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Amyloid- β and lysozyme proteotoxicity in *Drosophila*

Beneficial effects of lysozyme and serum amyloid P component in
models of Alzheimer's disease and lysozyme amyloidosis

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Cover: *Drosophila melanogaster*, amino acid sequence of A β ₁₋₄₂ and sketch of a secondary protein structure inspired by human lysozyme.

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*Till mormor
och alla andra krutgummor
som försvunnit in i demensens dimma*

If you don't know where you're going,
any road can take you there

Lewis Carroll
Alice in Wonderland

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Populärvetenskaplig sammanfattning

I den här avhandlingen har två olika sjukdomar som klassas som proteinfelveckningssjukdomar; Alzheimers sjukdom och lysozymamyloidos samt molekyler som skulle kunna ha en positiv effekt på dessa sjukdomar studerats med hjälp av *Drosophila melanogaster*, bananflugan. Trots att bananflugan är ett ryggradslöst djur så finns det stora likheter mellan människors och bananflugors fundamentala biologiska mekanismer. I början av 1980-talet kom de första transgena flugorna, det vill säga flugor där man placerat in humana gener i flugans egen arvs massa. Sedan dess har bananflugan varit en av de mest använda modellorganismerna för att studera effekten av överuttryckta humana proteiner i biologiska system; man kan se flugan som ett levande provrör. För majoriteten av proteinerna i vår kropp krävs det att de veckar sig till en tredimensionell struktur för att de ska fungera ordentligt. Men ibland blir det fel, och proteinet felveckas. Det kan till exempel vara på grund av mutationer som gör proteinet mindre stabilt. Ett felveckat protein kan då klumpa ihop sig, aggregera, med andra felveckade proteiner. I Alzheimers sjukdom, som är den vanligaste formen av demens, finns proteinaggregat i hjärnan hos patienterna. Dessa aggregat består av amyloid- β (A β) -peptiden, en liten peptid på runt 42 aminosyror som klipps ut från det större membranbundna proteinet A β PP av två olika enzymer, BACE1 och gamma-sekretas. I det första arbetet inkluderat i den här avhandlingen har två olika flugmodeller för Alzheimers sjukdom använts; A β -modellen som direkt uttrycker A β -peptiden samt A β PP-BACE1-modellen, där alla komponenter som behövs för att flugan ska producera A β -peptiden. De två olika flugmodellerna jämfördes och vi kunde se att det behövs en betydligt lägre mängd av A β i A β PP-BACE1-modellen för att få samma, eller till och med en större, toxisk effekt jämfört med A β -modellen. I det andra arbetet har dessa två flugmodeller för Alzheimers sjukdom använts igen, men nu för att studera huruvida lysozym, ett protein involverat i vårt medfödda immunsystem, kunde motverka den toxiska effekten som A β genererade i flugmodellerna. Vi fann att lysozym kan rädda flugorna från A β inducerad toxicitet, samt att A β och lysozym interagerar med varandra. Den andra proteinfelveckningssjukdomen som studerats i denna avhandling är lysozymamyloidos. Det är en ovanlig, ärftlig sjukdom där mutanta varianter av lysozym ger upphov till flera kilogram tunga aggregat som lägger sig runt njurar och lever, vilket tillslut leder till organsvikt. I avhandlingens tredje arbete har en flugmodell för lysozymamyloidos använts för att studera vad serum amyloid P-komponenten, SAP, ett protein som finns i alla proteinaggregat man hittar inom denna sjukdomsklass, har för effekt. Vi kunde se att SAP minskar toxiciteten som kom från att uttrycka den sjukdomsassocierade lysozymvarianten F57I i flugans centrala nervsystem (CNS). För att reda ut varför SAP har denna effekt så använde vi i det fjärde arbetet dubbeluttryckande lysozymflugor för att generera bättre sjukdomsfenotyper än de tidigare studerade enkeluttryckande flugorna i avhandlingens tredje arbete. Vi kunde då se att SAP minskar toxiciteten som kommer från att uttrycka F57I i flugans CNS och att SAP får lysozymvarianten F57I att bilda proteinaggregat med en mer distinkt amyloid karaktär. Sammanfattningsvis så visar den här avhandlingen att: i) A β genererad från A β PP klyvning i flugans CNS resulterar i högre toxicitet jämfört med direktuttryck av A β från transgenen, ii) lysozym kan motverka A β toxicitet i *Drosophila* och har därmed potential att kunna utvecklas som

behandlingsmetod för Alzheimers sjukdom och iii) i en *Drosophila*-modell av lysozymamyloidosis kan SAP motverka toxicitet från den sjukdomsassocierade lysozymvarianten F57I genom att främja bildandet av proteinaggregat med en distinkt amyloid karaktär; detta är viktigt att tänka på vid behandlingsformer av lysozymamyloidosis som innefattar en reducerad nivå av SAP.

Abstract

In the work presented this thesis, two different conditions that are classified as protein misfolding diseases: Alzheimer's disease and lysozyme amyloidosis and proteins that could have a beneficial effect in these diseases, have been studied using *Drosophila melanogaster*, commonly known as the fruit fly. The fruit fly has been used for over 100 years to study and better understand fundamental biological processes. Although the fruit fly, unlike humans, is an invertebrate, many of its central biological mechanisms are very similar to ours. The first transgenic flies were designed in the early 1980s, and since then, the fruit fly has been one of the most widely used model organisms in studies on the effects of over-expressed human proteins in a biological system; one can regard the fly as a living, biological test tube. For most proteins, it is necessary that they fold into a three-dimensional structure to function properly. But sometimes the folding goes wrong; this may be due to mutations that make the protein unstable and subject to misfolding. A misfolded protein molecule can then aggregate with other misfolded proteins. In Alzheimer's disease, which is the most common form of dementia, protein aggregates are present in the brains of patients. These aggregates are composed of the amyloid- β (A β) peptide, a small peptide of around 42 amino acids which is cleaved from the larger, membrane-bound, protein A β PP by two different enzymes, BACE1 and γ -secretase. In the first part of this thesis, two different fly models for Alzheimer's disease were used: the A β fly model, which directly expresses the A β peptide, and the A β PP-BACE1 fly model, in which all the components necessary to produce the A β peptide in the fly are expressed in the fly central nervous system (CNS). The two different fly models were compared and the results show that a significantly smaller amount of the A β peptide is needed to achieve the same, or an even greater, toxic effect in the A β PP-BACE1 model compared to the A β model. In the second part of the thesis, these two fly models for Alzheimer's disease were again used, but now to investigate whether lysozyme, a protein involved in our innate immune system, can counteract the toxic effect of A β generated in the fly models. And indeed, lysozyme is able to save the flies from A β -induced toxicity. A β and lysozyme were found to interact with each other *in vivo*. The second misfolding disease studied in this thesis is lysozyme amyloidosis. It is a rare, dominantly inherited amyloid disease in which mutant variants of lysozyme give rise to aggregates, weighing up to several kilograms, that accumulate around the kidneys and liver, eventually leading to organ failure. In the third part of this thesis, a fly model for lysozyme amyloidosis was used to study the effect of co-expressing the serum amyloid P component (SAP), a protein that is part of all protein aggregates found within this disease class. SAP is able to rescue the toxicity induced by expressing the mutant variant of lysozyme, F57I, in the fly's CNS. To further investigate how SAP was able to do this, double-expressing lysozyme flies, which exhibit stronger disease phenotypes than those of the single-expressing lysozyme flies previously studied, were used in the fourth part of this thesis. SAP was observed to reduce F57I toxicity and promote F57I to form aggregates with more distinct amyloid characteristics. In conclusion, the work included in this thesis demonstrates that: i) A β generated from A β PP processing in the fly CNS results in higher proteotoxicity compared with direct expression of A β from the transgene, ii) lysozyme can prevent A β proteotoxicity in *Drosophila*

and could thus be a potential therapeutic molecule to treat Alzheimer's disease and iii) in a *Drosophila* model of lysozyme amyloidosis, SAP can prevent toxicity from the disease-associated lysozyme variant F57I and promote formation of aggregated lysozyme morphotypes with amyloid properties; this is important to take into account when a reduced level of SAP is considered as a treatment strategy for lysozyme amyloidosis.

Table of contents

LIST OF PAPERS	1
CONTRIBUTION REPORT	2
ABBREVIATIONS	3
INTRODUCTION.....	5
IT IS ALL ABOUT PROTEINS.....	5
PROTEIN FOLDING	5
WHEN PROTEIN FOLDING GOES WRONG	8
ALZHEIMER'S DISEASE	11
DEMENTIA AND ALZHEIMER'S DISEASE	11
PROCESSING OF A β PP.....	11
THE METABOLISM OF A β	13
PHYSIOLOGICAL FUNCTION OF A β PP AND ITS CLEAVAGE PRODUCTS	15
MUTATIONS CONNECTED WITH AD.....	17
A β PP mutations.....	17
PSEN1 and PSEN2 mutations.....	17
Mutations increasing the risk of late onset AD.....	17
A β AGGREGATION	18
A β PATHOGENESIS.....	18
The amyloid cascade hypothesis	18
A β oligomers impair memory	19
Intracellular A β	20
A β and the endosomal-autophagy system	20
Toxicity induced by the interaction between A β and other proteins	21
Neuroinflammation in AD	22
TAU AND AD	24
DIAGNOSIS AND TREATMENT OF AD	24
Difficulties in diagnosing AD.....	24
Drugs available on the market today.....	25
Treatment strategies based on the amyloid cascade hypothesis	25
Alternative treatment strategies	27
Lifestyle and AD	28
LYSOZYME AMYLOIDOSIS	29
LYSOZYME	29
MUTATIONS CONNECTED TO LYSOZYME AMYLOIDOSIS.....	29
CLINICAL SYMPTOMS.....	30
LYSOZYME AGGREGATION AND TOXICITY	31
SERUM AMYLOID P COMPONENT	33
TREATMENT OPTIONS	33
MODELLING AMYLOID DISEASE.....	35
<i>DROSOPHILA MELANOGASTER</i> AS A MODEL ORGANISM.....	35
TWO DIFFERENT <i>DROSOPHILA</i> AD MODELS	35
LYSOZYME AMYLOIDOSIS MODELS	36

METHODOLOGY.....	39
<i>DROSOPHILA MELANOGASTER</i> IN RESEARCH	39
CONTROLLING PROTEIN EXPRESSION IN <i>DROSOPHILA</i> : THE GAL4/UAS SYSTEM	40
FLY LINES	42
PHENOTYPIC ANALYSIS OF <i>DROSOPHILA</i>.....	42
THE ROUGH EYE PHENOTYPE	43
LONGEVITY ASSAY	44
LOCOMOTOR ASSAY	44
PROTEIN DETECTION AND QUANTIFICATION	45
ANTIBODIES	45
WESTERN BLOT ANALYSIS.....	46
MESO SCALE DISCOVERY (MSD) PROTEIN QUANTIFICATION ASSAY	47
IMMUNOPRECIPITATION	47
FLUORESCENCE MICROSCOPY	47
IMMUNOHISTOCHEMISTRY	48
STAINING WITH OLIGOTHIOPHENES	48
DETECTION OF APOPTOTIC CELLS	49
SCANNING ELECTRON MICROSCOPY	49
AIMS	51
SUMMARY OF THE PAPERS.....	53
PAPER I	53
PAPER II	54
PAPER III	55
PAPER IV	56
DISCUSSION	59
Aβ TOXICITY IN <i>DROSOPHILA</i> AD MODELS	59
A β TOXICITY IN THE A β PP-BACE1 FLIES.....	60
A β TOXICITY IN THE A β FLIES	61
LYSOZYME PROTECTS FROM Aβ TOXICITY IN AD FLY MODELS	63
ANTI-TOXIC EFFECTS MEDIATED BY INTERACTION BETWEEN LYSOZYME AND A β	64
ANTI-TOXIC EFFECTS INDUCED BY LYSOZYME INDEPENDENT OF ITS INTERACTION WITH A β	65
SAP PROMOTES FORMATION OF ALTERNATIVE AGGREGATED LYSOZYME MORPHOTYPES.....	66
CONCLUDING REMARKS	71
FUTURE PERSPECTIVES.....	73
TACK.....	75
REFERENCES.....	79

List of papers

Paper I

A β PP processing results in greater toxicity per amount of A β ₁₋₄₂ than individually expressed and secreted A β ₁₋₄₂ in *Drosophila melanogaster*

Liza Bergkvist, Linnea Sandin, Katarina Kågedal and Ann-Christin Brorsson

Biology Open (2016) 15;5(8):1030-9. doi: 10.1242/bio.017194.

Paper II

Beneficial effects of increased lysozyme levels in Alzheimer's disease modelled in *Drosophila melanogaster*

Linnea Sandin/**Liza Bergkvist**, Sangeeta Nath, Claudia Kielkopf, Camilla Janefjord, Linda Helmfors, Henrik Zetterberg, Kaj Blennow, Hongyun Li, Camilla Nilsberth, Brett Garner, Ann-Christin Brorsson and Katarina Kågedal

The FEBS Journal (2016) 283(19):3508-3522. doi: 10.1111/febs.13830

Paper III

Serum amyloid P component ameliorates neurological damage caused by expressing a lysozyme variant in the central nervous system of *Drosophila melanogaster*

Linda Helmfors/**Liza Bergkvist** and Ann-Christin Brorsson

PLoS ONE (2016) 18;11(7):e0159294. doi: 10.1371/journal.pone.0159294.

Paper IV

Serum amyloid P component promotes the formation of distinct aggregated lysozyme morphotypes and reduces toxicity in *Drosophila* flies expressing F57I lysozyme

Liza Bergkvist, K. Peter R. Nilsson and Ann-Christin Brorsson

In manuscript. Submitted and under review, *Biochemical Journal*.

Contribution report

Paper I

A β PP processing results in greater toxicity per amount of A β ₁₋₄₂ than individually expressed and secreted A β ₁₋₄₂ in *Drosophila melanogaster*

- Liza Bergkvist (LB) planned, set up, executed, and analysed data from all experiments. LB wrote the paper together with supervisor Ann-Christin Brorsson and co-supervisor Katarina Kågedal.

Paper II

Beneficial effects of increased lysozyme levels in Alzheimer's disease modelled in *Drosophila melanogaster*

- LB planned, set up, executed, and analysed data from fly experiments. LB wrote parts of the paper concerning the fly work.

Paper III

Serum amyloid P component ameliorates neurological damage caused by expressing a lysozyme variant in the central nervous system of *Drosophila melanogaster*

- LB planned, set up, executed, and analysed data from immunohistochemistry and TUNEL experiments. LB wrote the manuscript together with supervisor Ann-Christin Brorsson and Linda Helmfors.

Paper IV

Serum amyloid P component promotes the formation of distinct aggregated lysozyme morphotypes and reduces toxicity in *Drosophila* flies expressing F57I lysozyme

- LB planned, set up, executed, and analysed data from all experiments. LB wrote the main part of the paper.

Abbreviations

A β	Amyloid- β peptide
A β PP	Amyloid- β precursor protein
sA β PP _{α/β}	Soluble N-terminal fragment from α/β - and γ -secretase cleavage of A β PP
AD	Alzheimer's disease
AICD	A β PP intracellular domain
ApoE	Apolipoprotein E
AVs	Autophagic vesicles
BACE1	β -site cleaving enzyme (β -secretase)
BBB	Blood brain barrier
C83	A β PP C-terminal fragment from α -cleavage
C99	A β PP C-terminal fragment from BACE1 cleavage
CNS	Central nervous system
CPHPC	R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid
CSF	Cerebrospinal fluid
DS	Down syndrome (trisomy 21)
F57I	Disease-associated lysozyme variant
FLIM	Fluorescent lifetime imaging
GI	Gastrointestinal
h-FTAA	Heptameric formic thiophene acetic acid
IDE	Insulin-degrading enzyme
IHC	Immunohistochemistry
LCOs	Luminescent conjugated oligothiophenes
LRP	Low-density lipoprotein receptors
LTP	Long-term potentiation
LTD	Long-term depression
MAP	Microtubule-associated protein
MCI	Mild cognitive impairment
MSD	Meso scale discovery
MVB	Multi vesicular body
NET	Neprilysin
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PET	Positron emission tomography
p-FTAA	Pentameric formic thiophene acetic acid
PS1/2	Presenilin 1/2
RAGE	Receptor for advanced glycation end-products

SAP	Serum amyloid P component
SEM	Scanning electron microscopy
TREM2	Triggering receptor expressed on myeloid cells 2 (microglial receptor)
UAS	Upstream activating sequence
UPR	Unfolded protein response
WT	Wild type

Introduction

It is all about proteins

The group of biomolecules collectively called proteins performs a variety of different biological tasks; these molecules range from large proteins like titin, consisting of around 30 000 amino acids, which acts like a molecular spring and connects the Z line to the M line in the sarcomere, to the small 36 amino acid neuropeptide Y, which functions as a vasoconstrictor in the sympathetic nervous system. Proteins with catalytic sites, enzymes, are able to speed up reactions in the cell that would take anything from minutes to years longer to occur in the absence of the enzyme. Millions of years of evolution have resulted in a biological system fine-tuned to perform its tasks. Even though the functions of proteins can vary dramatically, different combinations of the 20 structural units called amino acids make up almost all proteins. For most proteins, it is important that they fold properly into their native structure if they are to perform their specific task in an organism. A scientific mystery yet to be solved is how knowledge about the primary structure, the amino acid sequence, can be used to predict the structure of the native, folded protein.

Protein folding

All amino acids consist of an amino ($-\text{NH}_2$) and a carboxylic group ($-\text{COOH}$) and the different side chains determine their biochemical character. The amino group and the carboxylic group are connected via the α -carbon, from which the different side chains, characteristic for each amino acid, stems. The joining of amino acids through peptide bonds can be seen as putting beads on a string, creating the primary protein structure known as a polypeptide chain (Fig. 1A). To reach the 3D structure, that several proteins need in order to function properly, the backbone of the polypeptide chain interacts with itself first, forming secondary structures called α -helices and β -sheets (Fig. 1B). What holds these structures together are hydrogen bonds between specific atoms in the polypeptide backbone. Hydrogen bonds are created between two different polar groups due to electrostatic attraction. This often occurs when a hydrogen atom is covalently attached to an electronegative atom such as nitrogen (N) or oxygen (O). A common example used to illustrate hydrogen bonds is the bonding between water molecules. In a water molecule, two hydrogens are bound to one oxygen molecule, which is highly electronegative. One of the hydrogens (donor) can in turn interact with the oxygen (acceptor) of a nearby water molecule, creating a hydrogen bond between the two. In amino acids, there are multiple

hydrogens bound to highly electronegative atoms; the hydrogen bound to oxygen in the carboxylic group and the hydrogen bound to the nitrogen in the amino group, facilitating the folding into secondary structures. α -helices are the most abundant secondary protein structure, and can be compared to a loosely right-handed coiled coil (Fig. 1C). In β -sheets, the primary peptide chain is folded alongside itself, and is only stable when β -strands align, in a parallel or anti-parallel fashion (Fig. 1C). After the formation of a secondary structure, the folding continues and the proteins tertiary structure is formed (Fig. 1D). Here, interaction between the different secondary structural elements take place. One such interaction is the creation of sulphur bridges between the sulphur atoms present in the side chain of the amino acid cysteine. This is a covalent bond, and it involves the sharing of electrons between atoms. Ionic bonds take place between atoms with highly different electronegativity, e.g. the positively charged group found in lysine and the negatively charged group present in aspartate. Van der Waals forces involve attraction or repulsion between groups that are not bound neither covalently nor through ionic bonds, and can occur due to fluctuating dipoles in, for example, the large hydrocarbon groups found in some amino acids, e.g. Leu, Ile and Phe. Hydrogen bonding does also occur in the formation of the tertiary structure. Many proteins are an assembly of multiple, individual polypeptide chains and the quaternary structure of a protein reflects the number of folded subunits and how they are arranged (Fig. 1E). The nomenclature regarding multi-subunit assemblies, also known as oligomers, goes from monomer (one subunit), dimer (two), trimer (three) and so on.

