The influence of lysozyme and oligothiophenes on amyloid- β toxicity in models of Alzheimer's disease

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Cover picture of a healthy brain and a brain with Alzheimer's disease, illustrated by Samuel Westergren.

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Till mina pärlor

Better an oops than a what if.
Beau Taplin

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease and the most common cause of dementia worldwide. Apart from dominantly inherited mutations, age is the major risk factor and as life expectancy increases the prevalence for AD escalates dramatically. AD causes substantial problems for the affected persons and their families, and the society suffers economically. To date the available treatments only temporarily relieve the symptoms, wherefore the development of a cure is of utmost importance. The etiology of AD is still inconclusive but many believe that small aggregates (oligomers) of the protein amyloid- β ($A\beta$) are central for the onset of AD. The aims of this thesis were to investigate how different molecules affect the aggregation and toxicity of $A\beta$. In paper I and II, two oligothiophenes were studied; p-FTAA and h-FTAA and in paper III and IV the inflammatory protein lysozyme was explored. Differentiated neuroblastoma cells and *Drosophila melanogaster* were used as models of AD to address the issue.

The results show that p-FTAA rescues neuroblastoma cells from $A\beta$ toxicity when $A\beta$ is co-aggregated with lysozyme. Various biophysical studies show that the co-aggregation increases the formation of fibrillar $A\beta$ structures rich in β -sheets. Noteworthy, these $A\beta$ fibrils were more resistant to both degradation and denaturation, and less prone to propagate seeding from $A\beta$ monomers. Furthermore, h-FTAA, but not p-FTAA, was able to protect neuroblastoma cell toxicity when exposed to $A\beta$ with the Arctic mutation ($A\beta_{Arc}$), which probably reflects the weaker binding of $A\beta_{Arc}$ to p-FTAA, compared to h-FTAA.

Lysozyme levels were increased in CSF from patients that were both biochemically and clinically diagnosed with AD. In mice models of AD it was revealed that the mRNA increase in lysozyme correlates to increased $A\beta$ pathology, but not to tau pathology, indicating that $A\beta$ could drive the expression of lysozyme. To evaluate the effect for increased expression of lysozyme, co-expression of lysozyme was achieved in flies that expressed $A\beta$ in the retina of the eyes, or in flies that expressed $A\beta_{Arc}$ in the central nervous system. In all AD fly models, co-expression of lysozyme protected the cells from the $A\beta$ induced toxicity. Of note, flies that expressed the toxic $A\beta_{Arc}$ in

the CNS of the flies showed an improvement in both lifespan and activity. Finally, we demonstrate that A β aggregating in the presence of lysozyme inhibits the cellular uptake of A β and also the cytotoxic effect of A β .

The work included in this thesis demonstrates that the oligothiophenes p-FTAA and h-FTAA, and also lysozyme have the potential to be used as treatment strategies for sporadic AD, but remarkable, also in familial AD with the highly toxic Arctic mutation. The protective mechanism of p-FTAA seems to be attributed to the ability to generate stable A β fibrils with reduced seeding capacity, and that lysozyme inhibits the neuronal uptake of A β , which could prevent both the intracellular toxicity and cell-to-cell transmission of A β .

POPULÄRVETENSKAPLIG SAMMANFATTNING

Alzheimers sjukdom är den vanligaste formen av demens och uppträder ofta sent i livet om man inte är bärare av en ärftlig mutation som orsakar Alzheimers sjukdom. Sjukdomen leder till att hjärnans nervceller långsamt förtvinar, vilket ger upphov till både fysiska och psykiska funktionsnedsättningar som så småningom leder till döden. Det finns idag inget botemedel, utan behandlingen syftar endast till att tillfälligt lindra de symptom som uppstår. Orsaken till Alzheimers sjukdom tros bero på proteinet amyloid- β ($A\beta$) som klumpar ihop sig (aggregerar) och ger upphov till de senila plack som finns i hjärnan hos patienter med Alzheimers sjukdom. Tidigare ansågs placken vara orsaken till nervcellsödöden. Idag anses mindre aggregat, eller själva aggregeringsprocessen när små enskilda $A\beta$ peptider aggregerar och bildar större komplex, förstöra nervcellerna.

Syftet med avhandlingen är att undersöka hur p-FTAA, h-FTAA och lysozym binder till $A\beta$, påverkar aggregeringsprocessen av $A\beta$ och vilken effekt de har på hur giftigt $A\beta$ är. Genom att göra biofysikaliska experiment i provrör, modellera Alzheimers sjukdom i neuroblastomceller och bananflugor samt använda hjärnvävnad från avlidna patienter med Alzheimers sjukdom har detta studerats.

Delarbete I visar att $A\beta$ som aggregerar tillsammans med p-FTAA inte längre är giftigt; p-FTAA skyddar cellerna. Den skyddande effekten beror på att p-FTAA påskyndar aggregeringsprocessen av $A\beta$, och de stora komplex som bildas är mindre giftiga och mer stabila än $A\beta$ som aggregerar utan p-FTAA. I delarbete II undersöktes hur p-FTAA och h-FTAA påverkar $A\beta$ med den arktiska mutationen ($A\beta_{Arc}$), en mutation där bäraren av mutationen i tidig ålder utvecklar Alzheimers sjukdom. $A\beta_{Arc}$ som aggregerade tillsammans med h-FTAA motverkade giftigheten hos $A\beta_{Arc}$ medan p-FTAA inte hade någon effekt på $A\beta_{Arc}$ giftigheten. En trolig förklaring till detta är att det är en starkare bindning mellan $A\beta_{Arc}$ och h-FTAA jämfört med p-FTAA och $A\beta_{Arc}$. Delarbete III visar att nivåerna av lysozym, ett protein involverat i immunförsvaret, är förhöjt i hjärnan vid Alzheimers sjukdom. I olika musmodeller av Alzheimers sjukdom påvisades att uttrycket av lysozym stämde överens med förekomsten av $A\beta$ plack i mössens hjärnor, vilket tyder på att $A\beta$ kan vara orsaken

till ökningen av lysozym. För att undersöka vilken effekt höga nivåer av lysozym har vid Alzheimers sjukdom konstruerades alzheimersjuka bananflugor med höga nivåer av A β och höga nivåer av lysozym. Lysozym visade sig ha en positiv effekt på alzheimerflugorna då längre överlevnad och förbättrad aktivitet uppmättes i flygorna som hade höga nivåer av både lysozym och A β . I dessa flygor var A β bundet till lysozym, vilket påvisar att lysozym via direkt bindning till A β kan förändra dess giftighet. Det har tidigare visats att intracellulärt A β är mycket giftigt. Delarbete IV visar att A β som får aggregera tillsammans med lysozym hämmar det cellulära upptaget av A β och därigenom den giftiga verkan av A β . Således kan lysozym via sin A β -bindande förmåga förhindra cellulärt upptag och giftiga effekter av A β .

Sammanfattningsvis visar den här avhandlingen att p-FTAA, h-FTAA och lysozym via bindning till A β minskar A β s giftiga effekt. Dessa tre ämnen har därmed potential att i framtiden användas vid utvecklingen av nya behandlingsmetoder för Alzheimers sjukdom.

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman number I-IV:

- I. **The luminescent oligothiophene p-FTAA converts toxic A β ₁₋₄₂ species into nontoxic amyloid fibers with altered properties**
Livia Civitelli, **Linnea Sandin**, Erin Nelson, Sikander Iqbal Khattak, Ann-Christin Brorsson and Katarina Kågedal
Journal of Biological Chemistry 291(17):9233-43, 2016
- II. **The luminescent conjugated oligothiophene h-FTAA attenuates toxicity of amyloid- β with the Arctic mutation**
Linnea Sandin, Simon Sjödin, Livia Civitelli, Ann-Christin Brorsson and Katarina Kågedal
Manuscript
- III. **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, Aug 26, 2016
- IV. **Lysozyme attenuates the cellular uptake of amyloid- β**
Linnea Sandin, Liza Bergkvist, Claudia Kielkopf, Livia Civitelli, Karin Öllinger, Ann-Christin Brorsson, Sangeeta Nath and Katarina Kågedal
Manuscript

ABBREVIATIONS

Aβ	Amyloid- β
AD	Alzheimer's disease
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ANS	8-anilino-1-naphthalene sulfonate
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	β -site amyloid precursor protein cleaving enzyme 1
CD	Circular dichroism
CNS	Central nervous system
CSF	Cerebrospinal fluid
FINGER	Finnish geriatric study to prevent cognitive impairment and disability
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HFIP	Hexafluoroisopropanol
h-FTAA	Heptameric formic thiophene acetic acid
IDE	Insulin degrading enzyme
ISF	Interstitial fluid
LCO	Luminescent conjugated oligothiophenes
LRP	Lipoprotein receptor-related protein
MSD	Meso scale discovery
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PEN2	Presenilin enhancer 2
PET	Positron emission tomography
p-FTAA	Pentameric formic thiophene acetic acid
PS1 (2)	Presenilin 1 and 2
RA	Retinoic acid
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
TAMRA	Tetramethylrhodamine
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
ThT	Thioflavin T
UAS	Upstream activating sequence

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia and it is estimated that 36 million people are affected worldwide and 100 000 people in Sweden [1, 2]. AD was first described in 1907 by the German physician Alois Alzheimer who had a patient with profound memory loss and confusion. Upon brain autopsy of the patient Dr. Alzheimer discovered abnormal deposits in the cerebral cortex [3]. This kind of deposits, plaques, was 80 years later identified to be aggregated forms of the peptide amyloid- β ($A\beta$). $A\beta$ together with neurofibrillary tangles (NFT) which consist of hyperphosphorylated tau are now acknowledged as the main pathological hallmarks of AD [4, 5]. The $A\beta$ -plaques were previously considered to be the cause of the observed neuronal death. Today the general view is that smaller soluble aggregates of $A\beta$ and tau are the most toxic species, and that the accumulation of large aggregates appear later in the disease [6-8].

AD is characterized by progressive memory loss due to loss of synapses and neuronal cell death especially in the cortex and hippocampus, areas involved in thinking and formation of new memories. Diagnosis of AD is based on memory tests and clinical measurements. However, many dementia disorders have overlapping symptoms and AD can only be definitely diagnosed post-mortem after autopsy of the brain. Controversy remains about what initiates the disease process but there are many indications that the cause of the massive neuronal cell death is attributed to $A\beta$. One

of the main indications is that familial forms of AD are caused by mutations linked to A β . However, the majority of people with AD are suffering from the sporadic form, with the same pathological hallmarks as the familial form, but where the reason for the increased A β accumulation is not known despite many years of research.

Although age is the greatest risk factor to develop AD, other risk factors are of importance for disease development. Traumatic brain injuries and risk factors for cardiovascular diseases, such as smoking, obesity and type II diabetes, are associated with a higher risk of developing AD, whilst higher education, bilingualism and being socially and physically active are considered protective factors [9]. In addition, there is also genetic predispositions to develop AD [10]. Since age is the greatest risk factor and life expectancy is increasing, the prevalence of AD is rising dramatically causing great suffering for the affected and their families and enormous healthcare costs. The situation is urgent and it is therefore of great importance to reveal the pathological mechanisms that drive the disease in order to develop treatment strategies for AD.

APP and generation of A β

The amyloid precursor protein (APP) is a transmembrane protein encoded by a gene located on chromosome 21 [11]. Several isoforms exist but APP₇₅₁ is the most commonly expressed isoform throughout the body except for the brain where APP₆₉₅ is the main variant, which is situated in the plasma membrane of neurons [12]. The physiological role of APP is unclear but APP knock-out studies in mice show numerous defects that indicate functions in neurite outgrowth, synaptic plasticity, adhesion and regulation of neuronal excitation [13].

Processing of APP

The processing of APP can either occur by the amyloidogenic pathway where A β is produced and the non-amyloidogenic pathway where the p3 peptide is generated (Figure 1) [14]. In the amyloidogenic pathway A β is produced through processing by

the two proteases β - and γ -secretase. β -secretase initiates the $A\beta$ production by cleaving APP at the N-terminal of the $A\beta$ peptide and sheds of soluble $APP\beta$ (sAPP β). The remaining C-terminal fragment, β CTF, is further cleaved by γ -secretase within the membrane to release $A\beta$ and a small C-terminal fragment called APP intracellular domain (AICD) (Figure 1).

Figure 1. Overview of the processing of APP. In the amyloidogenic pathway APP is first processed by β -secretase where soluble APP β (sAPP β) is released, followed by γ -secretase cleavage to release $A\beta$. The APP intracellular domain (AICD) is left in the membrane. In the non-amyloidogenic pathway APP is cleaved by α -secretase within the $A\beta$ region of APP, thereby preventing generation and release of $A\beta$. Soluble APP α (sAPP α) is released and the remaining α C-terminal fragment (α CTF) can further be cleaved by γ -secretase to release the p3 fragment. After processing of APP by the secretases a short transmembrane part is remained; the AICD.

The activity of β -secretase has its optimum at pH 4.5 [15] and cleavage occurs predominantly in acidic organelles like endosomes [16]. γ -secretase is a protein complex that consists of four subunits: presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx-defective 1 (APH-1) and the presenilin enhancer 2 (PEN2) [17]. The subcellular site for cleavage of APP by γ -secretase in neurons is not completely determined. There are data indicating the presence of proteolytically active γ -secretase at the plasma membrane and in endosomal/lysosomal compartments, where also β -secretase is located, therefore it is conceivable that cleavage of APP by β - and γ -secretases occurs in the same cellular compartments [18]. In order for these secretases to interact with APP, APP needs to be internalized from the plasma membrane and to enter the endocytic pathway [19]. The plasma membrane, the endoplasmic reticulum (ER) and mitochondria are other proposed sites of APP processing [14]. Depending on where the imprecise γ -secretase cleaves, $A\beta$ peptides of different lengths are produced, ranging from 37-43 amino acids, where $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most abundant forms found in plaques [20].

