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Amyloid- β Protofibril Formation and Neurotoxicity

Implications for Alzheimer's Disease

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Abstract

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Alzheimer's disease (AD) is the most common cause of dementia. A characteristic feature of AD is the presence of amyloid plaques in the cortex and hippocampus of the brain. The principal component of these plaques is the amyloid- β (A β) peptide, a cleavage product from proteolytic processing of amyloid precursor protein (APP). A central event in AD pathogenesis is the ability of A β monomers to aggregate into amyloid fibrils. This process involves the formation of various A β intermediates, including protofibrils. Protofibrils have been implicated in familial AD, as the Arctic APP mutation is associated with enhanced rate of protofibril formation *in vitro*.

This thesis focuses on A β aggregation and neurotoxicity *in vitro*, with special emphasis on protofibril formation. Using synthetic A β peptides with and without the Arctic mutation, we demonstrated that the Arctic mutation accelerated both A β 1-42 protofibril- and fibril formation, and that these processes were affected by changes in the physicochemical environment.

Oxidation of A β methionine delayed trimer and protofibril formation *in vitro*. Interestingly, these oxidized peptides did not have the neurotoxic potential of their un-oxidized counterparts, suggesting that formation of trimers and further aggregation into protofibrils is necessary for the neurotoxic actions of A β . In agreement, stabilization of A β wild type protofibrils with the omega-3 (ω 3) fatty acid docosahexaenoic acid (DHA) sustained A β induced neurotoxicity; whereas in absence of DHA, neurotoxicity was reduced as A β fibrils were formed. These results suggest that the neurotoxic potential of A β is mainly confined to soluble aggregated forms of A β , not A β monomer/dimers or fibrillar A β .

Stabilization of A β protofibrils with DHA might seem contradictory, as ω 3 fatty acids generally are considered beneficial for cognition. However, we also demonstrated that DHA supplementation reduced A β levels in cell models of AD, providing a possible mechanism for the reported beneficial effects of DHA on cognitive measures *in vivo*.

Keywords: Amyloid- β , Neurotoxicity, Aggregation, Protofibrils, Alzheimer's disease

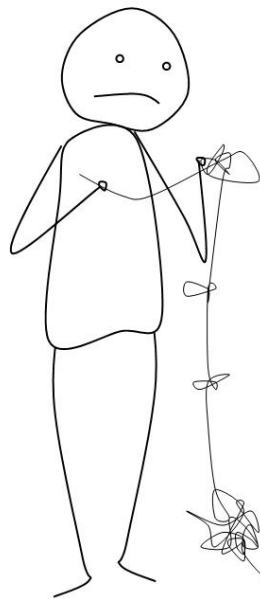
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It will all work out in the end

Cover image designed by Paul O'Callaghan

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I** **Johansson A-S**, Berglind-Dehlin F, Karlsson G, Edwards K, Gellerfors P and Lannfelt L.
Physiochemical characterization of the Alzheimer's disease related peptides A β 1-42Arctic and A β 1-42wt.
FEBS Journal (2006) **273**:2618-30

- II** **Johansson A-S**, Bergquist J, Volbracht C, Päiviö A, Leist M, Lannfelt L and Westlind-Danielsson A.
Attenuated amyloid- β aggregation and neurotoxicity owing to methionine oxidation.
Neuroreport. In Press.

- III** **Johansson A-S**, Garlind A, Berglind-Dehlin F, Karlsson G, Edwards K, Gellerfors P, Ekholm Pettersson F, Palmblad J and Lannfelt L.
Docosahexaenoic acid stabilizes soluble amyloid- β protofibrils and sustains amyloid- β induced neurotoxicity *in vitro*.
FEBS Journal (2007) **274**:990-1000

- IV** Sahlin C, Ekholm Pettersson F, Nilsson LNG, Lannfelt L and **Johansson A-S**.
Docosahexaenoic acid stimulates non-amyloidogenic APP processing resulting in reduced A β levels in cellular models of Alzheimer's disease.
Manuscript.

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Abbreviations

AD	Alzheimer's disease
A β	Amyloid- β
APP	Amyloid precursor protein
BACE	β -site APP cleaving enzyme
AICD	APP intracellular domain
FAD	Familial AD
ADDLs	A β derived diffusible ligands
CAA	Cerebral amyloid angiopathy
LTP	Long term potentiation
HCSM	Human cerebrovascular smooth muscle
ω 3	Omega-3
PUFAs	Polyunsaturated fatty acids
DHA	Docosahexaenoic acid
AA	Arachidonic acid
SEC	Size exclusion chromatography
MW	Molecular weight
ThT	Thioflavin T
TEM	Transmission electron microscopy
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
CMC	Critical micelle concentration
CSF	Cerebrospinal fluid
UV	Ultraviolet
PBS	Phosphate buffered saline
DMSO	Dimethyl sulfoxide
FTICR-MS	Fourier transform ion cyclotron resonance mass spectrometry

Introduction

Alzheimer's disease

Background

In 1906, the German psychiatrist and neuropathologist Dr. Alois Alzheimer described a medical condition of a woman in her early 50s. The patient, referred to as Auguste D, suffered from memory impairment, altered behavior as well as poor language skills and cognitive function. *Post mortem* neuropathological examinations revealed histopathological lesions, senile plaques and neurofibrillary tangles (Fig. 1), which still to this day are referred to as the pathological hallmarks of Alzheimer's disease (AD) [1]. Despite the early description of the disease, it was not until the late 1960s and early 1970s by the work of Tomlinson, Blessed and Roth that AD was recognized as the most common cause of dementia [2, 3]. During this time, it was also discovered that patients suffering from AD had severe loss of cholinergic neurons synthesizing the neurotransmitter acetylcholine [4]. As a result of this discovery, efforts have been made to increase the levels of acetylcholine in the brain. The most common drugs used to treat AD today are based on this idea. Later on it was shown that several other neurotransmitter systems are affected in AD providing an explanation for the modest effect of the existing drugs. In the last two decades, research has focused on the molecular mechanisms underlying the disease, and much knowledge has been gained by further examination and characterization of the brain lesions originally described by Dr. Alzheimer.

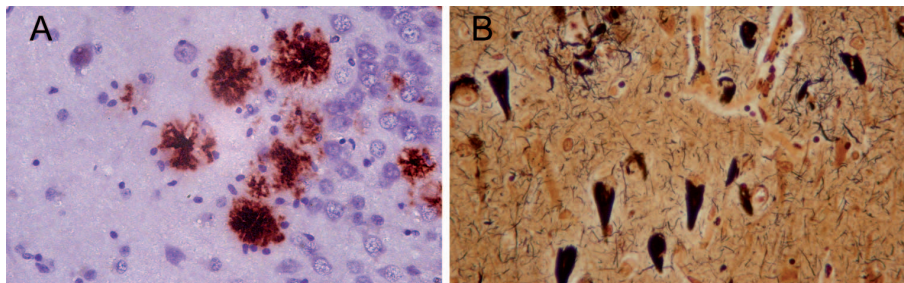


Figure 1. Histopathological lesions in AD. A: Human plaques B: Human neurofibrillary tangles. Pictures from Paul O'Callaghan and Hannu Kalimo.

Symptoms, diagnosis and treatment

The initial symptom of AD is typically impaired short term memory. Also language and spatial orientation can be affected at an early stage of the disease. Long term memory, general intelligence, vocabulary and reading abilities are generally not affected initially but as the disease progresses, symptoms like severe language problems, personality changes, not being able to remember family members and problems with daily life activities such as dressing and eating appear. Death occurs 5-15 years after disease onset, and most often results from secondary infections, like pneumonia or other infections unrelated to AD.

AD can only be diagnosed with 100% accuracy *post mortem* after brain autopsy. Clinical diagnosis of AD is made using medical history of the patient and neuropsychiatric testing complemented with physical examination to exclude other causes of reduced cognitive function, e.g. brain tumors or stroke. The most common tests used to evaluate cognitive function are mini mental status examination (MMSE) and the cognitive part of the Alzheimer's disease assessment scale (ADAS-cog). Physical examination includes assessment of neurological functions and can be complemented with brain imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT). In rare cases, positron emission transmission (PET) is used to measure decline in metabolic activity in the brain, a feature associated with AD. Studies where clinical diagnosis has been compared to neuropathological examination have shown that experienced clinicians can distinguish between AD and other types of dementia 80-90 % of the times. The need for early and accurate diagnosis is becoming increasingly important as future therapies will likely target early steps in the pathogenesis. (reviewed in [5]).

