bakom Alzheimers sjukdom och påvisat vikten av att studera sjukdomen på proteinnivå.

I människokroppen pågår mängder av kemiska processer och kroppens celler kan liknas vid miniatyrfabriker där det är ständig aktivitet: nedbrytning och omvandling av mat till energi, transport av näring och syre till kroppens alla celler samt uppbyggnad av kroppsvävnader och byggstenar, för att nämna några. Huvudkaraktärerna i alla dessa processer är proteiner. För att proteinerna ska kunna utföra sin funktion i cellen krävs att de antar en specifik tredimensionell struktur. Om proteinerna veckas på fel sätt förlorar de sin funktion och risken är stor att de börjar klumpa ihop sig, aggregera, med andra felveckade proteiner, vilket i sin tur ofta leder till sjukdom. En välkänd sådan "felveckningssjukdom" är Alzheimers sjukdom. Alzheimers är en sjukdom som i första hand påverkar minnet. I takt med att nervceller förtvinar och dör förstörs hjärnvävnaden och det sker en gradvis försämring av patienten. Detta leder till både fysiska och psykiska funktionsnedsättningar och resulterar så småningom i döden, då det i dagsläget inte finns något botemedel mot sjukdomen. En av huvudorsakerna till uppkomsten av Alzheimers tros vara proteinet Amyloid-beta, A β , som felveckas, klumpar ihop sig och bildar fibrer, så kallade plack, i hjärnan hos patienter.

Ett vanligt sätt att studera olika sjukdomar utanför människokroppen är att använda sig av ett modellsystem. Ett sådant modellsystem är bananflugan (*Drosophila melanogaster*). Flugan har förvånansvärt många likheter med människan och 75 procent av de identifierade sjukdomsgenerna hos människan återfinns i någon form hos flugan, vilket gör att den uppvisar många av de sjukdomssymptom människan får. Detta, tillsammans med den korta generationstiden och enkelheten att manipulera arvsmassan, har gjort bananflugan till en flitigt använd modell.

Avhandlingen är baserad på studier där bananflugan har använts för att studera Alzheimers sjukdom. Genom att uttrycka det humana A β -proteinet i flugan har vi studerat mekanismerna bakom aggregeringsförloppet och undersökt hur giftigt proteinet är för nervsystemet. Vi har även använt denna modell för att undersöka effekten av potentiella substanser som kan ha en positiv inverkan på sjukdomsförloppet. I det första och andra arbetet i avhandlingen har vi visat att längden av A β -proteinet är avgörande för dess skadlighet och benägenhet att aggregera. I det tredje arbetet har vi visat att A β -proteinet specifikt påverkar olika nervcelltyper. Avhandlingens sista arbete visar att kurkumin (gurkmeja) kan påverka A β -aggregeringen och på så sätt få flugan att leva längre.

Sammanfattningsvis har vi redogjort för att den skadliga effekten av A β i *Drosophila* är starkt beroende av vilken A β -variant som uttrycks, vilken struktur protein-aggregaten antar samt vilken celltyp som uttrycker proteinet. Vi har även påvisat potentialen som finns hos bananflugan när det kommer till att undersöka möjliga terapeutiska substanser.

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I-IV):

Paper I. **Systematic analysis of A β neurotoxicity in improved *Drosophila* Alzheimer's disease models reveals high toxicity for 1-42, 3-42 and 11-42 variants**

Maria Jonson, Malgorzata Pokrzywa, Annika Starkenberg, Per Hammarström and Stefan Thor
(2015), *PLoS One*; 10(7):e0133272

Paper II. **Systematic combinatorial A β expression in *Drosophila* reveals additive toxicity for the 1-42, 3-42 and 11-42 peptides and a mitigating effect of the 1-38 peptide**

Maria Jonson, Annika Starkenberg, Per Hammarström and Stefan Thor
Manuscript

Paper III. **Amyloid A β 1-42 is selectively toxic for neurons, whereas glial cells produce mature fibrils without toxicity in *Drosophila***

Maria Jonson, Sofie Nyström, Alexander Sandberg, Marcus Carlback, Wojciech Michno, Jörg Hanrieder, Annika Starkenberg, K. Peter R. Nilsson, Stefan Thor and Per Hammarström
(April 2017), *Submitted*

Paper IV. **Curcumin Promotes A-beta Fibrillation and Reduces Neurotoxicity in Transgenic *Drosophila***

Ina Caesar, **Maria Jonson**, K. Peter R. Nilsson, Stefan Thor and Per Hammarström
(2012), *PLoS One*; 7(2):e31424

Contribution report

Paper I: Maria Jonson (MJ) participated in the planning of the project, performed all the experiments, except the generation of the flies and the lifespan assay, and analyzed the results. MJ was the main author of the manuscript.

Paper II: MJ participated in the planning of the project, performed all the experiments, except the generation of the flies and the lifespan assay, and analyzed the results. MJ was the main author of the manuscript.

Paper III: MJ participated in the planning of the project, performed all the experiments, except the MS experiment, the fibrillation assay and the congo staining. MJ analyzed the results and was the main author of the manuscript.

Paper IV: MJ performed the MSD experiments and participated in analyzing the data. MJ participated in writing the manuscript.

Papers not included in this thesis:

Paper I. **Visualization of Oxidative Stress in Ex Vivo Biopsies Using Electron Paramagnetic Resonance Imaging**

Håkan Gustafsson, Martin Hallbeck, Mikael Lindgren, Natallia Kolbun, **Maria Jonson**, Maria Engström, Ebo de Muinck, and Helene Zachrisson
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Abbreviations

aa	amino acid
A β	amyloid β
A β PP	amyloid β precursor protein
AD	Alzheimer's disease
APOE	apolipoprotein E
APPL	amyloid precursor protein like
BACE1	β -site amyloid- β precursor protein cleaving enzyme 1
BBB	blood-brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
DAM2	<i>drosophila</i> activity monitor 2
DNA	deoxyribonucleic acid
GAL4	galactose-responsive transcription factor
GFP	green fluorescent protein
ER	endoplasmic reticulum
FAD	familial Alzheimer's disease
IDE	insulin degrading enzyme
LCO	luminescent conjugated oligothiophene
LTD	long term depression
LMW	low molecular weight
LRP	lipoprotein receptor-related protein
LTP	long term potentiation
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
MS	mass spectrometry
MSD	meso scale discovery
NFT	neurofibrillary tangle
NMDA	n-methyl-D-aspartate
PET	positron emission tomography
PSEN1 (2)	presenilin 1 and 2
RAGE	receptor for advanced glycation end-products
SAD	sporadic Alzheimer's disease
SEM	scanning electron microscopy
ThT	thioflavin T
UAS	upstream activating sequence

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Preface

A seed for this thesis emerged from the diagnosis of my grandma with Parkinson's disease. As I intended to learn more about her disease, an interest for neurological diseases in general was born. Since then I have gained tremendous knowledge, and the curiosity that arises from every new experiment has been a strong driving force. I continue to be fascinated by science and during the writing of this thesis it occurred to me many times that the vast majority of the immense research within the field of Alzheimer's disease has emerged during the course of my lifetime, from the identification of A β to improved imaging techniques and biomarkers facilitating diagnosis of the disease. I believe that the field will experience success toward a treatment of the disease within my lifetime.

This thesis summarizes the results obtained during my years as a PhD student. The research has focused on the Amyloid- β (A β) peptide, one of the causes of the most common form of dementia, Alzheimer's disease. My project aimed at investigating the toxicity induced by the A β peptide using *Drosophila melanogaster* (the fruit fly) to further our understanding of the underlying mechanisms of the disease. The first part of the thesis is intended to give the reader a general introduction to the field and to the findings essential for my research, as well as to the methods used during my PhD. Next, a brief summary of the findings and conclusions from the appended papers can be found, and finally, future perspectives and newborn questions are discussed.

I hope this thesis emphasizes the importance of continued research, and that it will increase your knowledge within the Alzheimer's field. Happy reading!

Linköping, Maj 2017

Introduction

1. Protein folding and misfolding

Proteins are essential for all life and have an extensive range of different functions. Processes where proteins are the main players are constantly ongoing in the human body. Some of their functions are; to build up a first line of defense (e.g. hair, nails, skin); to give cells and tissues their structure; to act as transporters and communicators between and within cells and to act as catalysts and enzymes facilitating chemical reactions, to mention a few. A protein consists of amino acid (aa) residues connected in chains by peptide bonds. Our cells possess 20 naturally occurring amino acids. The chain of amino acids builds up the primary structure of the protein. Proteins are synthesized by ribosomes from information encoded by the DNA. Every amino acid has different properties depending on the polarity, size, charge and hydrophobicity of the side chain of the amino acid. The interaction between different amino acids or stretches of amino acids, stabilized by hydrogen bonds, gives the protein its secondary structure. Common types of secondary structure are α -helix, β -sheet and random coil. The three-dimensional structure of a protein is given by the amount and arrangement of these structures [1].

To carry out their biological function, proteins are dependent on a correct three-dimensional fold, the so-called native conformation, which is encoded in their primary structure. For a given protein there is only one correct fold out of millions theoretically possible folds. However, the native fold seems to be the most energetically favorable state for the protein since the total amount of free energy is lower for the folded state, under the right conditions, than the unfolded state. The folding process is often described by an energy landscape resembled as a funnel where the protein travels from high energy and large degree of conformational freedom (entropy) at the top of the funnel, to low energy and stable conformation at the bottom of the funnel (**Fig 1**) [2], [3]. At the beginning of the folding process the unfolded protein fluctuates between large numbers of possible conformations. Moreover, the inside of the funnel is quite rough, increasing the risks of a partly folded intermediate to get stuck at local energy minima,

resulting in a decelerate folding process. As the formation of native-like interactions occur, the number of possible conformations is reduced along with a free energy reduction. When the protein reaches the bottom of the funnel it has adopted its most stable conformation under these conditions [4].

Figure 1. Protein folding described by an energy landscape illustrated as a funnel [2]. An unfolded protein travels from high energy and large degree of conformational freedom (entropy) at the top of the funnel, to low energy and stable conformation at the bottom of the funnel.

The cell is a crowded environment and when a protein folds, it is surrounded by a solution of proteins with a concentration as high as 350 g/l. This enhances the risk of making inappropriate contacts with other proteins or becoming partially unfolded or misfolded [5]. Misfolding can also occur due to mutations, changes in the environmental conditions or by chemical modifications [6]. The cell has several strategies of helping a protein to find its native structure. One of them being molecular chaperones - proteins that bind to folding intermediates and thereby prevents misfolding. If the protein does become misfolded the ubiquitin-proteasome system recognizes and degrades it thus minimizing the devastation of misfolding [3]. Nevertheless, failure of these prevention systems does occur, allowing proteins to maintain and propagate their misfolded state. These misfolded proteins become prone to aggregate, often resulting in several human diseases. The largest group of misfolding diseases, in total number of patients, is associated with the conversion of soluble proteins into organized fibrillar aggregates, generally described as amyloid fibrils or amyloid plaques [7].

2. Amyloid characterization and formation

The amyloid state was first observed more than 150 years ago in the context of systemic amyloidosis [8], and to date, 36 natively soluble and functional proteins are known to self-assemble and form amyloid and disease in humans. For example, A β peptides in Alzheimer's disease, α -synuclein in Parkinson's disease and the prion protein in Creutzfeldt-Jakob disease [9].

When a natively soluble and functional protein becomes partly unfolded or misfolded due to mutations, changes in the environmental conditions or chemical modifications, the protein begins to re-arrange into β -sheet rich structures, self-assemble, and aggregate [7].

The well-defined structure of an amyloid fibril is of low energy and can thereby be an alternative to the native state of a protein and an extended version of the folding landscape can be described (**Fig 2**). At certain critical concentrations of a protein, the protein can even be more stable in the amyloid state than in the native state. However, as long as there are high free energy barriers that hinder the transition into the more stable amyloid state the native state can persist [10].

Figure 2. An extended view of the protein folding energy landscape. The green area describes folding of native proteins via intermediates, and the red area describes misfolding of amyloid proteins via oligomers.

The current definition of an amyloid fibril protein is that it deposits as insoluble fibrils, mainly in the extracellular spaces of tissues and organs and gives rise to the disease syndrome. Additionally, the amyloid fibril must bind the dye Congo red and exhibit apple green birefringence when viewed in polarized light [9]. Congo red staining remains the gold standard,

but new promising conformation sensitive Luminescent Conjugated Oligothiophenes, LCOs have been introduced and may turn out to be very helpful in amyloid identification [11].

Amyloid deposits are primarily composed of a single protein, and even though there is no apparent similarity in the sequence, secondary structure or function of the group of disease-associated proteins, the corresponding fibrils all share a common quaternary structure [5]. The amyloid deposits are built up by 6-10 nm fibrils having a cross- β structure with the polypeptide chain arranged in β -sheets with their constituent β -strands perpendicular to the fibril axis [12]. The mature fiber consists of protofilaments that twist around each other and are stabilized by intra-chain hydrogen bonding along the fibril axis as well as specific interactions of side chains between the protofilaments giving rise to a specific cross- β fiber diffraction pattern with an interstrand spacing of 4.8Å and an intersheet spacing of 10Å [13], [14]. From a wide range of research, it is now apparent that the formation of amyloid structures is not a rare event associated with a small number of diseases, but rather a generic process that may be adopted by many, if not all, polypeptide sequences under the right circumstances [15].

Amyloid fibril formation

Amyloid fibril formation is known to be a nucleation-dependent process that can, for simplicity, be divided into three steps; the lag phase, growth phase and stationary phase (**Fig 3**). At first, the native monomer is destabilized and undergoes a re-arrangement into β -sheet rich partly unfolded structures. The lag phase is then continued by primary nucleation where interactions between the partly unfolded or misfolded monomers form a nucleus [16]. This is the rate-limiting step of the aggregation process. The length of the lag phase can hence be shortened or abolished by seeding from existing fibrils [17]. In the growth phase, monomers are continuously added to the previously formed nucleus, rapidly forming soluble oligomers and pre-fibrillar structures such as protofibrils. There is also a secondary nucleation step ongoing, where fragmentation of pre-formed aggregate species occurs, increasing the number of fibril ends that acts as new nuclei and generate elongation through monomer addition. The surface of already formed fibrils can also catalyze the formation of new oligomeric aggregates through interactions with monomers [18]. The stationary phase of the amyloid formation process is when mature fibrils are formed and an equilibrium between dissociation and association of monomer to the fibril is reached [10]. The aggregation process can be monitored *in vitro* by the fluorescent dye Thioflavin T (ThT), where increased fluorescence at 480 nm correlates with increased fibril formation [19]. The nucleation rate together with the growth rate of a fibril might influence the age of onset for amyloid-related diseases [20].

Figure 3. Fibrillation kinetics of an amyloid protein followed by ThT fluorescence. A monomeric protein forms oligomers and fibrils through a nucleation-dependent process in three main phases. The species illustrated in the lower panel are mainly formed in the respective phase, although they are all present throughout the entire process. (LMW = low molecular weight).

For most amyloid diseases, a gain of toxic function is the hypothesized mechanism of toxicity, still amyloid toxicity can also result from losing the function of a protein or from the sequestration or mislocation of other proteins [21]. Despite extensive research, it remains unclear which step of the amyloid formation cascade that is toxic, and additionally, this step may be different for the various amyloid diseases and for different fibril structures.

Amyloid oligomers

Fibril precursors or alternative assemblies of misfolded proteins comprising fewer protomers (structural units) are referred to as amyloid oligomers. Oligomers of a number of different proteins are thought to play an important role in many types of amyloid related degenerative diseases and nowadays most studies highlight the importance of these structures as the primary causative agent, exhibiting greater cytotoxicity compared to monomers or fibrils formed from the same protein [12], [22], [23]. Much information on these intermediate species is still missing, but due to their proposed toxicity, research is highly focused in this direction. Many different names have been given to these oligomers, reflecting their wide range of sizes, though they share some common characteristic: they, most commonly, are rich in β -sheet structure and they are all soluble, which means that they are not pelleted from physiological fluids by high-speed centrifugation. Studies done by electron microscopy and atomic force microscopy have identified spherical particles of 2.7-4.2 nm in diameter and structures called protofibrils, which represent strings of these spherical particles, that appear at early times of incubation and disappears as mature fibrils form [24].