In the non-amyloidogenic pathway, the α -secretase, a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), cleaves in the middle of the $A\beta$ domain and thereby prevents the formation of $A\beta$ (Figure 1). Apart from precluding the formation of $A\beta$ the activity of α -secretase has additional beneficial properties. α -secretase cleavage release a large N-terminal part, sAPP α , which after γ -secretase cleavage release the p3 peptide. sAPP α has been shown to have neuroprotective properties such as regulation of neurite outgrowth and synaptogenesis and protection of neurons against glutamate and $A\beta$ [21-23]. Hence, to increase the activity of α -secretase has been investigated as a potential treatment strategy.

Secretion of $A\beta$

Following APP processing in neurons $A\beta$ can subsequently be released into the extracellular space. This has been shown to be regulated by neuronal and synaptic activity, where increased activity leads to increased secretion of $A\beta$ [24, 25]. $A\beta$ is

present in synaptic vesicles but this only accounts for a fraction of the released A β [26]. Hence, the mechanism for release of A β was hypothesized to be closely associated to vesicle exocytosis. The process was later demonstrated to be dependent on endocytosis [27]. Upon synaptic activity, synaptic vesicles fuse with the plasma membrane and release their content, which subsequently results in increased endocytosis to recycle back the vesicular membranes. During this process APP is also internalized into the endocytic compartment where β - and γ -secretases are located, which in turn leads to increased A β generation and secretion [27]. In addition, activation of N-methyl-D-aspartate (NMDA) receptors, glutamate receptors involved in learning, memory and synaptic plasticity, inhibits α -secretase, thereby further promoting A β production [28].

In addition to extracellular A β , a large number of studies have shown the presence of intracellular A β [29-32], but whether the intracellular proportion of A β is due to intracellular generation, lack of secretion of A β or secretion and then re-uptaken remains elusive. The intracellular A β have been localized to intracellular compartments such as endosomes, lysosomes [33, 34] and mitochondria [35, 36].

Mutations linked to Alzheimer's disease

Besides increasing age, genetic mutations are the strongest risk factor for developing AD. AD is broadly classified into two forms depending on the onset age of disease. Early onset AD often strikes before the age of 60 and is in some cases due to rare familial, autosomal dominant mutations in genes related to A β production: *APP* [37, 38], *PS1* [39-41] and *PS2* [42]. All known mutations to date that cause familial AD either increase the production of A β or its propensity to aggregate. The other form of AD; late onset AD accounts for 99 % of all cases and is referred to as sporadic AD, where no obvious inheritance factor have been found. However, genetic predisposition still seems to play an important role [43]. One well established genetic risk factor for AD is carrying the $\epsilon 4$ allele of the apolipoprotein gene (*APOE*) [44]. ApoE exists in three isoforms, apoE2, apoE3 and apoE4, and is involved in lipid transportation and

clearance of A β from the brain. However, the apoE4 variant is supposed to bind A β less efficiently and thus the risk for developing AD is increased in people carrying this variant [45, 46]. One copy of the ϵ 4 allele increases the risk to develop AD threefold and two copies increase the risk 15-fold [47]. The ϵ 2 allele on the other hand seems to be protective as few AD patients carry this isoform [48]. This is further supported by the finding of later onset of AD in individuals with a familial mutation in *APP* who are heterozygous for *APOE2* [48, 49]. Another mutation that confers protection against AD and cognitive decline is a mutation in the *APP* gene (A673T) found on Iceland, located near the β -site cleavage in *APP* that results in lower A β production [50].

Further candidate genes that could be linked to AD have been identified with genome-wide association studies. These genes fall into clusters associated to three main pathways related to cholesterol and lipid metabolism, the immune system or endosomal vesicle cycling [10]. However, compared to *APOE4* the increased risk for AD in individuals carrying these susceptibility genes is modest.

There are a variety of mutations associated with early onset AD. The AD mutations used in the work of this thesis are the Arctic and Swedish mutations of *APP* and the M146V mutation of *PS1*.

The Arctic mutation of APP

The Arctic mutation of *APP* (E693G) is located within the A β sequence of *APP* and substitutes glutamic acid for glycine at codon 693. It was first described in a family of northern Sweden, and affected individuals develop AD at an early age [51]. The Arctic mutation is different from many other mutations in *APP*, as the amino acid substitution leads to increased stability of the formed toxic protofibrils.

The Swedish mutation of APP

The Swedish mutation of *APP* (APP KM670/671NL) is a double mutation located immediately adjacent to the β -secretase site of *APP* [52], where lysine and methionine

are replaced for asparagine and leucine at codons 670 and 671. This mutation leads to increased β -secretase cleavage resulting in increased production of total $A\beta$ and is commonly used in mouse models of AD, with the Tg2576 model being the most well characterized [53].

The M146V mutation of PS1

The M146V mutation substitutes methionine for valine at codon 146 of PS1 and has been found in at least 15 families worldwide and is traced to a family in Italy during the 17th century [41]. The mutation leads to increased levels of $A\beta_{1-42}$ and increased ratio of $A\beta_{1-42}/A\beta_{1-40}$ and is used in several mouse models of AD, either as single transgenic or as double transgenic in combination with the Swedish mutation of APP [54, 55].

The amyloid cascade hypothesis

Since the discovery of $A\beta$ as the main component in senile plaques and the identification of mutations in *APP*, *PS1* and *PS2* were found in families with early-onset AD, the amyloid cascade hypothesis has been central in the field of AD research [56, 57]. The hypothesis states that accumulation of $A\beta$, either due to increased production or reduced clearance of $A\beta$, is the initiating event in AD leading to formation of NFTs, inflammation, oxidative stress and finally neurodegeneration. As sporadic AD has a similar pathological phenotype as familial AD it was assumed that $A\beta$ could be responsible for both forms of AD. Additional genetic findings that supports the hypothesis are that individuals with Down's syndrome who carry an extra copy of the *APP* gene due to trisomy of chromosome 21, develop AD at an early age and that the Icelandic *APP* mutation (A673T) leads to decreased $A\beta$ production and protects against AD [50, 58]. However, it is not exactly clear how $A\beta$ leads to AD and whether $A\beta$ has a direct impact on the formation of NFTs, the other pathological hallmark of AD. Several studies have indicated that tau pathology is downstream of $A\beta$ accumulation [59-61], but in order for $A\beta$ to convey toxicity tau needs to be

present [62-64], which has raised concerns about the validity of the hypothesis. There are also studies showing substantial A β deposition in transgenic mice without evidence of neuronal loss [53, 65, 66]. In addition, there is low correlation between plaque load and neurodegeneration [67, 68]. This is in agreement with brain autopsies of elderly people that were cognitively normal, but had brains filled with amyloid deposits [69]. Instead the neuronal degeneration correlate better with the amount of NFTs [70]. However, emerging evidence suggests that the amount of soluble A β oligomers correlate well with synaptic loss and the degree of cognitive decline [71-73]. In particular, it is the intraneuronal accumulation of A β that is viewed as the most pathogenic form [31].

A β aggregation

For a protein to become functionally active it needs to fold into a three-dimensional conformation, which is determined by the amino acid sequence. This unique native state corresponds to the most stable conformation under physiological conditions. However, there are a tremendous amount of possible conformations for a polypeptide chain. Molecular chaperones are a group of proteins essential for correct and efficient folding of proteins to avoid aggregation *in vivo* [74]. Misfolding diseases arise when proteins adopt non-native conformations with an increased propensity to aggregate, which can be due to destabilizing mutations or extreme conditions of pH and temperature [75]. In AD aberrant folding of A β is involved, where the accumulation of misfolded protein probably overloads the chaperone capacity. The fibrillation of A β from monomers to fibrils and eventually plaques are a complex process that involves several intermediate steps that takes many years in the human brain. The definition of an amyloid protein is based on extracellular localization of fibrils with characteristic appearance in EM, staining with the dye congo red resulting in green birefringence upon illumination with polarized light and a typical X-ray diffraction pattern, revealing a cross- β -sheet structure [76] .

A β is released from APP processing as a monomer. This is a natural event which occurs in healthy individuals throughout life, with a continuous balance between A β production and clearance. The abnormal aggregation of A β occurs when the concentration of A β monomers is increased, either due to increased production or decreased clearance of A β [77]. *In vitro* studies have demonstrated that spontaneous aggregation of A β occurs at μ M concentrations [78, 79]. The concentration of A β in the extracellular space and cerebrospinal fluid (CSF) is in the lower nM range [80, 81] thus, spontaneous aggregation should not occur here under normal circumstances. However, it is possible that soluble A β can be taken up by cells and be concentrated in acidic vesicles, such as endosomes and lysosomes, where the low pH would favor aggregation [82]. In addition, plaques release A β which creates a local increase in A β concentration in the area surrounding the plaque, providing favorable conditions for aggregation [83]. During A β aggregation monomers self-associate to form soluble oligomers of various sizes and fibrils that eventually assemble into insoluble plaques in the human brain, although the exact mechanism for formation of plaques is still controversial (Figure 2) [84].

Figure 2. Schematic illustration of A β aggregation in AD. Monomers self-associate and form oligomers, protofibrils and fibrils that eventually lead to A β plaques in the human brain. Fibrils can also act as a template for oligomerization by secondary nucleation where monomers form oligomers on the surface of fibrils.

The aggregation of A β depends mostly on hydrophobic regions and since the A β_{1-42} isoform is more hydrophobic than A β_{1-40} it is more prone to aggregation [85]. The mechanisms of A β fibril formation remain unclear but it is believed that the fibrillization requires nucleation, the formation of a nuclei seed, where A β monomers convert from α -helical or random coil structure to β -sheet conformation [86]. This constitutes the lag phase and occurs when the monomers exceeds a certain critical concentration. This is followed by an elongation phase where the formed nuclei seed further aggregation and the fibril concentration increases rapidly until equilibrium is reached. It is now generally acknowledged that A β toxicity requires aggregation of native A β monomers, but the exact aggregation state of the toxic species have been difficult to characterize [6, 7]. Several different intermediate oligomeric assembly states with different size and characteristics have been reported: dimers, trimers, pentamers, hexamers, A β -derived diffusible ligands (ADDLs) which are oligomers ranging from 17-42 kDa and protofibrils [87]. In 2006, an A β form of 56 kDa, named A β^* 56, was found in transgenic AD mice, and proposed to be the toxic species [88], but this finding has been hard to confirm in human brains [89]. However, A β^* 56 correlates with neurodegenerative markers in the CSF of humans. In experimental settings A β assembly has been shown to vary depending on for example pH, temperature, salts, detergents, lipids, metal ions and fatty acids [87] but whether these have an effect *in vivo* remains to be elucidated. It is conceivable that metal ions can drive A β aggregation in AD since there are evidence of metal ions in A β plaques [90] and there are high concentrations of metal ions in and around synapses.

It is believed that plaques are inert within the brain but serves as reservoirs for oligomers [83]. Neurons in close association to plaques show signs of dystrophic neurites and synaptic loss [91]. This is supposed to be due to that plaques are in constant equilibrium with surrounding oligomers with constant association and dissociation, and partly through that A β fibrils can drive oligomer formation by secondary nucleation, where the surface of fibrils catalyze formation of new aggregates through interactions with monomers (Figure 2) [83, 92].

Although it is established that the aggregation of A β is essential for toxicity little is known about how it is initiated. It has been shown that injection of brain extract from old APP transgenic mice or AD brains containing various A β assemblies are able to induce A β aggregation in young APP transgenic mice [93-95]. This has raised the question if A β misfolding could spread like prion proteins, by inducing a conformational change in other A β peptides that it encounters.

A β toxicity

Although there is evidence for toxicity of extracellular A β depositions in the brain the AD field now agrees that small soluble A β oligomers are the primary neurotoxic agent in AD. The *in vivo* evidence for this is the poor correlation with plaque load and cognitive decline [67-69] and that neuropathological changes in AD transgenic mice occur long before A β deposits are apparent [96, 97]. In experimental studies A β oligomers are synaptotoxic [98, 99], neurotoxic [88] and have been shown to activate immune cells [100] and destroy cellular membranes [101, 102]. The exact mechanisms by how A β oligomers convey toxicity *in vivo* in humans are still unknown but a number of mechanisms have been proposed. A β is a sticky protein and has been shown to bind several transmembrane receptors, including the NMDA receptor, nicotinic acetylcholine receptors, the ApoE receptor and the cellular prion protein, thereby it might induce abnormal cell signaling that eventually leads to synaptic dysfunction and neuronal loss [103-105]. Another hypothesis is that A β binds to membranes of neurons and forms channels that disrupt the membrane integrity leading to unregulated Ca²⁺ influx as well as other ion disturbances in the end leading to neuronal death [105].

Moreover, several lines of evidence show that oxidative stress and the production of free radicals are involved in AD and contribute to neuronal death but whether it is a cause or consequence of the disease is not elucidated [106]. The increased level of oxidative stress could be associated with neuroinflammation [107, 108], since activated microglia produce nitric oxide which in turn gives rise to reactive oxygen species, but the high levels of redox-active metals in AD brains [109, 110] or

dysfunctional mitochondria [111] could also be responsible for the increased oxidative damage seen in AD. The central nervous system (CNS) is particularly vulnerable to oxidative stress due to high consumption of oxygen, low levels of antioxidants and the high content of polyunsaturated lipids susceptible for oxidation [112-114].