There are currently no treatments available to cure AD. The drugs used today are only symptomatic treatments as they do not affect the actual disease process. They provide some improvement in memory and cognitive function, perhaps for a period of 1-2 years. There are four registered drugs for AD in Sweden. Three of these are cholinesterase inhibitors, which increase the levels of acetylcholine at the synapse of cholinergic neuron and are given to patients with mild to moderate AD. The fourth, recently registered, is a NMDA (N-methyl d-aspartate) receptor antagonist that is thought to reduce the negative effects of glutamate in the brain and is administered to patients with moderate to severe AD.

Neuropathology

AD is manifested by extensive neuronal and synaptic loss [6], macroscopically observed by severe atrophy of hippocampus as well as temporal and parietal lobes of the cerebral cortex. However, the best correlate of cognitive

impairment appears to be loss of synapses, not cell death per se [7]. Senile plaques and neurofibrillary tangles are located predominately in areas displaying neuronal loss. i.e. the neocortex, hippocampus, amygdala and nucleus basalis [8]. Degeneration of cholinergic projection neurons located in the basal forebrain, e.g. nucleus basalis results in acetylcholine deficiency in the neocortex and hippocampus [9].

Senile plaques are extracellular structures containing fibrillar protein structures that stain with Congo red, a dye widely used to visualize various pathological fibrillar lesions in different diseases, referred to as amyloidoses [10]. Hence, AD belongs to this group of diseases, and the plaques are often referred to as amyloid plaques. In 1984, Glenner and Wong extracted amyloid from vessel walls in AD patients and identified the main protein component, later denoted amyloid- β ($A\beta$) [11]. $A\beta$ is produced by numerous cell types, including neurons, through enzymatic cleavage of amyloid precursor protein (APP). Several different variants of $A\beta$ are produced, the predominant species being $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ comprising 38, 40 and 42 amino acids respectively [12]. $A\beta_{42}$ is the most common species in the plaques found in the parenchyma of the brain [13], whereas $A\beta_{40}$ is more common in the amyloid deposits found in blood vessels [14].

Neurofibrillary tangles are intracellular deposits found in affected AD brain regions. These deposits have been found to be composed of hyperphosphorylated tau [15, 16], a microtubuli associated protein. It is generally believed that $A\beta$ precedes tau in the pathogenesis, i.e. $A\beta$ causes the tau pathology [17, 18].

The amyloid cascade hypothesis

The amyloid cascade hypothesis was formulated 15 years ago. This hypothesis states that amyloid deposition of the $A\beta$ peptide drives neurofibrillary tangle formation and various toxic events, which finally causes AD [19]. This theory was mainly based on genetic evidence. The amyloid cascade hypothesis has been the leading hypothesis in the field, but over the years it has also been challenged. A common criticism is the poor correlation between plaque load and dementia [7] and the fact that transgenic mice display AD-like neurological deficits prior to plaque deposition (reviewed in [20]). Even though the hypothesis is built around $A\beta$ as the central mediator of AD pathology, some modifications of the hypothesis has been proposed in recent years. This is due to the emerging evidence for the pathological role of soluble oligomeric $A\beta$ species, preceding plaque formation, and the role of intracellular $A\beta$ [21]. Soluble $A\beta$ correlates with synapse loss [22], the strongest correlate of memory impairment [7]. Some researchers even propose that plaques are inert end products, possibly protecting the brain from the toxic soluble $A\beta$ species. In a review by John Hardy and Dennis Selkoe in 2002 [23], the amyloid cascade hypothesis is scrutinized and more emphasis is put

on oligomeric species of A β . Basically, the hypothesis today states that increased A β 42 production caused by mutation or other factors such as faulty A β clearance lead to A β 42 oligomerization and deposition which eventually results in the pathogenic events associated with AD, such as neuronal/synaptic dysfunction, resulting in dementia.

APP processing

APP is a transmembrane protein widely expressed in different tissues. Proteolytic processing of APP is mediated by three different secretases, α -secretase, β -secretase and γ -secretase. The α -secretase cleavage site is located between residue 16 and 17 in the middle of the A β sequence, releasing α -APPs from the cell membrane. Left in the membrane is the C-terminal fragment C83. Alternatively, β -secretase cleaves APP into β -APPs and the C-terminal fragment C99. C83 and C99 are then cleaved by γ -secretase, producing p3 and A β respectively (Fig. 2). Hence, cleavage with α -secretase and γ -secretase does not produce A β . This pathway is therefore referred to as the non-amyloidogenic pathway. The amyloidogenic pathway producing A β is thus mediated by β -secretase and γ -secretase (reviewed in [24]).

β -secretase cleavage in the brain has been found to be mediated by one protein, the aspartyl protease β -site APP cleaving enzyme one (BACE-1) [25]. This transmembrane protein is transported through the secretory pathway to the plasma membrane, where it is clustered in lipid rafts (membrane microdomains rich in cholesterol) together with APP. Lipid rafts and endosomes are the primary sites of β -secretase activity [25, 26]. The optimum pH for BACE-1 activity is 5.5 [27], therefore the acidic environment of endosomes favors β -secretase activity. The biological relevance of BACE-1 has been unclear but recently, BACE-1 activity was found to be essential for myelination of axons [28]. BACE-2, a protein homologue of BACE-1, has been reported to perform β -cleavage of APP in glia cells, but not neurons [29].

γ -secretase cleavage is carried out by a protein complex consisting of four proteins; presenilin, nicastrin, APH-1 (anterior pharynx-defective phenotype 1) and PEN-2 (presenilin-enhancer 2) [30]. The γ -secretase complex cleaves within the transmembrane domain of APP. γ -secretase has been reported to be present and active in several cell compartments, including the endoplasmic reticulum, trans-Golgi network, endosomes as well as the plasma membrane ([31] and references therein) [32]. Like β -secretase, also γ -secretase has been found to be localized to lipid rafts [33]. In addition to the cleavage at γ -site, the γ -secretase complex also cleaves APP at ϵ -site, approximately ten residues downstream of the γ -site, releasing the APP intracellular domain (AICD) [34]. Recently, transmembrane protein 21 (TMP21) was identified as a new component of the γ -secretase complex, regulating γ -cleavage but not ϵ -cleavage [35].

The non-amyloidogenic cleavage mediated by α -secretase is not fully understood, but the metalloproteases ADAM 9, ADAM10 and ADAM17 (A disintegrin and metalloproteinase domain 9, 10 and 17) fulfill some of the criteria required of α -secretase. It seems there is an element of functional overlap between these candidates, suggesting that these enzymes can contribute to α -cleavage to different degrees, possibly depending on cell type (reviewed in [36]). In addition, BACE-2 has been reported to perform an α -secretase like cleavage three amino acid residues downstream of the α -cleavage site [37, 38].

The secretases involved in APP processing are important therapeutic targets for the pharmaceutical industry and much effort is put into finding β - and γ -secretase inhibitors. However, BACE-1 is not an easy target because of its rather large active site and localization to endosomes, as well as its possible involvement in myelination. γ -secretase inhibition is also associated with potential side-effects as the γ -secretase complex cleaves several other substrates, e.g. notch.

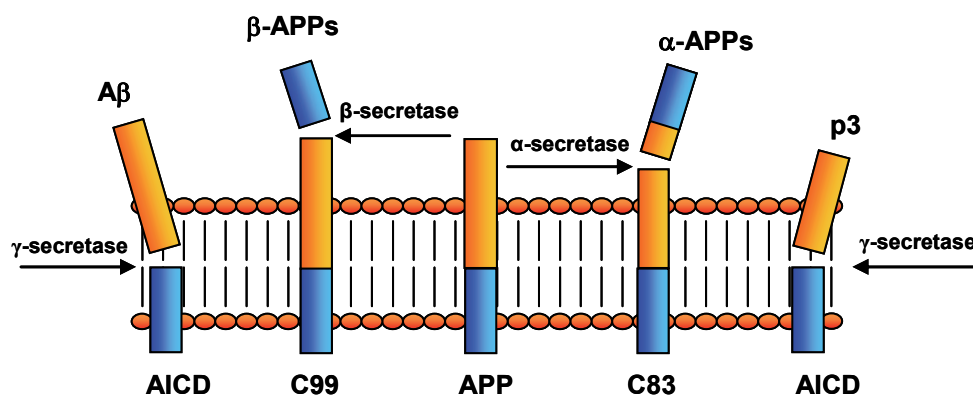


Figure 2. APP processing pathways. APP is cleaved by β -secretase and α -secretase, leaving C99 and C83 in the plasma membrane. C99 and C83 are substrates for γ -secretase, releasing A β and p3. Left in the membrane is AICD. Cleavage by α - and γ -secretase is referred to as the non-amyloidogenic pathway, whereas cleavage by β - and γ -secretase is referred to as the amyloidogenic pathway.