Figure 1. Peptide bonds and different protein structures. (A) Peptide bonds link different amino acids together creating a (B) polypeptide chain, also known as the protein's primary structure. (C) The formation of secondary structural elements (α -helices and β -sheets) occurs through hydrogen bonding between atoms in the polypeptide backbone. (D) Further interactions (sulphur bridges, hydrogen bonds, ionic bonds, van der Waals forces and the hydrophobic effect) between the different side chains of the amino acids give rise to the protein's tertiary structure. (E) Some proteins are assemblies of individual polypeptide chains, interactions between which create the protein's quaternary structure.

Considering the many possible interactions that can take place, how does folding into the correct structure, resulting in a native protein, occur within the time frame that is necessary for a functioning, biological system? This would obviously be an issue, should protein folding occur randomly; however, that is not the case. Due to the different interactions described above, as well as to the hydrophobic effect, which means that nonpolar side chains tend to aggregate or form hydrophobic cores in water soluble proteins or insert into the plasma membrane in the case of membrane bound proteins, some protein conformations are less energetically favourable than others. The energy landscape for protein folding is often depicted as a funnel with rough sides, giving rise to local minima on the pathway towards the stable, low energy, high order, native state at the bottom of the funnel (Fig. 2).

Figure 2. The protein folding energy landscape. The folding process, starting from a polypeptide chain, that gives rise to a native protein, which is a structure with low energy and entropy.

When protein folding goes wrong

The critical process of folding the primary amino acid sequence into a native protein can in some cases lead to a misfolded protein, meaning one that does not reach its stable, native state as depicted in Fig. 2. This can be due to mutations leading to changes in the amino acid sequence or to changes in the environment, e.g. the pH, protein concentration or temperature. Changes in the primary structure of the protein may lead to loss of function, change of function (e.g. decreased enzymatic activity) or the formation of aggregates. The native protein structure is not the only possible structural outcome in the protein folding landscape; an alternative, stable, low energy structure known as amyloid can also form. This type of folding may possibly represent a generic ground state structure for all proteins. Thus, the protein folding energy landscape can now be expanded: in addition to the native and intermediate states, where interactions occur within the same molecule (intramolecular interactions), it can also include structures in which intermolecular interactions take place, e.g. oligomers, protofibrils, amorphous aggregates and amyloid fibrils (Fig. 3).

Figure 3. An expanded view of the protein folding energy landscape. The different protein folding configurations, from intermediate states to the native structure. Misfolded states can lead, through intermolecular interactions, to the formation of oligomers, protofibrils, amorphous aggregates and stable, low energy amyloid aggregates.

Misfolding of a protein into amyloid fibrils is an event that is, in the medical context, involved in a disease classified as amyloidosis. Mutations in the gene encoding the protein may lead to an amino acid substitution, causing a change in the protein structure, exposing new hydrophobic surfaces and leading to the formation of an insoluble β -sheet structure, which is characteristic of amyloids. The amyloid formation process can be divided into three different phases (Fig. 4). During the lag phase, also known as the nucleation phase, the partially unfolded protein is able to interact with other partially folded proteins, creating oligomers, which grow from monomers, to dimers, to trimers and so on until an oligomeric nucleus has been formed. These oligomeric species are still soluble and have been proven to be neurotoxic in several amyloid diseases, e.g. Alzheimer's disease (AD) and Parkinson. (Dahlgren et al., 2002; He et al., 2012; Karpinar et al., 2009; Nimmrich et al., 2008; Winner and Jappelli, 2011; Zhang et al., 2014). In the elongation phase, more oligomeric species are rapidly added to the growing protofibril. In the third phase, a plateau is reached; by this stage, mature insoluble fibrils have been formed. This leads to the accumulation of both extracellular and intracellular protein deposits in different tissues and organs. Depending on where in the body these deposits accumulate, various symptoms may appear. Amyloid diseases can be systemic, affecting multiple organs, or local, affecting only a certain organ (Dobson, 2003). Today there are over 30 different proteins that have been shown to be connected with amyloid diseases; some of them are of frequent occurrence and well known, such as the A β peptide and its link with AD. Other proteins and the diseases connected with them are less frequently seen in the human population, e.g. lysozyme amyloidosis. Amyloid diseases

can be familial or sporadic, or occur in both forms, and mutations have been connected with familial amyloid diseases as well as sporadic cases. The amino acid sequences, the sizes and the native structures of proteins involved in amyloid diseases vary, but their insoluble fibrils share some common features, including a distinct β -sheet structure revealed by X-ray scattering, which can be stained with Congo red, displaying birefringence under polarised light (Dobson, 2003; Sipe et al., 2010).

Figure 4. The amyloid formation process. During the lag phase, misfolded intermediates interact with each other, forming different oligomeric species. In the elongation phase, oligomers are rapidly added to the growing protofibril. The rate of fibril growth decreases and a plateau is reached, by which stage mature amyloid fibrils have been formed.

Alzheimer's disease

Dementia and Alzheimer's disease

In our society, in which the longevity of the population has increased by 30 years since 1900, so has the prevalence of age related diseases (Christensen et al., 2010). Dementia is a syndrome caused by many different diseases, and it is not a normal part of the ageing process. Some of the most common diseases underlying dementia are AD, Lewy body disease and frontotemporal dementia (Karantzoulis and Galvin, 2011). People suffering from dementia in any form experience memory loss and personality changes and they will eventually need help to manage their everyday lives (Iqbal et al., 2013). Dementia affects around 47.5 million people all over the world, and AD is believed to be responsible for around 60-80 % of all dementia cases (Thies and Bleiler, 2013). Because the longevity of the population will continue to increase, the number of people suffering from dementia is predicted to be over 150 million by 2050, putting an enormous strain on society, from an economical perspective, as well as on the care-giving relatives of the patient suffering from dementia. It is therefore of great importance to study the diseases causing dementia, particularly AD as it is the major causative disease, in order to be able both to mitigate the symptoms and also, hopefully, to find ways of preventing the disease from ever occurring.

AD has been studied for over 100 years, since the German doctor Alois Alzheimer first documented the disease in 1906 after observing a patient who displayed personality changes, memory loss and irrational behaviour. When performing an autopsy of the deceased patient, he observed the pathology now well known to be correlated with AD: extracellular protein aggregates and neurofibrillary tangles (NFTs) in the brain of the patient (Serrano-Pozo et al., 2011). Accompanying the plaques and tangles is a high degree of neurodegeneration, characterised by loss of synapses and the presence of dystrophic neurons. The major component of the extracellular protein aggregate was later discovered to be the amyloid- β peptide, A β (Glenner and Wong, 1984), while the NFTs contain another protein, a hyperphosphorylated microtubule-associated protein called tau (Goedert et al., 1988). Years of intensive research have resulted in an increase in our knowledge about the disease mechanisms and proteins involved; however, as of today, no cure or method of prevention is commercially available.

Processing of A β PP

After the main component of the protein aggregates found in AD patients had been identified as the A β peptide in 1984, Kang et al. were able to report three years later that they had been able to clone a large protein containing the sequence encoding the A β peptide (Kang et al., 1987). This protein became known as the amyloid β precursor protein, A β PP. A β PP is a type I transmembrane protein and it can be found in three different isoforms: 695, 751 and 770 amino acids long. The shortest isoform, A β PP₆₉₅, is found in neuronal cells while the two longer isoforms are found mainly in peripheral cells (Ling et al., 2003). There are two major pathways for the processing of A β PP: the non-amyloidogenic and the amyloidogenic pathway, the latter resulting in the production of the A β peptide (Fig. 5) (Haass et al., 1993). In the non-amyloidogenic pathway, A β PP is first cleaved by α -secretase, a membrane

spanning metalloprotease (Esch et al., 1990). This results in the release of the extracellular N-terminal sA β PP $_{\alpha}$ fragment, and in an 83-amino acid C-terminal fragment, C83, which is still attached to the membrane. Further processing of the C83 fragment is carried out by γ -secretase, a multi-subunit integral membrane protein (Edbauer et al., 2003). γ -secretase cleaves single pass transmembrane proteins, and it has many identified substrates, including Notch receptors (Duggan and McCarthy, 2016). Cleavage of the C83 fragment by γ -secretase results in the production of the p3 fragment and the A β PP intra cellular domain (AICD). In the amyloidogenic pathway, β -secretase, also known as BACE1, cleaves the full-length A β PP, releasing the extracellular N-terminal sA β PP $_{\beta}$ fragment and producing a 99-amino acid C-terminal fragment, C99, still attached to the membrane (Vassar, 1999). The C99 fragment is further processed by γ -secretase, releasing the A β peptide as well as producing AICD. Cleavage by γ -secretase is sequential and can give rise to peptides of varying lengths, so A β peptides of different lengths are produced depending on how γ -secretase cleaves A β PP (Matsumura et al., 2014; Qi-Takahara, 2005). The most dominant isoform is the 40-amino acid peptide, A β ₁₋₄₀. However, even though the A β ₁₋₄₂ peptide, which is two amino acids longer than A β ₁₋₄₀, is secreted in an amount only approximately one tenth of the amount of the latter, the longer form is the main component of the amyloid aggregates found in AD brains (Gravina et al., 1995; Iwatsubo et al., 1994). Kinetic studies have shown that A β ₁₋₄₂ is more prone to aggregate and that it displays increased neurotoxic properties compared to A β ₁₋₄₀ (Bitan and Kirkitadze, 2003; Dahlgren et al., 2002; Jarrett et al., 1993). Both the amyloidogenic and the non-amyloidogenic pathway occurs as part of normal cell function, with the non-amyloidogenic pathway being prevalent in most cells (Ling et al., 2003).

Figure 5. The non-amyloidogenic and amyloidogenic pathways and the different cleavage products from A β PP processing by α - or β -secretase/BACE1 followed by γ -secretase cleavage.

The metabolism of A β

A β PP is ubiquitously expressed, thus the production of the A β peptide is not solely localised to the CNS; both muscle cells and platelets contribute to the pool of A β present in human plasma (Kuo et al., 2000; Li et al., 1998). The processing of A β PP takes place at the membrane of different cellular compartments and at the plasma membrane, since A β PP (Perez et al., 1999), as well as two of the proteases known to process it, are membrane bound. This leads to both extra- and intracellular production of A β . The relative abundances of the different secretases in different cellular locations determine which processing pathway predominates. Processing of A β PP by BACE1/ β - and γ -secretase takes place mainly in the early secretory pathways, as well in the endosomal pathway (Greenfield et al., 1999; Koh et al., 2005; Vetrivel et al., 2004). The presenilin proteins 1 and 2 (PS1 and PS2) can both act as the catalytic domain in γ -secretase (Shirotani et al., 2007). However, in contrast to PS1, which is present in many cellular compartments, PS2 contains a sorting sequence that guides it to late endosomes and lysosomes, where it contributes to the production of the intracellular pool of A β , producing mainly the longer A β_{1-42} peptide (Sannerud et al., 2016). BACE1 activity is at its highest in an acidic environment, around pH 4.5, which can be found in the vesicles that are part of the endosomal-lysosomal pathway (Vassar, 1999). Processing of A β PP by α - and γ -secretase takes place predominantly at the plasma membrane (Sisodia, 1992); however β -cleavage of A β PP also occurs at the plasma membrane to some degree.

For A β homeostasis to occur, the peptide must be cleared from the brain as it is produced. This clearance is carried out mainly by three different pathways. The first pathway is receptor mediated clearance across the blood brain barrier (BBB). Receptors known to transport A β across the BBB are low-density lipoprotein receptors (LRP) and the receptor for advanced glycation end-products (RAGE) (Zlokovic, 2004). Interaction between the receptor and A β can be direct or mediated through other proteins. ApoE, a cholesterol transporter, is known to bind A β and it can either mediate clearance across the BBB by binding to LRP (Kang et al., 2000) or promote accumulation and aggregation of A β , depending on the isoform (Castellano et al., 2012; LaDu et al., 1994). The gene coding for ApoE has been connected to sporadic cases of AD (Corder et al., 1993). After A β has crossed the BBB, it can be transported to the kidney and liver for degradation, which is the second pathway for clearance of A β . But degradation of A β also takes place in the brain; proteolytic degradation of A β is primarily carried out by insulin-degrading enzyme (IDE) and neprilysin (NEP). Both IDE and NEP can degrade intra- and extracellular monomeric A β , and NEP is also able to degrade oligomeric A β species (Iwata et al., 2000; Iwata et al., 2001; Kurochkin and Goto, 1994; Qiu et al., 1998). Another enzyme that cleaves intracellular A β is the endothelin-converting enzyme (ECE) (Eckman et al., 2001). ECE activity is induced by low pH and this enzyme therefore contributes to the degradation of monomeric A β in intracellular compartments such as endosomes and lysosomes where the environment is acidic. Cathepsin D and Cathepsin B, which are also present in intracellular compartments, have been shown to be able to degrade A β fibrils (Hamazaki, 1996; Mueller-Steiner et al., 2006). The maintenance system of the CNS involves microglia and astrocytes, and they are part of the third clearance pathway: clearance of A β via activated microglia and astrocytes. Microglia express scavenger receptors, which

in turn bind to A β and induce phagocytosis (Wilkinson and El Khoury, 2012). Astrocytes secrete matrix-metalloproteinases, MMP-2 and MMP-9, in proximity to amyloid aggregates, and both of these enzymes are able to degrade A β fibrils (Yin et al., 2006). NEP is also localised within the astrocytes, which have been found to be able to internalise and degrade different soluble species of A β (Carpentier et al., 2002). In summary, both the processing of A β PP leading to the production of A β and the enzymes and processes involved in the degradation of the peptide are complex (Fig. 6), thus small changes affecting either production or degradation could have major impacts on A β homeostasis.

Figure 6. Different A β clearance pathways. Receptors such as RAGE and LRP take part in the receptor mediated clearance of A β across the BBB. ApoE is involved in assisted receptor mediated clearance via LRP. A β degrading enzymes are present both extra- and intracellularly. Endosomal/lysosomal proteases can degrade both monomeric A β (IDE, ECE) and fibrillary A β (Cathepsin D, Cathepsin B). Microglia engulf A β via phagocytosis induced by the binding of scavenger receptors to A β . Astrocytes contain proteases that are able to degrade both monomeric and oligomeric A β and they can secrete matrix-metalloproteinases able to degrade A β fibrils.

Physiological function of A β PP and its cleavage products

The processing of A β PP results in a variety of different cleavage products (Fig. 5), and although the A β peptide has been strongly correlated with AD, the physiological functions of A β PP and its cleavage products, including the A β peptide, have remained elusive since A β PP was first discovered in 1987. A β PP knock out mice were found to be viable and fertile, but a significant reduction in body weight and brain mass, as well as reduced locomotor behaviour, was observed, indicating that A β PP plays a functional role in the CNS (Anliker and Müller, 2006). The structure of A β PP as well as the enzymes known to process it are very similar to those of the family of Notch receptors, supporting the theory that A β PP is a cell surface receptor (Selkoe and Kopan, 2003). The physiological function of the extracellular domains of sA β PP $_{\alpha/\beta}$, produced by cleavage of A β PP by either α -secretase or BACE1, has also been investigated. Both sA β PP $_{\alpha}$ and sA β PP $_{\beta}$ have been found to be involved in neurite outgrowth and neuronal proliferation, with sA β PP $_{\alpha}$ being a more potent stimulator of neuronal proliferation than sA β PP $_{\beta}$ (Chasseigneaux et al., 2011). sA β PP $_{\beta}$ was also found to bind to death receptor 6, triggering apoptosis in peripheral neurons (Nikolaev et al., 2009). In addition to the extracellular domain produced after cleavage by α -secretase or BACE1, an 83- or 99-amino acid long C-terminal fragment is formed. C99, which is produced after cleavage by BACE1, has been found to alter the activity of acetylcholinesterase; in mice overexpressing C99, an altered activity of the enzyme could be observed in specific parts of the brain, correlating with a decline in behavioural performance (Dumont et al., 2006). C99 has also been linked to the endosomal pathology observed in AD models. C99 is able to bind directly to human adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1, APPL1, which in turn recruits and pathologically activates Rab5, leading to cholinergic neurodegeneration in Down syndrome patient cells, i.e. cells carrying one extra copy of A β PP and thus expressing higher levels of the A β peptide (Kim et al., 2016). Furthermore, the amount of the C99 fragment has been shown to be higher in AD brains even though normal levels of A β PP were detected, and the same over-activation of Rab5 could also be observed in AD brains (Kim et al., 2016). The C-terminal fragment produced after cleavage by α -secretase, C83, has no known function but it has been implicated as being involved in neurodegeneration (Rockenstein et al., 2005). These C-terminal fragments are further processed by γ -secretase. In the non-amyloidogenic pathway, this results in the production of the p3 fragment as well as AICD. If p3 has a physiological function it is yet to be discovered. Around 20 proteins have been demonstrated to interact with AICD, one of them being Fe65 (Bórquez and González-Billault, 2012). Fe65 is able to bind A β PP and it acts as a potent modulator controlling the balance between the non-amyloidogenic and amyloidogenic pathways (Hu et al., 2005). AICD also functions as a transcription factor and regulates a number of cellular processes, one being a negative feedback loop, decreasing A β PP trafficking to the cell surface by binding to the promotor site for Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous protein 1, WAVE1 (Ceglia et al., 2015). WAVE1 belongs to a group of proteins that induce actin polymerisation, thus facilitating the transport of vesicles from the Golgi to the plasma membrane. AICD downregulates the levels of WAVE1, hence also reducing transport of A β PP to the plasma membrane. In the amyloidogenic pathway, cleavage of C99 by γ -secretase results in the production of AICD and the A β peptide. The A β peptide is one of the best studied peptides in the world due to its strong association

with AD, and a number of possible physiological functions have also been identified for it. One such function identified for the A β is that it depresses synaptic activity, guarding against excessive glutamate release and therefore also excitotoxicity (Kamenetz et al., 2003). A β_{1-42} was found to induce neurogenesis, while A β_{1-40} did not do so (López-Toledano and Shelanski, 2004). Platelets increase the secretion of A β_{1-40} when stimulated (Li et al., 1998), and A β can in turn induce platelet activation and aggregation *in vitro* (Shen et al., 2008). The fact that there seem to exist specific uptake, breakdown and clearance pathways indicates that the A β peptide plays a role in the normal function of the nervous system. The physiological functions of A β PP and its cleavage products are summarised in table 1.