Under physiological conditions A β is secreted as a heterogeneous mixture of different A β species, partly through differential cleavage of APP by γ -secretase but A β can also be post translationally modified, which in turn can influence the toxicity [115, 116]. APP processing generates an array of different A β isoforms, where A β_{1-40} is the most common form, followed by A β_{1-42} and small quantities of other A β isoforms ranging from 27 to 43 amino acids. A β_{1-38} has *in vitro* been shown to enhance cytotoxicity of A β_{1-40} , whilst acting in a protective fashion when together with A β_{1-42} [115]. A β containing pyroglutamic acid is increased in AD [117], which is a result of post-translational modification where residues 1 and 2 of A β are removed by proteolytic cleavage and thereafter cyclizes at residue 3 by glutaminy cyclase to a pyroglutamate. Pyroglutamylated A β aggregates faster and is more toxic than A β_{1-42} and have been proposed to trigger AD by inducing misfolding of A β_{1-42} [116].

The role of intraneuronal A β in AD is controversial, but there are indications for intraneuronal A β causing neurotoxicity. Evidence for disturbed autophagy has been demonstrated in AD patients, which exhibit swollen autophagosomes filled with undegraded material [118]. An impaired function of the lysosomal system could increase the levels of intracellular A β since lysosomes degrade A β . In addition, *in vitro* studies have shown that aggregated forms of A β_{1-42} are resistant to degradation which also could explain why A β_{1-42} accumulates in neurons [119, 120]. Lysosomal accumulation of A β_{1-42} has previously been reported to cause loss of lysosomal membrane stability with release of lysosomal hydrolases into the cytosol [121]. The leakage of lysosomal hydrolases, especially cathepsins, may in turn initiate apoptosis [122, 123] and contribute to neurodegeneration [124]. Not only lysosomes but also mitochondria has been shown to accumulate A β_{1-42} and signs of mitochondrial dysfunction are evident in early stages of AD [125-128].

A β clearance

According to the amyloid cascade hypothesis it is believed that AD is either caused by increased production or decreased clearance of A β . In sporadic AD, evidence suggests an abnormal A β clearance mechanism [77]. As neurons are post mitotic cells their survival relies on systems that can eliminate damaged cellular constituents or misfolded proteins since they cannot dilute the burden by cell division. The proteasome and the lysosomal system are the main degradation systems in the cell where the proteasome mainly degrades short-lived proteins and abnormal proteins marked with ubiquitin, and the lysosome degrades long-lived proteins, larger protein aggregates and organelles. There is crosstalk between the systems and they can to some extent compensate for loss of function for each other. Ubiquitinated proteins can be degraded by the lysosome, and upregulation of autophagy and the lysosomal system is evident upon proteasome inhibition [129, 130].

Disturbances in both major degradative systems have been reported in AD. The proteasome activity is decreased in AD patients compared to healthy individuals [131], and both plaques and tangles contain ubiquitinated proteins which indicate that the proteasome has become incapable of degrading the proteins [132]. In addition, it was assumed early on that lysosomes might be involved in AD as an accumulation of lysosomes were apparent in dystrophic neurites [133]. This accumulation was later confirmed to be due to an upregulation and/or dysfunction of the lysosomal system [134, 135]. In animal models of AD lysosomal accumulation of A β has been shown [136] and neurons exposed to A β show lysosomal accumulation of A β and AD-like phenotype with neurite destruction [124, 137]. It is believed that the acidic pH in lysosomes favors aggregation and accumulation of A β resulting in lysosomal membrane instability, leakage of lysosomal hydrolases and neurodegeneration [129].

Besides A β degradation by the proteasome or lysosome, an array of proteases are known to digest A β , with neprilysin and insulin degrading enzyme (IDE) being two of the main A β degrading proteases [138]. Neprilysin is almost exclusively expressed in neurons where it is able to degrade both monomeric and oligomeric A β when it is in

close association with membranes [139]. IDE is most abundant in the cytosol but it is also present in the extracellular space. It solely degrades monomeric A β since its structure only allows complete binding of small molecules [140].

A β can also be cleared from the brain through transport across the blood brain barrier, mainly by the low-density lipoprotein receptor-related protein 1 (LRP) [141]. LRP mediates clearance of both free A β and A β bound to apoE2 and apoE3, but not apoE4. Furthermore, soluble LRP in plasma is a major A β binding protein that prevents free A β to enter the brain. Free A β and A β bound to soluble LRP ends up in the liver or kidney for systemic clearance [141].

Treatment

Current treatments

Today there is no treatment to halt or reverse AD, rather the treatment regimens aim to relieve the symptoms of the neurodegeneration. During the disease progression synapses and cholinergic and glutamatergic neurons are degenerated which lead to disrupted levels of the neurotransmitters acetylcholine and glutamate [142, 143]. Acetylcholine is important for learning and memory and one treatment strategy is to inhibit acetylcholinesterase, an enzyme that catalyzes the breakdown of acetylcholine at the synapse [144, 145]. By prolonging the time acetylcholine is present at the synapse the signaling is extended which compensates for the loss of acetylcholine in the early stages of the disease. Glutamate and its binding to the NMDA receptor is important for the formation of new memories through long-term potentiation [146]. Under physiological conditions the NMDA receptor channels are blocked by Mg²⁺ but in response to glutamate the channels open and the intraneuronal concentration of Ca²⁺ increases [147]. Increased levels of glutamate are found in the brain in AD, with constant influx of Ca²⁺ and hyperpolarized neurons as a result [148]. Another treatment is therefore NMDA-receptor antagonists that reduce the entry of Ca²⁺.

Future treatment strategies

Prevention of Alzheimer's disease

Future treatment strategies aim to prevent the onset of AD or to slow or stop the disease progression. Many prevention trials regarding lifestyle, nutritional supplement and drug treatment (agents related to cardiovascular diseases, non-steroidal anti-inflammatory drugs and hormone replacement therapy) have been performed. None of these strategies have proven successful in clinical trials, which could be due to small group sizes and short trial periods [149]. However, one promising study is the Finnish Geriatric Study to Prevent Cognitive Impairment and Disability (FINGER), which is the first large long-time study which investigates the effect of multiple approaches to combat cognitive decline [150]. The people included were between 60-77 years and cognitively normal but at risk to develop dementia based on cardiovascular risk factors. The participants were either assigned to the control group receiving health advice or to the intervention group with regular healthcare meetings, physical exercise, cognitive training, social activity and a healthy diet. The latter group had improved or maintained cognitive function throughout a two-year study time. This study suggests that a healthy lifestyle is beneficial to reduce the risk of cognitive decline among older individuals and an extended follow-up study is currently ongoing.

A β directed therapies

To slow down or stop the disease progression of AD, a number of disease modifying trials that target A β have been conducted. The three main approaches are to 1) inhibit A β production, 2) change A β aggregation and 3) increase A β clearance [151]. To reduce A β production clinical trials using β - and γ -secretase inhibitors have been performed but the trials have failed due to off target effects. Apart from cleaving APP γ -secretase also cleaves several other proteins of which Notch is important for normal cell development. Consequently, inhibition of γ -secretase leads to severe side-effects. Thus, therapeutic strategies that modulate the γ -secretase to specifically target γ -secretase cleavage of APP are under investigation. The substrates for β -secretase are

not well known, but studies with β -secretase inhibitors in mice indicate involvement in myelination apart from cleavage of APP [151]. The side effects of β -secretase inhibitors have not been as serious as for γ -secretase inhibitors, and several trials are currently ongoing [152]. Another treatment strategy is to target A β aggregation, given that abnormal aggregation of A β is central in AD. Several small compounds that either inhibit the aggregation and keep A β as non-toxic monomers (e.g. resveratrol and epigallocatechin-3-gallate) or accelerate the aggregation towards less toxic fibers of A β (e.g. curcumin and O4) have been investigated *in vitro* [153-156]. Two clinical studies of A β aggregation inhibitors (3-amino-1-propanesulfonic acid (Tramiposate)) and scylloinositol have been performed, but although the compounds block A β aggregation in preclinical studies the effect in clinical trials showed no clear improvement in AD patients [157, 158]. In addition, since metal ions have been shown to enhance A β aggregation another aggregation targeting strategy is metal chelation. Two compounds have made it to clinical trials (Clioquinol and PBT2), but no clear beneficial effects were evident with either compound [159]. The treatment strategy to increase A β degradation by enhancing the activity of the A β degrading enzymes IDE or neprilysin has also been investigated but so far only in preclinical settings [160-162]. Furthermore, the most developed drug approach in the AD research field concerns A β clearance by active or passive A β immunotherapy [149, 151, 163-165]. Several trials are ongoing but one study is of particular interest, were a monoclonal A β antibody (Aducanumab) developed from healthy older individuals, which targets fibrils of A β , not monomers, and shows both amyloid removal and improved cognition [165]. In addition to Aducanumab, another promising A β antibody (BAN2401) which targets protofibrils of A β is being investigated [164].

Up to date many clinical trials in the field of AD have failed. Proposed explanations for failure of the clinical trials are that the patients recruited to the trials are heterogeneous groups of individuals with mild cognitive impairment, where a large proportion of the patients never develop AD or that the treatment was administered too late in the disease process, when irreversible brain damage already had occurred [166, 167]. Currently ongoing trials aim to start treatment at an early stage of AD. Positron emission tomography (PET) makes it possible to identify people in the

preclinical phase but the AD field is in great need of biomarkers that could be detected early in biological fluids. The Dominantly Inherited Alzheimer Network (DIAN) study aims to address this issue, where the purpose is to identify early biomarkers indicative for AD in individuals with a known autosomal dominant mutation for AD [168]. Another ongoing trial is the A4 study where an anti-A β antibody (solanezumab) is tested on healthy older individuals at risk for developing AD based on amyloid accumulation in the brain imaged by PET [149]. This is the first study made on a homogenous population, with only on PET positive individuals with no clinical symptoms of AD. Both these studies are expected to be completed by 2020.

Other therapies

Drugs targeting tau strive to increase stabilization of microtubules, inhibit tau phosphorylation and aggregation and increase the degradation of tau [169]. In addition, epidemiological studies have identified several compounds that might prevent AD such as non-steroidal anti-inflammatory drugs, antihypertensive treatment, hormone replacement therapy, ginkgo biloba, vitamin B, omega-3 fatty acids, flavanol and vitamin E, but none have proven successful in clinical trials so far [149].

Inflammation in Alzheimer's disease

Besides plaques and neurofibrillary tangles there is also prominent neuroinflammation in the AD brain but whether it is a cause or consequence of the disease is still unclear [170]. Many studies have reported on expression of inflammatory proteins and activated microglia at A β plaques [170] but there are indications of inflammation decades before symptoms and plaque pathology appear [171]. Moreover, several gene polymorphisms associated with inflammation have been linked to AD and epidemiological studies indicate that long-term use of anti-inflammatory drugs is associated with a reduced risk to develop AD [10, 172]. However, an inflammatory response could also be beneficial and act neuroprotective. The danger lies in a chronic

inflammation, when there is an imbalance between a pro-inflammatory and an anti-inflammatory response. Under normal conditions inflammation is terminated by resolution which attenuates inflammation by several means through the action of specialized pro-resolving mediators [173, 174]. It was recently published that several of these factors were reduced in AD patients, which indicate that the resolution of inflammation is disturbed in AD [175].

Microglia in Alzheimer's disease

Microglia are the resident macrophages of the CNS and their main role is to protect the brain by destroying pathogens and remove toxic debris. When microglia becomes activated they secrete an array of pro-inflammatory cytokines, chemokines, prostaglandins, nitric oxide and reactive oxygen species [170]. These substances do not only destroy the intended target but also the surrounding cells. Microglia also produces the anti-inflammatory cytokines IL-10 and TGF- β that regulates the pro-inflammatory response. Many of these inflammatory mediators have been found to have altered expression levels in AD patients compared to controls [176], and both APP and A β peptides have been shown to activate microglia [177]. In addition, there is also *in vivo* evidence for increased activation of microglia in AD patients [178]. This activation promotes phagocytosis and clearance of A β before plaques appear, and hence act protective early on in the disease [179]. In addition, activated microglia also secretes IDE and other proteases that degrade A β along with neurotrophic proteins which indicate that microglia activation is beneficial [180-182]. Although there is evidence for a protective role for microglia in AD, the reason why A β continues to accumulate and contribute to AD progression despite microglia activation is not clear. A possible explanation for microglia failure is that they become overloaded with non-degradable material over time due to excess A β and lose their ability to phagocytose, and become more pro-inflammatory [183].

Lysozyme

Lysozyme (Figure 3) is part of the innate immunity and is abundant in all body fluids [184, 185]. Lysozyme is secreted by microglia and astrocytes in the CNS and by endothelial cells and macrophages in the rest of the body. Lysozyme is a glycoside hydrolase that breaks down bacterial cell walls by hydrolyzing the bonds in the peptidoglycan layer of the cell wall [186]. Besides being bacteriolytic, lysozyme also has anti-oxidant and anti-inflammatory properties [187-189]. Lysozyme was earlier reported to be increased in the CSF during inflammatory conditions [190] and in AD [191]. *In vitro* experiments have shown that lysozyme is able to inhibit the aggregation of A β ₁₋₄₀, A β ₁₋₄₂ and A β ₁₇₋₄₂, and also to reduce the toxicity of A β ₁₋₄₀ in a cellular model and of A β ₁₋₄₂ in *Drosophila* flies [191-193]. These findings make lysozyme an interesting candidate for AD treatment.

Figure 3. Three-dimensional structure of lysozyme.

Luminescent conjugated oligothiophenes

Luminescent conjugated oligothiophenes (LCOs) are conformation sensitive optical probes that are used to study the structure and conformation of protein aggregates. LCOs have a defined length of the thiophene backbone and are named according to the number of thiophenes. In this thesis the two LCOs, p-FTAA and h-FTAA, were used (Figure 4A and B). The first letter indicates the number of thiophene rings (**p**-

FTAA; **p** indicates penta (five), **h**-FTAA; **h** indicates hepta (seven)). The second letter indicates the substitution of the end-capping groups (-FTAA means that the two end thiophene moieties are substituted by formic acid). The last two letters (-FTAA) indicates that the thiophene backbone have been substituted with acetic acid. The full names of the used LCOs are therefore pentameric formic thiophene acetic acid and heptameric formic thiophene acetic acid.