Genetics of Alzheimer's disease

Less than 5 % of all AD cases are caused by mutant genes inherited in an autosomal dominant fashion. These familial cases often have an earlier age of onset as compared to AD cases with no obvious genetic cause. AD causing mutations have been located in three different genes, the APP gene on chromosome 21, the presenilin 1 gene on chromosome 14 and the presenilin 2 gene on chromosome 1 (reviewed in [39]).

The APP mutations are located around the β - and γ -secretase cleavage sites, leading to disturbed APP processing favoring A β production [40-42]. A few of the APP mutations are located inside the A β sequence, affecting both APP processing and the aggregation properties of the A β peptide (*see section intra-A β mutations*). Moreover, patients with Down's syndrome carrying an extra chromosome 21 and thus an extra copy of the APP gene develop dementia at an early age [43]. Recently, inheritance of APP gene duplications has been linked to AD [44]. Mutations located in the presenilin genes are situated mainly in the transmembrane region of the proteins, and affect γ -secretase cleavage in such a way that A β 42 is overproduced [45].

In addition to the fully penetrant APP and presenilin mutations, there is also one known genetic risk factor for late-onset AD, the *APOE* (apolipoprotein E) *e4* allele located on chromosome 19 [46].

The A β peptide

Background

After the identification of the A β peptide as the main protein component of the amyloid plaques in AD, researchers started to characterize the properties of this peptide. As amyloid cores of senile plaques were extremely hard to dissolve and A β showed no homology to previous known proteins, the A β peptide was originally thought to be present only in senile plaques. However, studies revealed that A β is present in plasma in healthy individuals as well [47] and is produced in various types of cells in the body, not only neuronal cells [48].

Small parts of the A β amino acid sequence were found to form amyloid fibers *in vitro*, similar to the structures found in plaques *in vivo* [49]. Since the neuritic plaques mainly are composed of A β 42, even though A β 40 is the main A β species produced by neuronal cells [40], it was early recognized that the longer A β peptide seemed to be important in the etiology of the disease. In 1993, Jarret *et al* showed that the length of the carboxy terminus of A β was important for the rate of amyloid formation and that the aggregation of more soluble A β peptides could be "seeded", i.e. accelerated by addition of A β including the C-terminal residues [50].

The potential neurotoxic actions of the A β peptide *in vitro* were initially reported in the late 1980s. These studies were somewhat paradoxical, with both neurotrophic and neurotoxic actions described [51, 52]. Later on, the neurotoxic potential of synthetic A β was associated with A β aggregation [53-55], and the neurotoxic actions of A β was generally believed to entail fibril formation [56-58]. However, in recent years, soluble A β oligomers have emerged as the main neuropathological A β species [20, 59-65].

Neurotoxicity and aggregation experiments *in vitro* using synthetic A β peptides are associated with methodological difficulties because of the low solubility of these peptides and their high propensity to aggregate. Researchers have reported lot-to-lot variability [66, 67], and even variable results with the same peptide lot in different laboratories [68]. These methodological issues still remains a matter of concern. Recently, inconsistent neurotoxic properties of the A β peptide relating to peptide lot and choice of toxicity assay was reported [69].

Nucleation dependent polymerization

The fibrillization of monomeric A β into fibrils display several characteristics of a nucleation dependent polymerization process, a common type of process in biology. Other well studied examples are protein crystallization, microtubuli assembly and actin polymerization. For polymerization to occur, this process requires the formation of a nucleus. The growth of fibrils from the nucleus is often referred to as elongation. There are three characteristic features for nucleation dependent polymerization processes [70].

1. Lag phase – The time required for the nucleus to form, from which then fibrils can evolve. The lag phase is a thermodynamically unfavorable process, and therefore the rate limiting step.
2. Critical concentration – The concentration of monomers left after polymerization is complete. Below this concentration, no polymerization will occur.
3. Seeding – The addition of a seed, which will function as a nucleus, will speed up the polymerization process as a consequence of reduced lag time.

When it comes to A β , the exact number of molecules the smallest nuclei consist of is unknown, but work from David Teplows laboratory suggests that pentamer/hexamers might be the smallest unit required for fibrillization. A β 1-42wt have been shown to form these pentamers/hexamers early in the aggregation process, in contrast to A β 1-40wt which mainly consisted of monomers to tetramers [71].

The critical concentration required for A β fibrillization to occur is difficult to estimate. For synthetic A β peptides *in vitro* approximately 10 μ M is suggested to be the critical concentration, but this is not applicable for *in vivo* conditions, since the concentrations of A β in plasma and cerebrospinal fluid are in the nM/pM range. Probably, the local concentration of A β is higher in certain cell compartments, or unknown co-factors could enable a seed to form at a much lower A β concentration *in vivo*. Extracts from human AD brain have been reported to induce amyloidosis in APP transgenic mice [72, 73], suggesting that seeding can occur also *in vivo*. The lag time of a

of 3-6 nm [64]. Most importantly, they are soluble structures; i.e. they are not pelleted by centrifugation at moderate speed of 16-18000 x g. Development of a protofibril specific assay has enabled detection of protofibrils in cell media from APP transfected cells and brain homogenate from APP transgenic mice (*Englund et al - manuscript*). ADDLs are small soluble globular A β oligomers, ranging in size from trimers to 24-mers [60]. These oligomers have been detected in brain homogenates from AD patients, using an ADDL specific antibody [79].

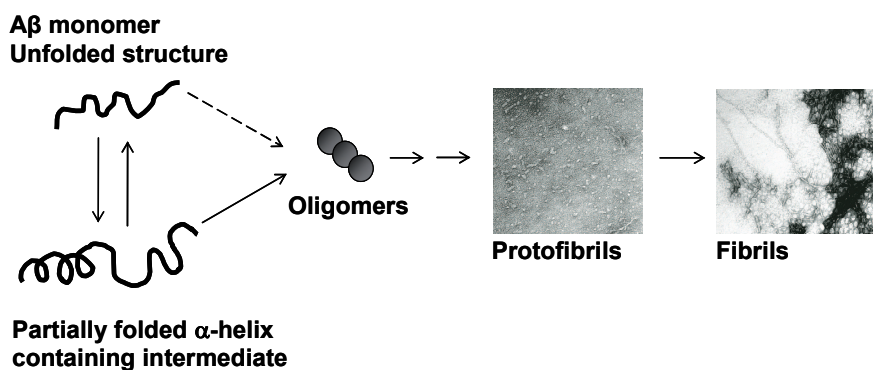


Figure 4. The A β fibrillization process. It has been suggested that the unfolded A β peptide can form a partly unfolded helix containing intermediate, from which fibril formation is accelerated. A β fibrillization entails the creation of oligomers, protofibrils and eventually insoluble fibrils are formed.

Fibrils are insoluble long structures >200 nm in length, with a diameter similar to that of protofibrils [77]. They are pelleted at moderate centrifugation speed, and bind β -sheet binding dyes such as Congo-Red and Thioflavin. A β fibrils have been reported to consist of 5-6 protofilaments with β -strands running perpendicular to the fiber axis, held together by hydrogen bonding (reviewed in Serpell *et al* [80]). Most likely, the strands are arranged in a parallel manner, forming a parallel β -sheet, with a turn at residues 25-30. Most of the amino acids in the core of the β -sheet are neutral and primary hydrophobic (Q15, L17, F19, A21, I31, M35 and V39), except aspartic acid in position 23, which forms a salt bridge with lysine in position 28, according to the fibril structure of Petkova *et al* [81]. A30, I32, L34, V36 and V40 form a hydrophobic face, whereas remaining amino acids are distributed along the bend, in the N-terminal segment and opposite to the hydrophobic face. Glutamic acid in position 22 can possibly form a salt bridge with lysine in position 16 when protofilaments associate with each other (Fig. 5).