While structural studies of amyloid fibrils have revealed that they are composed by parallel, in-register, hydrogen-bonded peptide strands, similar studies of oligomers are more difficult to conduct due to their polymorphic and time-dependent nature. For this reason it is still elusive if different proteins have similar structural properties or if they give rise to different oligomeric forms. However, the use of antibodies that specifically recognize soluble oligomers among all types of amyloidogenic proteins, except monomers and fibrils, have been employed. Such studies indicate that there is a common structure among oligomers, and hence, that they may share a common pathogenic mechanism [24], [25]. Still, oligomers recognized by either the oligomer specific antibody A11 or the fibrillar antibody OC have been identified, implicating two different conformational assemblies called prefibrillar- and fibrillar oligomers. Reported structural variability suggests that different amyloid oligomers prepared under different conditions could have subtle differences in structure [26]. Additional evidence of the diverse polymorphism of oligomer structure is demonstrated by the observed formation of oligomer structures independent from the fibril formation pathway, i.e. off pathway oligomers, that are not able to seed fibrillation [27], [28].

Structural studies of oligomers formed by the amyloid protein α B crystalline have identified a cylindrical barrel formed by six antiparallel protein strands, termed a cylindrin [29], that may represent a common structural element of oligomers of various sizes and origin. A similar oligomer model have been proposed for the A β peptide by Lendel et. al., with the formation of a symmetric hexameric β -barrel as the building blocks of protofibrils [30]. A more detailed discussion of the oligomer structure and toxicity can be found in Section; *Structural-related toxicity*.

Understanding how oligomerization and fibrillation occurs is a vital step in understanding and preventing many degenerative diseases, hence further research on the biochemical mechanisms of underlying fibrillation of different proteins is essential. One of the most studied amyloid fibril is the amyloid β (A β) fibril most commonly found in senile plaques in the brains of Alzheimer's patients and is the main subject discussed further in this report.

3. Alzheimer's disease

Alzheimer's disease (AD) is a neurological disorder that was first described in 1906 by the German psychiatrist Alois Alzheimer. His 51-years old patient suffered from rapidly declining memory function and autopsy showed both brain shrinkage, a great loss of neurons and a "peculiar substance" occurring as extracellular deposits, now known as amyloid plaques [31]. By the time, the disease was considered rare, but as of today AD has progressed into one of the most common forms of brain degenerating disorders. The disease accounts for about 60-70% of all cases of dementia, which is a general term for memory loss and loss of other brain abilities that affects your daily life. In 2016 about 47 million people worldwide lived with dementia, and

this number is continuously increasing, especially due to the increased life expectancy world wide [32]. Hence AD is a global public health issue, demanding huge amounts of resources, both economically and from a healthcare perspective. Thus, research in this field is of great importance through all perspectives of the society.

AD is an age-dependent disease, with high age as the major risk factor and incidence doubles every five years after the age of 65 [33]. The disease is subdivided into two forms; early-onset AD and late-onset AD. Early-onset AD is genetic, with disease onset before the age of 65 and accounts for about 5% of all cases. Late-onset AD is sporadic, with disease onset after the age of 65 and accounts for around 95% of all cases [34]. To diagnose AD a complete medical assessment is needed. This includes; a thorough medical history; various memory tests to measure cognitive impairment; physical and neurological examination and clinical measurements such as blood tests and brain imaging to exclude other forms of dementia. As of today the molecular pathogenic mechanisms causing the disease are still unknown and to get a definite AD diagnosis, post-mortem brain autopsy has to be performed [35]. There is currently no cure for the disease, although there are a few drugs that can provide symptomatic benefits, and to date there are approximately 100 therapeutics targeting the disease in phase 1, 2 or 3 clinical trials [36].

AD is characterized by features including memory impairment, disorientation, mood and behavior changes as well as difficulties in expressing yourself and understanding the spoken and written language [35]. As might be expected, the symptoms worsen along with disease progression. The physiological hallmarks of the disease are; brain atrophy with reduced brain volume, widening of sulci and enlargement of ventricles, loss of synapses and neuronal death [37]. At the time of death, the brain of an individual that suffered from AD may weigh two-thirds of the brain of an age-matched non-demented individual [38]. Microscopic hallmarks for disease include extracellular accumulation of amyloid plaques consisting of aggregated forms of the A β peptide [39], [40], and intracellular formation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein [41]. These protein accumulations are first seen in areas important for learning and forming new memories, and later progression to other brain regions can be observed. AD is a slowly progressive disease where plaque and tangle pathology begin more than a decade before symptoms emerge, complicating both diagnosis and treatment strategies [42]. Patients suffering from the disease can survive up to 20 years after diagnosis, with an average of eight - ten years [35].

The AD pathological process can be divided into five stages of the disease according to the Clinical Dementia Rating (CDR), from cognitively normal to severe dementia. In the pre-clinical/cognitively normal stage, changes in the brain related to AD have begun, but there are no signs of the disease. The second stage of disease is mild cognitive impairment (MCI) due to

AD, where mild changes in memory and thinking ability occur. AD is often diagnosed in the third stage of disease, mild dementia due to AD, and this stage together with the moderate and severe dementia due to AD stages is what people recognize as AD [43]. From the pre-clinical stage to the late stages of disease, the progression can be monitored using biomarkers and imaging techniques following A β and tau levels and deposition, together with brain neurodegeneration [44], illustrated in **Figure 4**.

Figure 4. The progression of Alzheimer's disease monitored by biomarkers and imaging techniques. CSF = Cerebrospinal fluid, PET = Positron emission tomography, MCI = Mild cognitive impairment. Re-drawn from [44].

Since both the A β peptide and the tau protein are normally found in healthy individuals, there is a great interest in understanding what triggers the abnormal aggregation resulting in the pathology of AD. This is a crucial step in the process to develop new treatment strategies that can delay or hopefully prevent the disease.

4. Molecular mechanisms of Alzheimer's disease pathogenesis

From the first description of senile plaques in 1906 it took almost 80 years until the major component of the plaques was identified. In 1984 Glenner and Wong isolated a peptide from cerebral vessel walls in the brain of AD and Down's syndrome patients - and within a year it was concluded that this was amyloid- β (A β), the main component of senile plaques in AD [39], [40]. More than 30 years have passed since this discovery, and several molecular mechanisms and other causative proteins have been proposed, some of them further discussed in this section.

The involvement of Tau in Alzheimer's disease

Major research regarding AD has focused on the various aspects of A β involvement, however, the second hallmark of AD is hyperphosphorylation (excessively phosphorylated) of the microtubule associated protein Tau and the formation of intracellular neurofibrillary tangles (NFTs) [41]. Tau was identified more than 40 years ago [45] and is predominantly expressed in neurons of the CNS, but has also been observed at low levels in CNS astrocytes and oligodendrocytes [46]. The main function of tau is to interact with tubulin to promote and stabilize microtubule assembly [47]. Due to alternative mRNA splicing of the *tau* gene found on chromosome 17, there are 6 isoforms of tau in human brain tissue. The isoforms differ from one another in both the N-terminal and C-terminal region of the protein. The N-terminal consists of either zero (0N), one (1N) or two (2N) insertions of 29 amino acids, while the C-terminal has three (3R-tau) or four (4R-tau) so-called microtubule-binding domains containing tandem repeats. This results in a length of 352 aa for the shortest isoform (0N3R) and 441 aa for the longest variant (2N4R) [48]. In the longest isoform of tau, there are 85 putative serine and threonine residues susceptible to phosphorylation, and research has shown that more than half of these sites are phosphorylated by the regulation of kinases (the most important being GSK-3 β) and phosphatases in normal tau [49]. When hyperphosphorylated, the tau protein detaches from microtubules and can self-assemble and form insoluble tangles observed to contribute to various neurodegenerative diseases, such as AD, frontotemporal dementia and other tauopathies [47]. Although not completely proven, this suggests that phosphorylation is the driving force of tau aggregation.

The exact mechanism behind the formation of intracellular NFTs is still unclear, but NFTs have been observed to interfere with axonal transport and damage cytoplasmic functions, eventually resulting in cell death. Research regarding the involvement of tau in AD has gained more attention in the last two decades; much due to findings that tau pathology correlates better with cognitive impairments observed in AD patients than amyloid pathology [50]. However, the main view of the amyloid cascade hypothesis (further described in the section; *A β aggregation*) is still dominating the field; i.e. that tau pathology is downstream of A β . This hypothesis has been supported by work in both *Drosophila* models and tau transgenic mouse models crossed by mice expressing mutant Amyloid- β precursor protein, A β PP, where the development of A β aggregates preceded that of NFTs [51], [52]. Moreover, mutations in the tau gene has been shown to cause familial forms of tauopathies, such as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), where tangle pathology is evident without the presence of amyloid pathology [53], indicating that tau phosphorylation is not the cause of amyloid deposition. A very recent publication of triple-transgenic mice models demonstrates that tau antibodies can effect both tau and A β assemblies, suggesting a crosstalk between the two molecules [54].

Amyloid- β precursor protein

AD has a complex pathology and probably reflects a variety of related molecular and cellular abnormalities, however, the most apparent common denominator so far is abnormal Amyloid- β precursor protein (A β PP) processing.

The human A β PP gene is located on chromosome 21 and was first identified in 1987 [55]. A β PP is a transmembrane protein with a large extracellular domain and a smaller intracellular domain. There are several isoforms of the protein, with the most common forms being; the 695 amino acid form, expressed predominantly in the neurons of CNS, and the 751 and 770 amino acid forms, expressed more ubiquitously [56]. A β PP is part of a gene family that is evolutionarily conserved across different species, A β PP, APLP1, APLP2 in mammals, APL-1 in *C. Elegans* and APPL in *Drosophila*. Of interest, only A β PP, none of the other genes, contains the sequence encoding the amyloidogenic A β -peptide [57].

Three decades have passed since the identification of A β PP and numerous investigations have been conducted, much owing to the fact that proteolytic cleavage of A β PP renders the A β -peptide which is the main player of the amyloid cascade hypothesis (further described in Section; *A β aggregation*) of AD. Tremendous progress has been made, but despite vast research the precise physiological function of A β PP is still not completely understood. However, there are research demonstrating that A β PP stimulates neurite outgrowth, facilitates cell adhesion, acts as a cell surface receptor and regulates synapse formation and cell division [58]-[60].

Processing of A β PP

The proteolytic processing of A β PP is likely to occur in multiple compartments of the cell since both A β PP itself and the secretases involved in the processing can traffic through both secretory and endocytic pathways. It has been observed that A β PP localizes to the plasma membrane as well as to the endoplasmic reticulum (ER), the trans-golgi network, and to endosomal, lysosomal and mitochondrial membranes [61], [62].

A β PP processing can occur either by the non-amyloidogenic pathway or the amyloidogenic pathway depending on the secretases involved [63] (**Fig 5**). A β PP molecules mature through the secretory pathway and due to the high abundance of α -secretase at the plasma membrane, the non-amyloidogenic processing is considered to occur predominantly at the cell surface [64]. This processing is initiated by α -secretase, rendering a large extracellular fragment termed sA β PP α . The cleavage is made in the middle of the A β region, between amino acid 16 and 17, thus preventing formation of the toxic A β -peptide. The remaining membrane bound fragment, α -CTF, is next cleaved by γ -secretase, releasing the pathologically irrelevant p3-fragment and the C-terminal fragment, ACID [63].

The processing in the amyloidogenic pathway is initiated at the N-terminal of the A β region within A β PP by a β -secretase, BACE1, releasing a large soluble fragment, sA β PP β . Following this, γ -secretase cleaves the membrane bound β -CTF, generating the toxic A β -peptide and a C-terminal fragment, AICD. BACE1 is mainly located to endosomal and lysosomal compartments where the secretase activity is optimized due to the acidic pH [65]. Data indicates that γ -secretase is present and active both at the plasma membrane and in endosomal and lysosomal compartments [66]. Thus, A β PP processing by the amyloidogenic pathway can occur at two locations, (1) the processing is initiated by the internalization of A β PP to intracellular compartments, where cleavage by β -secretase occurs and is finalized by γ -secretase either intracellularly or after transport to the plasma membrane, (2) due to the presence of A β PP in intracellular compartments, the cleavage can be initiated directly in the ER, trans-golgi network or at various membranes of intracellular compartments, with the generation of intracellular A β as a result [61] [62]. The γ -secretase cleavage is not precise and can occur at several sites, rendering a pool of various A β -peptides, between 37-43 amino acids long [63]. This is further discussed in Section; *A β length dependent toxicity*.

In neurons, where the abundance of BACE1 is greater, the amyloidogenic processing seem to be favored, while the non-amyloidogenic pathway is superior in all other cell types [63].

Figure 5. Overview of the proteolytic processing of A β PP. Cleavage by α - and γ -secretase releases the p3 fragment through the non-amyloidogenic pathway, while cleavage by β - and γ -secretase releases the toxic A β peptide through the amyloidogenic pathway.

The Amyloid- β peptide

Due to the findings by Glenner and Wong that the main constituent of amyloid plaques in AD patients is the 4.5-kDa A β peptide [39], it was initially assumed that the generation of the A β peptide itself was the pathological event. Although, further research implicated that the production and release of A β is a normal physiological process continuously occurring in healthy individuals under normal conditions [67], [68]. Thus, A β is only considered harmful when aggregated.

The physiological role of the A β peptide is not fully understood, but studies have reported that picomolar concentrations of A β can stimulate synaptic plasticity, regulate synaptic vesicle release and play an important role in hippocampal memory formation, hence indicating a dual effect of A β depending on its concentration; neurotrophic or neurotoxic [69]-[72]. Additionally, *in vitro* evaluations have demonstrated that A β can act as an antioxidant, being able to protect neurons from neurotoxicity in a concentration dependent manner [73].

Following A β PP processing in neurons, A β is secreted to the extracellular space by the regulation of neuronal and synaptic activity. Increased synaptic activity leads to increased production and secretion of A β [70]. Studies by Cirrito et. al. demonstrated an endocytosis-associated mechanisms as a link between synaptic activity and A β production. They showed that prior to processing of A β PP at the plasma membrane, the protein is reinternalized to endosomes where A β is generated. Following proteolysis, the endosome recycles to the cell surface, releasing A β . The levels of extracellular A β was demonstrated to be reduced by the inhibition of endocytosis [74].

Besides extracellular A β , several studies have demonstrated that a portion of the produced A β have been found in intracellular compartments such as endosomes, lysosomes and mitochondria. This is most certainly a result from various mechanisms, such as the intracellular generation of A β , the lack of secretion and secretion and than re-uptake of the peptide [38]. Further discussed in Section: *Intracellular A β toxicity*.

Mutations linked to Alzheimer's disease

Extensive research continues to emphasize the genetic role in, predominantly, early-onset AD but also in late-onset AD. Early-onset AD can result from mutations in one of three genes; A β PP, PSEN1 or PSEN2. More than 50 mutations in A β PP have been linked to familial AD (FAD), with the most pronounced being the Swedish mutation resulting in an increase of total A β [75]. Each of these mutations occurs either within, or flanking the A β -region affecting the generation of A β in two different ways. Mutations that occur within the A β -region enhance the oligomerization of A β , while the flanking mutations increase the total production of A β , or the production of the amyloidogenic A β 42 isoform, hence increasing the A β 42/40 ratio [76].

People with Down's syndrome, or trisomy 21, also show an increased risk of developing AD because they carry an extra copy of chromosome 21 and hence three copies of A β PP. This results in a higher production of A β , and a 6-time higher risk of developing disease compared to healthy individuals [77].

The most common cause of developing early-onset AD is mutations in the PSEN1 and PSEN2 genes. The proteins encoded by these genes are parts of the catalytic site of the γ -secretase involved in the processing of A β PP. To date, close to 300 mutations have been identified within these two genes, resulting in an altered processing of A β PP. In cell culture this leads to an overproduction of the longer, toxic form of A β and hence an increase of the A β 42/40 ratio [78], [79].