Figure 4. Chemical structures of the luminescent conjugated oligothiophens (LCOs) A) *p*-FTAA and B) *h*-FTAA.

In a conjugated system atoms are bound together by alternating single and double bonds and this is called π -conjugation. This system is flexible and can adopt different conformations depending on the structure it binds to, and give rise to different wavelengths of the absorbed and emitted light. This optical behavior can be used to discriminate between different protein aggregates. For instance, LCOs have been shown to spectrally discriminate between different prion strains [194] and the LCO *p*-FTAA can distinguish between A β deposits and NFT [195]. In addition, LCOs can be used to monitor the aggregation of different amyloid proteins. In the A β fibrillation

pathway LCOs that consists of five or more thiophene units, like p-FTAA or h-FTAA, can detect prefibrillar species not detected by ThT [196].

AIMS

The general aim of the studies included in this thesis was to investigate if p-FTAA, h-FTAA and lysozyme have an impact on A β aggregation and *if* and *how* these molecules affect A β toxicity.

Specific aims of paper I and II

- To study if the interaction between A β and p-FTAA changes the toxicity of A β_{1-42} .
- To biophysically characterize the effect of p-FTAA on A β_{1-42} toxicity.
- To assess whether the LCOs p-FTAA and h-FTAA could change the toxic effect of A β_{1-42} with the Arctic mutation.

Specific aims of paper III and IV

- To investigate the mRNA and protein expression of lysozyme in brain tissue and CSF from AD patients and non-demented individuals and in brain tissue from AD mouse models and wild type mice.
- To elucidate the effect of lysozyme in different *Drosophila* models of AD.
- To identify potential mechanisms for the effect of lysozyme on A β toxicity.

METHODOLOGY

Model systems used in the projects

Cells

In papers I, II and IV experiments were performed with the human neuroblastoma cell line SH-SY5Y. This cell line has been subcloned a number of times from the original cell line SK-N-SH which was derived 30 years ago from a bone marrow biopsy from a four-year old girl with neuroblastoma [197, 198]. The SH-SY5Y cells in this thesis were differentiated with retinoic acid to obtain cells with a more neuronal-like phenotype in aspects of ceased cell proliferation and induction of neuronal processes. The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with penicillin, streptomycin, glutamine and fetal bovine serum. Cultivation occurred at 37 °C and 5 % CO₂ in a humidified incubator. The use of cells was limited to passages 19-25 since neuronal characteristics are lost with increasing passage numbers.

Brain tissue

In paper III the mRNA levels of lysozyme in AD and control brain tissue from both human and mice were investigated by use of two publicly available microarray data bases (GEO accession number GSE44772 and GSE64398, respectively) [55, 199].

Mice brain tissues were used for co-localization studies of lysozyme and A β plaques, but also to study the protein expression of lysozyme in AD mice compared to wild

type mice. Fifteen months old transgenic mice expressing APP with the Swedish mutation were used for co-localization analysis. Animal care was performed in accordance with the Animal Care and Use Ethical Committee at the Linköping University (ethical permission 84-12). The tissue used in this thesis was frozen tissue from 2013. For protein expression analysis the APP-PS1 AD mouse model expressing chimeric mouse/human APP695swe/Swedish mutations (K595N/M596L) and mutant human PS1 (PS1/ Δ E9) was used. Animal ethics approval was from the University of Wollongong Animal Ethics Committee (ethical permission AE11/03) where the experiments were performed.

In order to study the protein expression of lysozyme in AD, human postmortem tissue from temporal cortex was used in paper III. Brains with different disease stage, according to established neuropathological criteria [200], were selected and analyzed with western blot. Brain tissues with Braak stages between V-VI were considered as AD and tissues with Braak stages between 0-IV were considered as controls (Table I). However, different postmortem times, the time between death and preparation, can influence the protein expression. The brain tissues were received from the Sydney Brain Bank at Neuroscience Research Australia and the New South Wales Tissue Resource Centre at the University of Sydney via a Material Transfer Agreement (Dnr IKE-2013-00056). Informed consent for the collection of material was obtained prior to donation of their brain and tissue use was approved by the University of New South Wales Human Research Ethics Committee (ethical permission HE10/327). Paper III was performed in collaboration with the research team of Professor Brett Garner with ethical permissions regulated via an Adhesion Agreement to Ethical permission HE10/327 (Dnr IKE-2013-00056).

CSF

Since CSF is in direct contact with the extracellular space of the brain, changes in the brain are mirrored in the CSF. In paper III CSF was analyzed for the protein expression of lysozyme. The patients were both biochemically and clinically diagnosed with AD. AD was assigned according to CSF biomarkers levels, using

cutoffs of P-tau_{181P}; 60 ng/l, T-tau; 350 ng/l and A β ₁₋₄₂; 530 ng/l (Table III). Age and gender matched controls had CSF biomarkers in the control range (Table II). The de-identified and archived CSF samples were obtained from the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden, according to regulations from the Swedish Central Ethical Board. The participants underwent lumbar puncture at the L3-L4 or L4-L5 interspace and the collected CSF was centrifuged, frozen, thawed and aliquoted and stored at -80 °C prior to analysis.

Table I. Patient information regarding age, Braak stage and postmortem delay of the brain tissues.

Gender	Age (years)	Braak stage	Postmortem delay (h)
Female	85	0	23
Female	93	0	21
Female	84	0	6
Male	66	0	23
Male	68	0	45,5
Male	69	0	52
Male	57	0	18
Male	64	0	17
Male	79	0	8
Female	104	I	27
Female	78	I	45
Male	63	I	24
Male	62	I-II	46
Female	85	II	10
Male	69	II	19
Male	91	II	16
Male	103	II	20
Female	81	III	28
Female	73	III	45
Female	92	III	14
Male	67	III	25
Female	83	II-IV	64
Female	98	IV	6
Female	92	IV	5
Female	94	V	7
Female	83	V	3
Female	100	V	3
Female	98	V	11
Female	84	VI	6
Female	80	VI	32
Female	85	VI	10
Male	68	VI	23
Male	69	VI	3
Male	67	VI	9

Table II. Demographics of CSF from control individuals.

Group	Gender	Age (years)	P-tau (ng/l)	T-tau (ng/l)	A β ₄₂ (ng/l)
Control	Female	64	38,5	217,0	553,5
Control	Male	66	62,0	288,5	860,0
Control	Male	48	44,0	153,5	621,5
Control	Female	48	55,5	206,5	746,0
Control	Female	65	46,5	253,5	616,0
Control	Female	61	40,5	165,0	588,9
Control	Male	68	44,2	196,9	750,0
Control	Female	58	57,1	273,9	833,7
Control	Female	63	36,0	144,4	623,2
Control	Female	46	41,7	162,2	851,1
Control	Male	66	46,4	216,1	862,1
Control	Male	80	59,3	290,7	663,3
Control	Female	63	40,4	155,9	685,1
Control	Female	64	40,8	153,3	754,8
Control	Male	57	59,0	263,0	1160,8
Control	Male	55	31,3	108,0	705,2
Control	Female	57	63,7	194,0	1405,0
Control	Male	66	60,9	248,2	1185,5
Control	Female	60	44,3	235,7	702,1
Control	Female	60	48,3	207,1	788,7
Control	Female	57	67,0	240,7	954,7
Control	Male	61	37,5	125,1	726,6
Control	Female	54	53,4	166,7	828,1
Control	Female	53	37,0	111,4	663,9
Control	Female	61	61,7	213,4	807,6

Table III. Demographics of CSF from Alzheimer’s disease (AD) patients.

Group	Gender	Age (years)	P-tau (ng/l)	T-tau (ng/l)	A β ₄₂ (ng/l)
AD	Male	77	98,0	556,5	310,0
AD	Male	77	102,0	1001,5	342,0
AD	Female	60	145,5	986,5	316,5
AD	Male	68	144,0	873,5	133,0
AD	Female	58	127,0	899,0	206,0
AD	Female	67	221,0	1638,0	352,0
AD	Male	64	188,5	1425,0	275,0
AD	Female	72	80,1	557,0	313,0
AD	Female	69	106,3	783,0	370,0
AD	Male	83	282,0	>1200	270,1
AD	Female	67	168,0	1314,4	360,1
AD	Female	74	107,2	713,9	326,0
AD	Female	74	121,7	794,1	206,0
AD	Female	68	118,4	729,8	273,7
AD	Male	73	125,5	754,2	243,0
AD	Male	66	86,1	463,7	354,7
AD	Female	60	106,5	642,9	338,7
AD	Female	60	239,7	>1200	378,9
AD	Female	54	143,6	1024,1	310,8
AD	Female	75	97,3	433,2	442,9
AD	Female	65	125,6	1015,7	376,4
AD	Male	58	93,9	765,7	428,4
AD	Female	76	89,3	525,3	441,3
AD	Male	51	144,8	>1200	441,3
AD	Female	56	100,7	1059,9	233,4

Drosophila melanogaster and the Gal4-UAS system

The fruit fly *Drosophila melanogaster* is an excellent model to study the effect of gene expression as it is an inexpensive methodology, has a short generation time and many genes are conserved between humans and flies. *Drosophila* has four pairs of chromosomes; one pair of sex chromosomes (X/X for females and X/Y for males) and three autosomes (2, 3 and 4). The genes of interest are usually inserted in chromosome 2 or 3. Transcription of the cloned gene is achieved by the Gal4-UAS system, where the yeast transcription factor (Gal4) binds a DNA sequence, the promotor upstream activating sequence (UAS), of the desired gene [201]. Hence, the transgenic fly only expresses the gene if Gal4 is present (Figure 5). Moreover, the driver Gal4 is only expressed in a tissue specific manner. In paper III, *gmr*-Gal4 and *elav*-Gal4 were used which direct the expression to the photoreceptors of the eyes and to the central nervous system, respectively. Control *w¹¹¹⁸* flies (only expressing Gal4) or flies that expressed UAS containing genes that encoded APP₆₉₅ (APP) or BACE1 were purchased from Bloomington *Drosophila* stock centre. UAS containing genes that encoded human wild type lysozyme [202], A β ₁₋₄₂ and A β _{Arc} [203] (all three containing a secretion-tag), were generated for this study.

In order to separate flies with the genotype of interest the flies are identified by different markers that give rise to different visual phenotypes, such as curly wings, more than two hairs on their shoulder, deformed eyes, short hair on their back etc. Homozygous expression of the markers is lethal and flies with this expression will never develop. Some of these markers can act as balancer chromosomes that prevent recombination, which occurs in female flies. In paper III two balancer chromosomes have been used; Cyo (curly wings) and TM6B (hairy shoulder). The gene of interest has to be heterozygous over a balancer chromosome if the gene is not homozygously expressed.

Figure 5. Illustration of the Gal4-UAS system. Cross breeding of a Gal4 fly with a fly that carries the cloned gene of interest. The Gal4 transcription factor binds to the upstream activating sequence (UAS) promoter in the gene of interest. Only the offspring that have both the gene for Gal4 and the cloned gene produces the protein.

Preparation of A β

A β is a challenging protein to work with. Small variations in pH, temperature, salt concentration, agitation etc. change the aggregation process and generate A β with different properties [204, 205]. Throughout all the papers A β was prepared to yield monomers, oligomers or fibrils. Both recombinant and synthetic A β have been used as well as different species; A β ₁₋₄₀, A β ₁₋₄₂ and A β ₁₋₄₂ with the Arctic mutation (A β _{Arc}). Several protocols for preparation of A β exist and we have used different approaches in the separate projects in the thesis.

In paper I A β ₁₋₄₂ was first dissolved and aliquoted in trifluoroacetic acid (TFA), which has been shown to render A β in a monomeric form [206]. TFA was removed by lyophilization and A β was re-dissolved in 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP) to remove any preexisting structures [207], lyophilized and kept in -80 °C pending experiment. In paper II A β _{Arc} was dissolved as A β ₁₋₄₂ was in paper I but frozen in liquid nitrogen before the lyophilization step to prevent aggregation and kept at -80 °C. Prior to experiments the A β peptides were dissolved in NaOH, since alkaline conditions inhibit A β aggregation [208], and then diluted in PBS (pH 7.4) to favor aggregation [207], and aggregated for different times at 37 °C.

In paper IV a well-established protocol for the preparation of oligomers was used [207, 209]. A β ₁₋₄₂ was purchased as a lyophilized TFA salt and was upon receipt dissolved and aliquoted in TFA and lyophilized. It was subsequently diluted in HFIP, lyophilized again and then stored in -20 °C until use. A β conjugated to the red-fluorescent tetramethylrhodamine (TAMRA) was directly dissolved in HFIP and aliquoted. For an oligomeric preparation the A β was first dissolved in a small volume of DMSO before diluted in PBS or HEPES with (pH 7.4) and sonicated for 2 min and then left to aggregate at 4 °C for 24 h. For a monomeric preparation A β was dissolved in DMSO and thereafter diluted in PBS or HEPES and sonicated for 5 min immediately before use. For preparation of fibrils A β was first dissolved in DMSO, diluted with PBS or HEPES and then left to aggregate at 37 °C for 24 h.

For each new batch of A β the protein concentration was analyzed and adjusted for. To ensure the proper aggregation state different methods were applied, including ThT kinetics, transmission electron microscopy and size exclusion chromatography (SEC). SEC separates molecules according to size, where larger molecules elute faster than smaller molecules since the smaller molecules migrate through the pores in the beads while larger species cannot penetrate the small pores and only enter the volume between the beads (Figure 6). A detector connected to a monitor is situated where the molecules elute from the column.