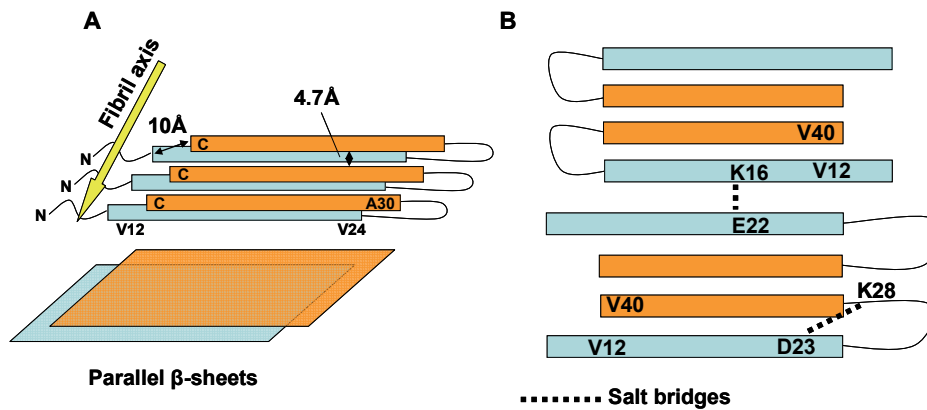


Figure 5. A β 1-40wt fibril structure according to Petkova *et al.* A: Amino acid 12-24 and 30-40 forms two β -strands with a bend comprising residue 24-30. The N-terminal part is unstructured. The strands are arranged in a parallel manner, forming parallel β -sheets. The distance between the sheets is 10 Å and the distance between the strands is 4.7 Å. B: Cross section of two A β 1-40wt protofilaments. D23 and K28 form a salt bridge in the hydrophobic core. K16 and E22 can form a salt bridge between the β -strands of two laterally associated protofilaments, forming the narrowest observed fibril. This would prevent electrostatic destabilization of the structure. Adapted from Fig. 4 and Fig. 5 in Petkova *et al* [81].

Intra-A β mutations

Pathogenic mutations within the A β peptide are associated with AD, Cerebral amyloid angiopathy (CAA) or both. CAA refers to the deposition of amyloid in cerebral blood vessels, resulting in stroke, cerebral hemorrhage or dementia. Five pathogenic intra-A β mutations have been identified so far, named after the ethnic origin of the affected families. All these mutations are located near the central hydrophobic cluster, in position 21, 22 and 23 (Fig. 3). Mutations in the N-terminal portion of A β (H6R and D7N) have lately been implicated in AD, but their penetrance is unclear [82, 83]. Recently, a C-terminal mutation in position 42 (A42T), thus only affecting the A β 42 peptide, was associated with AD and strokes [84].

All five intra-A β mutations, with the exception of the Flemish (A21G) mutation, increased the aggregation rate of A β [85-88]. Moreover, the Arctic (E22G) and Dutch (E22Q) mutations are associated with accelerated A β 1-40 protofibril formation [63, 77, 86], and intracerebroventricular injection of these peptides in rats have been reported to inhibit long term potentiation (LTP) [89], an electrophysiological correlate of aspects of learning and memory. Intra-A β mutations have also been shown to confer resistance against degradation by neprilysin [90].

It has been suggested that A β 1-40 peptides with a loss or change of charge enhance the pathogenic properties of the peptide by binding to the surface of human cerebrovascular smooth muscle (HCSM) cells, providing

an explanation for the vascular phenotype seen in the Dutch, Italian and Iowa kindred. Interestingly, the Flemish non charge-altering variant does not bind to the surface of HCSM cells [88, 91]. Nor does it increase A β 1-42wt induced toxicity in PC-12 (rat pheochromocytoma 12) cells, unlike the other intra-A β mutations [85]. The Flemish mutation has however been reported to increase A β production [88].

A β mediated neurotoxicity

As evidence for aggregated A β as a pathogen in AD is accumulating, the search for the most pathogenic A β aggregate has intensified. In studies with synthetic A β , both protofibrils and fibrils have been reported to be toxic to cultured neurons [59, 64, 92] as well as affect electrophysiological parameters [59, 64]. Neither of these effects were observed for monomeric or low molecular weight A β [59, 64, 92]. On the other hand, there are also reports of fibrillar A β being non-toxic [93, 94] or at least less potent [95] compared to oligomeric forms. One explanation for these conflicting reports could be that oligomeric and fibrillar A β induce neurotoxicity by different mechanism, as reported by Deshpande *et al* [95]. One interesting study by Wogulis *et al* suggests that A β mediated cell death is dependent on ongoing polymerization, rather than a single specific aggregate [69].

Approaches with natural oligomers, formed in cells or transgenic mice, have also been reported. For instance, intracerebroventricular injection in rats with naturally secreted oligomers from cell cultures inhibited LTP [65] and oligomers purified from old transgenic APP mice impaired spatial memory when injected in young rats [62].

Both necrotic and apoptotic features have been linked to A β mediated neuronal death *in vitro*. Most likely, both these toxic mechanisms are occurring. A number of toxic events inducing both necrotic and apoptotic measures have been reported in the literature, such as oxidative stress, calcium overload, mitochondrial dysfunction and pore/channel formation (reviewed in [96]). These mechanisms can be interrelated and occur simultaneously, and some of these events are probably secondary to the initial toxic insult. Mitochondrial dysfunction have been reported to be an early A β mediated toxic event taking place prior to cell death [95].

Lipids in Alzheimer's disease

Background

In the late 1920s researchers acknowledged the importance of certain lipids for the development and growth of humans and animals. The significance of

lipid composition for neuronal functions is not very surprising since the brains dry weight to one fourth consists of lipids, and the lipid content of neuronal membranes and myelin sheets amount to 50% and 70%, respectively. In the 1960s, some fatty acids were recognized as essential, as infants fed milk substitutes lacking certain fatty acids developed symptoms like skin lesions and growth retardation, now acknowledged as typical for essential fatty acid deficiency (reviewed in [97]). Omega-3 (ω 3) fatty acids, as well as ω 6 fatty acids are derived from essential fatty acids, i.e. they can not be synthesized *de novo*, but have to be ingested through the diet. Essential fatty acids are important for several brain functions like myelination [98], vision and developmental and cognitive functions [97]. Growing evidence suggest that ω 3 polyunsaturated fatty acids (PUFAs) are implicated in several brain disorders ranging from attention deficit and hyperactivity disorders (ADHD) to schizophrenia, depression and AD ([98] and references therein).

Fatty acid chemistry

Fatty acids consist of a polar carboxylic acid with a hydrophobic carbon chain. They are abbreviated according to the formula X:Y, where X states the number of carbons in the fatty acid chain and Y is the number of double bonds. ω 3, ω 6 or ω 9 refers to the position of the first double bond counting from the methyl group (the end of the carbon chain). All ω 3 and ω 6 fatty acids, e.g. docosahexaenoic acid (DHA, 22:6 ω 3) and arachidonic acid (AA, 20:4 ω 6) are derived from their respective precursors α -linolenic acid (LNA, 18:3 ω 3) and linoleic acid (LA, 18:2 ω 6). DHA and AA are examples of PUFAs since they contain more than one double bond. Monounsaturated fatty acids contain one double bond, whereas saturated fatty acids have no double bonds.

Because of the amphipathic nature of fatty acids, they tend to form micelles in an aqueous environment. Micelles are structures where amphipathic molecules arranged in a manner so that the polar headgroups are facing the water, whereas the hydrophobic parts are buried in a hydrophobic core. The formation of biological membranes is based on this principle.

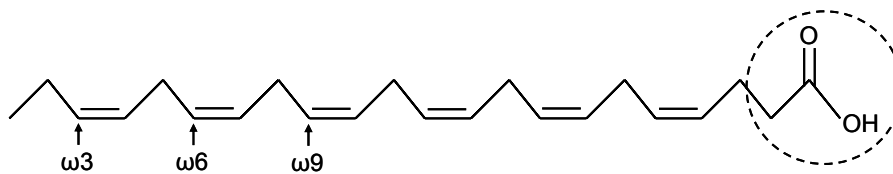


Figure 6. Chemical structure of docosahexaenoic acid (DHA, 22:6 ω 3). The structure contains a hydrophobic carbon chain with six double bonds and a polar head group (indicated by the dotted circle). The ω 3, ω 6 and ω 9 positions are indicated by arrows. As the first double bond of DHA is located in the ω 3 position, this fatty acid belongs to the ω 3 fatty acids.