In paper IV of this thesis we used flies expressing the A β peptide with the Arctic mutation. This mutation was identified in a Swedish family where individuals presented clinical features of early-onset AD. The mutation affects the middle part of the A β peptide where a substitution of a glutamate to a glycine has occurred at position 22 [80]. The resulting effect of this mutation is an increased propensity and faster rate of oligomer/protofibril accumulation leading to increased neurotoxicity.

The predominant form of AD is late-onset AD, or sporadic AD (SAD), with disease onset after the age of 65. Despite extensive research, no specific gene has been found responsible for the late-onset form of the disease. However, a person's risk of developing disease has been found to be increased having a specific isoform of the apolipoprotein E (APOE) [81]. APOE is a brain lipoprotein produced by glial cells that is present in three different forms, alleles; ϵ 2, ϵ 3 and ϵ 4. The most common form, found in over 70 % of the population, is ϵ 3 [82]. The ϵ 4 allele is a genetic risk factor in AD in a dose-dependent matter. People carrying one copy of the APOE ϵ 4 allele show a threefold increased risk of developing disease, and people carrying two copies have a 15-fold increased risk [83]. Though, it is important to remember that people with the APOE ϵ 4 allele does not inherit the disease itself, only an increased risk of developing the disease. Further, the ϵ 2 allele has been shown to have a protective effect in late-onset AD [82].

A β aggregation

Throughout life, A β is released from A β PP processing as monomers with a continuous balance between production, degradation and clearance. First when this balance is disturbed resulting in increased concentration of A β , a gradual accumulation and aggregation of the peptide is initiated. The aggregation of A β is a complex process during which a monomeric species self-assembles, forms oligomers and finally mature fibrils (**Fig 6**).

A β is an intrinsically disordered protein in its free soluble form with polar amino acids only within the first 28 positions [10]. An initiating step in the aggregation process is a

conformational change of the monomer, exposing hydrophobic surfaces and resulting in a β -sheet rich structure, driving the self-assembly of monomers into intermediate structures [84]. There is a wide range of intermediate structures preceding the formation of fibrils, including A β dimers, oligomers, amyloid-derived diffusible ligands (ADDL), globulomers and protofibrils [22], [85]-[87]. The interaction of these intermediates with one another, and/or with monomers eventually results in the build-up of mature fibrils composing amyloid plaques observed as a hallmark in AD, (**Fig 6**).

The aggregation of A β is believed to follow the process described in Section; *Amyloid fibril formation*, and is further discussed in Section; *Structural-related toxicity*.

Figure 6. A schematic illustration of A β aggregation. A monomer misfolds and forms soluble oligomeric intermediates and protofibrils that grow into insoluble fibrillar structures and amyloid plaques.

The amyloid cascade hypothesis

The amyloid cascade hypothesis was proposed in 1991 by John Hardy and David Allsop and is the most dominant hypothesis in the field of Alzheimer's disease pathogenesis, but lately also one of the most debated. The basic structure of the hypothesis is that the abnormal aggregation and fibril formation of the A β peptide is what initiates the cascade of events leading to pathological changes, neurodegeneration and cognitive decline in a quite linear pathway [88], [89]. The hypothesis is strengthened by observations that a majority of mutations linked to early-onset AD, with the most pronounced being the Swedish mutation, acts by increasing the total amount of A β , the ratio of the fibrillogenic A β 42 peptide compared to other alloforms and the levels of amyloid plaques [76], [79]. Furthermore, individuals suffering from Down's syndrome have three copies of chromosome 21, the location of A β PP, and develop advanced AD early in life [77]. Despite this, a growing number of studies have shown that the density of plaques in brain have a weak correlation with the degree of dementia, and that plaque formation also occur in healthy individuals [90]. For this reason, the amyloid hypothesis has been modified as evidence provides a strong correlation between soluble A β species, oligomers, and disease pathology [59], [91]. Research indicates that these toxic A β oligomers precedes fibril formation and are the species responsible for the cascade involving amyloid deposition, inflammation,

oxidative stress, and neuronal injury as well as the formation of neurofibrillary tangles [92]. Increasing evidence also suggests that intracellular A β is the initiating species in the development of disease [93].

The other side of the coin, suggests that A β is not sufficient to cause the complex pathology of AD and that there is more to the story than A β alone [94]. This is supported by several studies both in human and in mice, where addition of amyloid to healthy controls do not cause disease, or where removal of amyloid in diseased subjects do not prevent or cure the disease [90], [95]. Additionally, strong correlations have, in contrast to A β fibrils, been observed between the number of tau tangles and dementia [50]. In conclusion, many studies support the idea that AD pathogenesis most likely is immensely complex with many contributing factors enhancing the pathology, still an exclusion of the involvement of the A β peptide in pathogenesis is not proposed [94]. This, together with recent understanding of the physiological roles of A β is challenging the amyloid cascade hypothesis and points to a revised view of how we approach the development of therapies against Alzheimer's disease. Nonetheless, it is not a question of one hypothesis against another, more likely we have to consider multiple approaches to be able to conquer this devastating disease.

Mechanisms for A β toxicity

A majority of the studies in AD research follows the amyloid cascade hypothesis and points to the fact that A β is the primary causative agent underlying disease pathology [88], [89]. Still, the exact mechanism behind A β toxicity remains elusive. However, due to extensive research there are several theories covering the damaging effects of A β resulting in AD pathology. Neuronal cell loss is one of the hallmarks for disease, although, it is preceded by cognitive impairment due to neuronal dysfunction and synapse loss. A wide variety of underlying events have been proposed to result in this pathology.

A β length dependent toxicity

An imprecise cleavage by γ -secretase in the processing of A β PP through the amyloidogenic pathway renders a pool of different A β variants between 37 and 43 amino acids long [63]. The predominant A β form is the A β 1-40 variant, whereas only a fraction, about 10 %, is cleaved to be 42 amino acids long, A β 1-42. Despite that, the longer A β 42 species is more aggregation prone and is believed to be the toxic building block of A β oligomers [96], [97]. Hence the difference in length of the A β species is of great importance for understanding AD pathology.

Once A β PP is bound to the proteolytic site of γ -secretase a sequence of cleavages is initiated within the lipid membrane. It is suggested that the different cleavages are separated by approximately three amino acids, corresponding to one helical turn, starting with the ϵ -cleavage after amino acid 49 or 48 [98]. This is followed by ζ -cleavage after amino acid 46 or 45 and terminates by γ -site cleavage predominantly at amino acid 42 or 40, but also after amino acid 37, 38, 39 and 43 [63] (**Fig 7**).

Figure 7. Sequential cleavage of AβPP by γ-secretase, resulting in a complex pool of Aβ alloforms. The main alloforms produced, Aβ40 and Aβ42, are highlighted by a bold arrow. In addition, the upper sequence illustrates the Aβ peptide with main N-terminal truncations highlighted by grey boxes (amino acid 3 and 11).

Generally, longer Aβ peptides are more hydrophobic and are therefore considered more aggregation prone [99], [100]. As stated previously, the Aβ 1-42 is well known to be more aggregation prone than other Aβ peptides *in vivo*. Besides the 1-42 peptide, several studies, both *in vivo* and *in vitro*, have focused on the Aβ 1-43 peptide, showing it to be of importance to AD pathology [96], [101]-[103]. Mouse studies indicate that Aβ 1-43 is potentially amyloidogenic and pathogenic *in vivo*, and that it appears to be as prone to aggregate as Aβ 1-42 *in vitro* [100], [104]. Additionally, senile plaques from AD patients have been found to primarily be composed of Aβ 1-42 and 1-43 [102], [105].

Increased levels of Aβ 1-38 in cerebrospinal fluid (CSF) of AD patients has been reported placing this peptide in focus of further research [106], [107]. Contradictory results have been observed concerning the Aβ 1-38 peptide. In isolation, this peptide acts as the 1-40 peptide, exhibiting little cytotoxic potential. However, when the Aβ 1-38 peptide is mixed with Aβ 1-40 the mixture becomes highly toxic. On the other hand, when adding Aβ 1-38 to Aβ 1-42 a protective effect of the 38-peptide is observed [100]. Similar Aβ behavioral changes were seen *in vivo* using *Drosophila* to model AD. In this study, the soluble Aβ40 species was shifted to the insoluble fraction when combined expression of Aβ40 and Aβ43 was induced, resulting in defects in both climbing and lifespan [103]. These findings highlight the complexity of investigating the role of various Aβ peptides both *in vivo* and *in vitro*.

Moreover, a study by Portelius et. al. compared CSF levels of various Aβ isoforms in

individuals with sporadic AD (SAD) to individuals carrying the familial AD (FAD) associated presenilin 1 A431E mutation found in Mexican families. Results displayed a clear difference in the levels of A β 1-37, A β 1-38 and A β 1-39, with FAD individuals showing decreased levels compared to SAD patients. This indicates an alleviating effect of the C-terminally truncated isoforms. The authors speculated that the truncated isoforms may inhibit the oligomerization of A β 1-42 by forming heterocomplexes less prone to aggregate in SAD cases, hence the late onset of AD [108].

The sequential γ -secretase cleavage renders A β peptides with various C-terminals. To complicate the understanding further, research has shown N-terminally modified A β peptides, with the most prominent forms identified starting at amino acid 3 or 11 and possessing N-terminal pyroglutamic acid (pyroE), generated from glutamic acid by the enzyme glutaminyl cyclase [109], [110]. *In vitro* studies have shown that small N-terminal deletions accelerate A β aggregation into neurotoxic fibrils compared to A β 1-42 [111], [112]. In the case of pyroglutamylation, the increased propensity to aggregate might be due to the higher hydrophobicity of the peptide when losing one or two charges [113] and limited N-terminal degradation [109]. Moreover, several studies have demonstrated that the ratio of soluble oligomeric A β p3E-42 to A β 1-42 is proportional to the clinical phenotype and severity of AD [110]. In contrast to A β 1-42, where a physiological function of the peptide has been implicated in addition to the toxic properties of the peptide, current knowledge indicates that A β p3E is solely pathological, suggesting its potential as a therapeutic target.

Additional studies implicate that the A β 11-42 peptide has an early role in A β deposition into plaques [114], however further research is needed regarding the role of this peptide and the A β p11E-42 peptide in AD pathogenesis.

Structural-related toxicity

As described above, the length of the A β peptide influences toxicity, but even more so does the structure of the various intermediate and aggregated forms of the peptide. There is a close connection between these two parameters, where the length dependence results in various oligomer species mediating toxicity in slightly different ways.

The end products of the complex aggregation pathway of A β are fibrils and amyloid plaques. According to the initial amyloid cascade hypothesis, these fibrillar structures were long considered to be the main toxic species resulting in neurodegeneration [88]. However, extensive research demonstrated a poor correlation between the amount of insoluble fibrillar assemblies and neurodegeneration and cognitive decline. Although there is support for neurotoxicity of fibrillar aggregates, the main toxic process is ascribed to the numerous intermediate structures including A β dimers, oligomers, amyloid-derived diffusible ligands (ADDL), globulomers and protofibrils preceding fibril formation [22], [85]-[87].

Due to the difficulties of isolating or purifying these species reliable structural information of the intermediates is difficult to obtain and the debate on which form/forms that contribute the most to disease pathology is ongoing. The heterogeneous pool of A β oligomers is in a constant equilibrium with monomers, fibrils and other A β aggregates, making it even harder to analyze a specific form. This equilibrium might also be varied by concentration, pH, temperature, salt and other proteins, interfering additionally with *in vitro* and *in vivo* analyses [115].

Almost all soluble oligomers have been shown to alter long term potentiation (LTP) and long term depression (LTD) *in vivo* and may lead to the cognitive decline seen in AD [86], [116]. LTP is a long-lasting enhancement of synapses and results from an increase in synaptic activity. LTD is a long-lasting decrease in synaptic efficacy dependent on synapse activity. Both LTP and LTD are thought to play a role in learning and memory [117], [118] .

The minimal toxic species found in extracted brain tissue from AD patients have been suggested to be A β dimers [86]. Moreover, a transgenic mouse study reported a 56-kDa species (A β *56) corresponding to a dodecamer as the toxic species correlating with cognitive decline [85]. A third study, using transgenic mice, suggested that the presence of multiple assembly forms of A β throughout life was what caused synaptotoxicity and not a single A β species [119]. Taken together, all these studies, along with many others, present very different outcomes, demonstrating the difficulties in ascribing a specific A β species the toxic properties. Although, all studies support the existence of soluble A β species exerting synaptotoxicity. The generation of A β oligomers *in vitro* demonstrates both advantages and difficulties. You have greater control over the starting conditions compared to *in vivo* samples. Still, A β monomers are intrinsically disordered, representing a mixture of conformations that might end up in different aggregation pathways [120]. In parallel with this, a recent mouse study by Klementieva et. al. demonstrated that the physiological conformation of A β is altered before the formation of amyloid plaques, and that the A β peptide initially exists as a β -sheet rich tetramer. These results suggest a novel therapeutic approach, where stabilization of the tetramer, similar to that of transthyretin (TTR) in transthyretin amyloidosis, could inhibit the oligomerization and toxic effects of A β [121].

There are several different models of A β oligomer/fibril structure, with a general agreement on a hidden hydrophobic C-terminal sequence (30-40/42) and central region (15-25), with a partially accessible hydrophilic N-terminal region (1-10/12) [122]-[126]. All models presume that the hidden C-terminal and central regions make up β -strands, forming extensive hydrogen interactions between the hydrophobic side chains of adjacent peptides. There is a generally accepted view that the increased aggregation propensity of A β 1-42 compared to 1-40 is due to the two additional hydrophobic residues, Ile-Ala [127]. Implications have been made that the hydrophobicity *per se* of the C-terminal half of A β is the mere driving force of aggregation, rather than specific side-chain identity [128]. In contrast, reports have demonstrated that

A β 1-40 and 1-42 oligomerize through distinct pathways, where A β 1-42 forms pentameric or hexameric paranuclei with the ability to grow towards protofibrils, while A β 1-40 forms monomers, dimers, trimers and tetramers growing protofibrils less easily [99]. Moreover, a recently proposed oligomer model suggests the formation of a symmetric hexameric β -barrel as the building block of protofibrils. A C-terminal β -strand, residue 39-42, is paired to an antiparallel β -strand, residue 34-36, in an adjacent protomer. This suggest that the formation of these structures are dependent on specific residues [30].

Not only oligomers show structure diversity and maturation, so does A β amyloid plaques. *In vitro* and *ex vivo* mouse studies using LCOs have demonstrated a conformational rearrangement of A β amyloid deposits dependent on age. As the quantity of plaques in older APP/PS1 mice appears to reach a plateau, the core of the plaques seem to rearrange reflecting a second wave of core plaque formation resulting in fibril morphology changes over time [129]. *In vitro* analyses of A β 1-40 using the same LCO probes displayed fibrils transforming from solitary filaments ($\varnothing$ ~2.5 nm) into higher order bundled structures ($\varnothing$ ~5 nm) [130]. The authors hypothesize that the more mature and bundled structures are less toxic due to a more stable structure, hence fewer pieces shed off the already formed plaques being able to act as seeds for new aggregate formation [129].

This type of general protein aggregation behavior is described in a study investigating the bacterial protein HypF-N. They observed two similar types of oligomers during the fibrillation process; one being non-toxic and the other highly toxic. The suggested explanation for the differences in toxicity was that the toxic oligomer had a lower degree of hydrophobic packing compared to the non-toxic variant, resulting in a structural flexibility and hydrophobic exposure. This enables interactions with cell membranes and it might also result in trapping vital proteins [131].

Intracellular A β toxicity

The classical view in the A β field ascribed the observed toxicity of the A β peptide to, almost exclusively, extracellular A β . The first evidence that A β was generated not only at the plasma membrane but also intracellularly came in the early nineties [132]. Lately, emerging evidence has resulted in a revised view, and intracellular A β detected early in disease is considered to be as important and harmful as extracellular accumulations in the pathology of AD [93], [133].