Figure 6. Size exclusion chromatogram of a sample with oligomerized A β ₁₋₄₂ that contains both oligomers and monomers. Larger oligomers are eluted first followed by smaller monomers.

Protein expression and localization

Western blot

Western blot is a widely used technique to analyze protein expression in cells or tissues. In paper III this method was used for detection of lysozyme and/or A β in brain tissue from mice and humans. In western blot denatured proteins separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is analyzed. The protein separation is based on the molecular weight and the electrical negative charge of the proteins that is due to the reducing agent dithiothreitol (DTT). The proteins are then transferred to a nitrocellulose membrane. The proteins of interest are detected with primary antibodies, which in turn bind a secondary antibody linked to horseradish peroxidase (HRP), enabling detection for example by a digital camera when a chemiluminescent substrate is added. Protein expression is detected as dark bands

which correlate to the amount of protein. Densitometric analysis is used to measure the intensity of the band which makes it a semi-quantitative method that can be used to compare the expression between different samples, but an exact quantity is not possible to deduce. A protein that is constantly expressed by the cells such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a loading control, to ensure even loading between blots, allowing for comparison.

Dot blot

When the specificity of an antibody is known and only a few proteins have interacted *in vitro* the dot blot technique can be used to visualize the protein. Dot blot is a technique which resembles western blot but the proteins are not denatured and separated by electrophoresis before blotting. Since western blot is not an appropriate technique to study A β fibrils as the fibrils are too large to penetrate the electrophoresis gel, dot blot is a good alternative. In paper I the dot blot technique was used to investigate the proteolytic resistance towards proteinase K degradation of A β fibrils that have been aggregated alone or together with p-FTAA. Every hour for seven hours a small volume of each sample was dotted on a nitrocellulose membrane and detected by the A β specific antibody 6E10. The method was also used to show the presence of fibers with the anti-amyloid fibrils OC antibody and to demonstrate the amount of soluble/insoluble A β after aggregation with p-FTAA. The procedure is similar as for western blot, with incubation with primary antibody and HRP-labelled secondary antibody followed by detection by a light sensitive camera.

Immunohistochemistry

Immunohistochemistry is an antibody-based staining technique used to visualize a specific antigen in tissue samples. In paper III, A β deposits and lysozyme were detected with immunohistochemistry in brain slices from mice that expressed APP with the Swedish mutation (APP_{Swe}). The brain tissues were fixed in formalin, dehydrated in sucrose, sectioned and frozen pending analysis. The brain slices were

incubated with a blocking agent prior the primary antibody, to reduce the amount of unspecific binding. The primary antibody 6E10 recognizing A β was diluted in bovine serum albumin together with the permeabilization agent Triton-X and a HRP-polymer that binds the 6E10 antibody. When diaminobenzidine is added it gets oxidized by HRP and a brown color appears in 6E10 positive areas. To investigate whether lysozyme co-localizes with A β plaques the tissue slices were labelled with an anti-lysozyme antibody as well, followed by the chromogen Vina Green, that produces a green stain in the presence of HRP. The brain tissue slices were then placed on glass slides and visualized by light microscopy.

Fluorophore tagged proteins and organelle specific dyes

Immunocytochemistry is a common antibody-based method used to visualize cells and the subcellular expression, localization and co-localization of endogenous proteins. An alternative way to study proteins is to use fluorescently labelled exogenous proteins and add them to the cells to study the uptake and the subcellular location. In paper IV fluorescently labelled A β_{1-42} or lysozyme was used to study the sub-cellular localization of the proteins by confocal microscopy or the proportion of cellular uptake of the proteins by flow cytometry. A β_{1-42} was conjugated to the red-fluorescent tetramethylrhodamine (TAMRA) and lysozyme to the green-fluorescent Alexa-488. Prior visualization by confocal microscopy, the cells were fixed with the cross-linker paraformaldehyde and mounted with mounting medium that contained the DNA binding dye DAPI, to visualize the nuclei.

Meso Scale Discovery protein assay

The Meso Scale Discovery (MSD) protein assay was performed to quantify lysozyme levels in human CSF and the level of A β_{1-42} in heads of *Drosophila* flies in paper III. MSD is based on the ELISA technique in the sense that it is based on antibodies and performed in a microtiter plate. It is most commonly used in a sandwich setup where the plate is coated with a capture antibody that will bind the antigen of interest in the

sample followed by a detection antibody. The difference lies in the detecting antibody as antibodies that are used in MSD are Sulfo-tagged compared to antibodies conjugated to an enzyme that produce color when a substrate is added in a conventional ELISA. The Sulfo-tag label undergoes a redox reaction that generates light when electricity is applied to the plate, a process called electrochemiluminescence. The advantages compared to conventional ELISA are lower background, smaller sample volumes and higher capture capacity.

Protein aggregation analysis

Protein aggregation is a complex process that has been studied throughout this thesis by use of the different methods described below.

ThT

Thioflavin T (ThT) fluorescence is the most common way to study A β aggregation over time *in vitro* and was introduced in 1959 [210, 211]. ThT is a dye that binds to fibrillated proteins and has primarily been used for plaque staining in tissue sections. When unbound, ThT is flexible which quenches the signal but upon binding the structure is locked and fluorescent and the fluorescence increases as amyloid fibrils accumulate [212]. The fluorescence can be captured by fluorescent spectroscopy and results in a typical sigmoidal shaped curve when the aggregation is plotted over time (Figure 7). The aggregation process is characterized by three phases. The lag-phase constitutes the initial phase where smaller A β assemblies are generated but no fibrils are formed. These early formed A β species seed the growth phase where A β fibrils are formed, followed by the stationary phase where equilibrium is reached. One drawback with ThT is that it does not distinguish between different amyloid proteins.

Figure 7. The aggregation of $A\beta_{1-42}$ over time monitored by ThT that binds to fibrillar $A\beta$. The curve is characterized by a lag-phase (0-7 h) where no fibrils are formed followed by a rapid growth phase (7-30 h) that leads to the stationary phase (>30 h).

Luminescent conjugated oligothiophenes

Another way to monitor $A\beta$ aggregation is by the use of luminescent conjugated oligothiophenes (LCOs) [195, 196]. These are optical probes that are sensitive for conformational changes and give rise to different spectral properties depending on the structure they bind to. LCOs are named according to the number of thiophene units. In the projects included in this thesis the pentameric (= five units) p-FTAA and the heptameric (= seven units) h-FTAA have been used. Both p-FTAA and h-FTAA detect early formed species of $A\beta$ (Figure 8).

Figure 8. The aggregation of $A\beta_{1-42}$ monitored by *p*-FTAA (black line) and *h*-FTAA (grey line) that recognizes early formed species of $A\beta_{1-42}$.

ANS

The fluorescence of 8-anilino-1-naphthalenesulfonic acid (ANS) is sensitive to a hydrophobic environment due to the aromatic rings in aniline and naphthalene, which leads to intense fluorescence upon binding to hydrophobic residues [213]. Since the hydrophobicity increases as $A\beta$ aggregates, ANS can be applied to study $A\beta$ aggregation [214, 215] (Figure 9), as in paper I. Moreover, the sulfonate group in ANS has a negative charge which makes ANS sensitive to polarity in addition to hydrophobicity [213]. The binding of ANS to proteins require both these interactions. If a protein is completely denatured it exposes all its hydrophobic regions, but ANS binding is weak due to the loss of charged regions.

Figure 9. Changes of $A\beta_{1-42}$ hydrophobicity when aggregated over time, monitored by 8-anilino-1-naphthalenesulfonic acid (ANS).

Circular dichroism spectroscopy

Circular dichroism (CD) is a method to determine the secondary structure and folding property of a protein. This method is based on chirality, that all amino acids are chiral and therefore they will absorb left- and right- handed circular polarized light to different extent [216]. In the near UV-spectrum (between 250 and 320 nm) the absorbing molecules are the side chains of the aromatic amino acids. In the far-UV spectrum (between 190 and 250 nm) the absorbing molecules are the peptide groups located in asymmetric protein structures. Far-UV CD spectroscopy was applied in paper I to study the changes in secondary structure when $A\beta$ was aggregated in the absence or presence of p-FTAA. When irradiated in the far UV region the CD spectrum can differentiate between random coils (monomers), α -helices and β -sheets (fibrils) (Figure 10). The α -helical fold results in two minima at 208 nm and 222 nm, whereas the β -sheets structure has the minima at 218 nm. Random coil structures have the minima at 195 nm but otherwise follow the baseline [217].

Figure 10. Circular dichroism (CD) spectra of A β aggregated for 0 h and 12 h. After 12 h of aggregation β -sheet structures are apparent as evident by the negative peak at 218 nm.

Binding study of p-FTAA and h-FTAA to A β

In order to study the binding of p-FTAA and h-FTAA to A β_{Arc} , the shift in excitation wavelength that occurs when the oligothiophenes bind to A β_{Arc} was used. An excitation spectrum between 380-500 nm was recorded for p-FTAA and h-FTAA over time at the peak emission wavelength for p-FTAA and h-FTAA (515 nm and 545 nm, respectively). The excitation spectrum plotted at the start of the experiment and the end (24 h later) shows the highest fluorescent intensity when the oligothiophenes are unbound and free in solution and when bound to A β_{Arc} , respectively. This shift in excitation wavelength was plotted as the ratio of bound and unbound over time, resulting in a binding curve.

Uptake of A β

Flow cytometry measures fluorescent properties of individual cells that flow in a stream of fluid. When the cells pass a laser the cells will scatter the light. This will be measured by a forward light scatter detector and a side light scatter detector, which

provide information about cellular size and granularity. In addition, cells can be labelled with fluorescent dyes or antibodies that when excited emit light with varying wavelengths that are filtered to direct the light to the appropriate detector. Flow cytometry analysis was performed in paper IV to study cellular uptake of A β when aggregated in the presence or absence of lysozyme (Figure 11). Differentiated SH-SY5Y neuroblastoma cells were fed with monomeric or oligomeric A β that were coupled to the red-fluorescent dye TAMRA. TAMRA was excited by the 561 nm laser available on the flow cytometer and the emitted light passed the PE-filter (585/15 nm) before reaching the detector. Any A β that was stuck on the surface of the cells was removed by trypsin prior analysis to ensure that only internalized A β was analyzed.

Figure 11. Cellular uptake of A β studied by flow cytometry. Control cells (untreated cells) were used for gating (black). The cells that had higher fluorescence than the control cells were considered positive for A β (grey).

Transmission electron microscopy

Transmission electron microscopy (TEM) is a technique where electrons are fired and interacts with the specimen while it passes through. The resolution is dependent on the wavelength of the light source. The improved resolution with TEM compared to light microscopy is because of shorter wavelengths of electrons than photons. The faster the electrons travel, the shorter the wavelengths are and the more detailed the image will

be. Therefore high voltages are used to accelerate the electrons to high speed. Electrons move slowly through air and therefore vacuum is necessary. This technique was used to study the morphology of different A β aggregates in the absence and presence of p-FTAA and h-FTAA in paper I and II. The samples of interest were added on to carbon coated copper grids and thereafter contrasted with uranyl acetate. The grids were analyzed with TEM and the images were detected by a charge-coupled camera.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used in paper III to study the eye morphology of *Drosophila* flies. This technique uses a focused beam of electrons, but instead of passing through the specimen, as with TEM, the beam hits the surface, interacting with the atoms of the specimen and bouncing back. Depending on the energy exchange between the beam and the specimen, the reflected electrons will be different, either called secondary electrons (low-energy) or backscattered electrons (high-energy). These are registered and turned into an image. For this to occur, specimens need to be electrically conductive and are therefore coated with a metal. In paper III flies mounted onto aluminum specimen stubs were sputter coated with platinum, and the eye morphology was studied.

Measurements of toxic effects

Determination of cell proliferation and viability can be made in many different ways. Examining cells under a light microscope gives valuable information about cell morphology and structural changes occurring during cell death like condensation of the nuclei and formation of apoptotic bodies. This is often followed by a quantitative measurement of cell viability. Likewise, cell toxicity and viability in flies can also be evaluated by structural changes visualized by microscopy or by quantitative measurements related to survival or locomotion.

Cell viability test

In papers I, II and IV the XTT viability assay was used to determine cell viability upon A β treatment. The method is based on processing of the yellow tetrazolium salt XTT that is converted to an orange formazan salt in metabolic active cells. The change in XTT absorbance is measured and correlates to the number of viable cells. Metabolic changes are early events during cell death and the XTT method makes it possible to detect A β toxicity even before cell morphology is altered. In paper III the morphology of the fly eyes ommatidia, the hexagonal units of the eye comprising the photoreceptors, were the readout for cell toxicity since the proteins of interest were expressed in the eyes of the flies with the *gmr* driver. Healthy fly eyes have well organized ommatidia structures whereas a disturbed pattern is apparent when the proteins are toxic to the eye.

Survival assay of *Drosophila melanogaster*

A survival assay is a fast read-out to estimate the health effects of a transgene or pharmacological substance in *Drosophila* flies. In paper III this method was used to quantify the impact lysozyme expression has on flies expressing A β_{1-42} with the Arctic mutation (A β_{Arc}) in the central nervous system. For the survival assay 100 female mated flies of each genotype were separated into tubes with 10 flies each. Three times a week the flies were transferred to new fresh agar food supplemented with yeast and the number of living flies were calculated and analyzed by Kaplan-Meier log rank statistics. Lifespan trajectory plots of control flies (flies only expressing Gal4) and flies that expressed A β_{Arc} with or without one or two copies of lysozyme was created and the median survival (the time when half of the population for a genotype have died) was compared.