Protein/fragment	Physiological function	Reference
AβPP (full-length)	A cell surface receptor	(Anliker and Müller, 2006; Selkoe and Kopan, 2003)
sAβPP$_{\alpha}$ (α-cleavage)	Potent stimulator of neurite outgrowth and proliferation.	(Chasseigneaux et al., 2011)
sAβPP$_{\beta}$ (BACE1-cleavage)	Stimulates neurite outgrowth and proliferation to some extent. Triggers apoptosis.	(Chasseigneaux and Allinquant, 2012; Chasseigneaux et al., 2011; Nikolaev et al., 2009)
C83 (α-cleavage)	May be involved in neurodegeneration?	(Rockenstein et al., 2005)
C99 (BACE1-cleavage)	Affects acetylcholinesterase activity. Recruits and pathologically activates Rab5 leading to cholinergic neurodegeneration.	(Dumont et al., 2006; Kim et al., 2016)
AICD (α/β- and γ-cleavage)	Transcription regulator; affects A β PP trafficking etc.	(Bórquez and González-Billault, 2012; Ceglia et al., 2015; Hu et al., 2005)
p3 (α- and γ-cleavage)	No known function.	-
Aβ (β - and γ-cleavage)	Depresses synaptic activity, stimulates neurogenesis. Involved in platelet activation/aggregation. Strongly correlated with AD.	(Kamenetz et al., 2003; Li et al., 1998; López-Toledano and Shelanski, 2004; Shen et al., 2008)

Table 1. The physiological functions of A β PP and its cleavage products.

Mutations connected with AD

AβPP mutations

People with trisomy 21, also known as Down syndrome (DS), carry one extra copy of chromosome 21. The gene coding AβPP is located on this chromosome, thus individuals with DS produce a higher level of Aβ due to the extra copy of AβPP and have a six-fold greater risk of developing AD compared with individuals without DS (Head et al., 2012). This strongly links AβPP and mutations within the gene coding for the protein to AD. Several cases of familial AD, FAD, are caused by AβPP mutations (Weggen and Beher, 2012). AD related mutations located in the gene encoding AβPP have been divided into two classes: mutation within or outside the region coding for Aβ. The arctic mutation (studied in paper II) is an example of a mutation within the region of AβPP that codes for Aβ, where an amino acid substitution has occurred, replacing glutamate with glycine at position 22 in the Aβ amino acid sequence (Nilsberth et al., 2001). It was found in a Swedish family showing the clinical symptoms of FAD. Aβ levels in the plasma were found to be decreased and instead an increase in protofibrils could be detected, suggesting that the mutation enhances protofibril formation. The other AβPP mutation studied for the thesis (paper II) is the Swedish mutation, AβPP_{swe}, which is a double mutation adjacent to the cleavage site for BACE1, resulting in higher levels of secreted Aβ₁₋₄₀ and Aβ₁₋₄₂ (Mullan et al., 1992).

PSEN1 and PSEN2 mutations

Two other genes connected to AD are PSEN1 and PSEN2 (the catalytic subunits of the γ-secretase complex). Mutations in PSEN1 and PSEN2 are accountable for most familial cases of AD, with over 200 identified mutations. These mutations often result in an increased Aβ₁₋₄₂/Aβ₁₋₄₀ concentration ratio (Scheuner et al., 1996). In paper II, two different double-transgenic AD mouse models were used. In one, the AβPP_{swe} mutation and PSEN1dE9 (a deletion of exon 9, corresponding to early onset AD) were combined. The second double-transgenic mouse model combined AβPP_{swe} and M146V, a mutation in the PSEN1 gene connected to early onset AD.

Mutations increasing the risk of late onset AD

A variant of ApoE, a brain lipoprotein, has been shown to be a risk factor for developing sporadic, late onset AD. There are three isoforms of ApoE, encoded by ε2, ε3 and ε4. Both ε2 and ε3 encode proteins with a cysteine in either one or both of the positions 112 and 158. But ε4 codes instead for a protein with arginine in both these positions. In a study performed by Corder et al., 40% of the post-mortem brains from documented AD patients carried the ε4 gene (Corder et al., 1993). Another protein that has been connected to an increased risk of developing late onset AD is triggering receptor expressed on myeloid cells 2 (TREM2) (Guerreiro et al., 2013; Jonsson et al., 2012). TREM2, which is one of the most highly expressed receptors in microglia, is involved in activating microglial phagocytosis (Hickman and El Khoury, 2014). However, in contrast to the other mutations mentioned above, being a carrier of ApoE or TREM2 variants is not a predictor that the individual will develop AD.

A β aggregation

The aggregation process from monomeric A β to amyloid fibrils is believed to follow the same pattern as that shown in Fig. 4, where misfolded monomers are able to interact via exposure of hydrophobic surfaces and eventually form amyloid proteins. A β_{1-42} has been shown to be more aggregation prone compared to A β_{1-40} , probably due to its more hydrophobic character (Bitan and Kirkitadze, 2003). A β amyloid build up takes several years in humans, but it is believed to start when the A β monomer misfolds from an α -helical or random structure to a β -sheet structure (Fig. 7) (Zagorski, 1991). For fibril formation to take place, aggregation into nuclei, consisting of A β oligomers, is needed. A β oligomers are structurally diverse, and using different protocols, different A β species can be attained (Glabe, 2008). These oligomers then interact, creating protofibrils that eventually develop into mature amyloid fibrils.

Figure 7. Aggregation of A β . A β misfolds from an α -helical or random structure into a β -sheet structure. Several misfolded A β monomers interact, creating many structurally diverse oligomers. After formation of protofibrils, mature amyloid A β aggregates are created.

A β pathogenesis

The amyloid cascade hypothesis

A variety of physiological functions has been identified for A β and it seems that the peptide plays a role in the normal function of the nervous system, so when and how does the peptide become neurotoxic? The amyloid cascade hypothesis proposes that AD is caused by an imbalance in the production and clearance of the A β peptide, allowing the formation of neurotoxic A β species (Hardy and Selkoe, 2002; Pimplikar, 2010). This hypothesis was developed on the basis of the following discoveries:

- 1) A β – the peptide is the main constituent of the amyloid plaques that are characteristic of the disease (Glenner and Wong, 1984).
- 2) A β PP – the A β precursor protein. People with DS have an extra copy of A β PP and therefore runs a higher risk of developing AD (Head et al., 2012). The first genetic mutation causing AD was found in A β PP; since then more AD related mutations in the gene have been discovered (Weggen and Behr, 2012).
- 3) PSEN1/2 – mutations in the genes coding for PS1 and PS2 (the catalytic subunits of the γ -secretase complex) have been shown to alter the processing of A β PP, leading to an increase in the production of A β_{1-42} or a decrease in the production of A β_{1-40} (Scheuner et al., 1996).

Reports on the level of A β ₁₋₄₂ in human cerebrospinal fluid, CSF, have been very inconsistent but a majority of studies show a decrease in the levels of A β in CSF from AD patients, which could reflect the build-up of plaques and thus a decrease in the efflux of A β ₁₋₄₂ from the brain (Anoop et al., 2010). At first, the extracellular plaques found in AD brains were thought to be the main toxic species; however, the correlation between plaque load and disease progression in humans is poor (Arriagada et al., 1992), and mouse models of AD show a decrease in cognitive function long before any aggregates can be detected and oligomeric species induces cognitive function in a more potent manner than fibrillar A β (He et al., 2012; Knobloch et al., 2007; Lesné et al., 2006). In addition, in a study by McLean et al., the soluble pool of A β was found to correlate well with the severity of the disease manifested as cognitive decline, while the levels of insoluble A β only could discriminate between AD and control groups (McLean et al., 1999). Taking these findings together with the fact that many cognitively healthy people show robust plaque formation (Chételat et al., 2013; Nordberg, 2008), the focus has shifted from the insoluble plaques to the soluble A β peptides and oligomeric intermediates as the mediators of neurotoxicity.

A β oligomers impair memory

One of the clinical manifestations of AD is the patient's loss of memory. Long-term potentiation (LTP) is a process whereby the synapses are strengthened due to continuous activity and it is believed to be involved in the way memories are created. It involves the release of glutamate from the presynaptic cell and the activation of AMPA and NMDA receptors, and it results in a postsynaptic cell that is more sensitive to future stimulation. The opposite of LTP is long-term depression (LTD), activity-based reduction in synaptic sensitivity. LTP and LTD together govern the plasticity of the brain, controlling the strengthening and weakening of synapses. Increases in synaptic activity lead to an increase in the secretion of both A β ₁₋₄₀ and A β ₁₋₄₂ from neurons and an A β selective depression of excitatory synapses (Kamenetz et al., 2003), indicating a close relationship between brain plasticity and the A β peptide. A β dimers isolated from AD brain tissue induced memory impairment in rodents by potentially inhibiting LTP and enhancing LTD (Shankar et al., 2008). The enhancement of LTD was mediated through glutamate receptors (e.g. AMPA receptors), and the loss of dendritic spine, an indicator of synapse density, was attributed to the activation of NMDA receptors. Amyloid fibrils did not have this effect; only when they were denatured, in order to release smaller A β species, was the same effect achieved. In a study by Lesné et al., the accumulation of an extracellular oligomeric 56-kDa A β species impaired memory in mice over-expressing A β PP, which, when purified and administered to young mice, also induced disruption in memory (Lesné et al., 2006). Sub-nanomolar concentrations of small A β oligomers injected into rats impaired LTP and disrupted learned complex behaviour (Walsh et al., 2002). Taking these results together, the endogenous role of A β could be to protect nerve cells against excessive stimulation leading to cell damage or cell death, but in the pathological process of AD, excessive amounts of A β lead to loss of synapses and impairment of memory.

Intracellular A β

From the initial belief that the extracellular plaques were the main toxic species in AD, and the subsequent focus on the extracellular, soluble A β oligomers, research expanded to include studies on the effect of intracellular accumulation of A β . A β can be produced both at the cell membrane and in cellular compartments, e.g. endosomes and lysosomes (Koo and Squazzo, 1994), and the peptide is also known to bind to cell surface receptors, e.g. the α -7 nicotinic acetylcholine receptor, facilitating internalisation and thus uptake of A β (Nagele et al., 2002). The presence of intracellular A β has been observed to be non-age related and to occur in both AD and non-AD subjects (Grundke-Iqbal et al., 1989). A β oligomerisation starts intraneuronally, and by clearing intracellular A β deposits using A β antibodies, early tau pathology can be avoided in mice (Oddo et al., 2006). Amyloid plaques are often found to contain intracellular proteins, e.g. lysosomal proteases and molecular chaperones (Cataldo and Nixon, 1990; Wilhelmus et al., 2007). In different cell lines, internalised A β seems to localise to multivesicular bodies (MVB), which are a subgroup of endosomes (Friedrich et al., 2010). MVBs can be degraded if fused with lysosomes or released into the extracellular space if they instead fuse with the cell membrane. It has been suggested that the pool of internalised A β forms fibrils which eventually penetrate the vesicular membrane of the MVBs, ultimately leading to cell death and the release of aggregated intracellular A β to the extracellular space. This results in extracellular amyloid deposits which can be composed of A β as well as intracellular proteins (Friedrich et al., 2010). Accumulation of A β has been detected in other organelles in which all subunits of the γ -secretase complex have also been located, such as the mitochondria, where accumulation of A β has been associated with dysfunction of the respiratory chain, reducing oxygen consumption. Human neurons in AD sensitive areas accumulate A β ₁₋₄₂, and this accumulation can be detected before either NFTs or amyloid plaques are observed (Gouras et al., 2000). The effect of intracellular A β has been investigated using AD mouse models, in which intracellular A β deposits correlate well with memory impairment, and are observed before any extracellular plaques can be detected (Knobloch et al., 2007).

A β and the endosomal-autophagy system

Autophagy is a cellular process in which cytoplasmic material undergoes a catabolic process known as lysosomal degradation (Klionsky, 2000). The endocytic pathway merges with the autophagy system to degrade and recycle proteins (Cuervo, 2004). A β PP and the secretases that process it have been located to the endocytic pathway (Koh et al., 2005), hence also the intracellular production of different variants of the A β peptide within these endocytic vesicles (Koo and Squazzo, 1994). In AD mouse models, lysosomes are found to accumulate in the axons of neurons in proximity to the amyloid aggregates. These lysosomes are lacking soluble proteases, indicating that their ability to degrade cytosolic material is impaired (Gowrishankar et al., 2015). The accumulation of lysosomes and enlarged endosomes in neurons could then act as a pool of A β for the build-up of extracellular aggregates when the neurons eventually degenerate. In addition, BACE1 co-localises with LAMP-1 (a lysosomal marker), indicating that there is an increased level of BACE1 in the accumulated lysosomes. This could lead to an increase in A β production in proximity to the amyloid aggregates;

A β PP localises in the neurons surrounding these aggregates (Cras et al., 1991). However, abnormalities in the endocytic pathway arise prior to amyloid accumulation in the late onset form of AD (Cataldo et al., 2000). In a microarray analysis of hippocampal neurons, both Rab5 and Rab7, proteins connected to early and late endosomes, were up-regulated in patients with mild cognitive impairment (MCI) and AD (Ginsberg et al., 2010). The dystrophic swelling of axons observed in AD brains has been shown to be due to a build-up of autophagic vacuoles (AV), which are vesicular intermediates that are part of the autophagic-lysosomal pathway (Nixon et al., 2005). An accumulation of AVs can be seen in a wide range of neurodegenerative diseases; however, this pathology is far more pronounced in AD (Nixon and Cataldo, 2006; Nixon et al., 2005). The level of AVs is low in healthy neurons due to an efficient turnover process, which clears these intermediates rapidly (Boland et al., 2008). The underlying cause of accumulation of AVs in AD is believed to be a disruption in the proteolytic clearance of these vesicles by lysosomes. The C-terminal fragment produced after BACE1 cleavage of A β PP can pathologically activate Rab5, leading to swelling of endosomes (Kim et al., 2016). Neurons are postmitotic, leaving them more vulnerable to a potential toxic build-up from waste that the endosomes and lysosomes fail to degrade. This evokes the question of whether lysosomal dysfunction is a result of A β induced toxicity or whether the accumulation of lysosomes lacking proteases contributes to A β accumulation, or could it be a combination of the two (Nixon, 2016; Peric and Annaert, 2015)?

Toxicity induced by the interaction between A β and other proteins

Due to its sticky tendencies, A β is prone to bind to and interact with other proteins. Several studies have reported how A β is able to interact with different receptors, such as the NMDA (Snyder et al., 2005) and Frizzled (Magdesian et al., 2008) receptors, leading to downstream signalling, which could mediate A β neurotoxicity. NMDA receptors are connected with LTP and LTD, and hence also with brain plasticity. A β affects the levels of NMDA receptors on the cell surface and regulates their trafficking, where A β induces endocytosis and thus a reduction in the number of receptors at the cell surface (Snyder et al., 2005). This may potentially contribute to synaptic dysfunction and eventually to neuronal loss, which is characteristic of the disease. The Frizzled receptors are a family of cell surface receptors for a group of secreted glycoproteins encoded by several WNT genes (Gordon and Nusse, 2006). Wnt signalling controls many critical cellular processes, e.g. cellular differentiation, proliferation and synapse formation. The Wnt signalling pathway is inhibited upon binding of A β to the Frizzled receptors; the transcription of Wnt-target genes is decreased (Magdesian et al., 2008). The detergent-like properties of oligomeric A β , which allow it to bind to various other proteins, also permit it to penetrate the plasma membrane, creating pores and channels (Arispe et al., 1993a; Arispe et al., 1993b). The plasma membrane's integrity is crucial for cell survival; pores/channels created by A β in the plasma membrane can disrupt the cell's Ca²⁺ homeostasis (Kawahara et al., 2000). A β has been found to increase the activity of tau protein kinase I, TPK I, a kinase responsible for phosphorylating tau, thus promoting degenerative processes (Takashima et al., 1993). The exact mechanism for this increase in activity is unknown but increases in intracellular levels of Ca²⁺, due to disruption of the cell membrane, could be involved in increasing the levels of TPK I synthesis, leading to hyperphosphorylation of tau. Pores in the cell membrane could also induce the formation of free

radicals, resulting in an acceleration in the degeneration of neurons (Yatin et al., 1998). Amyloid aggregates can also sequester other proteins (Olzscha et al., 2011). The nature of the sequestered proteins has been investigated, and all were found to be multidomain proteins or proteins with high levels of unordered structure. This indicates that the proteins are multifunctional and/or act as hubs, i.e. connective proteins in large complexes (Ekman et al., 2006). A β induced cell toxicity could therefore be connected with the loss of function that occurs when proteins crucial for cellular functions are sequestered by the amyloid aggregates.

Neuroinflammation in AD

The inflammatory response in the CNS that is observed in AD pathology is mainly driven by microglia (Heppner et al., 2015). Microglia survey the CNS in search of pathogens and support CNS homeostasis as well as neuronal plasticity. There has been an increase in research surrounding the role of inflammation in AD during recent years, especially since mutations in the gene coding for TREM2, a microglial receptor protein, have been linked to an increased risk of developing late onset AD (Jonsson et al., 2012). However, inflammation has been known to be a characteristic of AD pathology for many years. In a study using microarray assays on AD brains, and in a separate bioinformatics study, up-regulation and activation of the immune response was found to both accompany and contribute to the disease pathogenesis (Blalock et al., 2004; Zhang et al., 2013). Increased levels of different inflammation markers, e.g. cytokines and other immune mediators, have been detected in both tissue and body fluids from patients suffering from AD or patients with early symptoms of the disease (Brosseron et al., 2014; Sudduth et al., 2013; Tarkowski et al., 2003). In the same study, the increased inflammatory response was found to correlate with early, cognitive symptoms of the disease, suggesting that the immune system is involved in the initial stages of disease progression. Thus, inflammation seems to play a role in AD pathogenesis. In other neuroinflammatory diseases, e.g. multiple sclerosis and encephalitis, it has been established that the immune response is disease-promoting (Kennedy, 2004); however, whether inflammation is beneficial or damaging in AD is still under debate (McCaulley and Grush, 2015). Microglia have been observed to engulf A β (Frackowiak et al., 1992; Pluta et al., 1999), indicating that activated microglia could be beneficial in AD. Microglia have receptors which both soluble and fibrillary A β have been shown to interact with, and in addition, receptor-independent interaction between oligomeric A β and microglia occurs. However, when microglia are exposed to A β , microglia become “primed”, making them more sensitive to secondary stimuli, leading to various phenotypical functions, one such being the continuous expression and release of cytokines, a pro-inflammatory function (Prokop et al., 2013). The continuous activation, priming, of microglia results in dystrophic microglia and eventual degeneration of neurons. The initial immune response may therefore be beneficial, degrading A β fibrils and decreasing the levels of soluble A β , however, in response to constant exposure to A β , microglia become more pro-inflammatory and less efficient at degrading A β species (Heppner et al., 2015).