Biological membranes

Biological membranes are composed of lipids, proteins and sugar. The major lipid components are phospholipids, glycolipids and cholesterol. Phospholipids are composed of a polar phosphate head group and a hydrophobic tail consisting of a saturated or unsaturated fatty acid. Glycolipids are arranged in a similar manner, but with a sugar instead of a phosphate head group. The relative amount of saturated and unsaturated fatty acids incorporated into the phospholipids is important for structural integrity and fluidity of the membrane, which in turn affect membrane protein activities. Unsaturated fatty acids increase the fluidity of the membrane, whereas saturated fatty acids make it more rigid. AA and DHA are the major PUFAs present in brain phospholipids, and high levels of DHA are found in synaptosomes, synaptic vesicles, mitochondria, microsomes and nerve growth cones [99]. Cholesterol is structurally very different from phospholipids and glycolipids, as it is composed of a steroid structure together with a hydroxyl group and hydrocarbon side chain. Cholesterol is inserted into the phospholipid bilayer, facilitating the packing of the fatty acid side chains, and hence making the membrane more rigid (reviewed in [100]).

DHA and cholesterol in clinical and pre-clinical studies

Several epidemiological studies have demonstrated an association between high intake of ω 3 fatty acids [101-104] as well as cholesterol lowering drugs [105-107] with a reduced risk of developing AD. Moreover, elevated levels of midlife serum cholesterol have been linked to increased risk of developing AD [108-110] and dementia [111, 112]. In support of these data, two small clinical trials have reported positive effects on cognitive measures in cognitively impaired patients treated with ω 3 fatty acids [113] or cholesterol lowering statins (Atorvastatin) [114].

The clinical and epidemiological data are supported by animal studies, as a diet enriched with DHA improved cognition in normal rats [115] and maintained learning abilities in rats infused with A β peptide [116]. In A β -infused rats on a DHA enriched diet, levels of both A β and cholesterol in lipid rafts were decreased [117]. Reduced A β levels have also been observed in cell cultures treated with DHA [118, 119]. Moreover, a diet enriched in DHA dramatically reduced amyloid burden in aged transgenic APP mice with biochemical studies demonstrating decreased A β levels and altered APP processing [120]. In addition, cholesterol lowering drugs have consistently been shown to reduce A β levels and increase α -APPs both in transgenic mice [121] and in several *in vitro* studies [122-125].

Aims

Overall aim

The overall aim of this thesis was to investigate the processes of A β aggregation, with special emphasis on protofibril formation. In addition, we wanted to explore how fatty acids affect these processes and the neurotoxicity of different A β species that are generated.

Specific aims

- Paper I: To investigate how the Arctic mutation and the physiochemical environment influences A β ₁₋₄₂ protofibril formation and aggregation.
- Paper II: To evaluate if oxidation of the single methionine residue in the A β sequence affects A β aggregation and neurotoxicity.
- Paper III: To study the effects of fatty acids on A β aggregation and neurotoxicity.
- Paper IV: To investigate if increased lipid content of DHA affects APP processing and A β production in cellular models of AD.

Results and Discussion

Defining A β species in size exclusion chromatography

Analysis of synthetic A β 1-42wt with size exclusion chromatography (SEC) using a Superdex 75 SEC column generated two peaks, one gel-included peak and one peak eluting in the void volume of the column (**Paper I**). The gel included peak is through out this thesis referred to as monomer, although this peak could contain a population of other low molecular weight A β species as well. Size determinations of the gel-included peaks for A β 1-42wt and A β 1-42Arc using globular weight standards on a similar column (TSK 3000) resulted in a molecular weight (MW) of 13-16 kDa, implying a trimer or tetramer (unpublished data). However, such size determinations are associated with methodological problems, as A β is a very hydrophobic linear peptide in a random coil structure and is thus very different from soluble globular proteins often used as size markers. As a consequence, the elution behavior of the peptide is affected, making exact size determinations difficult. However, linear standards, such as dextran, could possibly provide a more accurate estimate. Using dextran standards, the MW of the gel included peak for A β 1-40Arc has been calculated to 4.2 kDa using the Superdex 75 column [86].

The peak eluting in the void volume of the column contains a heterogeneous pool of various A β species with a MW exceeding the exclusion limit of the column, for Superdex 75 approximately 100 kDa, and for TSK 3000 approximately 600 kDa. Since this soluble pool of A β elute in the void when using both these columns, these A β species are fairly large, perhaps in the range of 600-1000 kDa. As they are not pelleted at a moderate centrifugation speed of 18 000 x g, they have a soluble nature distinguishing them from the pelleted material. In this thesis, these soluble A β species are referred to as protofibrils as they also have been in other publications [77, 126].

Pelleted A β species were Thioflavin T (ThT) positive, and the amount of peptide lost in the supernatant as measured by SEC was recovered in the pellet, demonstrating the mass balance of this system. Cryo-transmission electron microscopy (Cryo-TEM) demonstrated that the pelleted species consisted of long stretched thread-like structures, whereas the protofibrillar

species eluting in the void volume of the column were much smaller, and somewhat curvy in appearance (**Paper I**). Because of the ability of the pelleted A β species to bind ThT, as well as their size and structure, these species were identified as amyloid fibrils. A decrease in protofibril area can thus be interpreted as fibril formation.

A β aggregation

The A β 1-42wt peptide spontaneously formed protofibrils in a physiological phosphate buffer environment *in vitro*. This time-dependent increase of protofibrils was accompanied by a decrease in monomers. Introducing the Arctic mutation accelerated both protofibril- and fibril formation, in line with the increased protofibril formation previously described for A β 1-40Arc [63]. Furthermore, A β 1-42Arc formed protofibrils also in a basic pH environment in contrast to A β 1-42wt which formed very small amounts of protofibrils at basic pH (**Paper I**).

The early phase of the A β 1-42wt aggregation process, i.e. the rate of decrease in monomer peak area, was highly dependent on temperature and ionic strength, indicating significant conformational changes and hydrophobic interactions. In agreement, the activation energy for this process was calculated to 115 kJ/mole, i.e. fairly high. Surprisingly, this process appeared not to be dependent on A β concentration (**Paper I**). This suggests that the early stage of A β aggregation viewed as an isolated process does not depend on the formation of a nucleus. The formation of nuclei thus probably occurs later in the A β aggregation process, as suggested previously by Pallito *et al* who proposed a nucleation dependent mechanism for the late fibrillization phase but not for the early oligomerization of monomers and dimers [127].

The later phase of the A β 1-42wt aggregation process, i.e. the formation of fibrils from protofibrils was not affected by changes in ionic strength and temperature to the same extent as monomer assembly. The activation energy for this process was estimated to 50 kJ/mole, i.e. significantly lower than for the early aggregation process (**Paper I**). This indicates that fibril formation is associated with less conformational changes and hydrophobic interactions, compared to the assembly of monomers. However, A β 1-42Arc displayed a more obvious dependence on ionic strength, suggesting that the assembly of Arctic fibrils involves hydrophobic interactions to a higher extent than wild type fibrils.

Even though monomer assembly did not depend on A β concentration, the formation of protofibrils did, suggesting that protofibril formation is a nucleation dependent process (**Paper I**). The nuclei needed for the formation of A β protofibrils is most likely larger than a dimer, as A β 1-42wt and A β 1-40Arc with oxidized methionine formed dimers but not trimers or protofibrils (**Paper II**), as previously described for oxidized A β 1-40wt [128].

Paper I and **II** illustrate the importance of changes in amino acid sequence and the physiochemical environment for the aggregation behavior of A β . However, *in vivo*, also endogenous biological molecules could influence the A β aggregation process. In **paper III**, we demonstrated that the polyunsaturated fatty acids DHA and AA accelerated the assembly of A β 1-42wt monomers and stabilized protofibrils, hindering their conversion to fibrils. This suggests that these fatty acids can lower the energy threshold for monomer assembly, thus facilitating the formation of a nucleus. The saturated fatty acid arachidic acid did not have this effect, and A β 1-42Arc aggregation was unaffected by DHA and AA. The ability of DHA to stabilize A β 1-42wt protofibrils was concentration dependent, and appeared to be dependent on the formation of fatty acid micelles. Both α -synuclein [129] and tau [130] aggregation has previously been demonstrated to be induced by anionic micellar detergents and fatty acids. The need for micellar structures explains why arachidic acid did not stabilize protofibrils in our study, as the critical micelle temperature for this fatty acid is 75°C [131]. Cryo-TEM of A β structures stabilized with DHA displayed granular structures, which may represent DHA micelles. These granular structures are located in proximity to the soluble A β aggregates, indicating a direct interaction (**Paper III**). Possibly, DHA and AA micelles work as a protective agent and prevent intermolecular interaction and consequent aggregation into insoluble fibrillar aggregates, pelleted at 18 000 x g.