It has been observed that A β PP localizes to the plasma membrane as well as to the ER, the trans-golgi network, and to endosomal, lysosomal and mitochondrial membranes [61], [62]. As a consequence, neurons accumulate intracellular A β through several pathways, such as uptake of extracellular A β by binding to various membrane receptors and through the processing of A β PP in intracellular compartments [38]. Studies using C-terminal-specific antibodies against A β 40 and A β 42 have demonstrated that most of the intracellular A β observed is in the longer, more aggregation prone, form [134]. Recent work described a sorting signal in presenilin-2 (PS2)

(part of the γ -secretase) that directs the protease to endolysosomal vesicles, where the processing of A β PP renders a pool of intracellular A β , enriched with A β 42 [135]. Both *in vitro* and *in vivo* reports have displayed that the accumulation of intracellular A β precedes the build up of extracellular deposits [134] and AD transgenic mice studies have reported a correlation between levels of intracellular A β and A β -related synapse damage and memory impairment. A correlation that was not observed with plaque burden [136].

It has been widely accepted that various oligomers are the most toxic assembly form responsible for pathology. Both animal models and work on tissue derived from human brain have shown that the oligomerization of A β is initiated intracellularly rather than extracellularly. The secretion of these intracellular assemblies might act as seeds for further extracellular plaque formation, suggesting that intracellular A β is sufficient for the initiation of AD pathology [137], [138]. In addition to seeding extracellular formation of fibrils and plaques, intracellular A β in animal models has been demonstrated to; facilitate tau hyperphosphorylation by the activation of enzymes such as GSK3 β and multiple caspases [139]; cause proteasome impairment, resulting in build-up of both A β and tau [140]; disrupt mitochondrial function through membrane infiltration and the formation of reactive oxygen species [141]; cause disturbance of autophagy [142] and result in synaptic dysfunction and deficits in long term potentiation (LTP) [143], [144].

Receptor and membrane mediated toxicity

As A β and its assemblies are very “sticky” they tend to interact with various receptors, resulting in abnormal cell signaling and neuronal dysfunction [145]. The regulation of glutamate homeostasis within neurons is maintained by N-methyl-D-aspartate (NMDA) receptors. The binding of A β to these receptors have been observed to cause a constant influx of Ca²⁺, resulting in a excitotoxicity that impacts the neuronal plasticity [146]. Additionally, A β interacts with the α 7 nicotinic acetylcholine receptor, promoting the internalization of receptors and the accumulation of intracellular A β [147]. Studies have also showed that A β interaction to the Frizzled (Fz) receptor inhibit Wnt signaling, resulting in downstream processes causing tau phosphorylation and formation of NFTs [148]. These are a few examples of A β interactions with receptors, and the sometimes contradictory reports regarding A β interactions might be because different A β assemblies or conformations have different targets. Apart from binding to receptors, A β assemblies can bind to the plasma membrane. The importance of maintaining the plasma membrane integrity is crucial, since the exchange of material between the cell and its surrounding environment is controlled by the membrane. Insertions of A β assemblies into the membrane can cause an increase in membrane permeability and pore formation, leading to disruption of calcium homeostasis, which in turn promotes degenerative processes such as free radical formation and tau phosphorylation, accelerating neurodegeneration and eventually resulting in cell death [149], [150]. Moreover, extracellular aggregation of A β in the presence of metal ions e.g. Fe²⁺ and Cu⁺ generates reactive oxygen species that impair membrane proteins.

A β deposition is accompanied by microglia activation, resulting in an inflammatory response and the release of cytokines and chemokines into the extracellular space. This process can increase the production of amyloid, killing healthy neurons and reducing the ability of microglia to engulf amyloid plaques [151]. This topic is further discussed in Section; *Inflammation in AD - the role of glial cells*.

Some of the mechanisms regarding receptor binding, pore formation and microglia activation due to various oligomer structures are illustrated in **Figure 8**. Evidently, the mechanisms of toxicity proposed in this section are not mutually exclusive and any or all of them could contribute during AD pathogenesis, simultaneously or at different stages of the disease.

Figure 8. Overview of some of the extracellular toxic mechanisms caused by A β oligomers.

A β monomers aggregate into oligomers and fibrils that interact with pre- and postsynaptic receptors as well as microglia cells, triggering synapse impairment and cell death. Oligomers can also form membrane pores leading to disruption of calcium homeostasis and eventually neurodegeneration.

Redrawn from [145].

A β degradation and clearance pathways

The cause of AD is still not fully understood, but according to the amyloid cascade hypothesis, critical processes in disease pathogenesis are believed to be; increased production of A β and/or impaired degradation and clearance of the peptide. The two major systems responsible for the maintained homeostasis in the cell are lysosomes and proteasomes. These systems are able to degrade a wide range of proteins, including partially folded or misfolded proteins. When a protein is targeted for degradation it is labeled with a polyubiquitin chain as a signal for the

proteasome to degrade it. Several studies have implicated impaired proteasome activity as a part of the AD pathogenesis, mainly due to detection of ubiquitin-conjugates in the amyloid plaques and NFT's [152]. The A β peptide has been observed to bind to and inhibit the proteolytic activity of the proteasome without being degraded itself [153]. While proteasomes mainly degrade more short-lived proteins, lysosomes contain acid proteases and hydrolases that are able to degrade larger protein aggregates, long-lived proteins and even dysfunctional organelles [154]. During the early stages of AD, it has been demonstrated that neurons increase their production of lysosomal system components, supporting the important role of a functional degradation system [155].

The first indication that altered degradation and clearance was part of the pathology of AD came from the finding that the ϵ 4 allele of ApoE is a genetic risk factor for disease [83]. ApoE binds A β and either promotes aggregation (the ϵ 4 allele) [156] or facilitates clearance (the ϵ 2 or ϵ 3 allele) across the blood-brain barrier (BBB) depending on the encoding genotype [157]. ApoE plays an important role in mediating A β clearance through all mechanisms described below.

The main clearance pathways for the removal of A β from the brain include: (1) enzymatic digestion by insulin-degrading enzyme (IDE) and neprilysin (NEP), (2) receptor-mediated and chaperone-assisted clearance across the BBB and (3) microglial phagocytosis [158].

In normal metabolism, different amyloid degrading enzymes regulates the levels of A β in the brain. The primary enzymes involved in the degradation of A β being IDE or NEP [159], [160]. Both enzymes are zinc metalloproteases and IDE is found mainly in the cytosol, while NEP is located to the extracellular face of the plasma membrane [161]. The difference in location of these enzymes suggests that they are involved in the degradation of different pools of A β . It has been demonstrated that both IDE and NEP can degrade monomeric A β , while NEP also degrades oligomeric forms of A β [162]. Knockout mice models, lacking IDE and NEP, have proven to increase the A β deposition when crossed with APP transgenic mice, demonstrating the importance of this clearance pathway [159], [163]. A third enzyme that degrades monomeric A β is endothelin-converting enzyme (ECE). This protease primarily degrades intracellular A β [164].

In a second clearance pathway, cerebral A β can be transported across the BBB into the bloodstream by either direct or indirect binding to the scavenger receptor; low-density lipoprotein receptor-related protein (LRP) [158]. *In vivo* studies indicate that the direct binding of A β to LRP favors clearance of the A β 1-40 form over the more aggregation prone A β 1-42 and mutant A β [165], [166]. As a consequence, A β peptides that are more prone to aggregate are cleared less efficiently from the brain. During the indirect binding of A β to LRP, A β initially forms a complex with chaperones such as ApoE and α 2-Macroglobulin. This complex then binds to LRP and is transported across the BBB [167], [168]. Once A β is located in the bloodstream the peptide can either be transported to the liver and kidney for degradation, or it can reenter the brain through the receptor for advanced glycation end-products (RAGE).

Downregulation of RAGE has been observed to inhibit the influx of A β from the bloodstream into the CNS in animal models, supporting the physiological interaction between the receptor and A β [169]. An additional mechanism of reducing the influx of plasma A β into the brain is the binding of soluble LRP to free A β . This binding sequesters some 70 to 90 % of plasma A β peptides and delivers it to the kidney and liver for degradation [170].

More recent research has strengthened the evidence of microglial and astrocyte phagocytosis as an important clearance pathway. Both microglia and astrocytes produce A β degrading proteases, including IDE and NEP, release extracellular chaperones, such as ApoE and α 2-Macroglobulin, and phagocytose A β as their mechanism of clearance [171]. Microglia, the macrophages of the brain, are found in the vicinity of amyloid plaques and have been demonstrated to phagocytose both soluble and fibrillar forms of A β [172], [173]. The binding of A β by microglia receptors activates the cell, secretes neurotrophic factors and promotes phagocytosis. Additionally, astrocytes have been observed to secrete matrix metalloproteinases (MMPs) that are important for the degradation of both monomeric A β and fibrillar A β deposits [174]. In contrast to the important role of glial cells in the clearance pathway, it has been implicated that they can be detrimental by inducing brain inflammation.

Inflammation in Alzheimer's disease - the role of glial cells

Inflammation has long been a topic when discussing AD, however, the focus of the discussion has been shifted along the way. Initially, research described the neuroinflammation associated with AD as a secondary or even protective event in response to A β aggregation that was assumed to occur at late to end stages of the disease. More recent research emphasizes the dual role of the immune system; on one hand being protective by reducing the formation of A β assemblies and amyloid plaques, and on the other hand being detrimental by inducing brain inflammation. A current hypothesis is that AD is promoted by the inflammatory activity, increasing the production of amyloid, killing healthy neurons and reducing the ability of microglia to engulf amyloid plaques [151]. Neuroinflammation in AD, and other neurodegenerative diseases, is driven by the innate immune system as compared to neuroinflammatory diseases where the adaptive immune system is the driving force.

In the CNS there are four kinds of glial cells; *ependymal cells* - line the spinal cord and the ventricular system of the brain; *oligodendrocytes* - coat axons in the CNS and produce their myelin sheath; *microglia* - protect neurons of the CNS by destroying pathogens and removing dead neurons, and *astrocytes* - regulate the external chemical environment of neurons, link neurons to their blood supply and are a component of the BBB [175].

Research supports the involvement of all kinds of glial cells in AD pathology, but the inflammatory response is mainly driven by microglia cells [151].

The interest of microglia exploded in 2016, and researchers are working hard on understanding the interplay between microglia and A β . Microglia are the macrophages of the brain, constantly monitoring the environment, both in physiological and pathological conditions, and regulating tissue homeostasis and neuroplasticity through scavenging functions. It has been shown that increased levels of various inflammation markers are present in tissue, CSF and serum from patients suffering from AD [176]-[178].

Two main types of microglia have been described; resting and activated microglia. Depending on the extracellular signals that the microglia receive a transition from the resting to the activated form is achieved [179]. In AD, the activation of microglia may be causative, reactive and/or protective, probably varying with disease stage. The presence of A β can prime microglial cells, meaning that the cells become sensitive to a second stimulus and/or that they get activated. When activated, microglia are often classified in two states; the inflammatory (M1) and alternatively activated (M2) state [180]. In the M1 state, microglia cells release pro-inflammatory cytokines and cytotoxic substances promoting an inflammatory response that may induce neurotoxicity. In contrast, the M2 state results in a neuroprotective action by the release of anti-inflammatory molecules. During the progression of AD, there is an imbalance between the M1/M2 states, with the M1 being more pronounced at later stages of the disease [181]. At early stages of disease, activated microglia participate in the clearance of A β by receptor-mediated phagocytosis or by the release of IDE and other proteases able to degrade A β , hence acting beneficial. However, the consistent accumulation of A β results in a vicious circle due to the chronic activation of primed microglial cells [182] (**Fig 8**). Both soluble A β oligomers and fibrillar forms are able to bind various receptors expressed by microglia. This binding results in a constant production of inflammatory cytokines and chemokines, which in turn maintains the activation of the primed cells. A β oligomers are also able to form non-receptor-mediated interactions with microglia, resulting in reduced phagocytic capacity. Eventually these processes result in dystrophic microglia affecting the surrounding CNS cells and finally causes neurodegeneration and cell death [151], [182] (**Fig 8**).

A β PP is heavily expressed in neurons, where the majority of studies of the protein have been carried out. Despite this, it has been observed that both microglia and astrocytes have endogenous BACE1, A β PP, and A β [183]-[185]. A study in cultured primary astrocytes has demonstrated that a combination of expressed cytokines, e.g. TNF- α and INF- γ , can increase the levels of these endogenous proteins and stimulate the amyloidogenic A β PP processing in the activated astrocytes. The endogenous BACE1 and A β PP levels can additionally be increased by oligomeric and fibrillar A β 1-42. Hence, this indicates a cytokine- and A β 42-driven feed-forward mechanism that promotes astrocyte A β production [184]. Since activated microglia also releases the mentioned cytokines, they are likely to participate in this feed-forward mechanism [186]. The fact that astrocytes outnumber neurons might suggest that even low levels of A β in

these cell types could make a significant contribution to the total A β burden during neuroinflammation in AD. However, extended research regarding this is needed. Moreover, recent *Drosophila* studies of endogenous APPL, a highly conserved homolog to A β PP, in cortical glial cells demonstrates that the protein regulates sleep/wake cycles. The pathology in patients suffering from AD is associated with reduced and highly fragmented sleep that, according to this study, may be a result from disturbed A β PP function within both neurons and glial cells [187].

Recent research strengthens the involvement of the immune system in AD. Using genome-wide association studies (GWAS) researches have identified a number of risk genes for AD, one of them being Triggering receptor expressed on myeloid cells 2 (TREM2). Mutations in this gene have been observed to associate with microglial dystrophy, decreased phagocytosis and an increased pro-inflammatory reactive phenotype [188], [189]. This was the first time that the link between AD and immune alterations was supported on a genetic level.

Taken together, there is still an ongoing debate whether inflammation in AD is beneficial or damaging, and a better understanding of the role of inflammation in AD is needed. This knowledge may result in novel therapeutic strategies for controlling the disease and attractive biomarkers that are relevant for diagnostics.

Treatments of Alzheimer's disease

As discussed so far in this thesis, the exact molecular pathogenic mechanisms causing AD are still unknown, and despite the fact that extensive research and a large number of clinical trials has been conducted, current drugs provide symptomatic benefits, e.g. reduce symptoms of memory loss and confusion, but do not treat the underlying disease or delay its progression.

Generally, drug discovery processes usually begins by the identification of a target protein that is implicated to be involved in a certain human disease. Next, the search for a compound that can alter the disease-causing protein is initiated, usually by screening a large library of known compounds, and eventually the compound is tested in animal models. On average, it takes between 10-15 years for a drug to travel from the lab to a patient and only 5 in 5000 potential compounds that enter preclinical trials make it to clinical trials in human. Of these five tested in humans, only one is approved [190].

Current treatments of AD

During the last two decades only five drugs have been approved by the U.S. Food and Drug Administration (FDA) to treat the symptoms of AD. As the disease progress, the communication between neurons is impaired (at the synapses), connections are lost and eventually the neuron dies. This results in disrupted levels of the neurotransmitters acetylcholine and glutamate important for learning and memory [191], [192]. The approved drugs reduce the cognitive symptoms by two different mechanisms that enhance the communication at synapses [193]. *Cholinesterase inhibitors* (Donepezil, Rivastigmine, Galantamine) decrease the activity of

cholinesterase, resulting in an increase in the levels of acetylcholine and improved brain function. *NMDA receptor antagonist* (Memantine), regulates the activity of glutamate by partially blocking the NMDA receptors, thereby reducing the entry of Ca^{2+} into the neuron preventing hyperpolarization of the cell [146]. There is also one drug (Donepezil + Memantine) combining the two mechanisms. Cholinesterase inhibitors are prescribed in early to moderate stages of the disease and NMDA receptor antagonists in moderate to severe AD [193].

Challenges of therapeutic strategies against AD

To date, most phase II trials that end with a positive outcome do not make it in a phase III trial. The most common reasons for phase III failure is the lack of efficacy and severe side effects due to toxicity. The reason for this is, as one could expect, not known, but two very important challenges are; incorrect diagnosis and the significance to start treatment at the earliest stages of the disease [194]. Despite extensive research, a definitive diagnosis of AD requires post-mortem examination. Additionally, several forms of dementia display similar symptoms at early stages of disease. If part of the recruited patient group is misdiagnosed, e.g. with individuals of mild cognitive impairment (MCI) never resulting in AD, specific AD therapeutics will not have the expected outcome, and might end up in a discontinued trial [195]. In addition, the accumulation of A β has been observed to precede disease symptoms by 15 to 20 years [42], making it hard to start treatment before irreversible brain damage has occurred. Imaging techniques such as positron emission tomography (PET) and magnetic resonance imaging (MRI) provides images of brain functions aiding the early diagnosis. However, about 10-30% of non-demented individuals older than 75 years show amyloid positive PET-scans [196]. Hence, to administer treatment in time, the field is in great need of advanced techniques and biomarkers that could be detected in biological fluids at the earliest stages of the disease.