Overall, many different pathogenic pathways leading to the disease's characteristic neurodegeneration as well as the clinical symptoms have been investigated and proposed to involve A β ; some of them are summarised in Fig. 8. However, the prospect of pinpointing an exact mechanism for A β induced toxicity is distant due to the many cellular processes that are associated with this promiscuous peptide and its larger species.

Figure 8. Mechanisms for A β related toxicity. (A) A β can form pores in the plasma membrane, thus disrupting the membrane integrity. (B) By interacting with different cell surface receptors, A β can induce or inhibit downstream signalling. (C) When microglia are continuously exposed to A β , they become pro-inflammatory, hence inducing neuroinflammation. (D) Accumulation of intracellular A β appears before extracellular deposits can be detected and it has been shown to induce mitochondrial dysfunction. A disruption in the endosomal-autophagy system has been correlated with the early stages of AD pathology. (E) Amyloid aggregates can bind to other proteins, thus leading to a loss of function when these proteins are sequestered by the aggregates.

Tau and AD

The second hallmark of AD pathology, apart from the extracellular A β amyloid aggregates, are the NFTs consisting of a microtubule-associated protein (MAP) called tau (Serrano-Pozo et al., 2011). The normal physiological function for tau and other MAPs is to promote the assembly of microtubules and to stabilise them. This function is dependent on the degree of phosphorylation, where the optimal level of phosphorylation is 2-3 phosphate groups on each tau molecule. However, in the NFTs found in AD brains, tau is hyperphosphorylated. This hyperphosphorylation precedes tangle formation and it is believed that the increase in phosphorylation allows tau to interact with other normal tau molecules as well as other MAPs, leading to the formation of tangles. The loss of function, e.g. the breakdown of the microtubule network, when normal tau and other MAPs are sequestered by hyperphosphorylated tau into tangles, leads to a retrograde degeneration, loss of synapses and eventually neuronal cell death (Iqbal et al., 2005). Tau pathology correlates well with disease progression, i.e. dementia, in AD patients (Arriagada et al., 1992).

Diagnosis and treatment of AD

Today there are no drugs available to prevent or cure AD, only to mitigate its symptoms. A majority of the drugs in current, as well as discontinued, clinical trials are based on the amyloid cascade hypothesis, targeting the production of the A β peptide or the formation of amyloid aggregates. An important aim is to be able to identify people at risk of developing AD and intervene at the early stages of the disease, therefore developing tools for early diagnosis is of great importance.

Difficulties in diagnosing AD

In order to treat a disease, it is crucial to be able to make an accurate diagnosis as early as possible. The clinical symptoms such as dementia are likely to occur late in the progression of the disease (Tarawneh and Holtzman, 2012). AD is the major cause of dementia but there are other diseases that cause similar symptoms, e.g. frontotemporal dementia and Lewy body dementia (Karantzoulis and Galvin, 2011). Hence dementia patients may share the same symptoms, but the underlying causes of them may vary, and to use the most effective treatment, correct diagnosis is necessary.

Biomarkers for AD have been in the spotlight for many years. In a meta-analysis, PubMed and Web of Science were screened for articles about CSF, and plasma biomarkers reflecting neurodegeneration, and the data were extracted and analysed (Olsson et al., 2016). Taken together, in CSF, significantly lower levels of A β ₁₋₄₂ are found in AD patients compared to controls, whereas levels of A β ₁₋₄₂ in plasma do not differ between the two groups. However, as mentioned above, it is necessary to start treating AD before symptoms appear. MCI, which is the pre-dementia phase of AD, is a diagnosis given to patients if they perform poorly in one or several cognitive domains, taken into account their age and educational background, while not exhibiting symptoms of dementia and still being able to functionally perform (Albert et al., 2011). For MCI patients who go on to develop AD, A β ₁₋₄₂ levels have been found to be lower in CSF compared to those of stable MCI patients.

Positron emission tomography (PET) scans have also been used in order to distinguish different forms of dementias from one another and to predict which MCI patients will go on to develop AD (Marcus et al., 2014). FDG-PET uses the biologically active molecule ^{18}F Fludeoxyglucose (FDG), an analogue of glucose, to monitor changes in glucose metabolic rate. As we age, the rate of glucose metabolism decreases; at 78 years, the metabolic rate is 26% less than that at the age of 18 (Kuhl et al., 1982). In cases of dementia, glucose uptake is severely reduced in specific parts of the brain. FDG-PET has been shown to be able to predict which MCI patients will go on to develop AD (Yuan et al., 2009). Another strategy is to use PET to image amyloid accumulation in patients. In 2002, the first amyloid imaging agent, the C-Pittsburgh compound, was used on patients. It is a derivate of the amyloid binding fluorescent dye Thioflavin T (Mathis et al., 2002). However, due to its short half-life, around 20 min, additional probes have since been developed and in 2012 the US FDA approved the first amyloid imaging agent, ^{18}F -Florbetapir, to be used in the clinical assessment of dementia patients (Wong et al., 2010). The amyloid accumulation detected using ^{18}F -Florbetapir correlates well with amyloid load assessed post mortem. However, the binding of amyloid agents in AD patients does not correlate with clinical measurements of cognitive deficiencies. A negative PET scan only indicates a low probability of dementia being caused by AD and a positive PET scan does not result in the diagnosis of AD; approximately 25% of non-demented subjects older than 75 years give rise to amyloid positive PET scans (Nordberg, 2008; Villemagne et al., 2008). The issue with diagnosing different forms of dementia is reflected in the difficulties in finding a working treatment for AD; if the patients involved in the clinical trials are incorrectly diagnosed with AD, a treatment targeting $\text{A}\beta$ will probably not have the desired effect, confounding the results of clinical trials (Becker et al., 2008).

Drugs available on the market today

Today there are four US FDA approved drugs on the market to treat the symptoms of AD, all targeting the synapses and seeking to slow down the decrease in cognitive function seen in AD patients. Three of these drugs (donepezil, galantamine and rivastigmine) target the cholinergic system, inhibiting acetylcholinesterase, an enzyme that degrades the neurotransmitter acetylcholine released from the pre-synapse (Yiannopoulou and Papageorgiou, 2013). The fourth drug, memantine, acts as a low-to medium-affinity antagonist against the NMDA receptor and it is believed to reduce the effect of excitotoxic glutamate release (Robinson and Keating, 2006). All the compounds mentioned here are non-preventative drugs used for symptom relief.

Treatment strategies based on the amyloid cascade hypothesis

There have been three main different treatment strategies pursued for AD; they target either $\text{A}\beta$ production or the peptide itself (Fig. 9). The first strategy involves inhibiting γ -secretase, and thereby preventing $\text{A}\beta$ from being released from the C-terminal fragment of $\text{A}\beta\text{PP}$ created by BACE1 cleavage. The first γ -secretase inhibitor to reach a phase 3 clinical trial was Lilly's Semagacestat. The trial had to be discontinued due to an increased risk of skin cancer and infection and the finding that cognitive function actually worsened for all three different treatment groups (Doody et al., 2013). One reason for the failure to utilise γ -secretase inhibitors as a treatment strategy could be that γ -secretase

has more than 40 other known substrates, including Notch receptors. The C99 fragment created after BACE1 cleavage of A β PP has been shown to be toxic to neurons. One side effect of decreasing A β production is an increase in un-cleaved C99. Research therefore now focuses on γ -secretase modulators that can lower A β levels but still allow processing of the C99 fragment.

Figure 9. Treatment strategies based on the amyloid cascade hypothesis. (A) Inhibition of the secretases cleaving A β PP in the amyloidogenic pathway, thereby inhibiting A β production. (B) Immunotherapy targeting soluble A β , preventing oligomerisation, or targeting insoluble A β species, inducing degradation.

The second treatment strategy focuses on the other secretase that processes A β PP leading to the production of the A β peptide: BACE1. BACE1 does not have as many known substrates as γ -secretase and is therefore a more suitable candidate for inhibition. Since A β PP will still be able to be processed completely by α - and γ -secretase, inhibiting BACE1 should only shift the equilibrium towards the non-amyloidogenic pathway and not result in partially cleaved A β PP, as is the case when using γ -secretase inhibitors. As of today, three different BACE1 inhibitors are in phase 3 clinical trials. One of them, AZD3293 (Lilly and AstraZeneca), has shown great promise in its phase 1 clinical trial, with test groups showing tolerance towards a wide range of doses. A β levels are lowered in both plasma and CSF for a prolonged period of time and it is noteworthy that A β levels in the blood were found to be reduced with the drug being administered only once-a-week (Cebers et al., 2016). AZD3293 is now the subject of two large phase 2/3 clinical trials scheduled to end in 2019 and 2021. A phase 2/3 clinical trial involving Merck's BACE1 inhibitor Verubecestat was cancelled in February 2017, due to the fact that no positive effect was observed. The participants in this trial were diagnosed with mild to moderate

AD; however, in another ongoing trial using the same drug, where the patients include sufferers from MCI, results are expected by 2019.

A third approach for treating AD is immunotherapy, targeting soluble A β or the amyloid aggregates. Solanezumab (Lilly) is an antibody targeting soluble monomeric A β , thereby reducing the amount of soluble peptide that can act cytotoxically in synapses. In a phase 1 study, a significant decrease in amyloid load was found in enrolled patients; however, in the phase 3 EXPEDITION-3 clinical trial, no statistical effect on cognition was observed. Trials are still ongoing in which the test groups are part of the Dominantly Inherited Alzheimer Network (DIAN) study, where the drug is administered at earlier stages than in the failed phase 3 clinical trial.

Alternative treatment strategies

Since all clinical trials based on the amyloid cascade hypothesis have failed so far, what other potential treatment strategies could be pursued? Inflammation and AD are strongly connected; several inflammation related genes have been observed to be up-regulated in AD and microglia and astrocytes are activated. Non-steroid anti-inflammatory drugs (NSAID) have been in the spotlight since epidemiological studies began reporting that long-time users of these drugs, i.e. people suffering from chronic pain, have a lower incidence of AD (Vlad et al., 2008). Ibuprofen and naproxen, common over-the-counter pain killers, have been evaluated in clinical trials. In the case of ibuprofen, no significant beneficial effect on cognition could be observed. Three different trials involving naproxen have been carried out and in the first two trials, the drug did not slow down cognitive decline and in addition, an increased risk of negative cardiovascular side effects was observed. The third clinical trial is ongoing and scheduled to terminate in 2019.

Studies have shown that hypertension during mid-life increases the risk of developing AD (Qiu et al., 2005). For this reason, drugs that target neurovascular dysfunction are now being investigated to see whether they can also be beneficial in treating AD. Carvedilol (Procter & Gamble) is an adrenergic receptor antagonist and vasodilator, currently in a clinical trial to investigate its effect on patients suffering from mild AD.

Tau pathology correlates well with cognitive decline in AD patients and is, alongside the presence of amyloid plaques, one of the hallmarks of the disease (Iqbal et al., 2005). TPI 287 (Cortice Biosciences) is a microtubule-stabilising drug which is currently being evaluated as a treatment strategy for AD patients in a phase I clinical trial scheduled to end in 2017. However, a similar drug, BMS-241027 (Bristol-Myers Squibb), which also stabilises microtubules, underwent a phase I clinical trial in 2012-13 and was subsequently discontinued due to a lack of positive results.

Lifestyle and AD

In a large randomised controlled trial, the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER), a 2 year multidomain intervention including diet, exercise, cognitive training and vascular monitoring showed that the multidomain approach was able to maintain and even improve cognitive function in at-risk elderly subjects compared to a control group (Ngandu et al., 2015). The control group received general health advice while the intervention group received guidelines on food intake (Finnish Nutrition Recommendation) and a program for exercise which included a muscle strength programme (1-3 times a week) and aerobic exercise (2-5 times a week). The interventions group also received cognitive training and its members were encouraged to participate in social activities. After 24 months, the improvement in primary (overall cognition) and secondary (e.g. processing speed) outcomes were 25-150% better in the intervention group compared to controls. Lifestyle choices therefore seem to play a significant role when it comes to the risk of developing late onset AD.

Lysozyme amyloidosis

Lysozyme

Another amyloid disease, which does not occur as commonly as AD but is still well characterised, is lysozyme amyloidosis. The disease is systemic and inherited in an autosomal dominant mode. It is caused by the aggregation of a mutant variant of the protein lysozyme (Pepys et al., 1993). Lysozyme is a bacteriolytic enzyme and part of our innate immune system. The enzyme exerts its bacteriolytic mechanism by attacking the cell walls of bacteria, binding the peptidoglycan molecule in its active site and hydrolysing the glycosidic bond (McKenzie and White, 1991). Lysozyme is a well-studied enzyme and its protein structure was the first ever to be solved using x-ray diffraction (Blake et al., 1965). In healthy subjects, lysozyme is produced at high levels by monocytes and macrophages in different body fluids such as breast milk, tears and saliva (Lappin et al., 1986). In plasma, the lysozyme content has been measured as being between 4-13mg/l, but very low concentrations of the enzyme can be detected in urine (Osserman, 1966). This suggests a short lifetime for the enzyme and studies have shown that 75 % of the total plasma content of lysozyme is degraded within 1 hour. Around 500 mg of lysozyme is therefore produced every day.

Mutations connected to lysozyme amyloidosis

There are several disease-associated variants of lysozyme: Y54N, I56T, F57I, W64R, D67H, D68G, W82R, L84S and T70N/W112H (Fig. 10) with increased tendencies to aggregate compared to the wild type protein. The mutation studied in paper III and IV is the F57I mutation, in which phenylalanine at position 57 has been replaced with isoleucine due to a single base transversion (Yazaki et al., 2003). This mutation was first discovered in 2003 in an Italian Canadian family, where the affected family members suffered from renal failure. A renal biopsy confirmed amyloid formation; amyloid aggregates had replaced glomeruli within the kidney.

Figure 10. The protein structure of human lysozyme. Some of the disease-associated amino acids are shown as red balls. Pdb structure 2ZIL.

Clinical symptoms

The clinical symptoms of hereditary amyloid diseases vary depending on the mutations underlying the disease. It is not uncommon for symptoms also to vary within a family, even though the disease is caused by the same mutation. A case study involving two pairs of monozygotic twins showed that the onset of familial amyloid neuropathy type I (transthyretin amyloidosis) varied, being 12 years for one pair of twins and 4 years for the other pair. For the second twin pair, the aggressiveness of the disease also varied dramatically (Munar-Qués et al., 1999). This shows that non-genetic factors contribute to both onset and progression when it comes to systemic amyloid diseases. In lysozyme amyloidosis, the mutations affect the native structure of the WT protein in different ways, giving rise to several disease phenotypes, summarised in table 2. However, a hallmark of lysozyme amyloidosis is that the disease gives rise to massive amyloid deposits, with weights in the kilogram range. These amyloid deposits cause haemorrhaging in the liver and spleen, first seen in two English families (I56T and D67H). Renal failure has been seen in patients carrying the F57I or the W64R mutation (Yazaki et al., 2003). Sicca syndrome, i.e. dry mouth and dry eyes, is a symptom in several of the families suffering from lysozyme amyloidosis. In patients with the I56T mutation, capillaries bleed, leaking blood into the skin, causing red or purple spots called petechiae. As a result of a recently discovered lysozyme mutation, L84S, the clinical symptoms involve impairment of multiple organs, including the renal system; however, amyloid accumulation affects the heart as well as results in peripheral neuropathy (Nasr et al., 2017). Lysozyme amyloidosis has long been thought of as a rare disease, but emerging data suggest that all hereditary systemic amyloid diseases may very well be underdiagnosed; due to the many and varying symptoms of lysozyme amyloidosis. Many disease cases are probably incorrectly diagnosed as the more frequently occurring amyloid disease immunoglobulin light chain (AL) amyloidosis (Granel et al., 2006).

Mutation	Clinical symptoms	Organs affected	Reference
F57I	Renal failure	Kidney	(Yazaki et al., 2003)
D67H	Renal failure, petechiae	GI, liver, kidney spleen, skin	(Gillmore et al., 1999; Harrison et al., 1996)
Y54N	Sicca syndrome, weight loss, abdominal pain	GI, eye	(Girnus et al., 2012)
I56T	Petechiae, renal failure	Liver, kidney, skin, spleen	(Pepys et al., 1993)
W64R	Sicca syndrome, weight loss, renal failure	Kidney, eye, liver, salivary glands	(Jean et al., 2014; Valleix et al., 2002)
T70N/W112R	Renal failure	GI, kidney	(Röcken et al., 2006)
L84S	Renal failure, sicca syndrome, peripheral neuropathy, cardiac symptoms	GI, kidney, heart, eye, peripheral neurons	(Nasr et al., 2017)
D68G	Renal failure, sicca syndrome	Liver	(Woolliver, et al., 2008)

Table 2. A summary of the disease-associated lysozyme variants, the symptoms observed and the organs affected by the disease.

Lysozyme aggregation and toxicity

Lysozyme is a 130-amino acid, 14 kilo Dalton protein consisting of two domains, one α - and one β -domain. None of the mutations disrupts the structure of the native protein dramatically compared to WT lysozyme. The folding and unfolding of two different amyloidogenic lysozyme variants, I56T and D67H, compared to those of the WT protein, have been investigated (Canet et al., 1999). The two mutations are found in different parts of the protein; I56T is located in the interface between the two domains, and D67H is located in the β -domain. The mutations were found to affect the process of protein folding in different ways; I56T decreases the folding rate and D67H increases the unfolding rate. Both processes result in a decrease in the stability of the mutated variants compared to the native WT protein. The mutations increase the population of a partially folded, transiently occurring intermediate. In this intermediate, new aggregation-prone regions are exposed, leading to the self-association of these transiently occurring, partially folded species. Low pH in combination with high temperature and compression/decompression induce amyloid formation by both WT and mutant proteins *in vitro* (De Felice et al., 2004; Morozova-Roche et al., 2000). Pre-formed fibrils from both

disease-associated lysozyme variants and WT lysozyme can seed fibril formation *in vitro*, indicating that seeding could play an important role in the development of systemic amyloidosis (Morozova-Roche et al., 2000). Lysozyme consists of 130 residues, and residues 32-108 have been found to form the core region in mature amyloid fibrils *in vitro* (Frare et al., 2006). These residues correspond to the same area of the polypeptide chain that participates in the co-operative local unfolding, as observed in the disease-associated variants I56T and D67H, in which the β -domain and the adjacent C-helix unfold while the α -domain remains in its native state (Dumoulin et al., 2005). As I56T unfolds, starting with the β -domain and the C-helix, it is the presence of non-native, locally molten globular or unfolded states that drives the aggregation rather than the presence of non-native states with distinct and well defined properties. The unfolding of the β -domain and the C-helix allows residues in these structural segments to interact with each other, giving rise to the first intermolecular interactions, driving the aggregation process onwards (Buell et al., 2011).