A β neurotoxicity

Cortical neurons exposed to A β 1-42wt exhibited pyknotic/apoptosis-like morphological characteristics including shrunken nuclei with partially condensed, but not fragmented chromatin clumps. The A β 1-40Arc peptide induced more classical morphological characteristics of apoptosis including both shrunken nuclei with chromatin condensation and fragmentation (**Paper II**). Both A β 1-42wt and A β 1-42Arc induced toxicity in PC-12 cells, as measured by the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay (**Paper III**). Oxidizing methionine in A β 1-42wt and A β 1-40Arc, thereby keeping these peptides in a mainly monomeric/dimeric state, severely diminished the toxic action of both peptides as measured by cell counting, cytoskeleton integrity and metabolic activity (the MTT assay) (**Paper II**). Thus, the ability to form oligomers and protofibrils seemed to be essential for the neurotoxic properties of the peptide. In addition, pre-incubation of A β 1-42wt with DHA, which stabilized protofibrils, maintained the neurotoxic effect induced by A β in PC-12 cells (**Paper III**). This observation gives further proof for the proposed neurotoxic actions of A β protofibrils. Interestingly, DHA did not maintain A β 1-42Arc induced neurotoxicity, in line with the absent ability of DHA to stabilize

A β 1-42Arc protofibrils. When incubating A β with fatty acid vehicle only, both A β 1-42wt and A β 1-42Arc were toxic when added fresh but the neurotoxicity was reduced as the peptides were allowed to aggregate into fibrils, indicating a reduced neurotoxic potential for fibrillar A β , compared to oligomeric forms. The results from **paper II and III** suggest that the neurotoxic potential of A β is confined to soluble aggregated forms of A β , not A β monomer/dimers or fibrillar A β . However, it is possible that fibrillar A β can exert other neurotoxic effect not detected with the MTT assay, especially as oligomeric and fibrillar A β have been reported to induce neurotoxicity by different mechanism [95].

Significance for the *in vivo* situation

A β methionine could be oxidized in AD brains through exposure to free radicals produced and released by microglia, such as superoxide, which quickly forms reactive hydrogen peroxide [132]. In theory, the proportion of methionine oxidized A β could also increase through decreased activity of methionine sulfoxide reductase, which reduces methionine sulfoxide back to methionine. These mechanisms might constitute a means by which A β is rendered less toxic in the brain, if oxidation of methionine occurs *in vivo* to a significant extent.

The existence of DHA and AA micelles *in vivo* might seem unlikely, as the concentrations of DHA and AA in cerebrospinal fluid (CSF) (DHA concentration in CSF: ~100-200 nM) are well below the critical micelle concentrations (CMC) determined for these fatty acids *in vitro*. For example, a CMC in the range of 200 μ M has been determined for AA [130]. However, it is in this context important to note that the CMC for surfactants is well known to decrease in the presence of polymers [133]. In line with this, other amyloidogenic proteins have been shown to decrease the CMC for fatty acids dramatically [129, 130]. Moreover, DHA and AA can form mixed micelles with other fatty acids, lowering the CMC even further ([134] and references therein). In addition, brain trauma and ischemia have been associated with up to 6 fold elevated concentrations of free DHA and AA in human CSF [135, 136].

If DHA can form micelles and thus stabilize protofibrils *in vivo*, how is this linked to the proposed beneficial effects of DHA, reported in animal and epidemiological studies? Fatty acids are largely incorporated into membrane phospholipids, affecting interactions of membrane proteins, receptors and ion channels. The beneficial effects of ω 3 PUFAs are thus more likely attributed to such events. To investigate this issue, we supplemented APP transfected neuroblastoma cells with DHA and then analyzed the levels of several different APP processing fragments (**Paper IV**). This DHA treat-

ment increased the total lipid content of DHA six fold and decreased levels of AA seven fold resulting in a nearly 50 fold increase of the DHA/AA ratio.

The increased proportion of DHA in the cells increased the C83/C99 ratio and the α -APPs/ β -APPs ratio, thereby shifting the processing of APP towards the non-amyloidogenic pathway, lowering both intracellular and extracellular levels of A β . In addition, levels of AICD increased, an effect eliminated in presence of the γ -secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), indicating a γ -secretase dependent mechanism. AICD has been shown to translocate to the nucleus and activate transcription of the neprilysin gene [137], coding for one of the major enzymes responsible for A β degradation [138]. However, the observed increase in AICD levels did not affect levels of neprilysin in our system. Also, levels of cholesterol were not changed upon DHA treatment, suggesting an effect directly associated with the lipid content of DHA.

In conclusion, DHA administration stimulated non-amyloidogenic APP processing and reduced levels of A β , providing a mechanism for the reported beneficial effects of DHA *in vivo*. However, an increased level of AICD was also observed, highlighting the need to increase our knowledge about the relevance of this fragment in AD pathogenesis.

General Discussion and Future Perspectives

Since the discovery of the A β peptide as the principal component of the amyloid plaques, AD research has focused on events associated with A β production and aggregation. The most compelling evidence for the involvement of A β in AD pathogenesis come from genetics, as all AD causing mutations are located in the APP and presenilin genes and affects A β production and/or aggregation.

This thesis is focused on A β aggregation and neurotoxicity *in vitro*, with special emphasis on protofibril formation. As A β 1-40 with the Arctic mutation has been associated with increased protofibril formation, both Arctic and wild type A β peptides were investigated. In **paper I**, we demonstrated an accelerated protofibril formation also for A β 1-42 with the Arctic mutation, compared to A β 1-42wt. Furthermore, we characterized how aggregation of both A β 1-42wt and A β 1-42Arc is affected by changes in the physicochemical environment. The results from **paper I** indicated that the early aggregation phase, i.e. the assembly of monomers, was associated with a high degree of conformational changes and that this process was not a nucleation dependent process. The formation of a nucleus thus probably occurs later in the aggregation process. The smallest A β assembly capable of nucleating the A β aggregation process is not known. This is an important issue, as the most efficient way to reduce A β aggregation would probably be to slow down or prevent the formation of a nucleus. Results from **paper II** suggests that A β dimers are not large enough to function as nuclei, as oxidation of A β Met35 allowing for the formation of dimers, but not trimers, inhibited further aggregation into protofibrils. Interestingly, these oxidized peptides did not have the neurotoxic potential of their un-oxidized counterparts, suggesting that formation of trimers and further aggregation into protofibrils is necessary for the neurotoxic actions of A β .

In **paper III**, we demonstrated that polyunsaturated fatty acids, like DHA and AA, accelerated the assembly of A β 1-42wt monomers and stabilized soluble protofibrils, attenuating their conversion into insoluble fibrils. In agreement with the conclusions from **paper II**, DHA maintained A β induced neurotoxicity in cell culture, illustrating the neurotoxic potential of A β protofibrils. In absence of DHA, the neurotoxicity was reduced as A β aggregated into insoluble fibrils. These results suggest that the neurotoxic potential of A β is confined to soluble aggregated forms of A β , not A β monomer/dimers or fibrillar A β . However, different oligomeric species of

A β might exert different toxic mechanisms. So even if insoluble A β and monomeric/dimeric A β were not toxic in our experimental setup, A β might exert other toxic events not detected by the assays used in this thesis. The identification of the main pathogenic A β species is important for the development of therapies targeting A β aggregation and toxicity. Drugs targeted against the later stages of A β aggregation might be detrimental as A β species formed early in the aggregation process, possibly more pathogenic, could accumulate.

The stabilization of neurotoxic protofibrils with DHA (**paper III**) might appear contradictory, as DHA generally is considered beneficial for cognition. However, as polyunsaturated fatty acids are incorporated into membrane lipids, thereby affecting the interaction of membrane proteins, receptors and ion channels, we wanted to investigate this aspect as well. In **paper IV**, we demonstrated that by increasing the content of DHA in neuronal cell membranes, APP processing was shifted towards the non-amyloidogenic pathway, resulting in reduced A β levels. Interestingly, this was accompanied by increased levels of AICD. These results suggest that the reported beneficial effects of DHA *in vivo* could be mediated by altered APP processing resulting in reduced A β levels. Although the observed increased level of AICD is intriguing, it is difficult to evaluate the significance of this observation since the relevance of AICD in AD pathogenesis is poorly understood.