Locomotor assay of *Drosophila melanogaster*

The health of the fly can sometimes be misleading using lifespan studies, since it only takes into account if the fly is alive or not. However, the flies can have health problems and be unable to fly, but still be alive. Hence, the activity of the fly is a better determination of health. The iFly system is a three-dimensional approach with a camera and two mirrors that allows for locomotor behavior to be tracked and velocity and angle of movement to be calculated for individual flies [218]. Flies are strongly negatively geotactic which means that they move against earth's gravity to the top of the vial, but this behavior declines with advanced age [219, 220]. In addition, the movement of flies follows a straight vertical path from the bottom of the tube towards the top. However, unhealthy flies deviate from this path on their way towards the top. This method was applied for flies that expressed A β with the Arctic mutation with or without lysozyme in the CNS with use of the ELAV driver. Each genotype were aged in 29 °C and each day transferred to a measuring vial and subsequently dropped into the iFly instrument to ensure that flies started from the bottom before recording for 90 seconds. At each interval of 30 seconds the vial was dropped again to reactivate the locomotor behavior.

RESULTS

PAPER I: The luminescent oligothiophene p-FTAA converts toxic $A\beta_{1-42}$ species into nontoxic amyloid fibers with altered properties

The LCO p-FTAA was previously shown to bind prefibrillar toxic species of $A\beta$ [195, 196]. As any interaction with $A\beta$ might influence the aggregation pathway and hence the toxic properties of $A\beta$, the aim in paper I was to investigate if the LCO p-FTAA had an impact on the $A\beta_{1-42}$ aggregation pathway and toxicity.

Human neuroblastoma SH-SY5Y cells were exposed to $A\beta_{1-42}$ that was aggregated with or without p-FTAA for 0-5 h. The different aggregates of $A\beta_{1-42}$ all reduced the cell survival, with the highest toxicity observed at 0 h of aggregation. However, co-aggregation with p-FTAA conferred cytoprotection at all aggregation times examined.

Various biophysical methods were conducted to explore the mechanism behind the protective effect of p-FTAA. By dot blot measurements of the proportion of soluble and insoluble $A\beta$ species it was shown that $A\beta_{1-42}$ co-aggregated with p-FTAA, had increased proportion of insoluble $A\beta$ species (Figure 12A). Using TEM, $A\beta$ aggregated alone showed prefibrillar structures whereas $A\beta$ co-aggregated with p-FTAA contained dense fibrillar structures (Figure 12B). To further define the formed species an antibody, OC, that specifically recognizes amyloid fibrils was used. $A\beta$ was aggregated with or without p-FTAA for 0, 1 and 3 h before analysis with dot blot, which revealed that already at the start of the aggregation OC positive fibrils were

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formed when p-FTAA was present (Figure 12C). In the presence of p-FTAA these species also showed faster formation of β -sheet structures, as revealed by CD.

A

B

C

Figure 12. *p*-FTAA increases the aggregation of A β . A) Dot blot analysis of supernatant (soluble A β) and pellet (insoluble A β) from A β aggregated between 0-24 h with or without *p*-FTAA using the 6E10 antibody. B) Transmission electron microscopy of pellets from A β aggregated with or without *p*-FTAA for 3 h. Scale bar = 1 μ m. C) Dot blot analysis of A β aggregated with or without *p*-FTAA for different times using the fibril specific OC antibody.

Next, the stability of the formed fibrils was investigated. A β fibrils formed with or without p-FTAA were exposed to proteinase K for 0-7 hours and analyzed with dot blot for the presence of soluble A β . p-FTAA did not only accelerate the aggregation, but also increased the resistance of the formed A β fibrils towards proteolytic degradation by proteinase K (Figure 13). In addition, the fibrils formed in the presence of p-FTAA were less sensitive to denaturation with guanidine hydrochloride, as evident by less exposure of hydrophobic residues detected with ANS.

Figure 13. *p-FTAA prevents proteinase K proteolytic degradation of Aβ. Aβ was aggregated with or without p-FTAA for 24 h in 37 °C. Samples were then digested with proteinase K for the indicated times and the remaining Aβ peptides were visualized with dot blot using the 6E10 antibody.*

The propagating properties of Aβ fibrils formed in the presence of p-FTAA were investigated using ThT. Aβ fibril seeds formed with p-FTAA decreased the propagating properties of Aβ₁₋₄₂ as compared to Aβ seeds generated in the absence of p-FTAA (Figure 14).

Figure 14. *Aβ fibril seeds formed with p-FTAA decreases the propagation of Aβ. Seeding effect of 1 % of fibrillar Aβ formed with or without p-FTAA on Aβ aggregation analyzed with ThT.*

In summary, p-FTAA promotes formation of larger and less toxic Aβ aggregates with increased stability, and might therefore shift the equilibrium from soluble toxic Aβ species towards insoluble non-toxic Aβ species. Moreover, Aβ fibrils formed in the presence of p-FTAA display decreased propensity to propagate which suggests that p-FTAA could have the potential to reduce the spread of Aβ pathology in AD.

PAPER II: The luminescent conjugated oligothiophene h-FTAA attenuates toxicity of amyloid- β with the Arctic mutation

In this project it was investigated if p-FTAA has a similar protective effect against the highly toxic Arctic variant of $A\beta_{1-42}$ ($A\beta_{Arc}$) as for $A\beta_{1-42}$. The Arctic mutation in *APP* generates $A\beta$ with a high propensity to aggregate and form toxic prefibrillar species. In addition to p-FTAA, h-FTAA, another LCO known to bind $A\beta$ was also tested.

Exposure of human SH-SY5Y neuroblastoma cells to different $A\beta_{Arc}$ aggregates generated between 0-4 h of aggregation showed that $A\beta_{Arc}$ was toxic between 0-2 h as evident by the appearance of shrunken and fragmented cells and reduced viability using the XTT viability assay. The toxicity declined with time and no toxicity was evident after 4 h of aggregation. TEM analysis of $A\beta_{Arc}$ at 0 h of aggregation demonstrated small round-shaped species and at 3 h of aggregation larger species were formed. The morphology of $A\beta_{Arc}$ agrees with the toxicity data since smaller species of $A\beta$ are associated with toxicity while larger $A\beta$ species correspond to less toxicity. Next, $A\beta_{Arc}$ was aggregated for 0-2 h with the LCOs p-FTAA or h-FTAA before exposure to cells. h-FTAA was able to protect the cells at all times investigated (Figure 15A). However, h-FTAA did not exhibit a protective effect when added after the aggregation, which indicates that h-FTAA needs to be present during the aggregation to interfere with toxicity. By contrast, p-FTAA did not influence the toxicity at any time (Figure 15B). This indicates that the difference between p-FTAA and h-FTAA, where h-FTAA contains two more thiophene units than p-FTAA, assigns $A\beta_{Arc}$ different characteristics.

Figure 15. *h*-FTAA (A), but not *p*-FTAA (B), rescues the neuroblastoma cell toxicity of $A\beta$ with the Arctic mutation ($A\beta_{Arc}$). $A\beta_{Arc}$ was aggregated for 0–2 h, alone or together with *p*-FTAA or *h*-FTAA or with *p*-FTAA or *h*-FTAA added after the aggregation and analyzed with the XTT viability assay. Significant differences are denoted with ** ($p \leq 0.01$) or *** ($p \leq 0.001$). Error bars represent mean $\pm$ SD.

The kinetic behavior of $A\beta_{Arc}$ was monitored with *p*-FTAA and *h*-FTAA in order to study the aggregation process. During the first 5 h of aggregation, no difference in the aggregation behavior was detected for *p*-FTAA and *h*-FTAA. Thereafter, the curves for *h*-FTAA and *p*-FTAA started to separate. These results indicate that there are kinetic differences between *p*-FTAA and *h*-FTAA when bound to $A\beta_{Arc}$, but not during the first hour of aggregation when $A\beta_{Arc}$ is toxic.

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To investigate if the different ability of p-FTAA and h-FTAA to protect the cells could be due to different binding of p-FTAA and h-FTAA to $A\beta_{Arc}$, the shift in excitation wavelength that occurs when the LCOs bind to $A\beta_{Arc}$ was monitored over time, as explained in the materials and methods section. The ratio of bound and free h-FTAA over time for $A\beta_{Arc}$ showed a rapid increase followed by saturation at 10 h, while the interaction was more linear with no obvious plateau phase reached for p-FTAA (Figure 16). These results indicate a higher binding affinity of h-FTAA to the formed $A\beta_{Arc}$ aggregates than for p-FTAA and $A\beta_{Arc}$.

Figure 16. $A\beta_{Arc}$ binds stronger to h-FTAA than p-FTAA. Binding study of h-FTAA and p-FTAA to $A\beta_{Arc}$ where the ratio of bound and free h-FTAA and p-FTAA was followed over time. Shown are the excitation ratios of h-FTAA and p-FTAA when bound to $A\beta_{Arc}$ (at 480 nm and 450 nm for h-FTAA and p-FTAA, respectively) against free h-FTAA and p-FTAA at 450 nm and 400 nm, respectively. This was measured at the emission 545 nm or 515 nm for h-FTAA and p-FTAA, respectively.

Overall, our findings demonstrate that $A\beta_{Arc}$ cytotoxicity was prevented by h-FTAA but not by p-FTAA. We hypothesize that the reason for the cytoprotective effect of h-FTAA is due to a stronger binding of $A\beta_{Arc}$ to h-FTAA compared to p-FTAA.

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

Lysozyme has previously been shown to inhibit the aggregation of A β ₁₋₄₀, A β ₁₋₄₂ and A β ₁₇₋₄₂ *in vitro* [191-193, 221], which makes lysozyme an interesting protein to study in AD. The aim of this project was to investigate the lysozyme expression in brain tissue and CSF, both at mRNA and protein level, in different transgenic mice models of AD and AD patients and compare it to wild type mice and non-demented controls. Furthermore, the effect of increased lysozyme expression in different *Drosophila* models of AD was investigated.

Two microarray data-bases were used in order to analyze the mRNA expression of lysozyme in five AD mice models, either exhibiting A β pathology or tau pathology, and in AD patients. The amyloid transgenic mice expressed mutant human *APP*, *PSEN1* or *APP* and *PSEN1* and the tau transgenic mice expressed mutant human microtubule-associated protein tau (*MAPT*). Microarray was performed at 2, 4, 8 and 18 months in hippocampus, cortex and cerebellum and examination of the pathology was done at the same time. The lysozyme mRNA expression was increased in the hippocampus and cortex of the transgenic AD mice. However, the cerebellum was spared, which is consistent with the spread of A β pathology which starts in the areas of the cortex and hippocampus, but does not affect cerebellum until late in the disease progression [222-224]. The increase in lysozyme mRNA expression was associated with a concomitant increase in A β pathology. By contrast, the increased lysozyme expression did not cohere to the same extent with tau pathology. mRNA levels of lysozyme in humans was also studied. Microarrays of human AD brain and control individuals were made on homogenates from the visual cortex, prefrontal cortex and cerebellum. In agreement with the mice study lysozyme mRNA expression was increased in visual cortex and prefrontal cortex in AD patients, but the cerebellum was spared. Likewise, the protein level of lysozyme was increased in the temporal cortex and in the CSF from AD patients.

These results encouraged to further investigate the effect of lysozyme in AD by using three different *Drosophila* AD models. Two *Drosophila* drivers were used; *gmr*-Gal4

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and *elav-Gal4*, which express the genes of interest in the retina or in the CNS, respectively, of the flies. In the setup where the driver *gmr-Gal4* was employed two AD *Drosophila* models were used, one that expressed both APP and BACE1 and one that directly expressed and secreted A β_{1-42} . The morphological structure of the eyes was analyzed by SEM and in flies that expressed APP and BACE1 or A β_{1-42} the eyes had defects in the ommatidia structure. When the flies co-expressed lysozyme the eye phenotype was completely rescued, with ommatidia structures similar to control flies (Figure 17).

Figure 17. Lysozyme expression rescues the eye morphology in AD *Drosophila* models. Scanning electron microscopy of two AD *Drosophila* models that either express APP or APP-BACE1 in the eyes with and without lysozyme (Lys), or control (only *gmr*) and A β_{1-42} with or without lysozyme. Scale bars = 50 μ m.

Next, the impact of lysozyme on fly survival was investigated by using flies that expressed A β_{1-42} with the toxic Arctic mutation (A β_{Arc}) in the CNS, with or without co-expression of one or two copies of lysozyme. The lifespan analysis displayed a protective effect of lysozyme on the A β_{Arc} flies (Figure 18A). One copy of lysozyme

increased the mean survival with one day, and two copies extended the mean survival with two days. In addition, also the activity (velocity and angle of movement) of the flies was investigated by using the iFly technique. The velocity of the flies decreases with age or disease and before the flies die they are immobile and the velocity can no longer be recorded. A cut-off value of 4 mm/s was set as an indicator for dysfunctional locomotor behavior and $A\beta_{Arc}$ flies reached below this value after 9 days (Figure 18B). Co-expression of one or two copies of lysozyme postponed the toxic effect of $A\beta_{Arc}$ and the cut off value was reached after 12 and 15 days, respectively. The angle of movement is another way of measuring activity, since the angle increases as the flies age or get sick. A cut-off value of 80° was set as an indication for dysfunctional locomotor behavior, which $A\beta_{Arc}$ flies surpassed after 6 days while flies co-expressing one or two copies of lysozyme surpassed 80° after 10 days. These results show that lysozyme is protective against $A\beta_{Arc}$ induced toxicity.

Figure 18. Lysozyme is protecting AD transgenic flies. A) Longevity assay of controls and flies that express $A\beta$ with the Arctic mutation ($A\beta_{Arc}$) in the CNS with and without co-expression of one or two copies of lysozyme. B) Activity assay of controls and flies that express $A\beta_{Arc}$ in the CNS with or without one or two copies of lysozyme, where velocity (left) and angle of movement (right) were investigated.