In addition to studies focusing on the aggregation process, the toxicity of oligomeric intermediate species and of fibrils formed by lysozyme has been investigated. Malisauskas et al. studied the cytotoxic effect of both oligomers and fibrils formed by equine lysozyme using three different cell lines (primary neurons, fibroblasts and neuroblastoma cells) and observed that large, round-shaped soluble oligomeric species induced a concentration-dependent cell-death in all three cell lines, while smaller oligomers were less toxic (Malisauskas et al., 2006). The structure of oligomeric intermediates has been found to differ from those of both monomeric and fibrillary lysozyme; the polypeptide chain has been found to be more unfolded in oligomeric species than it is in monomers or in mature fibrils (Frare et al., 2009). This gives the oligomers a hydrophobic character. The non-core regions of lysozyme amyloid fibrils influence the toxicity of the amyloid: less ordered structures, which thus have larger non-core regions, result in greater toxicity. How these non-core regions and hydrophobic lysozyme species inflict cell damage and eventually cell death is still unknown. Using hen lysozyme, the impacts of oligomers and fibrils were investigated using neuroblastoma cells (SH-SY5Y) and both lysozyme species were found to induce a decrease in cell survival (Gharibyan et al., 2007). When the mechanism behind cellular death was investigated, fibrils were found to induce a non-specific, rapid necrotic-like cell death. Oligomers, on the other hand, induced a slower response, with increasing caspase activity, eventually leading to apoptosis. This was also observed for low concentrations of oligomeric species, indicating that a more specific mechanism underlies cell death caused by lysozyme oligomers. Their hydrophobic properties may allow both oligomers and the non-core regions of amyloid fibrils to interact with the plasma membrane and/or with different parts of the cellular machinery, disrupting crucial cellular processes. Toxicity could arise from disruption of the cell membrane through the insertion of oligomeric species into the lipid bilayer, a phenomenon which has also been observed for spherical α -synuclein protofibrils (Ding et al., 2002).

Serum amyloid P component

The protein serum amyloid P component (SAP) was first isolated and identified in 1974 (Binette et al., 1974). SAP can be found circulating in human plasma; it is produced by hepatocytes in the liver and is a member of the pentraxin family, a group of pentameric proteins with calcium dependent ligand binding (Pepys et al., 1978). The major role of proteins in the pentraxin family is to bind to pathogens during infections and function as the first line of defence, acting substantially faster than the antibody mediated response (Lu et al., 2012). It has been suggested that, by binding to specific phospholipids present on early apoptotic cells as well as to chromatin in permeable, late apoptotic cells, SAP is involved in managing apoptotic cells *in vivo*, facilitating their phagocytosis via macrophages (Bijl et al., 2003; Familian et al., 2001). SAP possesses chaperone properties; studies have shown that in the presence of SAP, the enzymatic properties of lactate dehydrogenase are preserved and the refolding yield increases (Coker et al., 2000). SAP was found to bind to amyloid fibrils (Pepys et al., 1979), and it is now recognised as a universal component of amyloid deposits; approximately 15% of the amyloid mass is composed of SAP (Dabbs et al., 2013; Pepys et al., 1994). However, due to the diverse roles identified for SAP so far, solving the question of SAP's involvement in amyloid disease is challenging. In AD patients, CSF levels of SAP are higher compared to controls (Hawkins et al., 1994), suggesting a correlation between the disease and SAP; however, in contradictory studies, SAP both suppressed and induced fibrillation of the A β peptide *in vitro* (Hamazaki, 1995; Janciauskiene et al., 1995). In studies using physiological levels of the A β peptide, SAP has been observed to both accelerate formation of amyloid aggregates *in vitro* and to stabilise them once formed (Mold et al., 2012). Furthermore, SAP prevents proteolysis of A β amyloid fibrils as well as fibrils formed from monoclonal immunoglobulin light chain, a protein connected to systemic amyloid disease, suggesting that it stabilises these aggregates, thereby preventing them from being degraded (Tennent et al., 1995). Depletion of SAP has also been shown to delay amyloid formation *in vivo* (Botto et al., 1997). In other studies, it has been proposed that SAP acts as a defence against the formation of toxic aggregates by binding to early intermediates and preventing them from forming toxic species *in vivo* (Andersson et al., 2013). Lysozyme and SAP are closely linked; ¹²³I-labelled SAP is used in clinical analyses to monitor amyloid load in patients suffering from lysozyme amyloidosis (Pepys et al., 1993).

Treatment options

Today there is no cure for lysozyme amyloidosis; only treatments for managing the symptoms are available. In patients suffering from the disease, the high amyloid load will eventually lead to organ failure, which can be treated temporarily by orthotopic liver transplant. However, an organ transplant does not cure the disease, and the new organs will eventually fail due to a continues build-up of amyloid aggregates throughout the patient's life. In one study, the recurrence of amyloid deposits was found as soon as five months after the transplant and within 6 years, a substantial build-up of amyloid deposits was detected (Sattianayagam et al., 2012). SAP and systemic amyloidosis have been linked to one another for a long time; radio labelled SAP is used to visualise systemic amyloid accumulation. Although SAP is a universal component of amyloid aggregates, its role in amyloid disease is still a subject of debate (Dabbs et al., 2013). In a SAP knock-out mouse model for secondary amyloidosis

(AA), amyloid formation was delayed in the absence of SAP (Botto et al., 1997). Using the same mouse model for AA, administration of the organic molecule R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid, CPHPC, decreased the endogenous level of mouse SAP in amyloid deposits by binding of the molecule to circulating SAP and inducing clearance of the complex by the liver (Pepys et al., 2002). In a phase I clinical trial, which included systemic amyloid light chain and apolipoprotein AI amyloidosis patients, a combinatorial treatment involving the drug CPHPC and an anti-SAP antibody reduced the level of SAP circulating in plasma and removed SAP from amyloid aggregates, leading to a decrease in amyloid load (Richards et al., 2015). A phase II clinical trial is now pending.

Modelling amyloid disease

Drosophila melanogaster as a model organism

Drosophila melanogaster, a species of fruit fly, is a popular model organism which has been used for over 100 years and is, genetically, one of the most well studied eukaryotes; it was the first complex organism to have its genome sequenced (Adams, 2000). The fruit fly has been used to increase our understanding of basic genetic processes that are similar in all eukaryotes, thus resulting in several Nobel Prizes awarded to scientists using the fruit fly; in 1933, Thomas Hunt Morgan, won the Nobel Prize in medicine or physiology “for his discoveries concerning the role played by the chromosome in heredity” and later, in 1995, Edward B. Lewis won a shared Nobel Prize in the same category “for their discoveries concerning the genetic control of early embryonic development”. The fly also provides a means of studying human diseases in a living biological system; when a systematic BLAST analysis of 929 human disease genes against the recently completed *Drosophila* genome was performed in 2001, homologues for 77% of the disease genes were found (Reiter et al., 2001), further validating the fly as a model system for medical research. By using transgenic flies over-expressing human disease genes, the *in vivo* consequences can be studied in a more time- and money- efficient manner than when using more advanced organisms, e.g. rodents or chickens.

Two different *Drosophila* AD models

To gain an increased understanding of the different pathways and mechanisms involved in AD, an appropriate disease model is necessary. Murine models have been the primary disease model used, often based on the over-expression of A β PP to generate the disease's characteristic extracellular amyloid plaques (LaFerla and Green, 2012). But limitations such as time and financial aspects, combined with the fact that many of the early murine models did not display cognitive deficits or neurodegeneration, two important features of the disease pathology observed in humans, resulted in the search for a complementary disease model.

Homologues for A β PP, α - and γ -secretase and BACE1 are present in the *Drosophila* genome (Carmine-Simmen et al., 2009; Hong and Koo, 1997; Luo et al., 1990; Rooke et al., 1996). Defective behavioural phenotypes arising from knocking out Appl, the fly homologue of human A β PP, can be rescued by expressing human A β PP, hence indicating functional conservation (Luo et al., 1992). Even though Appl lacks homology in the region corresponding to A β , flies have been shown to produce A β -like neurotoxic fragments, further indicating conservation of function between the homologues (Carmine-Simmen et al., 2009). In addition, fly γ -secretase is able to process human A β PP *in vivo* (Fossgreen et al., 1998; Greeve et al., 2004). In 2004, *Drosophila melanogaster* emerged as a potential candidate for modelling AD when Greeve et al expressed genes involved in the pathophysiology of AD, human A β PP and BACE1 (the A β PP-BACE1 fly model), in the fly retina and observed age-dependent neurodegeneration as well as the formation of A β -containing plaques (Greeve et al., 2004). Another approach using the fruit fly as a model organism for AD is to directly express the A β peptide (the A β fly model), together with a signal peptide, facilitating the secretion of the peptide to the extracellular

space (Crowther et al., 2005; Iijima et al., 2004). A decrease in longevity and behavioural phenotypes were observed in both studies. Behavioural phenotypes such as learning deficits and a decrease in locomotor activity were correlated with increased age in association with intracellular A β oligomers, prior to the development of extracellular deposits. During the past two decades, *Drosophila melanogaster* has become an established model organism for AD, with the A β fly model being predominantly used. This model gives rise to high levels of A β ₁₋₄₂, resulting in a high plaque load, making it possible to study amyloid formation *in vivo* (Berg et al., 2010; Caesar et al., 2012). The high levels of expression of the A β ₁₋₄₂ peptide in the A β fly model generates flies with a short life span, making it possible to perform screens for compounds interfering with the aggregation process with a relatively high throughput for an *in vivo* model. In the A β PP-BACE1 fly model, the levels of the A β peptides produced are much lower, shortening the flies' life span but not to the same, drastic, extent as in the A β fly model, giving rise to a disease model more closely resembling aged onset AD (Mhatre et al., 2014). In the A β fly model, only a few selected peptides can be co-expressed, compared to the A β PP-BACE1 fly model in which the processing of A β PP results in all possible cleavage products such as the A β PP C-terminal fragments as well as different versions of the A β peptide, e.g. A β ₁₋₄₀ and A β ₁₋₄₂ (Chakraborty et al., 2011).

Lysozyme amyloidosis models

To fully understand, and eventually also find treatments for, diseases, it is necessary to develop and use animal models before moving on to clinical trials involving patients. Today the only research ongoing in the world that employs a model organism to study lysozyme amyloidosis occurs at Linköping University, where *Drosophila* is used as a model. Flies express several variants of lysozyme, which are present at high levels in the digestive tract of the flies rather than in the fat body or the haemolymph, which are the invertebrate equivalents of mammalian liver or blood. The role of fly lysozyme is believed to be that it breaks down bacteria in the food ingested by the fly, rather than being an active player in the fly immune system, as it is in humans (Daffre et al., 1994). In 2012 Kumita et al. published a paper introducing a transgenic *Drosophila* model for expressing human WT lysozyme and disease-associated lysozyme variants. The lysozyme variants, F57I and D67H, trigger the unfolded-protein response and disrupt eye development when protein expression was directed to the retina of the flies. Some of the most commonly used *Drosophila* drivers direct protein expression to either the retina (*gmr*-Gal4) or the CNS (*elav*-Gal4) of the fly. Lysozyme amyloidosis is a systemic amyloid disease, resulting in the accumulation of amyloid aggregates around and within different organs in the human body; no cases of lysozyme amyloidosis affecting the CNS of patients have been reported so far. However, in a recently reported case of a new lysozyme mutation leading to lysozyme amyloidosis, peripheral neuropathy was observed (Nasr et al., 2017). The benefits of using drivers directing the protein expression to the CNS include being able to study the impact of protein expression on the survival of the flies and their locomotor behaviour, thereby gaining further insight into the *in vivo* behaviour and cytotoxic mechanisms of the disease-associated lysozyme variants. Another well-known *Drosophila* driver is *Act5C*-Gal4, which drives protein expression ubiquitously (Fyrberg et al.,

1983). It has however been shown that ubiquitous expression of WT lysozyme is toxic for flies during metamorphosis, probably due to the activity of the enzyme (Kumita et al., 2012).

Methodology

***Drosophila melanogaster* in research**

There are many reasons for the fruit fly's popularity; it has a relatively short life span, between 30-120 days depending on temperature, and it takes only 10 days at room temperature for an embryo to develop into a mature fly; making it possible to study several generations during a limited period of time. It is easy to distinguish females from males; females are often bigger and they lack the sex combs present on the front legs of the males. The genetics of the fruit fly are easy to keep track of since it only has four chromosome pairs (one sex chromosome and three autosomes). Chromosomes two and three are the most common targets for the insertion of transgenes when using the fruit fly as a model organism; chromosome four is only one-fifth of the size of the other chromosomes. But what really makes the fruit fly an outstanding model organism is the combination of the lack of recombination in males during meiosis with the use of balancer markers, making it possible to keep true-breeding stocks as well as to carry out crosses in which the researcher can easily keep track of invisible genotypes. If a fly is homozygous for the balancer marker, it gives rise to a lethal phenotype; however, there are markers that do not prevent recombination which are also widely used to track *Drosophila* genetics. In table 3, the different markers used throughout this thesis are presented. The use of the fruit fly as a model organism in research is not regulated to the same extent as that using more advanced organisms such as rodents or chickens; no permits are needed in order to use the fruit fly in research. There are many research tools available that facilitate the use of the fly, including genetic tools to manipulate the fly genome, as well as behavioural assays with which to investigate the effect of, e.g., the expression of transgenes. Transgenic flies can be purchased from the Bloomington Stock Centre, facilitating the use of the fruit fly even further.

Name	Chromosome	Balancer	Distinguishing phenotype
CyO (“Curly”)	2	Yes	Curly wings
Ife	2	No	Fused ommatidia
TM6b	3	Yes	Hairy “shoulders”
MKRS	3	No	Short hair, “shaved”
Sb (“Stubble”)	3	Yes	Short hair, “shaved”

Table 3. The markers used to control stocks and crosses throughout this thesis.

Controlling protein expression in *Drosophila*: the Gal4/UAS system

Drosophila has been widely used to study the consequence of over-expressing genes since Rubin and Spradling, at the beginning of 1980, published a paper in which they described the P-element technique for the generation of transgenic flies (Rubin and Spradling, 1982). The P-element is a class II transposon, also known as a jumping gene, which moves by a cut and paste mechanism, catalysed by a member of a specific class of enzymes, transposases. This trait has been exploited to create transgenic flies. This is done by cloning the P-element into a plasmid which is then transformed into and grown in bacteria. The gene coding for the P transposase is removed and replaced with the gene of interest. The plasmid, as well as DNA coding for the transposase, is injected into the fly embryo at an early stage, and insertion of the gene takes place. The insertion is random and once the gene is inserted, it will no longer be able to move because it lacks the ability to produce its own P transposase. The random insertion may damage the function of the host's own genes; microinjection of the plasmid is therefore carried out in a number of fly lines simultaneously in order to make comparisons, thus making sure that no essential genes have been disrupted by the insertion. This technique was further developed by Brand and Perrimon in 1993, and resulted in the protein expression control system which is used throughout this thesis: the Gal4/UAS expression system, Fig. 11 (Brand and Perrimon, 1993). Two different fly lines are needed; the first is called a reporter line. The reporter line is a fly line that is transgenic for the protein of interest. The gene encoding the protein of interest is placed downstream from a UAS (upstream activating sequence) domain. For the gene to be transcribed, a transcriptional activator, in this case Gal4, must bind to the UAS domain. In the absence of Gal4, the gene is silent. The second fly line needed is called the driver line. The driver line continuously expresses the transcriptional activator Gal4. By placing the gene encoding Gal4 downstream of a cell- or tissue-specific promoter, the expression of the Gal4 protein will be directed to a certain cell or tissue type. When the reporter line is crossed with the driver line, the offspring generated will be transgenic for the gene of interest as well as expressing Gal4, resulting in the cell- or tissue-specific transcription and translation of the protein of interest.

Figure 11. The Gal4/UAS system controls protein expression. Driver line with gene for Gal4 downstream of a tissue specific promoter (TSP) is crossed with reporter line containing gene of interest downstream of upstream activating sequence (UAS) domain. Offspring contain both gene encoding Gal4 and gene of interest, resulting in tissue-specific expression of the protein of interest.

Fly lines

In the papers included in this thesis, several different transgenic *Drosophila* fly lines were used, including both different driver and different reporter lines. In table 4, a summary of these fly lines is given.

Fly line	Genotype	Comment
Control	+/+;+/+	Background to A β ₁₋₄₂ (W ¹¹¹⁸) and lysozyme (A11) flies
AβPP	A β PP/CyO;+/+	Background to A β PP-BACE1 flies
BACE1	+/+;BACE1/TM6b	Background to A β PP-BACE1 flies
AβPP-BACE1	A β PP/CyO;BACE1/TM6B	Constructed from A β PP and BACE1 fly lines
LysWT	+/+;LysWT/LysWT	WT lysozyme
LysF57I	+/+;LysF57I/LysF57I	Disease-associated lysozyme variant (F57I)
SAP	+/+;SAP/TM6b	Serum amyloid P component
Aβ_{Arc}	A β _{Arc} /A β _{Arc} ;+/+	A β with the arctic mutation (E22G)
EGFP-Xbp1	Xbp1/Xbp1;nsyb/TM6b	Expresses EGFP when UPR is activated
gmr-Gal4	+/+;gmr/TM6b	Directs protein expression to the retina of the fly
elav-Gal4	X _{elav} /Y(X _{elav});+/+;+/+	Directs protein expression to the CNS of the fly (gene encoding Gal4 placed on the X chromosome)
nsyb-Gal4	+/+;nsyb/sb	Directs protein expression to the CNS of the fly (postmitotic neurons)

Table 4. The different fly lines used in the papers included in this thesis.

Phenotypic analysis of *Drosophila*

Drosophila melanogaster is a powerful tool with which to study the *in vivo* consequences of protein expression. During the more than 100 years for which *Drosophila* has been used to study genetic processes and later, by using transgenic flies expressing human proteins, study human diseases, a number of assays have been developed in order to analyse the phenotypes that are induced. Behaviour assays have been developed for both larvae and adult flies, so that, for example, locomotor behaviour can be studied. Adult flies are known to react to a number of stimulants, e.g. gravity, odours, light, temperature and sound (Vang et al., 2012), which have all been applied when creating assays to evaluate the flies' behaviour, and thereby gain information on the effect of a certain drug or how the

expression of transgenes impacts behaviour. In transgenic flies, by directing the protein expression to different cells or tissues, different phenotypical assays can be used to study any possible toxic effects.