Even though A β has been the focus of AD research for many years, important questions regarding this peptide still remain. Some view A β as a destructive byproduct from APP processing, but the possibility of a physiological function for A β monomers or dimers should not be ignored. Aggregation could in theory result in a loss of function that might contribute to the pathology. As APP processing is a central part of AD pathology, investigating the physiological role of these processing events is important. While the pathogenic mechanisms of familial AD are more straightforward, the mechanisms of sporadic AD remain elusive. If disturbed APP processing and A β production is the cause of also sporadic AD, what are the mechanisms for these events? Research focused on the involvement of fatty acids, cholesterol and other aspects of lipid homeostasis in AD pathogenesis might shed some light on this issue.

Methodological Considerations

A β aggregation *in vitro*

Size exclusion chromatography

Chromatography is a technique for separating different components in a sample. The sample is transferred via a mobile phase through an immobile stationary phase. Depending on the chemical nature of the stationary and mobile phase, the components can be separated based on their physio-chemical properties or size.

SEC separates the components of a sample according to their size, or more accurate, their hydrodynamic volume. The stationary phase consists of a porous material (often referred to as the gel matrix) composed of a polymeric organic compound, like dextran, agarose or silica. When the sample is eluted through the SEC column, small molecules will enter the pores of the gel matrix, whereas large molecules will run directly through the matrix, eluting in the so called void volume of the column. The smaller the molecule, the larger the elution volume will be since small molecules will enter more pores. The eluted components of the sample can be detected in various ways. Common detection methods are ultraviolet (UV) absorbance, refractive index (RI) and light scattering (LS).

The elution volumes of the sample components are essentially logarithmically proportional to their molecular hydrodynamic volume (size). Therefore, by using MW standards, the MW of an unknown sample can be calculated. MW determinations using SEC are however not uncomplicated, as the results are influenced by the molecular three dimensional structure of the components. In addition, non-specific interactions with the column matrix and viscosity of the sample can affect the accuracy of the results.

In this thesis, the Superdex 75 column was mainly used (**Paper I-III**). This column contains a gel matrix comprising a composite of dextran and cross linked agarose. The optimal separation range of this column is 10-70 kDa and the exclusion limit is 100 kDa, according to the manufacturer. Samples were eluted with phosphate buffer at a flow rate of 0.08 ml/min and detected with UV absorbance at 214 nm. To minimize column interactions,

especially for the highly hydrophobic A β 1-42Arc peptide, Tween-20 was added to the elution buffer. Using this elution buffer, recovery of A β 1-42Arc and A β 1-42wt were 70%, as determined by injection with and without the column (data not shown). Unfortunately, Tween-20 also affected the aggregation process of A β 1-42wt, in a way that monomer assembly was accelerated and protofibrils were stabilized. However, Tween-20 was not present when incubating the samples, but only during the SEC run.

The TSK-3000 column was used for MW determinations. This is a silica based column with an optimal separation range of 10-500 kDa. Usually, the flow rate of the mobile phase is higher when using silica based columns, as compared to columns containing dextran or agarose. In this case, we used a flow rate of 0.4 ml/min. When using this column, Tween-20 did not improve the elution behavior of the A β peptides, and was therefore omitted from the mobile phase.

Dissolving and aggregating synthetic A β

Dissolving the synthetic A β peptide is complicated because of its hydrophobic nature and propensity to aggregate. Various solvents are used, both organic solvents and aqueous media. In **paper I and III**, dilute sodium hydroxide (NaOH) was used, as the solubility of A β is increased at basic pH. Aggregation was induced by neutralizing the A β /NaOH solution with phosphate buffered saline (PBS). In the SEC experiments in **paper II**, A β 1-42wt was dissolved in 1/10 DMSO (dimethyl sulfoxide), and further diluted in water. The aggregation behaviour in 10% DMSO was however similar to when using PBS in **paper I and III**. When dissolving the A β 1-40Arc peptide for SEC in **paper II**, we used water as the absence of the two C-terminal hydrophobic amino acids isoleucine and alanine makes it more soluble than the A β 1-42 peptide. Also in this case, the peptide was aggregated in PBS. For the mass spectrometry experiments in **paper II**, the A β peptides were dissolved in ddH₂O or in acetonitrile:H₂O (50:49) with 1 % acetic acid. The results from these two solutions were similar.

One important aspect of dissolving A β peptides for aggregation and neurotoxicity experiments is the existence of pre-existing aggregates. These pre-formed aggregates, initially present in the peptide preparation, could work as seeds, and thus accelerate the aggregation of the peptide. If these aggregates are not dissolved properly, experimental inconsistency and lot-to-lot variability will occur. As we experienced some variability between different lots, pre-existing aggregates might not have been fully dissolved, even though freshly dissolved A β 1-42wt produced a single peak in SEC, using the TSK-3000 column and an elution buffer with no Tween-20. However, these aggregates can be very hard to detect, since they constitute a very small fraction of the sample.

Transmission electron microscopy

Transmission electron microscopy (TEM) is a powerful microscopic technique where a specimen can be magnified up to 300 000 x. This high magnification capacity is due to the use of electron beams instead of light, as in normal light microscopy. A beam of electrons is passed through a thin specimen, creating an electron diffraction pattern based on the electron density of the sample. The diffraction pattern is then processed into an image.

Biological samples contain atoms of low electron density, i.e. carbon, nitrogen, hydrogen and oxygen. The samples are therefore usually treated with electron dense chemicals (negative staining), e.g. heavy metals like lead or uranium, to increase the contrast. However, contrast chemicals or other chemicals used to fixate the sample (e.g. glutaraldehyde) can create artifacts. It can thus be hard to determine if a structure is an artifact or actually from the specimen itself. By rapid freezing of a specimen at extremely low temperatures, the water in the specimen forms vitreous ice preserving the sample and creating a snapshot of its solution state. This technique is known as cryo-TEM, used in **paper I and III** to create TEM images of A β fibrils and protofibrils. For these experiments we used liquid ethane held just above its freezing point of -183°C to vitrify the sample.

A drawback of this technique is relatively low contrast, making details more difficult to distinguish as compared to negative staining.

Fourier transform ion cyclotron resonance mass spectrometry

Mass spectrometry is the analysis or separation of charged particles, such as atoms and molecules, based on their mass-to-charge ratios. Most mass spectrometers are comprised by three principal parts. Ions are produced in an *ion source* and subsequently separated or analyzed in a *mass analyzer* before detection by a *detector*. All of these, or at least the analyzer and detector, are inside a vacuum system. In Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), the mass spectrometry technique used in **paper II**, particles are trapped by a magnetic and electric field, making them move in spirals (cyclotroning). This amplifies the signal and makes the technique more sensitive than conventional mass spectrometry. The mass measurement errors are typically on the low ppm level and mass spectra have been recorded consuming only 140 zmol (10^{-21}) of standard proteins. The cyclotron frequency is a function of the m/z ratio and the strength of the magnetic field. By measuring the cyclotron frequency, a mass spectrum can be derived after performing a mathematic procedure, called a Fourier transform.

There are several different approaches to ionize the sample in mass spectrometry. In **paper II**, electrospray ionization (ESI) was used; a technique especially suitable for macromolecules as multiple charges can be imposed on the molecules, lowering the m/z ratio. This is important since molecules

with a high m/z ratio might not reach the detector, because of their low speed. In addition, fragmentation is largely avoided. Using FTICR-MS with electrospray, $A\beta$ species ranging from monomers up to decamers can be detected.

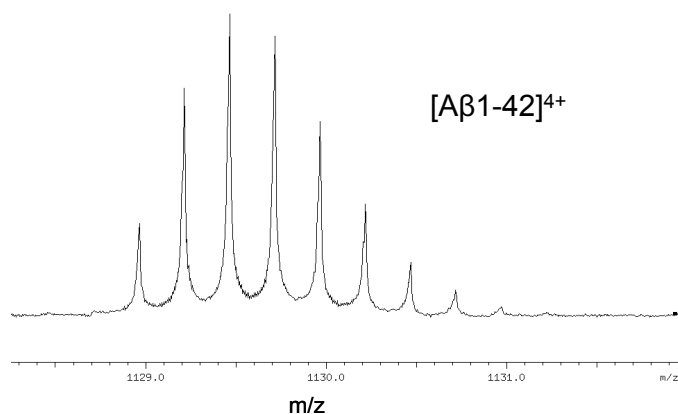


Figure 7: Mass spectra of $A\beta 1-42$ wt (MW: 4514 Da) at charge state 4^+ . Picture from Jonas Bergquist.