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In an attempt to elucidate if the protective effect of lysozyme was due to changed generation and/or turnover of $A\beta_{1-42}$ the levels of soluble and insoluble $A\beta_{1-42}$ were analyzed. Co-expression of lysozyme changed neither the soluble or insoluble level of $A\beta_{1-42}$ in flies that expressed $A\beta_{1-42}$ or APP and BACE1. However, the levels of both soluble and insoluble $A\beta_{1-42}$ were substantially increased in flies that expressed $A\beta_{1-42}$ compared to flies that expressed APP and BACE1. Hence, the protective effect is not due to degradation of $A\beta_{1-42}$. Another possible way lysozyme could confer protection is through interaction with $A\beta_{1-42}$. This hypothesis was tested by immunoprecipitation on extracts from flies that expressed APP and BACE1, with or without co-expression of lysozyme, which was run on a column with a lysozyme antibody. The eluate was subsequently analyzed by the MSD technique for $A\beta_{1-42}$, which revealed that APP-BACE1-lysozyme flies had a higher signal of $A\beta_{1-42}$ compared to APP-BACE1 flies. Hence, $A\beta_{1-42}$ and lysozyme interact in flies that express APP and BACE1, and this interaction makes $A\beta_{1-42}$ less toxic.

In summary, lysozyme levels are increased in AD and this increase is protective in different *Drosophila* AD models. The protective effect can be attributed to an interaction between $A\beta_{1-42}$ and lysozyme.

PAPER IV: Lysozyme attenuates the cellular uptake of amyloid- β

This project was conducted to further explore the mechanisms of the protective effect associated with lysozyme on different AD model systems. The aim was to elucidate if lysozyme has any effect on the cellular uptake of A β .

One way lysozyme could confer protection is through decreased uptake of A β . To investigate this, human neuroblastoma cells were exposed to monomeric A β_{1-40} or A β_{1-42} , both conjugated to the red fluorophore TAMRA, with or without lysozyme for 1.5 h. The uptake was thereafter measured as cellular red fluorescence detected by flow cytometry. When lysozyme was present a reduction in cellular uptake of A β_{1-40} and A β_{1-42} was evident (Figure 19A and B). However, the reduction was more prominent for A β_{1-42} than for A β_{1-40} .

Figure 19. The cellular uptake of monomeric A β_{1-40} and A β_{1-42} is reduced by lysozyme. Flow cytometry analysis of cellular uptake of 0.1, 0.2, 0.3 and 0.5 μ M monomeric A) A β_{1-40} -TAMRA or B) A β_{1-42} -TAMRA with and without 1 μ M of lysozyme. Significant differences are denoted with * ($p \leq 0.05$), ** ($p \leq 0.01$) or *** ($p \leq 0.001$). Error bars represent mean $\pm$ SD.

In addition, lysozyme also reduced the uptake of oligomeric A β_{1-42} . To address if the presence of lysozyme during the oligomerization process was of importance for the reduced uptake of A β_{1-42} two approaches were employed; one where lysozyme was co-aggregated together with A β_{1-42} (co) and one where lysozyme was added after (post) the aggregation, before exposure to cells. The reduction of A β_{1-42} uptake when lysozyme was added post aggregation was not as effective as when lysozyme was

RESULTS

added at the start of the aggregation process (Figure 20A). Finally, the cellular toxicity of A β ₁₋₄₂ when lysozyme was present, either during the aggregation or added post aggregation was investigated. Similar to the uptake study, lysozyme that co-oligomerized with A β ₁₋₄₂ showed the most effective protection (Figure 20B).

Figure 20. The cellular uptake and toxicity of oligomeric A β ₁₋₄₂ is reduced by lysozyme. A) Flow cytometry analysis of cellular uptake of 0.5 μ M oligomeric A β ₁₋₄₂-TAMRA either co- or post-oligomerized with 1 μ M of lysozyme. B) Viability analysis of neuroblastoma cells exposed to 0.5 μ M oligomeric A β ₁₋₄₂-TAMRA either co- or post-oligomerized with 1 μ M of lysozyme. Significant differences are denoted with ** ($p \leq 0.01$) or *** ($p \leq 0.001$). Error bars represent mean $\pm$ SD.

In order to investigate if the extracellular A β not taken up by cells in the presence of lysozyme, instead is secreted into the body, the level of A β in the body of the flies that expressed A β ₁₋₄₂ with the Arctic mutation (A β _{Arc}), with or without co-expression of one or two copies of human lysozyme in the fly CNS was measured. The level of A β in A β _{Arc} flies was increased in the body, as compared to control flies, which demonstrates that A β was secreted into the body. Co-expression of A β _{Arc} and one copy of lysozyme did not change this level, but two copies of lysozyme decreased the level of A β _{Arc} in the body.

In summary, lysozyme reduces the cellular uptake of oligomeric $A\beta_{1-42}$ which could explain the cytoprotective role against $A\beta_{1-42}$ induced toxicity. This beneficial effect was more pronounced when lysozyme was present from the beginning of the aggregation process compared to when lysozyme was added after the aggregation.

DISCUSSION

The influence of p-FTAA and h-FTAA on A β aggregation and cell toxicity

The AD brain is characterized by abundant deposition of insoluble A β plaques and loss of neurons which eventually leads to severe loss of cognition. In paper I it is shown that the A β ₁₋₄₂ toxicity in a cellular model is greatly reduced when the LCO p-FTAA is present during the aggregation process. By kinetic experiments it was demonstrated that p-FTAA binds to A β species formed before detection by ThT, which suggests that p-FTAA binds to the earliest formed soluble A β species that are toxic and prevent their toxicity. One way p-FTAA could confer protection is by interacting with the toxic soluble pool of A β and thereby change its conformation to less reactive insoluble A β .

It was recently reported in mice that there are two broad classes of A β oligomers: prefibrillar and fibrillar oligomers, referred to as type 1 and type 2 oligomers, respectively [225]. Type 1 oligomers appear early, are widely spread throughout the brain, correlate to cognitive deficits and can be stained with the oligomeric specific antibody A11 [84]. In contrast, type 2 oligomers have the structure of A β fibrils with parallel β -sheets, appear after plaques are formed through secondary nucleation, are in close vicinity to the plaques, are relatively innocuous and are stained by the antibody OC which detects amyloid fibrils [225]. Our results indicate that p-FTAA induces formation of oligomers of type 2 that are rich in β -sheet structure, since A β together

with p-FTAA was OC-positive already at the start of the aggregation process, as revealed by dense dots, whereas A β aggregated alone only displayed a faint OC signal. In addition, A β in the presence of p-FTAA accelerated the shift from random coil structures towards ordered β -sheet, compared to when A β was aggregated alone, as revealed by CD analysis. However, although not confirmed by the antibody A11, A β aggregated alone were most probably transformed to type 1 oligomers (Figure 21A). In conclusion, we believe that p-FTAA binds early formed A β species and convert them to more insoluble oligomers of type 2 rich in β -sheet (Figure 21B), which in turn leads to reduced A β cytotoxicity.

It was previously shown that p-FTAA was able to decrease the infectiousness of prion aggregates [226, 227], which prompted us to investigate the seeding effect of A β species formed in the presence of p-FTAA. Many studies have shown pathologic similarities between prion disease and neurodegenerative diseases and indicate protein templating in the induction and spreading of disease [228, 229]. A possible prion-like transmission of A β from neuron to neuron has been suggested. It is clear that aggregation of A β can be exogenously initiated *in vivo* by brain extracts containing aggregated A β [94, 230, 231]. Our results demonstrate reduced propagation properties of A β aggregated in the presence of A β -seeds formed together with p-FTAA compared to A β -seeds formed without p-FTAA (Figure 21C and D). In addition, the final ThT signal of the A β species formed with p-FTAA was higher, which indicates species of more fibrillar states. Speculatively, this could enable p-FTAA to reduce the cell-to-cell transmission and toxicity of A β . This mechanism of action appears similar to the orcein-related compound O4 and curcumin that previously were shown to enhance the aggregation of A β and thereby reduce toxicity [155, 156]. Moreover, p-FTAA was previously demonstrated to stabilize prion proteins [226]. A β aggregated with p-FTAA exhibited both an increased resistance towards degradation with proteinase K and a high resistance to the harsh denaturant Gdn-HCl, which suggest a more compact formation of the A β fibrils. Exposure of hydrophobic amino acids is associated to increased cytotoxicity [232]. However, according to Campioni *et al.* the toxicity could be reduced if the toxic oligomers were tightly packed with the hydrophobic amino acids incorporated within the protein [233]. Since our data

indicates a more compact formation of A β fibrils aggregated with p-FTAA we hypothesized that these fibrils were less hydrophobic than A β fibrils formed without p-FTAA. The hydrophobicity of the A β fibrils generated with p-FTAA, using the hydrophobic sensitive probe ANS, were less hydrophobic than when A β was aggregated alone. Noteworthy is that high ANS fluorescence did not correlate to high toxicity, since the highest ANS fluorescence occurred at a stage where A β was less toxic. Therefore, besides hydrophobicity, also the size of the formed A β oligomers seems to be of importance, where small hydrophobic aggregates are more toxic than larger hydrophobic aggregates.

Figure 21. Suggested mechanism for p-FTAA influence during Aβ aggregation. A) Aβ monomers aggregates and oligomers, protofibrils and fibrils are formed. The different species are in equilibrium to each other, which is indicated by the arrows pointing in both directions. B) Aβ monomers together with p-FTAA speeds up the aggregation and increase the stability of the formed fibrils, which is indicated by the smaller arrow pointing to the left. C) Aggregation of Aβ monomers together with preformed Aβ fibrils that seed the aggregation. D) Aggregation of Aβ monomers together with preformed Aβ fibrils formed in the presence with p-FTAA display less seeding capacity compared to fibrils formed without p-FTAA, as indicated by the smaller arrow.

In paper II the heptameric oligothiophene h-FTAA was introduced in addition to p-FTAA since it has been shown to detect a broader subset of protein aggregates and also to discriminate between different strains of protein aggregates [196, 234, 235]. It was demonstrated that h-FTAA, but not p-FTAA, reduced the $A\beta_{Arc}$ toxicity. This effect was operative in the beginning of the aggregation as adding h-FTAA after the aggregation of $A\beta_{Arc}$ did not reduce the $A\beta_{Arc}$ toxicity, which indicates that interaction with early formed species is crucial for the protective action. In order to scrutinize the different effects of p-FTAA and h-FTAA on $A\beta_{Arc}$ toxicity the binding of these LCOs with $A\beta_{Arc}$ was investigated. Kinetic measurements of $A\beta_{Arc}$ were employed by ThT, p-FTAA and h-FTAA in order to study the nucleation behavior. Of note, during the first 5 h of aggregation there was no difference in the fluorescence curves between p-FTAA and h-FTAA. This is surprising considered that the toxicity of $A\beta_{Arc}$ lasts up to 2 h of aggregation. Consequently, the distinct protective behavior of p-FTAA and h-FTAA during this time cannot be explained by kinetics. However, after 5 h of aggregation the curves of p-FTAA and h-FTAA were separated and h-FTAA and ThT appeared to detect the same $A\beta_{Arc}$ structures whereas p-FTAA detected other forms of $A\beta_{Arc}$. Another possible explanation could be that p-FTAA and h-FTAA affected the aggregation process differently and therefore gave rise to separate aggregation curves. The binding of $A\beta_{Arc}$ to p-FTAA and h-FTAA was further investigated by comparing the ratio of bound and unbound $A\beta_{Arc}$ over time, by recording the shift in excitation wavelength. Interestingly, this method revealed differences already during early aggregation, where $A\beta_{Arc}$ showed better binding affinity to h-FTAA compared to p-FTAA. Hence, the mechanism by which h-FTAA confers protection against $A\beta_{Arc}$ but not p-FTAA remains elusive, but our data points toward less efficient binding with p-FTAA. In conclusion, p-FTAA and h-FTAA are potential tools as therapeutics for AD and early onset AD with the Arctic mutation.

A β triggers the expression of lysozyme

p-FTAA binds A β and speeds up the aggregation, which reduces the cellular toxicity of A β (paper I). Lysozyme was also shown to bind A β and reduce the toxicity, but in contrast to p-FTAA instead reduce the aggregation of A β [191]. Indications of that lysozyme is involved in AD have been found both *in vitro* and *in vivo* [191-193], but its role is not yet clear. Paper III demonstrates that lysozyme mRNA expression is increased in the brain of different AD mice models, and in brains from AD patients. The expression of lysozyme in AD transgenic mice correlated well with A β pathology and the A β pathology had a spatial similarity with the increase in lysozyme expression, while the tau transgenic mice only showed an upregulation of lysozyme at the end stage of disease and did not correlate with the age-related increase in tangle load. These results suggest that A β , but not tau, might trigger the increased expression of lysozyme, which is in agreement with previous findings where A β exposure of glioma and neuroblastoma cells triggered increased levels of lysozyme [191]. Furthermore, increased levels of lysozyme were found in CSF from AD patients, which probably mirror the increased expression of A β in the affected brain regions. An increased level of lysozyme in CSF was previously demonstrated in two cohorts of biochemically diagnosed AD [191], but this is the first study of AD patients that are both biochemically and clinically diagnosed.