The rough eye phenotype

It has been estimated that two thirds of the vital genes in *Drosophila* are required for eye development; it is likely that many of these genes are specific to eye development, but some of the vital genes are probably also required for other cellular processes (Thaker and Kankel, 1992). Thus information gained by studying phenotypical differences in the *Drosophila* eye may be applicable to other gene functions and therefore also other cell types (Thomas and Wassarman, 1999). The fact that the eye is a non-vital organ for the fly has led to its being used to study many biological processes including apoptosis and neuronal connectivity (Karim et al., 1996). Expression of a protein can be directed to the retina of the fly using the *gmr*-Gal4 driver (Li et al., 2012). Large differences in eye phenotype can be evaluated using a normal light microscope; however, higher resolution images are needed in order to quantify the impact of protein expression on eye phenotype. In the work described in this thesis, protein expression was directed to the eye of the fly in order to investigate toxicity in A β PP-BACE1 and A β flies (paper I) and to study whether co-expression of lysozyme could rescue the induced toxicity in these AD fly models (paper II). The eye phenotypes were evaluated using scanning electron microscopy (SEM) to obtain high resolution images in which the fate of each separate ommatidium, a hexagonal structure containing photoreceptors and pigment cells (Fig. 12A), could be evaluated (Müller 2006) and quantified. When several ommatidia are fused to one another it gives rise to a rough eye phenotype; however, the degree of roughness varies depending on the toxicity induced by the transgene (Fig. 12B, C).

Figure 12. *Drosophila* eye phenotypes. (A) SEM image displaying the hexagonal structures, containing photoreceptors, that are known as ommatidia. The degree of roughness can vary; using light microscopy large differences can be detected, such as those between the normal retina seen in (B) and the severe rough eye phenotype with all ommatidia fused to one another shown in (C).

Longevity assay

A simple, yet elegant, way of monitoring any deleterious effects of expressing disease-associated proteins in *Drosophila* is to perform a longevity assay by directing protein expression to the CNS of the flies (Linford et al., 2013). This approach can also be used to investigate any possible positive effect on longevity induced by disease modulating compounds (Caesar et al., 2012; Chakraborty et al., 2011; Hermansson et al., 2014). Longevity assays are commonly used in many model organisms in addition to *Drosophila*, e.g. *C. elegans* and rodents (Park et al., 2017; Spindler, 2012). To perform a longevity assay, fly crosses are set up at an appropriate temperature. It is crucial to find a temperature at which the progeny expressing the disease-associated genes are able to hatch; lower temperatures tend to decrease protein expression, allowing flies that are highly affected by it to eclose. On the day of eclosion, flies with the required genotype are singled out and sorted into vials containing fly food with 10-20 flies in each. Approximately 100 flies of each genotype are collected, and the number of flies alive is counted every second day until all flies have died, generating a survival curve. Median survival, corresponding to the time when 50% of the flies have died, is often used to compare the survival curves generated from different fly lines using Kaplan-Meier statistics.

Locomotor assay

Adult flies respond to multiple stimuli and one trait that has been widely used is the flies' tendency to move against gravity, known as (negative) geotaxis. How the flies move depends on many variables, such as sex, age and environmental factors (Martin et al., 1999). It is therefore important to use flies of the same sex (usually female flies) and the same age when performing locomotor assays. Young flies tend to move in straighter lines and at higher velocity than old flies do. One of the first assays constructed to study flies' behaviour was the simple climbing assay. The principle of the assay is straightforward; the collected flies are placed in a vial which is then tapped to send them to the bottom of it, and during a set period of time, the number of flies that climbs to the top of the vial is counted. In this thesis, a locomotor assay was used that can be seen as a further developed, semi-automated version of the original climbing assay. In this locomotor assay, a software package called iFly is used to analyse different parameters of fly movement (Jahn et al., 2011). When performing the experiment, flies are manually tapped to the bottom of the vial every 30 seconds for 1.5 minutes, but after each tapping, the vial is positioned in the iFly equipment which records the flies' movement, generating a video clip. This video can then be analysed by the iFly software, which applies a 3D algorithm to calculate the parameters used for analysing locomotor behaviour: the velocity of the flies and their angle of movement. The velocity (mm/s) of the flies is simply the speed at which they move. As the flies age or become affected by the toxicity induced by transgene expression, their velocity tends to decrease and eventually they will stop moving, though this does not necessarily mean that they have died. The cut off value was set at 4 mm/s to indicate severe motor dysfunction. The angle of movement (degrees, °) describes how the flies' movement deviates from a straight line. If the flies move in a completely straight line, the angle of movement will be zero, because no deviation from the line can be observed. However, flies do not move in completely straight lines and they tend to move both backwards and forwards. Completely random movement corresponds to an angle of movement of 90°.

Young flies have an angle of movement between 50-70°, but as they age or experience toxicity from the expression of a transgenic protein, the angle of movement tends to increase, eventually resulting in values greater than 90°, indicating an actively staggering movement back and forth (Jahn et al., 2011).

Protein detection and quantification

The phenotypical assays described above provide numerical values representing the behavioural effects of expressing transgenic proteins in *Drosophila*, however, in order to elucidate why these effects are observed, biochemical methods are an important tool. In this thesis, methods for both the detection and the quantification of specific proteins have been used, and many of them rely on the use of antibodies.

Antibodies

Antibodies are commonly used in a number of biochemical assays; some of them are included in this thesis and will be described below (Goldman, 2000). The reason for the popularity of antibodies lies in some of their unique properties. Antibodies were first mentioned in 1890 by Behring and Kitasato, and they have since received much attention and are now known to play an important part in our immune system. Antibodies are secreted by B cells, part of our adaptive immune system, as a response to foreign substances, e.g. viruses and bacteria. Antibodies are Y-shaped, heterodimeric proteins. Each antibody, or immunoglobulin, consists of two heavy chains and two light chains and can be divided into two domains with different functions, the variable domain and the constant domain. The variable domain binds tightly to its antigen, and is highly specific for an epitope presented on the antigen. The constant domain of the antibody can then attract the components that induce the complementary cascade. These traits have been used in various biochemical assays, in which antibodies are produced in order to target a specific protein. (Schroeder and Cavacini, 2010) This is done by first purifying the antigen against which the antibody is to be raised, then immunising animals with it followed by screening and retrieval of the antibodies. Antibodies can be monoclonal or polyclonal; a monoclonal antibody is specific towards only a single epitope presented on the antigen, while a polyclonal antibody recognises several epitopes on the same antigen. These two types of antibody come with advantages and disadvantages. Monoclonal antibodies are more expensive and time-consuming to produce, but due to their high specificity, monoclonal antibodies usually give rise to less background (Liu, 2014). Polyclonal antibodies, on the other hand, are easier to produce and since they recognise several epitopes, they bind with high affinity, thus amplifying the signal if the sample contains a low concentration of the antigen. However, background signal also tends to increase when using polyclonal antibodies (Lipman et al., 2005). In many biochemical assays, multiple antibodies are used in combination. The first, or primary, antibody binds to the protein that present the correct epitope. The secondary, labelled, antibody binds to the primary antibody and detection is achieved through a variety of different visualisation methods, e.g. chemiluminescence and fluorescence. The constant part of the antibody makes it possible to use the same secondary antibodies, often with fluorophores connected to

them, to bind to multiple primary antibodies so long as they are all raised in the same animal, and thus also have the same constant part, which is then recognised by the secondary antibody.

Western blot analysis

Western blotting (WB) is an immunoassay technique used to detect a small quantity of a specific protein due to the ability of antibodies to bind with high specificity to a protein presenting the matching epitope. This is done by first separating the proteins present in the sample according to molecular weight using gel electrophoresis. Before applying the protein samples to the gel, the proteins are denatured. Denaturation is often done by first adding sodium dodecyl sulphate (SDS), an anionic surfactant which breaks all non-covalent bonds present in the protein structure and induces a negative charge proportional to the size of the polypeptide chain. By adding β -mercaptoethanol to the protein sample, any existing covalent bonds are broken. The proteins in the sample have now lost their tertiary and secondary structure and have gained a negative charge proportional to their size. The sample is then loaded onto the gel. An electric field is applied across the gel, forcing the negatively charged proteins to migrate towards the positive anode. Separation depending on size will be achieved due to the fact that larger peptide chains will be retained while smaller molecules will be able to move through the pores in the gel. After gel electrophoresis, the separated proteins are transferred from the gel to a nitrocellulose or PVDF membrane (Towbin et al., 1979). Usually the transfer is performed using electroblotting, whereby an electric field allows the polypeptide chains to migrate from the gel to the membrane, resulting in a membrane replica of the gel. This is usually followed by blocking of the membrane, using bovine serum albumin (BSA) or milk powder. Blocking decreases the nonspecific binding of the antibodies to the membrane. By using antibodies against specific proteins that are of interest, it is now possible to probe for them, revealing if they are present in the sample (Renart et al., 1979). The first, or primary, antibody binds to those proteins that present the correct epitope. The secondary, labelled, antibody binds to the primary antibody and detection is achieved through a variety of different visualisation methods; chemiluminescent detection was the principal method used in the papers included in this thesis. Western blot analysis is mainly used as a qualitative method; however, semi-quantitative data using western blotting can be generated by normalising band intensity for the protein of interest with respect to a normalising control. Housekeeping genes, i.e. genes that are constantly expressed at high levels (e.g. beta-tubulin and Hsp70), are commonly used as normalising controls.

Meso Scale Discovery (MSD) protein quantification assay

The need not only to detect, but also to quantify the amount of, a specific protein, has led to the development of many different variants of immuno-based methods. One of the most well-known methods is the enzyme-linked immunosorbent assay (ELISA). This assay utilises the antibody-antigen interaction to detect and quantify one or several specific proteins present in a sample. There are different variants of ELISA, but the typical sandwich ELISA set-up uses a capture antibody to capture the antigen and a second antibody linked to an enzyme for the detection step, often using a 96-well plate where the capture antibody is coated at the bottom of each well (Leng et al., 2008). The protein quantification assay used throughout this thesis is the MSD set-up, which can be compared to a sandwich ELISA, except that detection is not enzyme linked. Detection is instead based on electrochemiluminescence (ECL), which is produced when electrochemical reactions occur in the wells. High-energy transfer reactions by the electrodes create excited species emitting light, which is then detected. Most ECL based systems use tris(bipyridine)ruthenium(II) chloride ($\text{Ru}(\text{bpy})_3^{2+}$) as the emitting species together with a co-reactant, which often differs depending on the manufacturer (Miao, 2008). The advantages of the MSD protein assay are that it has a lower signal to noise ratio and is more sensitive than the traditional ELISA method.

Immunoprecipitation

To explore any possible interaction between A β and lysozyme, immunoprecipitation (IP) was used in paper II. IP is used to extract a specific protein from a solution by having antibodies against the specific protein covalently attached to beads, in this case sepharose beads. To investigate whether A β and lysozyme were interacting *in vivo* in the A β PP-BACE1 fly model, co-precipitation of the two proteins was used. Sepharose beads with a lysozyme specific antibody were employed, and the level of co-precipitated A β was analysed using the MSD protein assay.

Fluorescence microscopy

Fluorescence is a powerful tool on which many biochemical assays rely for detection; the three methods described below all use fluorescence microscopy. Fluorescence microscopy is based on the ability of a molecule to emit light, i.e. photons, when its orbital electrons relax back to its ground state from an excited state. In fluorescence microscopy, lasers emitting light of a specific wavelength, filtered through an excitation monochromator, are concentrated on the sample cell. The laser is able to excite the orbital electrons present in the fluorophore, which then relaxes and emits light of a higher wavelength, a process known as the Stokes shift. (Combs, 2010) The samples analysed in the papers included in this thesis are cryo sections of *Drosophila* brain sections, in which the presence and any possible co-localisation of different proteins and protein structures were investigated.

Immunohistochemistry

Immunohistochemistry (IHC) is a method that not only allows the detection of proteins but also makes it possible to determine their location in a tissue and reveal whether different proteins co-localise. Tissue sections, in this case *Drosophila* brain cryo sections, around 10-20 μm in thickness, are analysed using antibodies against specific proteins. There are many different variations of IHC staining protocols, but certain steps are always included in one form or another. The fixation of the sections is performed before antibody staining. The purpose of the fixation is to prevent the tissue from decomposing, maintaining the morphology of the tissue and the epitopes of the proteins. In paper IV, a permeabilisation step was added to the IHC protocol after the fixation stage. A Tween-20 concentration of 0.2-0.5% is able to solubilise the plasma membrane, allowing antibodies to come in contact with and detect proteins present intracellularly and in different cellular compartments. The subsequent steps are very similar to those described for both western blot and protein quantification analysis; a primary antibody is added and using a secondary antibody with a fluorophore connected to it, the detection and localisation of a specific protein can be accomplished using fluorescence microscopy.

Staining with oligothiophenes

Luminescent conjugated oligothiophenes (LCOs) are a group of molecules designed to bind to, and thus allowing fluorescent detection of, amyloid aggregates. LCOs are conjugated systems whose flexible thiophene backbone can adopt distinct conformations when binding to different structures, and these conformations can be observed as alterations in the optical properties, e.g. the emission spectra and the fluorescence lifetime, of the dye (Arja et al., 2013; Åslund et al., 2009; Magnusson et al., 2014). Two LCOs were used in the papers included in this thesis: p- and h-FTAA. In paper I, p-FTAA was used to detect any amyloid aggregates formed by the A β peptide *in vivo* in *Drosophila*. p-FTAA has previously been used to detect A β aggregates both *in vitro* and *in vivo*, and it can bind to pre-fibrillar species that are non-thioflavin T binding. The probe also gives rise to distinct spectral signatures depending on what pre-fibrillar or aggregated species it is bound to (Åslund et al., 2009; Hammarström et al., 2010). In paper IV, both p- and h-FTAA were used to study lysozyme structures formed *in vivo* in *Drosophila*. p-FTAA has previously been used to study the process of lysozyme aggregation *in vitro*; the probe was able to detect thioflavin T-negative pre-fibrillar lysozyme species before eventually giving rise to characteristic double-peak emission spectra when bound to mature amyloid fibrils (Hammarström, 2010). h-FTAA has been used to detect deposits in tissues from systemic amyloid patients (Sjolander et al., 2015), and it has been shown to be more sensitive than Congo red (Sjolander et al., 2016).

Detection of apoptotic cells

A TUNEL assay was used in paper III to investigate whether the expression of an amyloid disease related protein directed to the CNS of the fly induced cell apoptosis. TUNEL is short for terminal deoxynucleotidyl transferase dUTP nick end labelling. In a TUNEL assay, fragmented DNA, indicating apoptosis or severe DNA damage, is detected by the enzyme terminal deoxynucleotidyl transferase (Tdt). Tdt is able to catalyse the addition of dUTPs to the fragmented DNA ends. These dUTPs are secondarily labelled with a fluorescent probe, allowing the detection of apoptotic or damaged cells using fluorescent microscopy.

Scanning electron microscopy

SEM uses a high-energy electron beam to produce signals that give information about the shape and the structure of a specimen. In paper I and II, SEM was used to investigate the eye phenotypes of the flies. SEM is based on the fact that the electrons in the high-energy beam are either backscattered when they hit the sample or force the atoms they hit to emit electrons. This puts restraints on the sample being analysed; it needs to be conductive. The flies were therefore coated with platinum prior to SEM analysis.

Aims

The general aim of the work presented in the papers included in this thesis was to further investigate two different amyloid diseases, Alzheimer's disease and lysozyme amyloidosis and proteins that could have a potential beneficial effect in these diseases using *Drosophila melanogaster* as a disease model.

Specific aims for paper I and II

- Create and characterise a new AD fly model, the A β PP-BACE1 fly model.
- Investigate A β proteotoxicity in the A β PP-BACE1 and the A β fly model.
- By using this new A β PP-BACE1 fly model along with other AD models, investigate the *in vivo* impact of lysozyme on A β toxicity.

Specific aims for paper III and IV

- Investigate whether SAP could rescue toxicity induced by a disease-associated lysozyme variant expressed in the *Drosophila* central nervous system.
- Further investigate the mechanism underlying how SAP is able to prevent toxicity in the *Drosophila* central nervous system observed in double-expressing lysozyme flies.

Summary of the papers

Paper I

Background and aim

Over the years, two different AD *Drosophila* models have been used to study the *in vivo* consequence of expressing disease related proteins as well as to screen for compounds that can modulate A β production or aggregation. In the A β fly model, the A β peptide is over-expressed with a signal peptide directing it to the extracellular space. In the A β PP-BACE1 fly model, the amyloid β precursor protein (A β PP) is expressed together with BACE1, which in combination with fly endogenous γ -secretase results in the production of the A β peptide as well as other cleavage products. The aim was to generate a new A β PP-BACE1 fly model and then perform a number of experiments in parallel with the commonly used A β fly model in order to characterise the A β PP-BACE1 fly model and to investigate A β proteotoxicity in both fly models.

Methods

- 1) By using phenotypical assays (longevity, locomotor and rough eyes), toxic effects could be monitored in the different AD fly models.
- 2) Biochemical assays were used to investigate the levels of sA β PP $_{\beta}$ and different A β peptides.
- 3) The amyloidogenic probe p-FTAA was used to detect amyloid aggregates in *Drosophila* brain cryo sections.

Main results

- 1) Both AD fly models displayed decreased longevity and dysfunctional locomotor behaviour compared to control flies. However, a rough eye phenotype was observed only in A β PP-BACE1 flies.
- 2) High levels of A β ₁₋₄₂ were detected in the A β flies, while both A β ₁₋₄₀ and A β ₁₋₄₂ were observed at low levels in the A β PP-BACE1 flies.
- 3) Massive amyloid accumulation was observed in the A β flies while only small aggregates were detected in the A β PP-BACE1 flies

Discussion and conclusion

- 1) Both AD fly models generate the A β peptide and display dysfunctional behaviour, and are thus suitable AD models.

2) A greater toxic effect can be seen in the A β PP-BACE1 flies although they generate lower levels of A β ₁₋₄₂ compared to the A β flies. Thus, the toxic mechanism must differ between the two AD models. We speculate that the toxicity in the A β PP-BACE1 flies may arise from A β generated intracellularly while the toxicity in the A β fly model could originate from the huge amount of A β ₁₋₄₂ peptides located extracellularly.

3) It is important to take into account that the A β PP-BACE1 flies possess all the components necessary to produce different cleavage products from A β PP processing, which could contribute to the observed toxicity in these flies.

Paper II

Background and aim

It has been established that neuroinflammation accompanies the disease-characteristic amyloid plaques and tau tangles observed in AD brains, and several inflammation genes have been shown to be up-regulated during the initial phases of the disease. Lysozyme, an enzyme part of our innate immune system, is up-regulated in the CSF of AD patients, inhibits A β oligomerisation *in vitro* and co-expression of lysozyme in the fly CNS rescues both the reduction in median survival and the locomotor dysfunction of the A β fly model. The aim of this study was to further investigate the role of lysozyme in AD.

Methods

1) Gene expression databases were used to investigate the correlation between lysozyme mRNA levels and AD pathology in a mouse model of AD and in brain tissue from autopsied AD patients.

2) Human brain tissue and CSF were analysed to investigate whether lysozyme protein levels were altered in samples from patients diagnosed with AD.

3) Three different *Drosophila* AD models, *i*) the A β PP-BACE1 fly model, *ii*) the A β fly model and *iii*) the A β _{Arc} fly model (which expresses the A β ₁₋₄₂ peptide with the arctic mutation) were used to investigate the *in vivo* consequences of co-expressing lysozyme using phenotypical assays and biochemical analyses.