Thioflavin T

Thioflavin T (ThT) is a benzothiazol salt used as a histopathological stain for amyloid plaques. ThT has fluorescent properties, i.e. this compound can be excited by certain wavelengths of light, producing a spectrum of emitted wavelengths. When ThT binds to β -sheet structures, the maximum of the emitted wavelength spectra is shifted 100 nm, compared to ThT alone. This property of ThT allow for measurements of $A\beta$ aggregation kinetics *in vitro* as this process is accompanied by increased β -sheet content. In **paper I**, we used this technique to verify the β -sheet structure of pelleted $A\beta$ peptide.

ThT is generally considered to measure fibril formation but since soluble oligomers of amyloidogenic proteins also contain β -sheet, ThT should bind these aggregates as well. In my experience, soluble $A\beta$ species increases the amount of emitted light at 485 nm, but not to the same extent as fibrillar $A\beta$ (unpublished observations). This is in agreement with the published data of Walsh *et al*, where protofibrils increased the ThT signal, but not to the same extent as fibrils [64].

Cell models

In this thesis, three different cell models were used. For the toxicity experiments in **paper III**, we used PC-12 cells. This cell line is derived from a rat pheochromocytoma (PC), a neuroendocrine tumor of the medulla of the adrenal glands. These cells can be differentiated with nerve growth factor to induce a neuron-like phenotype. However, in **paper III**, we used undifferentiated PC-12 cells. PC-12 cells are known to be sensitive to A β induced toxicity, and they are often used in toxicity experiments together with the toxicity assay MTT (See below).

In **paper II**, we used primary cortical neurons from mice to evaluate the neurotoxicity of A β . Primary cells are derived from dissected tissue and propagated in cell culture dishes. These cell cultures are generally considered to be closer to the *in vivo* situation than tumor cell lines.

SH-SY5Y is a cell line derived from a human neuroblastoma. These neuronal cells can also be further differentiated to an even more neuron-like phenotype with more extended neurites, using retinoic acid. In **paper IV**, SH-SY5Y cells differentiated with retinoic acid and stably expressing APP were used to study the effect of lipid enrichment with DHA. These cells have previously been shown to incorporate DHA into phospholipids, by simply adding DHA to the culture media [139].

Neurotoxicity

Cell counting

The most straightforward way to determine degree of neurotoxicity is counting the number of dead neurons in a microscope. By using DNA binding dyes, one can distinguish between apoptotic and necrotic cells, as fragmented and condensed DNA is an event associated with apoptosis. In **paper III**, cells were double-stained with the DNA binding dyes H-33342 and SYTOX green. H-33342 is cell permeable and emits blue fluorescence when bound to DNA. SYTOX green is a plasma membrane impermeable dye, thus cells stained with SYTOX are in a necrotic phase, as the plasma membrane is not intact. Apoptotic cells are stained only with H-33342, and exhibit fragmented and/or condensed nuclei.

The MTT assay

The yellow tetrazolium salt MTT is reduced by living cells to a water insoluble purple formazan product. This colometric reaction is the basis for the MTT assay. After dissolving the formazan crystals, the degree of MTT

reduction (reflecting number of viable cells) can be assessed by measuring absorbance at 570 nm.

The reduction of MTT requires an electron donor. Originally, MTT was believed to receive its electrons from the oxidation of succinate by succinate dehydrogenase, an enzyme in the mitochondrial respiratory chain. However, in recent years, the electron donors NADPH (nicotinamide adenine dinucleotide phosphate) and NADH (nicotinamide adenine dinucleotide), present in the cytoplasm, have been shown to be important mediators of MTT reduction, accounting for more than 90% of the total cellular MTT reduction [140]. Other electron donors like pyruvate and lactate might also be involved. In general, cellular reduction of MTT reflects metabolic activity, and thus measure the proportion of metabolically active (i.e. viable) cells in the cell culture.

Since MTT is a membrane impermeable molecule, it is taken up by living cells through endocytosis. The reduced formazan product is then transported out of the cell through exocytosis, forming needle-like crystals on the cell surface [141]. When working with A β , one should be aware of reports demonstrating that A β enhances exocytosis of the formazan product, increasing the formation of formazan crystals at the cell surface [142, 143]. This could possibly interfere with endocytosis of MTT, and thus inhibit MTT reduction. As a consequence, inhibited MTT reduction induced by A β peptides could reflect enhanced exocytosis of formazan, and not reduced cell viability per se. However, even if this was true the assay is still relevant, as exocytosis is an important feature of cellular functions like intracellular trafficking and neurotransmitter release.

Ways of minimizing the exocytosis of formazan are reduced A β concentration and incubation time as well as reduced incubation time with MTT. In **paper II**, we used A β concentrations of 2.5-40 μ M for 48-72 hours, i.e. fairly high A β concentrations and incubation times. However, the incubation time with MTT was only 1 h, probably reducing the amount of formazan crystals at the cell surface. In **paper III**, the cells were incubated with MTT for 4 h. Nevertheless, in this paper, the A β concentration was 5 μ M and the cells were exposed to A β for only four hours, which likely reduced the effect of A β on exocytosis. However, since we did not evaluate crystal formation at the cell surface, we do not really know if this contributed to the inhibited MTT reduction.

Cytoskeletal and nuclear staining

In **paper II**, we evaluated cellular integrity by fluorescent staining of tubular structures and nuclei. Cytoskeletal alterations were visualized by immunostaining with an antibody specific for neuronal anti- β -III-tubulin. β -tubulin is a component of the microtubule, a cellular structural element important for several functions, like mitosis and vesicular transport. Nuclei were stained

with propidium iodide, a fluorescent marker for DNA (deoxyribonucleic acid). Propidium iodide is membrane impermeable and can thus not enter non-viable cells. Using this marker, apoptotic alterations of the nuclear DNA can be detected.

SDS-PAGE and Western blot

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique for separating proteins according to size using porous gels, somewhat similar to SEC. However, unlike SEC, this is a denaturing method, as it includes a denaturing step where the samples are boiled in SDS and β -mercaptoethanol. SDS denatures the protein and adds negative charges, whereas β -mercaptoethanol breaks up disulfide bonds. This procedure leaves the proteins in a rod like structure with negative charges along their polypeptide chains. When applying an electric field, the negatively charged proteins will migrate through the gel towards the anode. Since the SDS and β -mercaptoethanol treatments result in all proteins having essentially the same negative charge and structure, they will separate according to size, with small proteins migrating faster through the gel than large proteins. The proteins in the gel can then be visualized with various staining techniques, like coomassie blue or silver staining (used in **paper III**).

Another way to detect proteins in a gel is Western blot (**paper III and IV**). By forcing the negative proteins to migrate out of the SDS-PAGE gel to a membrane using an electric field, a protein of specific interest can be identified by adding a primary antibody specific for that protein. This antibody can then be detected by a secondary antibody, conjugated with e.g. a fluorescent marker or an enzyme capable of catalyzing a reaction producing light (chemiluminescence). The emitted light can then be detected on a photographic film, whereas fluorescence can be detected in a fluorescence scanner. The advantage of using fluorescence (used in **paper III and IV**) instead of chemiluminescence is increased linear range, as the linear range of the photographic film is limited.

In **paper III and IV**, a 10-20% tris-tricine polyacrylamide gel was used for SDS-PAGE. Tris-tricine gels are especially suitable for small proteins and peptides, as they separate these from SDS micelles which can be a problem in tris-glycine gels. The polyacrylamide gradient from 10-20% gives the gel a wide separation range. This gel is thus suitable for the separation of low molecular weight A β species (**paper III**), but one can also separate C83 (10 kD) and C99 (12 kD) and a large protein like APP (100 kD) can enter the gel (**paper IV**).

When analyzing A β oligomers with SDS-PAGE, only oligomers that are resistant to the SDS treatment are detected. Other oligomers that might have been present in the sample originally will denature into A β monomers.

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