Lysozyme prevents A β cytotoxicity via binding interactions

One way lysozyme could be protective is through interaction with A β_{1-42} . It was recently discovered that lysozyme keeps A β_{1-40} in a state that prevents the formation of A β fibrils and it was suggested that lysozyme acts like a molecular chaperone [192]. Lysozyme's substrate binding site appeared to interact with monomeric A β_{1-40} at the N-terminus but also hydrophobic interactions were evident at the C-terminal part of A β . It is therefore conceivable that lysozyme blocks hydrophobic residues important for A β aggregation. Moreover, lysozyme was recently shown to bind to monomeric A β_{1-42} , to co-localize with A β plaques in human AD brain and with A β

aggregates in A β ₁₋₄₂ overexpressing *Drosophila* flies [191]. In paper III we show co-localization of lysozyme to A β plaques in APP mice, which indicates that lysozyme is in close association to fibrillary A β . We also show an interaction between A β ₁₋₄₂ and lysozyme in the APP-BACE1 flies. Helmfors *et al.* demonstrated that this interaction turned A β ₁₋₄₂ less toxic to cells and reduced the toxicity in a *Drosophila* AD model that expressed A β ₁₋₄₂ in the CNS [191]. This is in line with Luo *et al.* who recently demonstrated reduced neuroblastoma cytotoxicity of A β ₁₋₄₀ with lysozyme [193]. In paper III the beneficial effects of lysozyme was confirmed in *Drosophila* models of AD, and in paper IV in neuroblastoma cells. One hypothesis is that the protective action of lysozyme could be due to increased cellular degradation of A β ₁₋₄₂, which was previously demonstrated in A β ₁₋₄₂ flies that co-expressed lysozyme in the CNS [191]. However, in paper III there was no difference in the amount of either soluble or insoluble A β ₁₋₄₂ between flies that expressed A β ₁₋₄₂ or A β ₁₋₄₂ co-expressed with lysozyme, or APP-BACE1 flies and flies with APP-BACE1 that co-expressed lysozyme. The discrepancies between these studies could be that the degradative capacity of the brain is different compared to the eye, but also that the interactions between A β ₁₋₄₂ and lysozyme are different in the two systems.

Discrepancies between A β ₁₋₄₂ and APP-BACE1 fly models

It was discovered in paper III that A β ₁₋₄₂ flies had an increased expression of A β ₁₋₄₂, both in the soluble and insoluble fraction, as compared to APP-BACE1 flies. Yet the toxicity of the ommatidia was greater in the APP-BACE1 flies. The underlying reason for this could be that the localization of A β and the A β isoforms generated are of importance rather than the quantity of produced A β . In the A β ₁₋₄₂ fly, the produced A β ₁₋₄₂ is secreted due to a secretion tag on the A β ₁₋₄₂ construct. This secretion tag is not present in the APP-BACE1 constructs. Although this has not been investigated, it could be speculated that the APP-BACE1 flies have a higher level of intracellular A β compared to the A β ₁₋₄₂ flies. The role of intracellular A β ₁₋₄₂ is still debated, but several studies have indicated that it is central for neurotoxicity in AD pathogenesis [32, 236]. Moreover, in the APP-BACE1 flies, part from the production of A β ₁₋₄₂, a range of A β

isoforms was most likely produced from processing of APP. Post-translationally modifications such as amino-terminal truncations and pyroglutamylation generates A β that are more toxic than A β_{1-42} . These variants are abundant in plaques and have been suggested to trigger AD by induction of a highly toxic conformation of A β_{1-42} [116]. In addition, it has been reported that mixtures of various A β peptides can change the cytotoxic behavior of the other isoforms present in the mixture [115]. It was shown that mixtures of co-aggregated A β_{1-38} and A β_{1-40} were highly toxic whereas A β_{1-38} added to A β_{1-42} instead had a cytoprotective effect [115]. Hence the more abundant degeneration of the ommatidia in APP-BACE1 flies compared with A β_{1-42} flies could be due to both the location and the mixture of A β species generated in the APP-BACE1 flies rather than the level of A β_{1-42} .

Lysozyme prevents A β toxicity via attenuation of A β uptake

Paper IV shows that the cellular uptake of monomeric A β_{1-42} was more reduced than the uptake of A β_{1-40} . This indicates that hydrophobic interactions might be involved in the interaction between lysozyme and A β resulting in more efficient binding of lysozyme to A β_{1-42} than A β_{1-40} , which hinders the uptake of A β_{1-42} . This is in agreement with molecular docking simulation studies of lysozyme and A β_{1-40} which demonstrates that hydrophobic interactions are involved [192]. A β_{1-42} co-oligomerized with lysozyme reduced the uptake more than when lysozyme was added post-oligomerization. The degree of A β uptake also reflected the toxicity and as previously demonstrated, the cellular uptake of A β is closely associated to toxicity [105]. The toxicity mechanisms behind intracellular A β are not completely understood, but several studies have demonstrated progressive accumulation of A β in multivesicular bodies, lysosomes and mitochondria along with defects in autophagy, the proteasomal system and mitochondrial function [105]. In addition, loss of function of lysosomal hydrolases might in turn lead to overloaded lysosomes with destabilized membranes resulting in lysosomal rupture and neuronal cell death [237]. Interestingly, it was previously shown that extracellular addition of A β induces the endogenous intracellular production of A β [238], which probably amplifies the toxic effect of A β .

Since lysozyme is present from the beginning during co-oligomerization, it is reasonable to believe that lysozyme binds to monomers and prevents the formation of toxic oligomers. Post-oligomerization on the other hand allows for oligomers to form and although lysozyme binds the oligomers and neutralizes them, it is conceivable that there are free oligomers available, which would explain the increased uptake and less protection, compared to the co-oligomerization. These *in vitro* results demonstrate that lysozyme inhibits the uptake of A β , hence lysozyme has the potential to stop the neuron-to-neuron transmission of A β_{1-42} but also the intracellular toxic actions of A β_{1-42} . The mechanisms behind A β uptake is not known to date, but several receptors are believed to be implicated [239]. The reason for the decreased uptake could therefore be due to A β having an impaired ability to bind to the receptors when lysozyme is present.

Soluble A β can be cleared by many ways from the interstitial fluid (ISF) of the brain when not taken up by neurons for intracellular degradation. These clearance systems include transport across the blood-brain barrier and blood-CSF barrier, ISF bulk flow where ISF proteins are directly cleared into the CSF and CSF absorption where proteins are exported into the circulation or lymphatic systems for systemic clearance by the liver and kidneys [240]. Furthermore, A β not taken up by neurons are available for intracellular degradation by microglia or degradation by extracellular IDE. It is conceivable that the reduced neuronal uptake of A β leads to an increased proportion of A β -lysozyme complex in the extracellular space that could be cleared by these mechanisms *in vivo*. Paper IV demonstrated that A β can be drained into the body of the flies, but there was no difference between flies that expressed A β_{Arc} alone or one copy of lysozyme. However, two copies of lysozyme decreased the level of A β in the body, and it remains to be elucidated whether this is due to increased degradation of A β in the body or if the secretion of A β into the body was attenuated. There is no evidence for involvement of proteolytic activity of lysozyme against A β . However, it was previously reported that lysozyme has properties that goes beyond its enzyme activity, since inactivated lysozyme still possesses antimicrobial activity as well as the ability to neutralize murine norovirus [241]. Hence, lysozyme in itself could be important through other potential mechanisms than enzymatic activity.

Potential mechanisms for how lysozyme could prevent the toxic effects of Aβ

The results in paper III and paper IV show that lysozyme influence the uptake and toxicity of Aβ. Potential scenarios of how this could be achieved are illustrated in Figures 22-24 below.

Figure 22. Secreted monomeric Aβ (Aβ_m) or Aβ_m and oligomeric Aβ (Aβ_o) released from plaques could bind to lysozyme. This interaction could prevent Aβ oligomerization in the extracellular space and inhibit the cellular uptake of Aβ and Aβ toxicity. Paper IV demonstrates that co-aggregation of Aβ_m and lysozyme prevents the cellular uptake of Aβ_o. The binding of lysozyme to extracellular Aβ_m is a possible mechanism for the protective effect of lysozyme in Aβ₁₋₄₂ flies and in flies with the Arctic mutation of Aβ₁₋₄₂ in paper III.

Figure 23. Internalized APP is cleaved by β- and γ-secretases in the endosomes/lysosomes which yields Aβ. The acidic pH in endosomes/lysosomes and the high concentration of Aβ

favor $A\beta$ aggregation and the accumulation of oligomeric $A\beta$ ($A\beta_o$). It is possible that $A\beta_o$ could be released extracellularly where it binds to lysozyme which prevents the complex from reuptake. Paper IV demonstrates that post-aggregation of $A\beta_o$ and lysozyme prevents the cellular uptake of $A\beta_o$. The lysozyme protection in APP-BACE1 flies seen in paper III could be explained by this mechanism.

Figure 24. Lysozyme could bind to the $A\beta$ region in APP when located at the plasma membrane. Internalized APP bound to lysozyme could then be cleaved by β - and γ -secretases in endosomes/lysosomes, and monomeric $A\beta$ ($A\beta_m$) might be generated in association with lysozyme. This association will prevent oligomerization of $A\beta$ and $A\beta$ cytotoxicity (1). In addition, internalized APP could be cleaved by β - and γ -secretases in endosomes/lysosomes, and the generated $A\beta_m$ could in turn bind lysozyme within the endosome/lysosome since lysozyme is a lysosomal protein. This will prevent $A\beta$ oligomerization and thus the $A\beta$ toxicity will also be prevented (2). These are possible mechanisms for the lysozyme protection seen in APP-BACE1 flies in paper III.

CONCLUSIONS

The work presented within this thesis has focused on the impact of the thiophene-based fluorescent probes p-FTAA and h-FTAA and the immune related protein lysozyme on aggregation and toxicity of $A\beta_{1-42}$. The following conclusions can be drawn from the studies:

- p-FTAA reduces the levels of prefibrillar $A\beta$, protects the cells from $A\beta$ toxicity and increases the stability of the formed $A\beta$ fibrils.
- h-FTAA protects neuroblastoma cells against $A\beta_{Arc}$ induced toxicity.
- The level of lysozyme expression in the brain and CSF is increased both in AD mice models and in AD patients and the increased expression of lysozyme correlates with the expression of $A\beta$ pathology but not to tau tangles.
- Increased expression of lysozyme is protective *in vivo* as demonstrated in three *Drosophila* models of AD.
- The protective effect of lysozyme is not attributed to degradation of $A\beta_{1-42}$ or to a change in the level of soluble and insoluble $A\beta_{1-42}$, but rather to an interaction between $A\beta_{1-42}$ and lysozyme.
- Lysozyme protects neuroblastoma cells against $A\beta_{1-42}$ induced toxicity and reduces the cellular uptake of both monomeric and oligomeric $A\beta_{1-42}$. These beneficial effects are most effective when lysozyme co-aggregates with $A\beta$.

FUTURE PERSPECTIVES

It is now generally accepted that high molecular weight species of A β are less toxic than low molecular weight species. Hence, treatment strategies that affect the aggregation process towards fibril formation could be beneficial. The results from paper I agree with this theory, where the compound p-FTAA induces formation of larger A β species together with reduced cytotoxicity. To have a treatment strategy that promote fibril formation of A β is controversial since formation of larger A β species in turn could develop into plaques after several years in the human brain and the outcome of that is not yet known. However, it would be highly interesting to investigate this *in vivo* by injecting p-FTAA in the hippocampus of AD transgenic mice of different ages and study the memory function and brain pathology over time. Indeed, this would reveal if A β is accumulating more in the brain in the presence of p-FTAA and if p-FTAA is protective *in vivo*.

Paper II demonstrates that h-FTAA is protective against cellular toxicity induced by A β_{Arc} . It is important to investigate if the protective mechanism is similar to that found in paper I with p-FTAA, or if other mechanisms are involved. Moreover, to further elucidate the different interaction between p-FTAA and h-FTAA to A β_{Arc} would be interesting in order to understand the difference in cellular protection mechanisms between the two oligothiophenes. Furthermore, to also do intracranial injections of h-FTAA in AD transgenic mice with the Arctic mutation, in a similar way as described

for p-FTAA above, would give important information of the significance of h-FTAA for the treatment of this patient group of AD.

As paper III and IV demonstrate that lysozyme has a protective role against $A\beta_{1-42}$ induced cytotoxicity in cultured cells and in different fly models of AD, also the *in vivo* effects of lysozyme in a more advanced organism would be intriguing to evaluate. The same approach as for p-FTAA also applies for lysozyme; to inject lysozyme in the hippocampus of AD transgenic mice followed by memory function studies along with microscopic examinations of the brain pathology.

The effects of p-FTAA, h-FTAA and lysozyme when administered via intra-venous injections would also be interesting to investigate. However, the blood-brain-barrier could prevent the substances to enter the brain and from exerting their effect. A mode of application that could be tested is to perform ultrasound sonication of the mice prior administration of p-FTAA, h-FTAA and lysozyme, to facilitate entrance of the molecules. This approach was recently shown to transiently open the blood-brain-barrier and enhance removal of $A\beta$ [242]. Moreover, to couple p-FTAA, h-FTAA and lysozyme to carriers specialized to deliver therapeutic agents over the blood-brain-barrier could also be a possible course of action. Regarding lysozyme, another potential way could be to treat the mice with a substance that passes the blood-brain-barrier and induce production of lysozyme inside the brain, without production of other pro-inflammatory cytokines.

Lysozyme inhibits the uptake of oligomeric $A\beta_{1-42}$ in neuroblastoma cells. It would also be highly interesting to study the uptake of $A\beta_{1-42}$ in neurons and microglia from rat, to investigate if the uptake inhibition with lysozyme is also seen in primary cell cultures. If lysozyme is inhibiting the $A\beta$ uptake in primary neurons as well, lysozyme has the potential to stop the neuron-to-neuron transmission of toxic oligomers of $A\beta_{1-42}$. This could be explored *in vivo* in mice by lysozyme added in different ways as mentioned above, and then study the spread of $A\beta$ pathology.

The work within this thesis demonstrates promising results of oligothiophenes and lysozyme as potential treatment of AD. The suggested experiments above would be

interesting to do in order to unravel if p-FTAA, h-FTAA and lysozyme could be used as treatment options for AD in the future.

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