Main results

1) A correlation was observed between lysozyme mRNA levels and AD pathology in transgenic mouse models. In brain tissue from AD patients, a significant increase in lysozyme mRNA levels was observed in visual and prefrontal cortex.

2) Lysozyme protein levels were up-regulated in CSF and human brain tissue from AD patients.

3) Co-expression of lysozyme rescued the rough eye phenotype observed in both A β PP-BACE and A β ₁₋₄₂ flies. The levels of the A β peptide remain unchanged in both models with or without co-expression of lysozyme; however lysozyme and A β were observed to co-precipitate. In A β _{1-42Arc} flies,

co-expressing one or two copies of lysozyme increased longevity in a dose-dependent manner and reduced locomotor dysfunction significantly.

Discussion and conclusion

- 1) The up-regulation of lysozyme (both mRNA and protein levels) in AD mouse models and in AD patients indicates that lysozyme is involved in the disease.
- 2) Co-expression of lysozyme in different AD fly models reduces toxicity, and lysozyme and A β interact in the A β PP-BACE1 flies. We therefore speculate that lysozyme is able to bind to A β , thereby preventing it from exerting its toxic properties.
- 3) Our findings that lysozyme can rescue AD pathology in *Drosophila* point towards that lysozyme could have a beneficial role in AD.

Paper III

Background and aim

SAP is a universal component of amyloid aggregates, but its role in the disease is still a subject of debate. Contradictory data have shown that SAP both induces and inhibits the aggregation of various amyloid-related proteins. In lysozyme amyloidosis, radiolabelled SAP is used to visualise amyloid aggregates *in vivo*; huge aggregates of mutated lysozyme accumulate around the kidney and the liver, eventually leading to organ failure. The aim of this study was to investigate the role of SAP in lysozyme amyloidosis using a fly model of the disease, expressing WT lysozyme or the disease-associated variant F57I.

Methods

Protein expression was directed to the fly CNS and several experiments were carried out:

- 1) Longevity assay.
- 2) Probing for apoptotic cells (TUNEL assay).
- 3) Quantification of protein levels.
- 4) Immunohistochemistry to examine the localisation of lysozyme and SAP in the flies.

Main results

- 1) Co-expression of lysozyme with SAP in the fly CNS rescued the toxic effect induced by F57I.
- 2) SAP also reduced the number of apoptotic cells in the F57I flies to levels comparable to those of WT lysozyme flies and control flies.
- 3) Co-expression of lysozyme with SAP decreased the accumulation of insoluble lysozyme species for both WT and F57I.
- 4) High levels of lysozyme were found in the WT flies and co-localisation with SAP was observed. F57I could only be detected in aged flies, without co-expression of SAP.

Discussion and conclusion

- 1) SAP can rescue *in vivo* toxicity which arises from expressing the disease-associated lysozyme variant F57I in the fly CNS.
- 2) We speculate that if the insoluble F57I species or intermediate species formed on the pathway towards insoluble species are cytotoxic and contribute to the observed cell death and reduction in the median survival time for the F57I flies, the ability of SAP to prevent accumulation of these species may contribute to the rescue effects detected in the longevity and TUNEL assays when SAP was co-expressed with F57I.

Paper IV**Background and aim**

To further investigate SAP's involvement in lysozyme amyloidosis, double-expressing lysozyme flies (F57I-F57I and WT-WT, with and without the presence of SAP) were created in order to generate flies with a stronger disease phenotype than observed in the single-expressing lysozyme flies previously used (Helmfors et al., 2016).

Methods

- 1) A longevity assay was carried out.
- 2) Immunohistochemistry using two different sets of anti-lysozyme and anti-SAP antibodies was performed.
- 3) Staining using two different amyloid-binding LCOs (h- and p-FTAA) was used to investigate the presence of amyloid aggregates.
- 4) Spectral properties (emission spectra and fluorescence lifetime imaging, FLIM) of the LCOs were investigated.

Main results

- 1) Co-expression of SAP in F57I-F57I flies increased median survival time by 14 days, while it had no effect on WT-WT flies, which also showed a significant decrease in survival time compared with controls.
- 2) The two different anti-lysozyme antibodies detected lysozyme in different areas of the brain. The ab36362 antibody detected lysozyme in WT-WT and WT-WT-SAP flies, while the ab108508 antibody detected lysozyme in all lysozyme fly genotypes $\pm$ SAP.
- 3) h-FTAA positive lysozyme species were detected in all fly lines, while p-FTAA only detected lysozyme in F57I-F57I $\pm$ SAP flies. The signal from the LCOs was observed in the same area in which the anti-lysozyme ab108508 antibody detected lysozyme.
- 4) The recorded emission spectra for the p-FTAA positive lysozyme species detected in F57I-F57I-SAP flies gave rise to the double peak spectra characteristic of amyloid aggregates whereas no such spectra were observed for F57I-F57I flies.

Discussion and conclusions

- 1) Co-expression of SAP in F57I-F57I rescues toxicity by significantly increasing the median survival time of these flies.
- 2) The anti-lysozyme antibody ab108508 detects the same lysozyme species as the amyloidogenic probes h-and p-FTAA, indicating that it detects insoluble lysozyme species.
- 3) Co-expression with SAP in F57I-F57I results in changes in the spectral properties observed for p-FTAA; the presence of SAP promotes formation of more amyloid-like lysozyme morphotypes.
- 4) Our data suggest that SAP is able to convert intermediate species formed by F57I into alternative structures with amyloid properties, thereby reducing the toxicity induced by the F57I intermediate species.

Discussion

A β toxicity in *Drosophila* AD models

AD has been modelled in *Drosophila* for nearly two decades, mainly using two different models, the A β and the A β PP-BACE1 fly model. Both models have been reported to express the disease-associated A β peptide, generating amyloid aggregates and displaying behavioural impairment, as a direct consequence of the transgenic over-expression (Crowther et al., 2005; Fossgreen et al., 1998; Greeve et al., 2004; Iijima et al., 2004). Both soluble A β oligomers and insoluble amyloid fibrils have been shown to possess toxic properties; however, it is now generally accepted that low molecular weight A β oligomers are the main toxic species rather than larger A β assemblies (Nimmrich et al., 2008; Zhang et al., 2014). In the *Drosophila* models of AD, both soluble and insoluble A β species are generated, as seen in paper II.

In paper I, two AD fly models, the A β and the A β PP-BACE1 model, were investigated in parallel. Both these fly models displayed similar reductions in the median survival time and dysfunctional locomotor behaviour compared to their respective control flies. When the total levels of A β_{1-42} were analysed, low levels were detected in the A β PP-BACE1 flies, while significantly higher levels of the peptide were observed in the A β flies. The same was observed when staining for amyloid aggregates; a low amyloid load was observed in the A β PP-BACE1 flies whereas the amount of amyloid aggregates was much higher in the A β flies. Thus, neither the total level of A β_{1-42} nor the amount of amyloid aggregates correlate with the toxicity induced in the two AD fly models. Low levels of A β result in the same, or an even higher, toxic effect, in the A β PP-BACE1 flies compared to that in the A β flies, where high levels of the peptide were detected. This indicates that the toxic mechanism must differ between the two AD models where the toxicity observed in the A β PP-BACE1 flies seems to be dependent on where the peptide is located rather than on the total amount. We speculate that the toxicity in the A β PP-BACE1 flies may arise from A β generated intracellularly while the toxicity in the A β fly model could originate from the huge amount of A β_{1-42} peptides located extracellularly. A summary of the data presented in paper I for the different AD fly models can be seen in table 5.

AD fly model	Reduction in median survival (days)	Rough eye phenotype	Total A β in the fly head	Total A β in the fly retina	Amyloid load	A β proteotoxicity
A β	10	No	32	7900	+++	+
A β PP-BACE1	14 (9)	Yes	0.82	7	+	+++

Table 5. A summary of some of the data from the parallel studies performed using the A β and the A β PP-BACE1 fly models. Reduction in median survival time compared to respective controls. For A β PP-BACE1 flies, reduction in median survival is shown compared to flies only expressing A β PP or BACE1 (A β PP (BACE1)). Observation of rough eye phenotypes when protein expression was directed to the retina of the fly. The total levels of A β ₁₋₄₂ (pg/fly) in the head of the fly when using *elav*-Gal4 or in the retina of the fly using *gmr*-Gal4 as a driver. Amyloid load and A β proteotoxicity categorised as (+) = low and (+++) = high.

A β toxicity in the A β PP-BACE1 flies

It is possible that production of A β in the A β PP-BACE1 flies could take place in intracellular compartments. In endocytic vesicles, pH decreases as the vesicles move towards lysosomes. In these relatively small, acidic compartments, BACE1 activity increases and as a consequence, the concentration of the A β peptide could also increase. If sufficiently high concentrations of the A β peptide accumulate, aggregation can occur, creating oligomeric species of A β that leads to malfunction of the endosomal/lysosomal degradation pathway. In AD brains, accumulation of autophagy-lysosomal intermediate vesicles has been found to induce the swelling of axons that form part of dystrophic neurons (Nixon et al., 2005). Nerve cells are post mitotic, and therefore more sensitive towards any waste that builds up due to a malfunctioning degradation system. If autophagy-lysosomal intermediate vesicles accumulate, these vesicles could eventually erupt, releasing the toxic, oligomeric A β species inside the cell. This would support our hypothesis that intracellular, endosomal/lysosomal oligomeric A β species contribute to the toxic effect observed in the A β PP-BACE1 flies.

In the A β PP-BACE1 flies both A β ₁₋₄₂ and A β ₁₋₄₀ (ratio 3:4 in the fly head) were detected which could influence toxicity. The aggregation and toxicity of different A β ₁₋₄₀:A β ₁₋₄₂ ratios was previously investigated and it was found that different ratios of A β ₁₋₄₂ to A β ₁₋₄₀ resulted in alterations in the process of forming oligomeric species affecting A β neurotoxicity (Kuperstein et al., 2010; Pauwels et al., 2012). An A β ₁₋₄₀: A β ₁₋₄₂ ratio of 3:7 resulted in increased nucleation and aggregation kinetics, while fibril formation was delayed, indicating that at this A β ₁₋₄₀: A β ₁₋₄₂ ratio the prefibrillary species formed are maintained in their oligomeric state unlike those prefibrillary species that are formed solely from the A β ₁₋₄₂ peptide. Thus, the co-existence of A β ₁₋₄₂ and A β ₁₋₄₀ in the A β PP-BACE1 flies could contribute to the toxic effect detected in these flies.

Production of A β peptides in the A β PP-BACE1 flies can also occur at the plasma membrane resulting in extracellular deposition of the peptide. Small quantities of amyloid aggregates were detected in the brain of the A β PP-BACE1 flies. Amyloid aggregates of A β have shown to possess toxic properties (Lorenzo and Yankner, 1994; Petkova, 2010); however, it is unlikely that the small amount of amyloid aggregates detected in the A β PP-BACE1 flies is responsible for the toxicity observed in these flies.

Another important aspect of the A β PP-BACE1 fly model is that since all components necessary to produce the A β peptide are present, the flies can also produce other cleavage products of both the non-amyloidogenic and the amyloidogenic pathway; fly intrinsic α -secretase as well as γ -secretase can process human A β PP *in vivo* (Fossgreen et al., 1998; Greeve et al., 2004). The N-terminal fragment that results from α -secretase cleavage of A β PP has been observed to promote neurite outgrowth and proliferation; sA β PP $_{\beta}$ produced from BACE1 cleavage has also been seen to have these effects, but in a much less potent manner (Chasseigneaux et al., 2011). sA β PP $_{\beta}$ can bind death receptor 6, inducing apoptosis (Nikolaev et al., 2009). The levels of sA β PP $_{\beta}$ were quantified in the heads and bodies of A β PP-BACE1 flies when protein expression was directed to the CNS where significant levels were found only in the bodies of the flies. Hence, the toxic effects observed in the A β PP-BACE1 fly model are likely not due to the generation of sA β PP $_{\beta}$ in these flies. Apart from sAPP $_{\beta}$, a 99-amino acid C-terminal fragment is also produced by BACE1 cleavage of A β PP. The C-terminal fragment has been linked to neurotoxicity and pathological activation of Rab5 leading to cholinergic neuronal death (Dumont et al., 2006; Kim et al., 2016). In a western blot analysis, an increased amount of the C-terminal fragments was detected in the A β PP-BACE1 flies compared to A β PP flies. Thus, some toxicity may arise from the C-terminal fragment produced after BACE1 cleavage. This could be a drawback when it comes to using the A β PP-BACE1 model to specifically study A β toxicity; however, the model resembles the process that takes place in patients suffering from AD more than the A β fly model with respect to the combined effect of cleavage products arising from A β PP processing

A β toxicity in the A β flies

In the A β fly model, high levels of the peptide are expressed with a secretion tag, which guides the peptide out of the cell. However, when Crowther et al. characterised A β ₁₋₄₂ flies used as the A β fly model, they observed intracellular A β accumulations (Crowther et al., 2005). This indicates that a fraction of the expressed A β peptide is either not secreted and remains intracellular, or is secreted but then taken up by the cell again, either by endocytosis or directly through the cell membrane since the peptide possesses detergent-like properties (Arispe et al., 1993a; Arispe et al., 1993b). If the A β peptide is taken up via endocytosis, it might end up in small compartments with low pH, which can promote aggregation of the peptide, leading to a cascade of cytotoxic events. Aggregation of the peptide might also occur before the peptide is secreted within intracellular compartments such as the ER or Golgi. Another possible scenario is that the extracellular accumulations of amyloid aggregates, detected in these flies, disrupt the surrounding nerve cells and their interactions with each other, thus contributing to toxicity. Amyloid aggregates have been found to sequester other proteins crucial for cell functions,

thereby inducing toxicity (Olzscha et al., 2011), thus toxicity in the A β flies could arise when the amyloid aggregates sequester proteins crucial for cell survival.

Taking these findings together, we believe that the A β induced toxicity in the A β fly models is caused by high levels of extracellular A β aggregates; however, intracellular aggregation of A β into toxic species cannot be ruled out for this AD fly model. The toxicity in the A β PP-BACE1 fly model is most likely caused by intracellular, oligomeric A β but other potential cleavage products, produced by A β PP processing, may also contribute to the toxicity observed in this AD fly model. It is important to take the different aspects presented above into account when choosing which AD fly model to use when studying disease mechanism or therapeutic possibilities for AD.

Lysozyme protects from A β toxicity in AD fly models

Drosophila AD models have previously been used to investigate how proteins and synthetic compounds can counteract the toxicity induced by over-expressing disease-associated proteins (Caesar et al., 2012; Hermansson et al., 2014). These AD models have been proven suitable to use for the evaluation of novel compounds that may have beneficial effects on disease progression.

The relationship between AD and inflammation has been studied; several immune genes have been found to be up-regulated in the early stages of the disease (Brosseron et al., 2014; Tarkowski et al., 2003). Lysozyme is expressed by astrocytes and microglia in the CNS and it has been shown to possess anti-inflammatory properties (Lee et al., 2009; Ogundele, 1998). In paper II, the role of lysozyme in AD was investigated. A strong correlation between AD pathology and lysozyme mRNA levels was observed in transgenic AD mice, and increased lysozyme levels were found in AD brains compared to controls. This is the first time lysozyme levels have been investigated in CSF from a cohort of patients who have been both biochemically and clinically diagnosed with AD; it has previously been observed that lysozyme levels are increased in the CSF from biochemically diagnosed patients (Helmfors et al., 2015). Co-expression of lysozyme was found to rescue the rough eye phenotype induced in both AD fly models (the A β and the A β PP-BACE1 fly model), and to increase the median survival time and reduce the dysfunctional locomotor behaviour in A β _{Arc} flies when protein expression was directed to the fly CNS. Thus, it is clear that lysozyme plays a protective role against A β induced toxicity, according to the results achieved from investigations using the different AD fly models. One possible explanation for this protective effect could be that lysozyme facilitates degradation of A β . In a paper previously published by our group, in which the A β fly model was used, expression of the protein was directed to the CNS using *elav*-Gal4. Co-expression with lysozyme reduced A β toxicity, which was observed as an increase in longevity and improved locomotor behaviour, and a significant reduction in the accumulation of insoluble A β species in flies that were aged for 20 days (Helmfors et al., 2015). These data point toward that lysozyme can promote degradation of A β in *Drosophila*. In paper II, no reduction of insoluble A β species was observed in either the A β PP-BACE1 or the A β fly model when the protein expression was directed to the retina of the flies indicating that lysozyme does not affect the degradation of A β in the fly retina. However, when using *elav*-Gal4, significantly lower levels of A β in flies co-expressing A β with lysozyme compared to flies only expressing A β were detected only at day 20 and not at day 0. When the *gmr*-Gal4 driver was used, the A β levels were only analysed at day 0, for consistency with SEM data, it is therefore not possible to rule out that lysozyme can have an effect on the A β degradation in the fly retina that could be detected in aged *gmr*-Gal4 flies. In addition, different drivers were used in the two experiments; thus protein expression was directed to different cell types (retina and CNS) that could have different abilities to degrade A β . From the immunoprecipitation assay it was found that A β and lysozyme interact *in vivo* in the A β PP-BACE1 flies pointing towards that the antitoxic effect of lysozyme could be due to the binding of lysozyme to A β leading to a rescue of the rough eye phenotype.

For the A β _{Arc} flies, co-expressing one or two copies of lysozyme in the fly CNS resulted in a dose-dependent rescue. A β levels were analysed 10 days after eclosion and no differences in A β _{Arc} levels were observed when it was co-expressed with one or two copies of lysozyme. The arctic variant of A β has been shown to be more aggregation-prone than A β ₁₋₄₂ (Nilsberth et al., 2001), thus, rapidly forming species that could be difficult for the cell to degrade. The rescue observed in the flies co-expressing A β _{Arc} with lysozyme could come from lysozyme binding to the toxic A β _{Arc} species (as found for the A β PP-BACE1 flies), thereby reducing A β _{Arc} toxicity rather than promoting degradation of the peptide. Lysozyme has been observed to prevent fibrillation of A β ₁₋₄₀, acting as a molecular chaperone (Luo et al., 2013), and lysozyme binds to monomeric A β ₁₋₄₂ *in vitro*, thereby reducing the amount of toxic A β ₁₋₄₂ species formed (Helmfors et al., 2015). Overall, the protective effect of lysozyme against A β induced toxicity can be correlated with lower insoluble A β levels, but decreased A β levels are not necessary; lysozyme interacting with A β *in vivo* is enough to result in a rescue effect.