Ongoing clinical trials and future treatment strategies of AD

Currently, more than 100 clinical trials are actively investigating new treatments for AD [36]. The majority of this ongoing development of new drugs aims to modify the potential underlying drivers of disease. The main therapeutic targets under investigation in current studies include; the A β peptide and its various aggregation stages, secretase inhibitors (BACE and γ -secretase), the tau protein and inflammation markers [197].

Due to the wide acceptance of the amyloid cascade hypothesis, the majority of clinical trials focus on; (1) decreasing the production of A β , (2) inhibiting the aggregation process of A β and/or (3) upregulating the clearance of A β [197]. To decrease the production of A β , clinical trials have approached the inhibition/modulation of secretases generating the A β peptide. However, γ -secretase is the proteolytic enzyme of several other proteins apart from A β PP, with the most notable being the Notch protein regulating cell development [198]. As a consequence clinical trials of first-generation inhibitors of γ -secretase rendered severe side effects and had to

be discontinued. A second-generation of γ -secretase inhibitors designed to modulate its activity and avoiding the influence on the Notch protein are in current clinical trials. A second secretase involved in the processing of A β PP to generate A β is BACE1. Inhibition of BACE1 displays less severe side effects, still only a handful of compounds representing this mechanism have reached clinical trials [199]. A promising BACE1 inhibitor, AZD 3293 administered by Astra Zeneca in cooperation with Eli Lilly, showed positive results in a phase I trial and caused prolonged A β reductions in both plasma and CSF [200]. Currently the compound is in phase II/III and phase III trials that are set to run until 2019 and 2021.

One of the most attractive and developed approaches in the treatment of AD is immunotherapy, bringing together two of the most complex biological systems in the human body; the immune- and the nervous system. Immunotherapy, both active and passive, aims to bind A β , facilitate its degradation and clearance and block the toxic effect [197], [201]. Since it is still unclear which form of A β that causes the pathogenesis of AD the variety of immunization has uttermost importance. Studies have shown that passive immunization can affect cognition without affecting plaque burden in the brain, possibly due to the efflux of soluble A β from the brain into the plasma via the peripheral sink mechanism. Currently there are four clinical trials using passive immunization, the most widely developed approach, in phase III. Three of them targeting A β aggregates, while the fourth (solanezumab) targets monomeric, soluble toxic species of A β [202]-[205].

Owing the fact that more than a decade has passed since the last approved treatment of AD, suggests that it is time to broaden the approach to other strategies than A β directed therapies. The second hallmark of AD is the hyperphosphorylation of the microtubule protein tau and the formation of intracellular NFTs. Apart from clinical trials focusing on A β directed therapies there are a few ongoing phase I/II clinical trials targeting the tau protein, using either small molecules or active- or passive immunization. These therapies aim to; inhibit tau phosphorylation and aggregation, increase the stabilization of microtubule and enhance the degradation of phosphorylated tau [206]. Recent data from mice models injected with tau-antibodies demonstrated reduced total and hyperphosphorylated tau in the brain, together with decreased levels of A β PP and cleared A β plaques [54]. Although far from clinical trials, these results highlight the fact that finding a compound that reduces both A β and tau pathology while improving cognition, have a much higher potential for successfully treating AD than those targeting either pathology alone.

Prevention trials against AD

As stated above, the best opportunity to modify the course of AD will be prior to widespread damage and clinical symptoms. Hence, AD research has shifted toward secondary prevention, i.e. prevention of the onset and progression of clinical symptoms in individuals in whom the pathophysiological processes of neurodegeneration has already started [207].

Studying individuals at risk of developing AD can approach this. These prevention trials have the potential of indicating when in disease progression AD treatment is to be initiated to give effect. Worldwide, four prevention trials are currently being conducted. In the Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease (A4) trial, clinically normal older persons with evident risk of developing AD due to amyloid accumulations (observed by PET imaging) are investigated using monoclonal anti-A β antibody therapies [208]. The TOMORROW trial includes asymptomatic individuals with a risk of developing AD, some with the genetic risk factor ApoE ϵ 4 allele. The trial will investigate whether an anti-diabetes drug, pioglitazone, can prevent MCI due to AD [209].

The therapeutic strategies described so far have targeted disease progression in sporadic late-onset AD. In contrast, the Dominantly Inherited Alzheimer Network Trial Unit (DIAN-TU) hopes to delay or stop the progression of disease in autosomal dominant Alzheimer's disease individuals. The participants in this trial have a known mutation in one of the three genes (A β PP, PSEN1 or PSEN2) causing early-onset AD, but are still asymptomatic. Two monoclonal anti-A β antibodies, gantenerumab and solanezumab, designed to remove excess A β in the brain, are currently being tested in this trial [210]. An additional trial testing the monoclonal anti-A β antibody therapies in individuals with known disease causing mutations is The Alzheimer's Prevention Initiative (API) [211].

These prevention trials can hopefully result in a foundation for combinational design of therapeutic agents targeting more than one mechanistic target in preclinical AD. Moreover, an intriguing aspect of the prevention trials in individuals with early-onset AD is that despite the genetic background, the clinical and pathological phenotype is similar to that of late-onset AD. Thus, a successful outcome of these trials has the potential to delay and/or prevent disease progression in the sporadic form of the disease as well.

5. *Drosophila Melanogaster* as a model system

Many landmark discoveries in genetics have been made using *Drosophila melanogaster*, one example being the concept that heritable traits are carried on the chromosomes [212] and during the past 20 years, two Nobel prizes have been awarded to *Drosophila* researchers. In 1990 *Drosophila* was chosen to be one of the model organisms to be studied under the guidance of the Human Genome Project, and in the year 2000 the fly was the first major complex organism to have its entire genome sequenced [213]. Hence, *Drosophila* research has been, and still is, an important source of understanding human development and disease, and although flies are evolutionary divergent from humans, 75% of human disease related genes have orthologs, or genes with similar functions, in the fly [214]. Many fundamental aspects of cell biology are similar between *Drosophila* and humans; e.g. cell cycle regulation, membrane trafficking,

synaptogenesis and cell death [215]. Despite this, the fly is not a miniature person and one has to remember that there are also important differences that cannot be robustly mimicked between the fly and humans; the circulatory system is much simpler, cognitive processes are less complex and flies do not have an adaptive immune system [212], [216]. However, the degree of conserved physiology and biology positions *Drosophila melanogaster* as an extremely valuable tool in the understanding of human disease and drug discovery processes.

The modeling of disease in *Drosophila* tends to fall into various stages, depending on how much is known about the disease. Initially the creation of a model is necessary, where the expression of a particular protein linked to disease cause a phenotypic behavior. These parameters can include lifespan or activity alterations as well as microscopic or biochemical observations in the fly tissue. The creation of a fly model can be approached in different ways, with the most common being; (1) direct expression of a known human disease causing gene, or (2) loss- and/or gain of function of the endogenous fly homolog of human disease genes. Once a model has been created, various alterations of the causative proteins or genetic screens to identify enhancer and/or inhibitors of the induced phenotype may be approached, strengthening the understanding of the disease [217].

During the last two decades there has been an increased use of *Drosophila* as a model of neurological dysfunction, including traumatic brain injury, brain tumors, stroke, epilepsy, dementia and neurodegeneration [212], [216]. Models established for neurodegenerative and amyloid disorders are presented in **Table 1**.

Table 1. Neurodegenerative and amyloid diseases modeled in *Drosophila melanogaster*.

Human Disease	Gene	Reference
Alzheimer's disease	A β , A β PP, Presenelin, Tau	[127], [218], [219]
Parkinson's disease	α -synuclein, Parkin and Pink	[220]
Huntington's disease	Huntingtin	[221]
Familial polyneuropathy	Transtyretin	[222], [223]
Lysozyme amyloidosis	Lysozyme	[224]
Amyotrophic lateral sclerosis (ALS)	TDP-43, FUS	[225]
Prion disease	Prion protein	[226]
Tauopathies	Tau protein	[227]
Spinocerebellar ataxia	Ataxin 3	[228]

***Drosophila* models of Alzheimer's disease**

AD has been modeled in *Drosophila* for over a decade with successful contributions to better understand the complex pathology of the disease [229].

Most of the genes involved in AD have homologs in *Drosophila*, such as the A β PP and γ -secretase. The fly homolog to A β PP is termed APP-like or APPL. Flies lacking APPL are viable, but show behavior abnormalities that can be strongly suppressed by expression of a human A β PP transgene, strengthening the conserved function of the gene between the two species [230]. A recent study demonstrates that a knockout of APPL mimics the neurodegenerative phenotype seen in A β expressing flies, such as behavior deficit, longevity phenotypes and eye degeneration. This indicates that APPL-knockdown can be used as an alternative to study the normal function of APPL, and due to the conservation also A β PP, in neurodegenerative diseases in the fly [231].

More importantly, the APPL gene lack the sequence of the human A β , disabling the investigation of endogenous processes linked to A β -derived pathology in AD [230]. However, recent evidence indicates that processing of APPL by endogenous secretases in the fly renders a 64 residue A β -like peptide that is neurotoxic in the fly brain and the eye [232].

There are predominantly two transgenic fly models used to study AD in *Drosophila*. The first model expresses human A β PP and BACE1 to mimic the complete proteolytic process resulting in the generation of A β and the subsequent aggregation [233]. In the second model, the human A β gene fused to a N-terminal secretion signal peptide is expressed directly, facilitating the study of the aggregation process [218], [219]. Although this latter model bypasses the secretase based generation of A β and may not localize A β in the correct sub-cellular compartment, this approach is crucial for a controlled and systematic investigation of various isoforms of the peptide [103], [127]. Despite the model used, pathological characteristics observed in humans, have been demonstrated in the fly and the progression of AD-like neurodegeneration in *Drosophila* mimicks the progression seen in humans (**Fig 9**). Such phenotypes include; impaired locomotor behavior, A β deposition, neuronal degeneration and reduced longevity [218], [219], [234], [235].

Figure 9. The progression of Alzheimer's disease like neurodegeneration in *Drosophila*. A β 1-42 expression in the fly CNS results in A β accumulation and aggregation prior to impairments in activity and lifespan, mimicking events in human disease progression.

As described above for general modeling of diseases in *Drosophila*, studies of A β expression using the fly have probed neurotoxicity in two ways. The first strategy has investigated what properties of the peptide and which aggregated species are responsible for the toxic phenotype observed. For example, in studies by Luheshi et. al. the systematic substitution of single amino acids revealed different toxicity profiles compared to their predicted aggregation propensity [127], [236]. In the second strategy a genetic modifier screen has been used to analyze A β neurotoxicity. For example the importance of metal metabolism in mediating A β toxicity has been investigated [237], [238].

Not only A β , but also tau, have been used to model AD in *Drosophila*. Wildtype *Drosophila* has a single endogenous tau gene, which can accumulate in axons in a similar manner as the mammalian tau protein [239]. Among other results, studies using tau expressing flies with an up-regulation of shaggy, the fly orthologue of GSK3 β , have supported the role of kinase dysregulation in the development of tau pathology [240]. Due to the frequent use of the rough-eye phenotype and the ease of interpreting the results, a wide range of modifier screenings and RNAi studies have been conducted in tau expressing flies [241]. This approach has even found genes that were later shown to possess significance in AD genome-wide association study (GWAS) meta-analysis [242].

Drosophila models of AD have also been used as a platform for testing potential drugs and therapies. This approach can enable testing of risky therapies that may not be tested in mice. Additionally it can save time by selecting the compound that works the best *in vivo* before

moving to more expensive mouse trials.

An indication that results from invertebrate model systems might be well transferred to higher organisms is that studies of A β 1-42 expressing flies fed by the drug memantine, a drug used to treat the symptoms of dementia in AD patients, prevented memory loss induced by the A β 1-42 peptide [243]. This positive effect was also apparent in double transgenic AD mice [243], [244]. Likewise, studies where human tau was expressed in flies demonstrated destabilization of the cytoskeleton and both loss of function and gain of toxic function of endogenous tau. These phenotypes could be reversed by the treatment of LiCl, a drug capable of suppressing tau phosphorylation [51].

Cell types of the nervous system in *Drosophila*

The developmental stages in the fly include 4 main stages: *the embryo* - consisting of 17 stages; *the larva* - with 3 stages; *the pupa* - 15 stages; and *the adult*. At 25°C a fertilized *Drosophila* egg begins neurogenesis about 3-4 hours after egg lay and continues until larval hatching into a first-instar larva with a functional nervous system in only 24 hours. After about four days in the larval stage, the puparium is formed and after additional 4 days the adult fly hatches [245].

The principal cellular element of the *Drosophila* nervous system is neurons. Neurons can be divided into three functional types: sensory neurons, association neurons and motor neurons. *Drosophila* neurons share similar processes and morphologies as their mammalian counterpart; they fire proper action potentials, they release conserved neurotransmitters from synaptic vesicles through highly conserved mechanisms, and they pass information between neurons at synapses with common protein architecture [246]. The CNS can be further divided into two histological regions; the neuronal cell cortex – composed of all neuronal cell bodies; and the neuropil – the location of axon and dendrite projection and neural circuit formation [247].

To direct expression of a gene of interest to a specific cell type in *Drosophila*, the Gal4-UAS system is employed (further described in the methodology, Section; *Controlling protein expression: the Gal4-UAS system*). A frequently used tool for neuronal expression in *Drosophila* is the pan-neuronal driver *Elav-Gal4*. ELAV (Embryonic lethal abnormal visual system) is a RNA binding protein, expressed in neurons, that is necessary for the formation and development of the embryonic and adult nervous system [248], [249]. A second neuronal driver is *n-syb-Gal4* [250]. Neuronal Synaptobrevin (n-Syb) is a vesicular protein that is specific to neurons and play a key role during synaptic vesicle exocytosis.

Apart from neurons, the CNS is composed of glial cells, accounting for about 5-10% of the total number of cells within the CNS [251]. As for mammals, glial cells in *Drosophila* constitute a support system for neurons and are critical regulators of nervous system development, function and health. Hence, *Drosophila* glia seems well suited to provide additional insights into glial biology that will be relevant to glial functions in mammals. In *Drosophila*, three main classes of

glial cells have been described from embryonic to adult stage: surface glia - form a layer around the CNS or peripheral nerves; cortex glia - ensheath neuronal cell bodies; and neuropil glia - associate directly with the neuropil [247], [251]. One of the earliest known markers for cells that will become glia is the transcription factor *Reversed polarity (Repo)* that is responsible for the differentiation and maintenance of glial cell function. Repo-positive glia cells become detectable at embryonic stage 11 and are very useful for labeling glial cells early in development as well as in the adult fly [251]. The total number of glial cells in the adult *Drosophila* brain can be estimated using the pan-glial driver *Repo-Gal4* [252].

A functional nervous system requires a close interplay between neurons and glial cells and *Drosophila* provides a great model to elucidate the fundamental mechanisms and to study how these affect animal behavior.

The compound eye of *Drosophila* has been widely used to study a variety of molecular, genetic and cell biological processes. About two thirds of the vital genes in *Drosophila* have been estimated to be required for the assembly and development of the fly eye. Thus, studies of the eye can give valuable insights in gene functions in other tissues as well as for the eye [253]. As important is the fact that the eye is not necessary for the viability or fertility of the fly, enabling studies of toxic proteins, gene disruption or gene mis-expression. Each fly eye is a simple repeating pattern of more than 700 precisely arranged identical units called ommatidia. Each ommatidium consists of eight photoreceptor neurons, which project axons that synapse in the optic ganglia of the brain [254]. The defined structure of the ommatidia can be disrupted by, for example, the expression of toxic proteins or genetic alterations, resulting in a rough eye phenotype easily detected by light microscopy or scanning electron microscopy [255]. A frequently used tool to study eye specific expression is the *GMR-Gal4 driver*. GMR (glass multiple reporter) is an eye-specific transcription factor required for normal photoreceptor development expressed in all cells of the developing eye [256].