Anti-toxic effects mediated by interaction between lysozyme and A β

For lysozyme and A β to interact, they need to co-localise. In the A β PP-BACE1 flies, there are two different sites where A β can be produced from BACE1 and γ -secretase cleavage of A β PP: 1) at the plasma membrane or 2) inside cellular compartments, such as endosomes/lysosomes, after endocytosis. Lysozyme is expressed with a secretion sequence, which guides it out of the cell, therefore the majority of the lysozyme should be present extracellularly. Lysozyme has been shown to bind to the N-terminal of A β ₁₋₄₀, while some hydrophobic interaction with the C-terminal part of A β has also been observed (Luo et al., 2013; Luo et al., 2014). The N-terminal of A β is not buried in the plasma membrane, thus it should be possible for lysozyme to interact with the A β sequence while it still is a part of A β PP (Fig. 13A). If lysozyme binds before BACE1 has cleaved A β PP, this could disrupt BACE1's ability to process A β PP. However, no difference in A β levels with or without lysozyme could be observed in the study described in paper II, indicating either that lysozyme binds full-length A β PP and BACE1 is still able to process it (Fig. 13A), or that lysozyme binds the C99 fragment produced after BACE1 cleavage (Fig. 13B). After cleavage of the C99 fragment by γ -secretase, A β is released already bound to lysozyme and lysozyme can prevent aggregation into toxic oligomeric species and therefore reduce A β induced toxicity (Fig. 13C). It is also possible that the extracellular lysozyme binds monomeric, extracellular A β after it is cleaved from A β PP (Fig. 13C). This is applicable for the A β fly model in which both A β and lysozyme have secretion tags, placing them both extracellularly where lysozyme can interact with monomeric A β , thus reducing the number of A β monomers that can form toxic oligomers (Fig. 13C). However, if the toxicity is caused by intracellular A β , lysozyme must be able to prevent A β toxicity intracellularly. A β PP can be internalised via endocytosis; if lysozyme binds to the A β sequence extracellularly before internalisation, lysozyme will be present in these cellular compartments and be able to bind monomeric A β , preventing it from oligomerisation (Fig. 13D). It is also possible that lysozyme is not secreted, and ends up in the lysosomes, where it may bind internalised A β PP (Fig. 13E).

Figure 13. Possible sites of interaction between lysozyme and A β . (A) Extracellular, secreted lysozyme could bind full-length A β PP, interacting with the N-terminal part of the A β peptide. (B) Lysozyme could either bind to the C99 fragment after BACE1 cleavage of A β PP, or be still bound to A β PP when BACE1 processes it. Thus, lysozyme will interact with monomeric A β when it is released from C99 by γ -secretase cleavage. (C) In the A β flies, both A β and lysozyme have secretion tags, placing them extracellularly, where lysozyme can interact with A β . (D) If A β PP is internalised with lysozyme already bound to it, this places both A β and lysozyme in the same intracellular compartments, thus preventing toxicity arising from A β . (E) A β PP can also be internalised without lysozyme bound to it; however, lysozyme could end up in the same intracellular compartments when it is transported from the ER/Golgi.

Anti-toxic effects induced by lysozyme independent of its interaction with A β

Co-expression of lysozyme in the flies could also result in beneficial effects, reducing toxicity, independent of its interaction with A β . Both inflammation and oxidative stress have been connected with AD in humans (Heppner et al., 2015; Huang et al., 2016). Lysozyme possesses anti-inflammatory properties; *in vitro* studies have shown that lysozyme inhibits activation of the complementary system in a dose dependent manner, thus reducing the inflammatory response (Ogundele, 1998). Hen egg lysozyme can also reduce expression of pro-inflammatory cytokines; this was observed in a porcine model of inflammatory bowel disease, a chronic inflammation of the gastrointestinal tract (Lee et al., 2009). Cytokines, which are mediators in immune processes, are conserved in evolution and immune signalling pathways that respond to cytokines are remarkably well preserved from flies to humans (Vanha-aho et al., 2016). Another potential physiological function for lysozyme is to reduce oxidative stress caused by reactive oxygen species (ROS). Lysozyme contains an 18-amino acid domain which

binds to advanced glycation end products that generate ROS. In a study using mice transgenic for hen lysozyme, an increased level of survival of severe oxidant injury induced by exposure to paraquat, an organic herbicide which is known to cause oxidative stress and neuronal death, was observed for the lysozyme transgenic mice compared to controls (Liu et al., 2006; McCarthy et al., 2004). Pre-incubation with lysozyme suppressed oxidant stress induced by exposing hepatocytes to paraquat (Liu et al., 2006). Oxidative stress has also been investigated in *Drosophila*, where several of the enzymes associated with the process, e.g. superoxide dismutase and catalase, have been found and increased levels of oxidative stress occur naturally as the flies age (Le Bourg, 2001). Taking these observations together, lysozyme may contribute to other beneficial effects in addition to those related to its interaction with A β in both AD fly models, i.e. by having positive impacts on both inflammation processes and ROS induced stress that potentially occurs in the flies.

SAP promotes formation of alternative aggregated lysozyme morphotypes

In paper III and IV, the focus shifted from AD to another protein misfolding disease, lysozyme amyloidosis, with the aim of investigating SAP role in this disease. SAP is a universal component of amyloid aggregates and its role in amyloid diseases is still not completely understood. In an earlier publication from our group, a *Drosophila* model of lysozyme amyloidosis was used to investigate the effect of expressing WT lysozyme and two disease-associated variants in the retina of the fly (Kumita et al., 2012). Expression of the lysozyme variants F57I and D67H gave rise to disruptions in the arrangement of ommatidia and an increase in the unfolded protein response. To further investigate the toxicity induced by the F57I variant and the impact of co-expressing lysozyme with SAP, in paper III, protein expression was directed to the CNS of the flies. Expression of F57I in fly CNS led to a decrease in median survival time and an increase in apoptotic cells compared to WT expressing flies and control flies. When F57I was co-expressed with SAP, SAP reduced the toxicity to levels comparable with that in control flies. We therefore speculated that the F57I lysozyme variant resulted in an alteration in the process of lysozyme aggregation, resulting in the formation of cytotoxic species, and that SAP is able to protect nerve cells from damage caused by F57I by preventing accumulation of toxic F57I structures. To achieve a better understanding of how SAP actually prevents apoptosis and restores the lifespan of F57I expressing flies, double-expressing lysozyme flies (WT-WT and F57I-F57I, $\pm$ SAP) were generated and studied in paper IV. Co-expression with SAP was able to rescue toxicity induced in F57I-F57I expressing flies, but not in WT-WT expressing flies. Two different anti-lysozyme antibodies were used, ab36362 and ab108508. Ab36362 detected lysozyme only in WT-WT $\pm$ SAP flies while ab108508 detected lysozyme in all flies. The locations of the different lysozyme species detected using the two anti-lysozyme antibodies differed; ab36362 detected lysozyme in the whole fly brain but showed accumulation and co-localisation with SAP in the SMP (superior medial protocerebrum). The lysozyme species detected by ab108508, on the other hand, accumulated in the medulla. This is also where the two amyloid specific LCOs detected lysozyme aggregates in WT-WT $\pm$ SAP (h-FTAA) and F57I-F57I $\pm$ SAP (both h- and p-FTAA), indicating that ab108508 is actually detecting

insoluble/aggregated lysozyme species. The schematic fly brain in Fig. 14 shows the different areas of the fly brain where the lysozyme antibodies and LCOs detected a signal.

Figure 14. Schematic fly brain showing where ab36362 (black square), and ab108508 as well as both LCOs (dotted square), detected lysozyme species.

The two different anti-lysozyme antibodies are both monoclonal antibodies, raised in mouse (ab36362) or rabbit (ab108508). However, ab36362 was raised using full-length native, human lysozyme purified from urine as the immunogen, while ab108508 was raised using a synthetic peptide, corresponding to a fragment of lysozyme (amino acid 50- 70). Thus ab36362 may be specific for native lysozyme while the epitope for ab108508 may be exposed mainly in aggregated lysozyme species. A summary of the different fly genotypes and the signals detected by antibodies and LCOs and their spectral properties is given in table 6.

Genotype	Median survival time (days)	ab36362 signal	ab108508 signal	p-FTAA (double peak emission spectra)	h-FTAA (fluorescence decay, ps)
WT-WT	29	++	+	-	+ (250-425)
WT-WT-SAP	29	++	++	-	+ (250-450)
F57I-F57I	21	-	+	+ (no)	+ (200-350)
F57I-F57I-SAP	35	-	++	+ (yes)	+ (250-400)

Table 6. Summary of median survival times, anti-lysozyme antibody signal, p- and h-FTAA positive signal and spectral properties. Median survival times in days. Lysozyme signal from ab36362, categorised as (-) = no signal and (++) = strong signal. Lysozyme signal detected by ab108508 categorised as (+) = some signal and (++) = strong signal. The p-FTAA positive signal was categorised as (-) = no signal, (+ (no)) = p-FTAA positive signal but no double peak emission spectra observed and (+ (yes)) = p-FTAA positive signal and double peak emission spectra observed. The h-FTAA positive signal was categorised as (+) = signal detected followed by the fluorescence decay time in ps.

The spectral properties of the LCOs differ depending on the structure to which they are bound as well as the environment surrounding them. When the fluorescence decay of h-FTAA was analysed, it was observed that co-expression with SAP increased fluorescence decay lifetime for F57I as well as for

WT lysozyme, but not to the same extent. Changes in the fluorescence decay lifetime indicate that co-expression with SAP results in structural alterations of the lysozyme species detected by h-FTAA. Fluorescence decay also differed between F57I and WT h-FTAA positive species, indicating that the aggregation process of the two lysozyme variants results in structurally different h-FTAA positive aggregates. Fluorescence decay data for the different genotypes can be seen in table 6. FLIM analysis of two different mouse models for prion disease has been performed using h-FTAA, and h-FTAA was found to give rise to distinct fluorescence decay lifetimes depending on what prion strain the aggregates were associated with (Magnusson et al., 2014). Interestingly, a shorter fluorescence decay was observed for the h-FTAA positive prion aggregates in the sheep scrapie mice compared with the fluorescence decay observed for aggregates detected in a mouse model for chronic wasting disease (CWD). The terminal onset for scrapie mice occurs much earlier than for CWD mice (Sigurdson et al., 2007), indicating that there is a greater toxic effect in the scrapie mice, in which h-FTAA fluorescence decay was observed to be shorter compared to that observed in the CWD mice (Magnusson et al., 2014). Since the shortest fluorescence decay was observed for the lysozyme species detected in the F57I-F57I flies, this strengthens our hypothesis that these species possess toxic properties. For the other LCO, p-FTAA, binding to mature amyloid aggregates gives rise to double peak emission spectra, which were observed for the p-FTAA positive species in F57I-F57I-SAP flies, whereas no such spectra could be observed in F57I-F57I flies. This indicates that co-expression of SAP changes the structure of the p-FTAA positive species, promoting the formation of aggregates that were more amyloid characteristic and could be detected in F57I-F57I flies in the presence of SAP. SAP has been found to induce the formation of A β fibrils and, at low concentrations, to accelerate the formation of, and stabilise, amyloid aggregates formed by A β ₁₋₄₂ (Hamazaki, 1995; Mold et al., 2012). In the absence of SAP, amyloid formation is delayed in a mouse model of AA amyloidosis (Botto et al., 1997). Thus, SAP can promote the formation of lysozyme aggregates with a more amyloidogenic character, thereby reducing toxicity in F57I-F57I flies.

Overall, we suggest that the toxicity observed in the F57I-F57I flies is due to the ab108508-, h-FTAA- and p-FTAA positive species and when SAP is co-expressed in these flies, it results in the aggregated lysozyme species having an alternative aggregation morphotype with a more amyloidogenic character, being less toxic. For WT-WT flies, a reduction in median survival time compared to control flies was observed with and without the presence of SAP. Ubiquitous expression of WT lysozyme during metamorphosis has previously been shown to be lethal, probably due to lysozyme's enzymatic properties (Kumita et al., 2012). Thus the reduction in median survival in both WT-WT and WT-WT-SAP flies could be due to the presence of high levels of native lysozyme that may possess enzyme activity that can induce toxicity that cannot be rescued by SAP.

The question of whether smaller, non-amyloidogenic species or mature amyloid aggregates are the main toxic species in amyloid disease has been extensively investigated during recent years; however, evidence has been presented suggesting that both possess toxic properties. In our *Drosophila* model of lysozyme amyloidosis, the toxic effect observed probably arises from intermediate species of F57I

formed on/off the pathway towards the more amyloidogenic-like lysozyme aggregates observed in the presence of SAP. Toxicity studies have shown that larger oligomeric lysozyme species induce toxicity in different cell cultures (Malisauskas et al., 2006), and both oligomers and fibrils have been observed to cause cell death, albeit via different mechanisms (Gharibyan et al., 2007). Less structured lysozyme aggregates, i.e. aggregates with a larger non-core region, have been found to induce a higher level of toxicity compared to more structured aggregates (Mossuto et al., 2010). The evidence reported for the role of SAP in amyloid disease is also contradictory; SAP has been found to both accelerate and suppress aggregation of the A β peptide and to bind to and stabilise amyloid aggregates (Hamazaki, 1995; Janciauskiene et al., 1995; Mold et al., 2012; Tennent et al., 1995). From the results obtained using our *Drosophila* model of lysozyme amyloidosis, SAP's role seems to be to promote the aggregation of non-amyloidogenic F57I species into more amyloid-like structures and thereby reduce the toxicity induced by the non-amyloidogenic intermediates formed on/off the pathway towards the more amyloid lysozyme morphotypes. A general mechanism for SAP's role in amyloid diseases could be that it promotes the formation of stable, amyloidogenic aggregates, thus decreasing the toxic impact of non-amyloidogenic species. However, in lysozyme amyloidosis, the main pathological event is the huge accumulation of amyloid aggregates, which eventually cause organ failure, implying that SAP promotes disease progression and that reduced SAP levels could be beneficial in patients suffering from systemic amyloidosis. Indeed, in an on-going clinical trial, the organic molecule CPHPC in combination with an anti-SAP antibody is used as a potential treatment strategy for systemic amyloidosis, targeting, and lowering the levels of, SAP both in plasma and in the amyloid aggregates (Richards et al., 2015). Positive results have been observed in a clinical phase I trial, where two injections of anti-SAP antibody in combination with CPHPC dramatically reduced the amyloid load in patients suffering from light chain and apolipoprotein AI amyloidosis. However, if the same toxic effects due to non-amyloidogenic lysozyme variant species observed in the F57I lysozyme flies occur in humans, depleting SAP may result in increased levels of intermediate lysozyme variant species that can possess cell toxicity.

Concluding remarks

The work included in this thesis has focused on further increasing the understanding of two amyloid diseases, AD and lysozyme amyloidosis, using *Drosophila* as a disease model. The effect of co-expressing other proteins that may impact the toxicity induced by over-expressing disease-associated, amyloidogenic proteins in *Drosophila* has also been investigated. Conclusions that can be drawn from the work presented in this thesis are:

- **A β proteotoxicity is higher in the A β PP-BACE1 fly model compared to the A β fly model.**
 - A β generated from A β PP processing in the fly CNS results in higher proteotoxicity compared with direct expression of A β from the transgene
 - It is important to take into consideration where and how the A β peptide exerts its toxicity when using *Drosophila* as a model organism for AD.
 - In addition to the A β peptide, the A β PP-BACE1 flies possess all the components necessary to produce other cleavage products than A β_{1-42} from A β PP, and such products may also contribute to the toxicity observed in these flies.
- **Lysozyme could have a beneficial role in AD.**
 - Lysozyme mRNA levels correlate with AD pathology in transgenic mouse models and lysozyme protein levels are increased in brain tissue and CSF from AD patients.
 - Lysozyme rescues A β induced toxicity in *Drosophila*.
 - A dose-dependent rescue by co-expressing lysozyme with A β was observed in A β_{Arc} flies.
 - Lysozyme is able to bind to A β which could prevent the peptide from exerting its toxic properties in *Drosophila*.
 - Lysozyme could potentially be used as a treatment strategy for AD.
- **SAP counteracts F57I induced toxicity in the fly CNS.**
 - Intermediate F57I species formed on the pathway towards insoluble species could be cytotoxic and contribute to the toxic effects in the F57I flies.
 - SAP reduces the decrease in longevity and the observed cell death in F57I flies.
 - SAP is able to convert intermediate species formed by F57I into alternative structures with amyloid properties, thereby reducing the toxicity induced by the F57I intermediate species; this is important to take into account when a reduced level of SAP is considered as a treatment strategy for lysozyme amyloidosis.

Future perspectives

The mechanism of A β induced toxicity in the A β and the A β PP-BACE1 fly model should be further investigated. A first step would be to investigate where A β species are located in the two different models; is A β located intracellularly, and if so, does it accumulate in specific cellular compartments?

The A β PP-BACE1 fly model is a suitable candidate in which to investigate the effects of substances that affect BACE1 processing of A β PP. In addition, many natural products have been proven to possess neuroprotective properties (Ansari and Khodagholi, 2013), e.g. rosmarinic acid (present in culinary herbs) and quercetin (present in apples, tea, wine etc.) and it would be valuable to investigate their effects in the two different *Drosophila* AD models.

The second hallmark of AD, apart from the amyloid aggregates, is the presence of NFTs consisting of hyperphosphorylated tau. A *Drosophila* tau homologue has been identified and a high degree of conservation between *Drosophila* and human tau was observed (Heidary and Fortini, 2001). It would be interesting to investigate how *Drosophila* endogenous tau behaves in both AD fly models; does over-expression of A β or A β PP and BACE1 affect the cellular location of *Drosophila* tau or its degree of phosphorylation? How does modulating *Drosophila* tau levels impact the phenotypes observed for the two AD fly models? This might give further insights into the interplay between tau and A β in AD.

To further investigate the mechanism behind how lysozyme is able to rescue A β induced toxicity, it would be of value to co-express lysozyme in the A β PP-BACE1 fly model by directing the protein expression to the CNS of the fly, but also to investigate the effect of co-expressing lysozyme in different variants of the A β fly model. A β peptides of various lengths have been observed to induce different levels of toxicity when expressed in *Drosophila* (Jonson et al., 2015). Tandem A β flies, expressing a dimeric A β construct, have shown an increase in soluble A β species with a corresponding increase in toxicity compared to single-expressing A β flies (Speretta et al., 2012); it would therefore be instructive to co-express lysozyme in these flies to further investigate how lysozyme is able to rescue A β induced toxicity.

Using the lysozyme amyloidosis *Drosophila* model, it would be interesting to further investigate the toxic properties of the different lysozyme species detected in the double-expressing lysozyme flies; could fly brain homogenates induce toxicity in cell cultures? It would also be of value to look at human

tissue samples from patients suffering from lysozyme amyloidosis and investigate the interaction between the LCOs and the lysozyme aggregates and how their spectral properties change upon binding, to further validate the results presented in paper IV.

To further study how SAP affects lysozyme aggregation and the structure of the amyloid aggregates formed, *in vitro* studies investigating lysozyme aggregation with and without the presence of SAP could be carried out. Mutant lysozyme can be expressed and purified from *Aspergillus niger* and *Pichia pastoris* (Canet et al., 1999; Johnson et al., 2005). The lysozyme species formed could be analysed with LCO staining and electron spectroscopy. The mutations associated with the disease-associated lysozyme variants have been observed to differ in the ways in which they affect the stability and aggregation process compared to the WT protein (Canet et al., 1999; Dumoulin et al., 2005); it would therefore be interesting to investigate SAP's interaction with the different lysozyme variants and its effects on the aggregation process.

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The reason that we work with flies is not because they are cute,
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