Aims of the research

By understanding A β aggregation and neurotoxicity in *Drosophila Melanogaster* models of Alzheimer's disease, we hope, in the end, to further our understanding of what causes Alzheimer's disease in humans.

Our overall approach has been to use transgenic *Drosophila* models of Alzheimer's disease to investigate how alterations of the A β peptide and its expression pattern affect the overall toxicity and aggregation behavior in the fly.

More specific aims of my thesis were to:

- Characterize how A β -induced toxicity is associated with varying **A β length** and amino acid composition and to examine if the observed toxicity by A β 1-42 could be altered by the co-expression of a second A β variant.
- Investigate **cell type** specific A β -toxicity and aggregation by expression of the A β 1-42 peptide and characterize the possible differences between varying expression genotypes.
- Analyze whether different substances can function as protective **compounds** against amyloid induced degeneration.

Methodology

1. *Drosophila* as a research tool

Drosophila Melanogaster, or the common fruit fly, can be described as a living test-tube and has been frequently used as a model organism in medical and scientific research for over a century. Thomas Hunt Morgan first described the fly as a tool in molecular genetics in 1910. His discoveries of the white-eyed mutation refined the theory of inheritance and established that genes were found within chromosomes [257].

There are several advantages of using *Drosophila* as your model organism, with the most pronounced being the low number of chromosomes, four pairs; which makes it easy to genetically modify the fly genome. The fly has a very rapid lifetime, and the short generation time and reproduction cycle are other reasons for using flies as your model system. The time from an egg to an adult fly is 10 days at 25°C. This enables the opportunity to perform experiments on several generations in a short period of time. You can also study a large number of flies fairly inexpensively and since flies are invertebrates there is no need for any ethical permissions to start your work [229].

Drosophila has one pair of sex chromosomes (X/X for females and X/Y for males) and three autosomes (2, 3 and 4) and it is the ratio of X chromosomes to autosomal sets that is the base for sex determination [258]. Chromosome two and three are almost exclusively used for insertions of genes of interest. An essential feature of fly genetics is that recombination in males is totally absent. In females on the other hand, you have to be aware that recombination during meiosis occur all the time. To control recombination in females balancer chromosomes are used. Except for preventing recombination, an additional advantage of using a balancer is that they contain markers, such as physical characteristics, making it easy to recognize their presence in the fly. Consequently, their transmission to progeny can be tracked unambiguously [258]. Markers allow you to distinguish flies carrying an “invisible” gene of interest from non-transgenes.

Examples of physical characteristics used as markers are; wing shape, eye color and hair length. To be able to make comparisons between experiments it is important to consider that the environmental conditions are the same every time you conduct your experiment, i.e. temperature, humidity, light-dark cycles and food composition. Since it can be hard to have control over all these parameters, comparisons should be made within an experiment, or after repeated experiments with similar results. Keep this in mind when comparing results from different laboratories.

Controlling protein expression: the Gal4-UAS system

A powerful and pioneering technique to control protein expression in *Drosophila*, the Gal4-UAS system, was developed and published by Brand and Perrimon in 1993 [259]. This genetic tool made *Drosophila* an even more appealing model to use for modeling diseases. The system allows you to express a certain protein of interest in a specific tissue or cell type. By expressing your protein of interest in only a subset of cells you may get a better understanding of e.g. mechanisms of toxicity. The system consists of two parts; the Gal4 gene and the Upstream Activating Sequence (UAS). The Gal4 gene encodes a yeast transcription factor, Gal4, and is under the control of a promotor/driver gene that directs expression to a certain tissue of choice. These flies are described as driver flies. The UAS enhancer region is arranged upstream of any gene of interest, and flies with this insertion represent the transgenic reporter line. Protein expression of your gene of interest is initiated first when the Gal4 protein binds to the UAS region. This makes it possible to keep stable stocks of the parental driver- and reporter flies separately for an indefinite period of time. If the UAS is not present, the Gal4 protein has no or little effect on the flies and vice versa. When cross breeding a female Gal4 driver line with a male UAS reporter line, you will generate an offspring that contains the UAS-controlled transgene in every cell, however, expression of this transgene is limited to the cells expressing Gal4 (**Fig 10**).

Figure 10. A schematic illustration of the Gal4-UAS system. Cross breeding of a female driver fly and a male UAS-reporter fly result in tissue-specific expression of your gene of interest in the offspring flies. Here depicted by A β 1-42.

The Gal4 expression is temperature sensitive, with increasing expression at higher temperatures. Hence, the expression is more active at 29°C than at 20°C. This is utilized in Paper II, where we express two variants of the A β peptide, generating very high levels of toxicity. To be able to conduct the desired experiments we had to lower the temperature and hence prolong the lifespan of the flies.

An extension of this system includes the use of a temperature sensitive yeast Gal80 transcriptional repressor. Gal80 blocks the binding of Gal4 to the UAS in a temperature dependent manner. As temperature is increased (to around 29°C) this block is relieved, thus the activation of Gal4 is introduced and expression of your gene of interest is initiated. This allows for a temporal control and can be used to introduce expression of a highly toxic protein first after eclosion, avoiding the toxicity during different developmental stages [260]. Moreover, Gal4 and Gal80 lines with overlapping expression pattern can be used to direct expression to only a subset of certain cells [261].

Fly lines

To direct expression to the tissue/cell type of your choice it is important to select the appropriate driver. Throughout the work of this thesis 5 different Gal4-driver lines have been used. The well studied driver *C155-elav-Gal4* directs expression to mature neurons of the CNS [248], [249], but has also been found to be transiently expressed in embryonic glial cells [262]. The *n-syb-Gal4* driver is an improved and stronger driver generated by the Thor lab (Paper I). This driver also directs expression to the neurons of the CNS, and is turned on first after neurogenesis [250].

The *D42-Gal4* driver express your protein of interest in motor neurons and sensory neurons [263]. The widely used *Drosophila* glial driver *repo-Gal4* directs expression to all glial cells in the CNS except the midline glia [252], [264]. The fifth driver used in this thesis is the well studied *GMR-Gal4* driver directing expression to the developing eye cells of the fly [256]. All drivers used are summarized in **Table 2**.

To be able to study the aggregation mechanism and toxicity of A β in different ways we have used 31 different A β UAS-reporter lines and two different control UAS-reporter lines throughout this thesis. The used UAS-lines are summarized in **Table 3**.

In paper I we generated a stronger UAS-A β transgenic line optimized for high-level expression. The A β construct was inserted to a pUASattB vector [265] and landed at position 65B on chromosome 3. This identical chromosomal insertion, where the gene of interest is landed at the same chromosomal position in every transgene, removes any transgenic position effects and facilitates the interpretation of the results. These constructs were also used in paper II and III.

Table 2. Summary of driver lines used throughout the PhD-studies.

Fly line	Expression	Paper
C155-elav-Gal4	Gal4-driver line directing expression of the transgene to the neurons of the CNS [248], [249]	III, IV
n-syb-Gal4	Gal4-driver line directing expression of the transgene to the neurons of the CNS [250]	I, II, III
D42-Gal4	Gal4-driver line directing expression of the transgene to the motor neurons and sensory neurons [263]	III
Repo-Gal4	Gal4-driver line directing expression of the transgene to the glial cells [252], [264]	III
GMR-Gal4	Gal4-driver line directing expression of the transgene to the developing cells of the eye [256]	III, IV

Table 3. Summary of UAS-reporter lines used throughout the PhD-studies.

Fly line	Expression	Paper
w ⁻ , control	Offspring from w ⁻ and a driver line expresses Gal4	IV
Oregon-R, control	Offspring from Oregon-R and a driver line expresses Gal4	I, II, III
A β 1-42	Transgenic for human A β 1-42 peptide [219]	IV
A β 1-42;A β 1-42	Transgenic for a double insert of human A β 1-42 peptide [219]	IV
A β 1-40	Transgenic for human A β 1-40 peptide [219]	IV
A β 1-42 E22G	Transgenic for human A β 1-42 with the Arctic mutation [219]	IV
Tau	Transgenic for human Tau peptide [266]	IV
A β 1-37	Transgenic for human A β 1-37 peptide [250]	I
A β 1-38	Transgenic for human A β 1-38 peptide [250]	I
A β 1-39	Transgenic for human A β 1-39 peptide [250]	I
A β 1-40	Transgenic for human A β 1-40 peptide [250]	I
A β 1-41	Transgenic for human A β 1-41 peptide [250]	I
A β 1-42	Transgenic for human A β 1-42 peptide [250]	I, II, III
A β 1-43	Transgenic for human A β 1-43 peptide [250]	I
A β 3-42	Transgenic for human A β 3-42 peptide [250]	I
A β 3-43	Transgenic for human A β 3-43 peptide [250]	I
A β 11-42	Transgenic for human A β 11-42 peptide [250]	I
A β 11-43	Transgenic for human A β 11-43 peptide [250]	I
A β 3-42 E3A	Transgenic for human A β 3-42 E3A peptide [250]	I
A β 11-42 E11A	Transgenic for human A β 11-42 E11A peptide [250]	I
A β 1-42 A42D	Transgenic for human A β 1-42 A42D peptide [250]	I
A β 1-42 A42R	Transgenic for human A β 1-42 A42R peptide [250]	I
A β 1-42 A42W	Transgenic for human A β 1-42 A42W peptide [250]	I
A β 1-42;A β 1-37	Transgenic for human A β 1-42 and A β 1-37, unpublished	II
A β 1-42;A β 1-38	Transgenic for human A β 1-42 and A β 1-38, unpublished	II
A β 1-42;A β 1-39	Transgenic for human A β 1-42 and A β 1-39, unpublished	II
A β 1-42;A β 1-40	Transgenic for human A β 1-42 and A β 1-40, unpublished	II
A β 1-42;A β 1-41	Transgenic for human A β 1-42 and A β 1-41, unpublished	II
A β 1-42;A β 1-42	Transgenic for human A β 1-42 and A β 1-42, unpublished	II
A β 1-42;A β 1-43	Transgenic for human A β 1-42 and A β 1-43, unpublished	II
A β 1-42;A β 3-42	Transgenic for human A β 1-42 and A β 3-42, unpublished	II
A β 1-42;A β 3-43	Transgenic for human A β 1-42 and A β 3-43, unpublished	II
A β 1-42;A β 11-42	Transgenic for human A β 1-42 and A β 11-42, unpublished	II
A β 1-42;A β 11-43	Transgenic for human A β 1-42 and A β 11-43, unpublished	II
nGFP	Expresses green fluorescent protein in cell nuclei [267]	I, III
Rpr	Expresses the pro-apoptotic gene reaper [268]	III

2. Phenotypic studies of *Drosophila*

Common features of the phenotypic assays described in this section are that they are conducted on live flies and that they are often used as a first indication of alterations in the fly caused by transgenesis. If performed under a controlled manner using the same procedure, these assays are highly reproducible and reliable.

Longevity assay

A straightforward read-out of general organismal protein toxicity or of the effect of a pharmacological substance is the longevity assay, i.e. the lifespan of a fly. Having established a robust transgenic disease-associated model with a significant phenotype, a first step is an investigation of the effect on lifespan. This assay will give you a first indication of the health status of your transgene compared to a control fly (not expressing any transgene but only Gal4) or other transgenes.

In general; during the course of the experiment 100 mated female flies per genotype, in vials of 20, are collected and three times a week the flies are transferred to fresh vials, containing agar food supplemented with yeast paste, and the number of surviving flies is recorded throughout the lifetime of all flies. Online software is readily used to track flies in lifespan experiments using barcode-labeled vials and when an experiment is terminated collected data can be analyzed using Kaplan-Meier log rank statistics [269] and tested for statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A common measurement extracted from the plot is the median lifespan, i.e. when 50 % of the flies of a genotype have died, of your various transgenes and is widely accepted as a measure of general health.

Locomotor assay

As flies age they naturally display a decrease in activity, however, this decrease is minimal compared to the impairment seen when expressing a toxic peptide or protein in the CNS of the flies. A locomotor assay can be seen as an extension of the lifespan assay and gives you a more detailed picture of the effects of toxicity upon the function of the nervous system [270]. By using solely a lifespan assay, two different transgenes can have the same lifespan even though it is obvious that they are not as healthy. An impairment of the fly's locomotor behavior does not mean that the fly cannot be alive, and this is where the locomotor assay gives you additional information.

There are several assays to study the fly activity, the most common being the climbing assay. In this assay you manually record the time it takes for flies to climb from the bottom of the vial to a certain point of the vial [271]. Another frequently used assay to examine the fly activity is the *Drosophila* Activity Monitor 2 (DAM2) system, which allow you to characterize the locomotor

and behavior rhythms of flies using an infrared light beam. This assay was used in paper IV. Flies are placed in narrow horizontal tubes, and as the fly walks back and forth in the tube, the beam is broken and thus movement is recorded. The experiment is conducted over a period of 24 hours, which enable you to observe differences in both the activity and the circadian rhythm [272]. One of the drawbacks of this assay along with the climbing assay is that it is one-dimensional, limiting the amount of information given.

For a more sensitive behavioral assay (paper I-III) we turned to the recently developed fly activity imaging system iFly (Jahn et al. 2011). This system allows for probing the negative geotactic movement of flies, i.e. the walking movement of the fly in response to the force of gravity, and to calculate several behavioral parameters using a 3D-tracking algorithm (velocity, slope of end-to-end distance, angle of movement and interaction angle). In our work, we choose to analyze the parameters velocity and angle-of-movement. Velocity simply describes how fast the flies move from the bottom of the vial to the top of the vial in mm/s. As the flies get neurologically impaired the velocity decreases, and shortly before the flies die they become immobile and their velocity cannot be recorded. Thus a cut-off value of 4 mm/s was set as an indication of disability. The parameter angle-of-movement describes the deviation from a straight vertical line when flies move from the bottom of the vial to the top. As flies age or get sicker they tend to move in a more disordered pattern, resulting in an increase of the angle-of-movement. This increase indicates that mobility functions, such as orientation and movement direction, of the flies are impaired, and the cut-off value was set to 80°. A screenshot of an iFly movie is displayed in **Figure 11**.

For every genotype examined the assay consists of three vials with 10 newly eclosed flies in each vial. A movie of 90 s is recorded for each vial, and every 30 s the flies are tapped to the bottom of the vial to ensure the same starting point in each movie. You end up with nine movies of 30 s for each genotype and time point. Movies are recorded three times a week until the flies become to immobile to be able to record. During the course of the experiment the assay vials are replenished to compensate for dead flies during ageing. The movies are processed using the iFly software and the parameters velocity and angle of movement are calculated. The output data is plotted in GraphPad Prism (GraphPad Software, San Diego, CA, USA) and compared between different genotypes.

Figure 11. Screenshot of an iFly movie illustrating the differences of control flies and flies expressing A β 1-42. Control flies move to the top of the vial following a straight line with high velocity, while A β 1-42 flies move in a more disordered pattern with decreased velocity.

3. Protein detection and quantification

Antibodies

In our immune system antibodies are constantly active to detect and neutralize pathogens such as bacteria and viruses. Antibodies are Y-shaped proteins that consists of two heavy chains and two light chains. They are produced by B-cells to recognize and bind surface molecules, antigens, of pathogens making it easier for white blood cells to fight the infectious agent. The part of an antigen that binds the antibody is called epitope and consists of a short amino acid sequence. There are two kinds of antibodies; monoclonal- and polyclonal antibodies. A monoclonal antibody binds to a specific epitope of an antigen, whereas a polyclonal antibody is a mixture of multiple antibodies that bind to different epitopes of the same antigen [273].

In research, purified antibodies have been used for more than half a century, most commonly to identify and locate proteins from crude samples or tissues by applications such as immunohistochemistry, western blot and immunoassays. In all four papers in this thesis we used different monoclonal antibodies to capture and detect the A β peptide in a sandwich immunoassay from Meso Scale Discovery (MSD). In paper IV we also use a monoclonal antibody against the A β peptide to stain sections of fly heads in an immunohistochemistry set up.

Protein quantification assay

A primary factor determining aggregation rates and hence presumed toxicity, of e.g. A β , is the protein concentration, which can be measured using various techniques, for example different immunoassays or western blot. Immunoassays are antibody-based assays to measure the presence or concentration of a target protein in a crude sample. The assay was first developed in

1959 by Rosalyn Sussman Yalow and Solomon Benson who examined plasma insulin in human subjects [274].

Throughout this thesis a sandwich immunoassay from MSD was used to be able to compare the protein levels in different *Drosophila* genotypes. The two key features of a sandwich immunoassay are; the recognition and binding of a capture antibody to a specific protein, in our case A β , and a way to produce a measurable signal in response to that binding. The latter often involves a chemically linked antibody with a detectable label. The most commonly used sandwich immunoassay is the ELISA assay, where enzymes are used to develop a signal [275]. In the MSD assay, a microtiter plate with high binding carbon electrodes is used and detection is achieved by the electrochemiluminescent principle. A capture antibody against the target protein is adsorbed onto the well, the sample is added and binding of the target protein to the antibody is achieved. To enable detection, a Sulfo-Tag-conjugated detection antibody is added. When electricity is applied to the plate electrode by an MSD instrument light emission is generated by the short distance between the surface carbon electrode and the ruthenium containing Sulfo-Tag:ed antibody. The light intensity is then measured to quantify the amount of protein in the sample (**Fig 12**). For every microtiter plate run, a standard curve using known concentrations of synthetic A β was prepared to be able to compare with our unknown samples.

There are several advantages of using an MSD immunoassay over e.g. a standard immunoassay. The most pronounced are; better sensitivity and a broader dynamic range, higher signal to background ratio and it requires less amount of sample.

Figure 12. A schematic illustration of the MSD immunoassay. A capture antibody binds to the protein that is detected by a sulfo tag-conjugated antibody. Electricity is applied to the plate resulting in light emission by the chemical reaction of the sulfo-tag. Emission is recorded at 620nm.

Sample preparation

To be able to measure the quantities of A β in our flies we have to extract the protein from the fly brain. Due to the proposed theories of A β toxicity not only the total protein concentration is of interest in this thesis, but also the ratio soluble/insoluble protein. We divided our head extracts into a *soluble* fraction composed of fly heads homogenized in a Hepes buffer (pH 7.3), and an *insoluble* fraction obtained from heads homogenized in a Hepes buffer pelleted and resuspended in a Hepes buffer containing 5M GuHCl before addition to the MSD plate.

Antibodies used for capture and detection throughout this thesis (Nordic Biosite and MSD):

6E10 - recognizes the N-terminal of A β with an epitope within amino acids 3-8 of the A β peptide

4G8 - recognizes the mid-region of A β with an epitope within amino acids 18-22 of the A β peptide

12F4 - recognizes an epitope including amino acid 42 (the C-terminal) of the A β peptide

82E1 - recognizes an epitope including amino acid 1 (the N-terminal) of the A β peptide

Fluorescence microscopy

To further analyze protein expression, location and aggregation, fluorescence microscopy was used. In the light of this thesis, this technique makes it possible to visualize; the location of a certain protein in a tissue, aggregation patterns of a protein and co-localization between different fluorescent probes.

A fluorescent substance absorbs light of a particular wavelength and energy, and then emits light of a longer wavelength and lower energy. A sample can possess the fluorescent properties either intrinsically or by the addition of a fluorescent dye, a fluorophore. The mission of the fluorescence microscope is to excite a sample with light of a specific wavelength, and then to separate the emitted light of a longer wavelength to make it visible for the user [276].

In this thesis the most frequently used imaging technique has been confocal fluorescence microscopy, which allows you to examine thick, intact specimens, like e.g. a whole fly brain. In a confocal microscope, excitation of your sample is carried out point-by-point, together with the use of a pinhole, which eliminates out-of-focus signals. This gives you a much better optical resolution compared to conventional fluorescence microscopy where the whole sample is flooded with light at once [277]. By using optical slicing varying the imaging depth, instead of physical slicing, of your sample you obtain a three-dimensional image with a more physiologically relevant representation of the analyzed structure/tissue. For example you can get a better understanding of the architecture of aggregates, i.e. if they are spanning several optical slices, and/or where in the tissue they are located.

Tissue samples used throughout this thesis were produced in two ways. Either decapitated fly heads were embedded in Tissue-Tek O.C.T. and cut in thin sections (10-20 μm) using cryosectioning, or whole fly brains were dissected and analyzed three-dimensionally. When preparing the tissue for histology analyses you commonly start by fixing your sample to ensure that the tissue is stabilized and maintains its cellular structure. If you intend to use antibody staining to visualize your protein of interest, as in paper IV, you need to block your sample to minimize unspecific staining before the addition of a primary antibody. This is followed by the addition of a labeled secondary antibody, which enables detection through a fluorescent signal.

Luminescent Conjugated Oligothiophenes, LCOs

To detect and visualize A β aggregates in this work, we made use of the properties of LCOs when binding amyloid forming proteins causing protein misfolding diseases, such as Alzheimer's disease. Previous work has shown that these probes are well suited for the detection of amyloid deposits in *Drosophila* brains [235]. LCOs are well-defined thiophene based molecules that bind several amyloid forming protein aggregates. Due to their conjugated backbone and their ability to twist or become more planar they possess different fluorescent properties depending on the conformation of their target. Because of this conformation sensitivity upon binding, the LCO gives rise to a specific emission profile facilitating the identification of protein aggregates and their different structures [278]. For example, instead of using two different antibodies targeting A β plaques and Tau tangles, the hallmarks for Alzheimer's disease, you can use a single LCO to discriminate between the two. In this case, A β aggregates binding the LCO p-FTAA will give rise to a green emission, while Tau tangles binding the same LCO, in the same sample, will display a yellow-red emission [278]. Another advantage of using LCOs instead of, or complimentary to, antibodies is the ability of LCOs to bind aggregates. An antibody is great when detecting a monomeric protein, but as soon as the aggregation process starts, the binding capacity of an antibody becomes reduced due to the fact that the epitope can become hidden within the aggregate. The LCO on the other hand binds to a specific conformation rather than a specific epitope, resulting in a more precise binding to protein aggregates. Compared to more conventional used amyloid stains, like Congo-red or ThT, the LCOs are able to detect early formed pre-fibrillar oligomeric states as well as mature fibers, making it possible to follow the amyloid fibrillation pathway [279].

LCOs are named according to the number of thiophene units in their backbone together with their chemical modifications. In this thesis the LCO p-FTAA (**Fig 13B**) was most frequently used. p- for penta, the number of thiophene units, F- for formic acid as the end group, T- for thiophene and AA- for acetic acid as the side group. In paper III we used two additional LCOs for spectral discrimination of the aggregates formed in different cell types. These were q-FTAA (four thiophene units) and h-FTAA (seven thiophene units) (**Fig 13A and C**).

Previous studies by Nyström et. al. have shown that q-FTAA tends to bind more mature and bundled fibrils, while h-FTAA also binds earlier formed aggregates, pre-fibrillar species and monofilamentous fibrils. The hypothesis is that the q-FTAA binding aggregates are less toxic due to a more stable structure, hence fewer pieces, able to act as seeds for new aggregate formation, shed off the already formed plaques [129].

Figure 13. Chemical structures of the LCOs used throughout the papers included in the thesis.

(A) q-FTAA, (B) p-FTAA and (C) h-FTAA.

In addition to the LCOs mentioned above, a library of probes [280] with different backbone length (4-7 thiophene units) and end groups (hydrogen or formic acid) have been evaluated for their staining of A β 1-42 aggregates in day 5 *n-syb-A β 1-42* brain sections. As displayed in **Figure 14**, all probes did stain A β 1-42 aggregates, however, LCOs possessing formic acid as an end group displayed slightly better staining with less background.

Figure 14. A library of LCOs analyzed in day 5 n-syb-A β 1-42 *Drosophila* brain sections. The backbone of the probes ranges between 4-7 thiophene units. Each spectrum is an average of 21 regions of interest in three individual brains. Images were acquired using excitation at 405 nm for the tetramers and 488 nm for all other LCOs. The magnification was 20x.

4. Scanning Electron Microscopy

To examine the toxic effect of A β in the retina we looked at the eye structure. The *Drosophila* eye is composed of more than 700 precisely arranged ommatidia [281] and when a toxic protein is expressed, the eye development can be affected and the structure of the ommatidia will be disrupted and can thus be analyzed for a rough eye phenotype [254]. A frequently used method to analyze the eye structure is scanning electron microscopy, SEM. An electron beam scans the sample and electrons interact with the atoms of the sample. Depending on the various depths

within the sample the produced signals enable the build up of an image of the surface topography and composition. When using SEM, specimens must be electrically conductive. Hence when imaging biological samples that are nonconductive it is necessary to coat your sample with an ultra thin coating of electrically conducting material, e.g. gold, gold/palladium or platinum to be able to collect a signal. In paper III, flies were mounted onto aluminum specimen stubs and air-dried for 24 hours before sputter coated with a few nm thick coat of platinum and analyzed for a rough eye phenotype.

Summary of papers

Paper I: Systematic A β analysis in *Drosophila* reveals high toxicity for the 1-42, 3-42 and 11-42 peptides, and emphasizes N- and C-terminal residues and

Paper II: Systematic combinatorial A β expression in *Drosophila* reveals additive toxicity for the 1-42, 3-42 and 11-42 peptides and a mitigating effect of the 1-38 peptide

Aim: The aim of the first and second paper was to systematically analyze if specific A β peptides may differentially contribute to the A β -induced toxicity observed in Alzheimer's disease and if the observed toxicity by A β 1-42 could be altered by the co-expression of a second A β variant. This was addressed through the generation of 16 transgenic *Drosophila melanogaster* strains specifically expressing a specific form of the A β peptide and 11 transgenic strains co-expressing A β 1-42 with a second A β variant, in the CNS of the fly.

Results and discussion: Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide and since the identification of the A β peptide as the main constituent of amyloid plaques more than 30 years ago, major research has focused on the aggregation and toxicity behavior of this peptide. Due to the proteolytic processing of the A β PP, a complex pool of different A β peptides (37-43 amino acids long) is generated, with the most studied being A β 1-40 and the more aggregation prone A β 1-42. Several studies provide increasing evidence that such variable A β peptides may be significant in AD pathogenesis and that the combinatorial effect of a mixture of different A β peptides display high complexity. We addressed A β peptide neurotoxicity by systematically investigating different A β peptides, both independently and in mixtures, in *Drosophila* models of AD.

A first indication of the impact of expressing different A β peptides in the *Drosophila* CNS was observed in lifespan and activity assays. The expression of single A β peptides displayed a severe reduction of lifespan and activity if the peptide ended at amino acid 42, with the most pronounced results for A β 1-42 and 3-42 peptides. This toxic effect could be mostly abolished by the addition of a C-terminal amino acid (A β x-43) or completely abolished by the removal of one or several C-terminal amino acids (A β 1-37 to 1-41) (**Fig 15A**). N-terminal substitutions of amino acid 3 and 11 (E3A and E11A), revealed no apparent change for E3A when compared to wild type, whereas E11A showed reduced effects. Surprisingly, the substitution of amino acid 42 (A42D, A42R, A42W) led to a clear reduction in toxicity and increased performance in the activity assay compared to A β 1-42 wt (**Fig 15B**).

Co-expression of A β 1-42 with a second A β peptide displayed an additive toxicity for the 1-42, 3-42 and 11-42 variants and a mitigating effect of the 1-38 peptide (**Fig 15C**). The toxic effect was much stronger than for previous data comparing single A β 1-42 and double inserted A β 1-42 (Paper IV and [219]).

Figure 15. Expression of A β peptides ending at amino acid 42 shortens the lifespan of *Drosophila* AD models. Summary of the median lifespan of flies expressing (A) a single A β peptide of various length, (B) a N- or C-terminally mutated A β variant, and (C) co-expressing A β 1-42 with a second A β peptide, in the CNS of the flies. Flies reported in paper I (A and B) were kept at 29°C, while flies reported in paper II (C) were kept at 20°C due to the severe toxicity. Hence a difference in median lifespan of the single expressing A β 1-42 flies is obvious in (A) and (B) vs (C).

Next, analysis of A β levels and aggregation pattern was addressed. For single expressing A β variants (Paper I), these results were in agreement with the lifespan and activity assay, with the exception of A β 11-42 and 1-42 A42W expressing flies. The highest level of insoluble A β was seen for the 1-42 and 3-42 variants, followed by extensive staining with the protein aggregate-specific luminescent conjugated oligothiophene (LCO) p-FTAA over time. Rather unexpectedly, A β 11-42 expressing flies displayed quite low levels of A β considering their rather severe lifespan and activity phenotype, and p-FTAA positive staining was almost absent. A similar pattern was seen for the A β 1-42 A42W expressing flies.

When co-expressing A β 1-42 with a second A β peptide, all genotypes examined displayed moderate to high levels of A β . The additive effect seen for the *in vivo* toxicity of flies expressing 1-42 or 3-42 as a second A β peptide was evident also in the protein level and histological analysis (Paper II). As for the single expressing A β 11-42 flies (Paper I), protein levels and p-FTAA positive staining did not correlate with lifespan and activity phenotypes for flies co-expressing A β 1-42 and 11-42 (Paper II). Both A β levels and p-FTAA staining in these flies were similar to that of single expressing A β 1-42 flies despite the difference in toxicity. C-terminal elongation or truncation of A β 1-42, resulted in similar or slightly lower levels of A β as the single expressing A β 1-42 flies. Histological examination of these flies displayed similar or increased aggregate load compared to single expressing A β 1-42 flies over time. The C-terminal truncated variants displayed increased levels of aggregated protein (p-FTAA positive staining) compared to A β 1-42, 11-42 expressing flies at day 5 post eclosion, despite the difference in median lifespan of up to ten days, indicating a structural difference of the aggregates detected in the A β 1-42, 11-42 flies.

In summary, we demonstrate that A β peptides ending at amino acid 42 show high neurotoxicity in *Drosophila* (Paper I and II). This deleterious effect can be mostly or completely abolished by the addition or removal of a single amino acid or by the substitution of Alanine in position 42 (Paper I). The results for the various genotypes are in general agreement; decrease in lifespan and locomotor activity parallels increase in A β accumulation and total A β content and aggregation (**Fig 16A and B**). By co-expressing A β 1-42 with a second A β peptide (Paper II), we demonstrate an additive toxicity for the 1-42, 3-42 and 11-42 variants and a partially mitigating effect of the 1-38 peptide, (**Fig 16C**). This mitigating effect of A β 1-38 is in line with previous studies, suggesting a slightly altered aggregation pathway of the A β 1-42 in the presence of A β 1-38.

Both paper I and II highlights the efficiency of rapid oligomerization of A β 1-42 to form specific stable neurotoxins, and supports the strategy aimed at decreased formation of A β x-42 peptides as a relevant therapeutic strategy.

Figure 16. Median active lifespan plotted versus total Aβ accumulation (ng/ml per fly). Flies expressing (A) a single Aβ peptide of various length, (B) a N- or C-terminally mutated Aβ variant, and (C) co-expressing Aβ 1-42 with a second Aβ peptide, in the CNS of the flies. Median active lifespan is; (the median lifespan + the day were the activity reaches the cut-off value) / 2. Flies corresponding to paper I (A and B) were kept at 29°C, while flies corresponding to paper II (C) were kept at 20°C due to the severe toxicity. Hence the difference in median lifespan of the single expressing Aβ 1-42 flies in (A) and (B) vs (C) is obvious.

Paper III: Amyloid A β 1-42 is selectively toxic for neurons, whereas glial cells produce mature fibrils without toxicity in *Drosophila*

Aim: In the third paper we aimed to investigate whether the A β 1-42 toxicity and aggregation pattern observed in CNS neurons of *Drosophila* can be observed in other cell types, or if the toxic effects are cell type specific. We generated five transgenic models expressing A β 1-42 in various cell types of the CNS in *Drosophila*.

Results and discussion: *Drosophila melanogaster* has, during the last decade, become a widely used model system to study Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder with memory loss being characteristic to the disease. Hence, it is often assumed that neurons are the sole target cell in AD patient's brain, but other cell types are also affected, such as glial cells. To our knowledge, there are no studies where a systematic expression of the A β 1-42 peptide (one of the main causative agents in AD) in different cell types of *Drosophila* has been investigated. To approach this, we turned to five different driver lines directing expression of A β 1-42 to the; CNS neurons; motor neurons; glial cells; and developing cells of the fly eye.

One of the hallmarks of AD is the formation of A β aggregates, including amyloid fibrils. Hence, in a first analysis of our transgenes we turned to the aggregate specific luminescent conjugated oligothiophene (LCO), p-FTAA, to examine aggregate load in different cell types. We found that all genotypes expressing A β 1-42 were able to form p-FTAA positive aggregates, however to a very different extent (**Fig 17**). Flies expressing A β 1-42 in the CNS neurons displayed moderate to high levels of aggregates, depending on the driver line used (weak or strong). Motor neuron expressing flies also revealed p-FTAA positive staining, although to a lower extent than the two global CNS neuronal expressing models. Rather unexpectedly, glial cell expressing flies revealed immense amounts of fibrillar amyloid structures, already at early time points. At day 5, these flies had higher amounts of aggregates than the stronger CNS neuronal expressing flies had at day 10; the very end of their lives. Additionally, when examining sections of flies expressing A β 1-42 in the eye, we also found extensive amounts of aggregates in the retina. However, quite surprisingly, we did not see a disruption of the eye structure when analyzed by scanning electron microscopy. Analysis of A β 1-42 levels were in good agreement with the histological findings, indicating that high concentration of A β 1-42 results in a large aggregate load.

Gal4 - A β 1-42

Figure 17. Histological staining reveals A β 1-42 aggregates in all genotypes. Whole *Drosophila* brain staining with the amyloid specific LCO, p-FTAA (green) and cell nuclei stain ToPro3 (red) at day ten post eclosion. (A) strong CNS neuronal expression, (B) weak CNS neuronal expression, (C) motor neuron expression, (D) glial cell expression and (E) eye disc expression, of the A β 1-42 peptide.

Next, we examined whether the various expression levels and aggregate loads of the A β 1-42 peptide had an impact on behavioral parameters such as lifespan and activity. For flies expressing A β 1-42 in CNS neurons and motor neurons we found a good correlation between toxicity and levels of A β 1-42 and amount of aggregates, with the stronger CNS neuron transgenes displaying the strongest phenotype. Unexpectedly, expression of the A β 1-42 peptide in glial cells resulted in a minor reduction in lifespan and activity compared to control flies, and a much longer lifespan than any of the other genotypes expressing A β 1-42 (**Fig 18**). Hence, these flies show a very mild toxicity despite the high levels of deposited A β 1-42.

Figure 18. Expression of A β 1-42 in neurons shortens the lifespan. Lifespan trajectories of A β 1-42 expressing flies and control flies using (A) the strong CNS neuronal driver, (B) the weak CNS neuronal driver, (C) the motor neuron driver and (D) the glial cell driver. (E) a measure of the toxicity level comparing the median lifespan of the respective control to their A β 1-42 counterpart. A high value represents high toxicity. 1 = no toxicity.

Because of these quite contradictory findings, we turned to a novel assay for amyloid fibril maturation staging combining two LCOs, q-FTAA and h-FTAA, for fluorescence microscopy. The emission spectral ratio from 499 nm and 540 nm serves as markers of various amyloid fibril structures, with a high ratio indicating a mature fibrillar structure. As for the behavior analyses, flies expressing A β 1-42 in CNS neurons or motor neurons displayed a similar low ratio, with a predominance of the h-FTAA fluorescence spectrum. The morphology of the aggregates in these flies was mostly perinuclear in ring-tangle like structures. In glial cells and in the eye disc, the aggregate morphology was extended with fibrous appearance, and the 499/540 ratio was significantly higher, suggesting a higher proportion of mature amyloid fibril structure (**Fig 19**).

A Gal4 - Abeta 1-42

Figure 19. Hyperspectral imaging reveals more mature aggregates in glial expressing and eye disc expressing A β 1-42 flies. (A) Sections of fly heads stained with q-FTAA and h-FTAA revealing morphological differences of aggregates among the examined genotypes. (B) Emission spectral ratios (499/540 nm) of the deposited aggregates using the two LCOs, q-FTAA and h-FTAA at different time points. A high ratio indicates a mature fibrillar structure. **** represent a p-value of less than 0.0001.

In summary, we demonstrate that the expression of A β 1-42 results in aggregation in all cell types examined, however the toxic properties of the expression is selectively neurotoxic.

For flies expressing A β 1-42 in the CNS neurons or motor neurons we found a good correlation between lifespan and locomotor activity impairment and the levels of insoluble protein and aggregates. However, this was not apparent in glial cell and eye disc expressing flies. We ascribe these differences to the localization and maturation of the observed aggregates. Neuronal aggregates have a more perinuclear appearance, indicating intracellular aggregates already in the secretory pathway. In contrast glial and eye disc expression shows extended fibrous appearance away from the soma. Moreover, spectral profiles of glial and eye disc A β 1-42 aggregates indicates significantly more mature fibrils than for the three neuronal drivers. These data strongly suggest that the toxic species in *Drosophila* are immature nascent A β 1-42 fibrils formed intracellularly.

Paper IV: Curcumin Promotes A-beta Fibrillation and Reduces Neurotoxicity in Transgenic *Drosophila*

Aim: The aim of the forth paper was to design a compound trial and investigate the protective roll of the extensively researched compound Curcumin against amyloid induced neurodegeneration in transgenic *Drosophila melanogaster*. Curcumin has been proposed to modulate the A β amyloid cascade and facilitate clearance of toxic amyloid in Alzheimer's disease, and thereby this project would give us a deeper understanding in the underlying molecular mechanism of the disease.

Results and discussion: Curcumin has been subject for extensive research in a wide range of diseases and has been observed to have both anti-inflammatory and anti-oxidative effects, as well as to inhibit A β oligomer and fibril formation. To investigate the protective effect *in vivo* we used four different A β expressing *Drosophila* lines (from the Crowther lab [219]) and one human Tau expressing *Drosophila* line (from the Feany lab [266]), all expressed in the CNS and the eye of the fly. This study was performed prior to the development of our new fly model (Paper I-III). Flies were fed various concentrations of curcumin (0-0.01% w/w in yeast paste). To address the effects of curcumin upon AD-related symptoms, both behavioral and histological analyses were conducted. A rather unexpected finding from the lifespan assay was that curcumin rendered a reduced lifespan in control flies in a concentration dependent manner. On the contrary, flies exhibiting a strong neurodegenerative phenotype displayed a clear effect upon curcumin treatment, with an improvement in median lifespan of up to 75 % for A β 1-42 E22G expressing flies (**Fig 20**). The results were similar in the activity assay, only here; the tau-expressing flies displayed the largest increase in activity of all genotypes following curcumin treatment.

Figure 20. Curcumin affects *Drosophila* longevity as a function of genotype. Survival trajectories of transgenic *C155-Gal4 Drosophila* expressing amyloidogenic A β or tau in the CNS of the flies. Flies were treated with various amounts of curcumin; no curcumin added (black lines), 1, 10, and 100 mg curcumin per g yeast paste represented in yellow, orange, and red lines respectively.

To evaluate if the alleviating effect on toxicity was due to alterations in the aggregation behavior, histological and spectral analysis were performed. The use of anti-A β /anti-tau antibodies in combination with the amyloid specific luminescent conjugated oligothiophenes (LCO), p-FTAA, demonstrated a different staining pattern in treated vs. untreated flies. Curcumin treated double inserted A β 1-42 expressing flies displayed extended amyloid staining by p-FTAA compared to antibody staining at day 10 in contrast to untreated flies. This suggests an increase in fibril formation dependent on treatment. Further, the p-FTAA emission spectrum of amyloid deposits differed depending on the treatment, enabling spectral analysis of the fluorescence intensities at 508 nm and 612 nm. Hyperspectral imaging of these flies strengthened the theory of promoted fibril formation, where a high ratio of 508/612 nm indicates a more well structured amyloid fibril. A spectral shift over time was clearly evident for double inserted A β 1-42 expressing flies, with an increased amyloid fibrillation index (508/612 ratio values) after 10 days of treatment. Additionally, *in vitro* analysis confirmed the hypothesis that curcumin interacts with A β 1-42 and promotes a more rapid conversion of soluble oligomers into insoluble more well organized fibrils.

In summary, Paper IV demonstrates a pharmacological effect of curcumin in transgenic *Drosophila* models of AD on both lifespan and activity. The positive effect was directly dependent on genotype, rendering the strongest positive effect following curcumin treatment for *Drosophila* AD models exhibiting the worst phenotype. This was specifically evident for flies expressing the Arctic mutation of A β 1-42. Histological results, combined with *in vitro* fibrillation of recombinant A β and quantification of soluble and insoluble A β levels in flies in absence or presence of curcumin, shows that curcumin does not inhibit amyloid formation, nor enhance clearance of the peptide. On the contrary, curcumin rather accelerates amyloid fibril conversion, and hence reduce the pre-fibrillar species of A β (**Fig 21**). This suggests that the reduction of pre-fibrillar species underlies the observed mitigated neurotoxicity in *Drosophila* models of AD. The main drawback for curcumin as a potential drug for AD treatment appears to be the poor bioavailability and stability in solution. With that in mind, it is encouraging that curcumin analogues are synthesized as candidate drugs towards AD.

Figure 21. Schematic illustration of A β aggregation in *Drosophila*. The aggregation from soluble A β monomer to A β oligomers (step 1) and further maturation to A β amyloid fibrils (step 2) is dependent on age. The Arctic mutation (E22G) favors the formation of oligomers. In the presence of curcumin, the aggregation into A β amyloid fibrils (step 2) is favored.

Conclusions

The work presented within this thesis has focused on the A β -induced toxicity and aggregation properties observed in *Drosophila melanogaster* models of Alzheimer's disease. From these studies, the following conclusions can be drawn:

Overall, we demonstrate that parameters affecting A β toxicity in *Drosophila* are; **Peptide length; Concentration; Cell type; and Conformation.**

- In general, the expression of A β 1–42 in neurons resulted in a concentration dependent severe neurodegenerative phenotype, where a reduction in lifespan and an impaired locomotor activity was in good agreement with high protein levels and aggregation load.
- Results from all four papers strongly emphasize the importance of pre-fibrillar species of A β as the causative agent for neurotoxicity in *Drosophila* models of AD.
- In AD, a complex pool of A β peptides is present. By a systematic investigation of the A β peptide we demonstrate that the toxicity is highly affected by the length of the peptide, with the most aggregation prone and toxic peptide being an A β variant ending at amino acid 42. Interestingly, this toxicity can be mostly or completely abolished by either addition or removal of single C-terminal amino acids. Additionally, we provide *in vivo* evidence for the crucial importance of an Alanine in position 42, suggesting that this quite small, hydrophobic amino acid highly stabilizes the formation of soluble toxic intermediates within the aggregation pathway.
- A β 1-42 toxicity observed in *Drosophila* can be partially mitigated by the co-expression of A β 1-38, while an additive toxicity was displayed by 1-42; 3-42; and 11-42 co-expression. Due to similar aggregation patterns and levels of insoluble A β , we hypothesize that A β 1-38 interact

with A β 1-42 and either; i) alters the aggregation pathway, resulting in a mixture of toxic and non-toxic oligomers, or ii) promotes fibrillation, resulting in lower amounts of toxic oligomers, and thus contribute to the improved phenotype observed in the A β 1-42, 1-38 flies.

- Little is known about A β expression in glial cells. Despite massive amounts of A β 1-42 aggregates, a minor effect was found on lifespan and activity behavior in flies expressing A β 1-42 in glial cells. These aggregates display a more mature fibril structure compared to the aggregates found in neuronal expressing A β 1-42 flies. Hence, the neurotoxicity observed in *Drosophila* is ascribed to immature fibrillar deposits. Moreover, the process of A β 1-42 fibrillation is influenced by cell type expression. Our data indicate that glial cells in *Drosophila* are insensitive to A β 1-42 amyloid, implicating that these cells could spread and replicate A β 1-42 fibrils without being harmed.
- The design of small molecules affecting the aggregation pathway of A β is a highly studied area of therapeutic strategies. By feeding transgenic A β 1-42 expressing *Drosophila* models with curcumin, our work supports the potential of this molecule. Strikingly, the pharmacological effect was directly dependent on the genotype, rendering the strongest positive effect following curcumin treatment of the worst phenotype. *In vivo* and *in vitro* analysis provide evidence for curcumin to accelerate amyloid fibril conversion, and hence reduce the pre-fibrillar species of A β , resulting in a mitigating neurotoxicity in *Drosophila*.

Future Perspectives

During my time as a PhD student many intriguing conclusions have been made. Nevertheless, with every successful, or unsuccessful, experiment conducted a new research question is born, hopefully leading us one step closer to understanding the devastating pathology of Alzheimer's disease. Thanks to this, there is still much work to be done in the light of the findings of this thesis.

Paper II indicates a partial mitigating effect of co-expressing A β 1-42 with A β 1-38. In the papers included in the thesis, a strong correlation between pre-fibrillar species and toxicity was demonstrated. Hence, a more thorough investigation of the interaction between the two A β peptides could further elucidate the structural properties essential for the altered toxicity observed in these flies. Is co-aggregation of A β 1-42 and A β 1-38 responsible for the mitigating effect? If so, are the oligomeric species formed trapped in a less-toxic conformation? Or, is the aggregation process shifted toward more mature species?

While the majority of data points to a correlation between increased aggregation, increased protein levels and increased toxicity, a contradictory finding in Paper I and II was the fact the flies expressing A β 11-42 in the CNS neurons showed high toxicity in lifespan and activity assays despite low levels of A β and aggregate load. Studies implicate that the A β 11-42 peptide has an early role in A β deposition into plaques [114], and hence additional information of the underlying mechanisms would be of great importance. Could the formation of toxic oligomers off-pathway towards fibrils be responsible for the observed phenotype? If so, could these oligomers still seed aggregation of other A β species into fibrillar structures and plaques?

Further analysis combining our findings in paper I and III would be of interest. In addition to A β 1-42, flies expressing A β 3-42 and 11-42 display high toxicity. Particularly A β 3-42 have been shown to accelerate A β aggregation into neurotoxic fibrils *in vitro* [111] [112]. Thus, could the expression of this peptide or A β 1-42 A42W have a toxic effect on glial cells in our fly model?

Studies expressing tau in glial cells of *Drosophila* demonstrate the formation of NFTs, a reduced lifespan and non-cell-autonomous neuronal cell death [282]. This is in stark contrast to our findings of A β 1-42 in Paper III. Thus a proteomics approach, investigating differences in protein expression, would be highly informative to analyze why glia cells are resistant to A β 1-42 and not towards Tau protein. Are specific proteins up/down-regulated in the respective model? If so, how do they affect the formation and toxicity of A β 1-42 aggregates?

A potential study design would be to isolate aggregates from flies expressing A β in neurons (immature aggregates) and glial cell (mature aggregates) respectively and further elucidate their structure to get a better understanding of the structural differences of the aggregates.

Despite the usefulness of *Drosophila* as a model in AD research, the fly is not a miniature person. One step toward a more comprehensive picture is the use of mouse models. It would be of interest to inject the above-mentioned aggregates into mouse brains and follow the potential propagation and toxicity. This approach has been used by Brouillette et. al. to investigate the induced toxicity of soluble low-molecular-weight A β 1-42 oligomers [283].

The fact that *Drosophila* displays hallmarks of A β induced neurodegeneration that is not readily detected in rodent models offers an advantage when conducting compound screening. This, combined with our findings in Paper IV that curcumin alleviates A β toxicity, opens up for a more thorough compound screen using our new *Drosophila* model of AD.

A compound screen in combination with the Gal80 driver to regulate protein expression could answer several important questions. When in the disease progression does a compound have to be introduced to show effect? Can the neurodegenerative phenotype of flies be delayed if a compound is introduced prior to A β expression? If A β expression is initiated and then turned off using the Gal 80 driver, can the neurodegenerative phenotype be rescued due to a prolonged treatment period?

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