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Pathogenic Mechanisms of the Arctic Alzheimer Mutation

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Abstract

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder, neuropathologically characterized by neurofibrillay tangles and deposition of amyloid-β (Aβ) peptides. Several mutations in the gene for amyloid precursor protein (APP) cause familial AD and affect APP processing leading to increased levels of Aβ42. However, the Arctic Alzheimer mutation (APP E693G) reduces Aβ levels. Instead, the increased tendency of Arctic Aβ peptides to form Aβ protofibrils is thought to contribute to the pathogenesis.

In this thesis, the pathogenic mechanisms of the Arctic mutation were further investigated, specifically addressing if and how the mutation affects APP processing. Evidence of a shift towards β-secretase cleavage of Arctic APP was demonstrated. Arctic APP did not appear to be an inferior substrate for α-secretase, but the availability of Arctic APP for α-secretase cleavage was reduced, with diminished levels of cell surface APP in Arctic cells. Interestingly, administration of the fatty acid docosahexaenoic acid (DHA) stimulated α-secretase cleavage and partly reversed the effects of the Arctic mutation on APP processing.

In contrast to previous findings, the Arctic mutation generated enhanced total Aβ levels suggesting increased Aβ production. Importantly, this thesis illustrates and explains why measures of both Arctic and wild type Aβ levels are highly dependent upon the Aβ assay used, with enzyme-linked immunosorbent assay (ELISA) and Western blot generating different results. It was shown that these differences were due to inefficient detection of Aβ oligomers by ELISA leading to an underestimation of total Aβ levels.

In conclusion, the Arctic APP mutation leads to AD by multiple mechanisms. It facilitates protofibril formation, but it also alters trafficking and processing of APP which leads to increased steady state levels of total Aβ, in particular at intracellular locations. Importantly, these studies highlight mechanisms, other than enhanced production of Aβ peptide monomers, which could be implicated in sporadic AD.

Keywords: Alzheimer's disease, Arctic mutation, Amyloid precursor protein, Amyloid-β, APP processing, Aβ oligomers, Docosahexaenoic acid

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List of papers

This thesis is based on the following papers, which will be referred in the text by their roman numerals:

I **Stenh C.***, Nilsberth C.*, Hammarbäck J., Engvall B., Näslund J. and Lannfelt L. The Arctic mutation interferes with processing of the amyloid precursor protein. *Neuroreport*; **13**, 1857-1860, 2002.

*These authors contributed equally to this work.

II **Sahlin C.**, Lord A., Magnusson K., Englund H., Almeida C.G., Greengard P., Nyberg F., Gouras G.K., Lannfelt L. and Nilsson L.N.G. The Arctic Alzheimer mutation favors intracellular Aβ production by making APP less available to α-secretase. *Journal of Neurochemistry, in press.*

III **Stenh C.**, Englund H., Lord A., Johansson A-S., Almeida C.G., Gellerfors P., Greengard P., Gouras G.K., Lannfelt L. and Nilsson L.N.G. Amyloid-β oligomers are inefficiently measured by enzyme-linked immunosorbent assay. *Annals of Neurology*; **58**, 147-150, 2005.

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

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Abbreviations

AA	Arachidonic acid
αAPPs	Alfa-APP soluble
Aβ	Amyloid-β
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
ADDL	Aβ-derived diffusible ligands
AICD	APP intracellular domain
APH-1	Anterior pharynx defective-1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	Beta-site APP-cleaving enzyme
βAPPs	Beta-APP soluble
CAA	Cerebral amyloid angiopathy
CSF	Cerebrospinal fluid
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
HEK	Human embryonic kidney
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IDE	Insulin degrading enzyme
IP	Immunoprecipitation
NICD	Notch intracellular domain
NMDA	N-methyl-D-aspartate
PAGE	Polyacrylamide gel electrophoresis
PEN-2	Presenilin enhancer-2
PS	Presenilin
RP-HPLC	Reverse-phase high-performance liquid chromatography
SDS	Sodium dodecyl sulfate
SEC-HPLC	Size exclusion chromatography high-performance liquid chromatography

Introduction

Alzheimer's disease

Background

Alzheimer's disease (AD) is the leading cause of dementia, affecting approximately 5% of the European population over 65 years of age[1]. This neurodegenerative disorder was first described by Alois Alzheimer, a German physician and scientist, at a meeting in 1906 and was published a year later[2]. He identified changes in brain tissue of a woman, Auguste D, who had died in her fifties of an unusual mental illness with memory loss, disorientation and hallucinations. In the histopathological examination of the brain he used a novel silver impregnation technique developed by Bielschowsky and found abnormal clumps, now called amyloid plaques, and tangled bundles of fibers, now called neurofibrillary tangles, which today constitute the neuropathological hallmarks of the disease (Fig. 1). The importance of AD neuropathology was demonstrated in the late 1960s when high prevalence of AD pathology was found in an aged cohort of clinically well characterized dementia patients[3, 4].

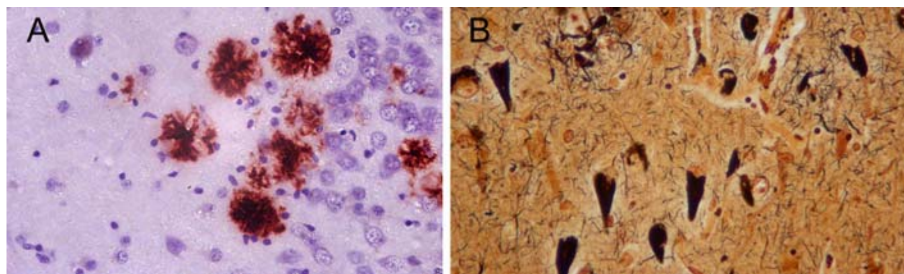

Figure 1. The neuropathological hallmarks of Alzheimer's disease are **(A)** senile plaques, and **(B)** neurofibrillary tangles. Pictures from Paul O'Callaghan (A) and Hannu Kalimo (B).

Risk factors and symptoms

The prevalence of AD is about 1-2% at the age of 65, although this number varies among populations studied. After the age of 65 the risk of developing AD increases dramatically, since age is the strongest risk factor for AD. Other risk factors are a family history of dementia, gender (women have a higher risk) and head trauma[5].

AD is a progressive disorder with an insidious onset. Loss of memory for recent events is often the first sign of this disease. Patients fail to recall names of both objects and familiar persons at an early stage and they can show reduced judgment and changed personality. With time other symptoms such as disorientation, aphasia and a general cognitive decline arise. During this period the patient will become more and more dependent upon caretakers to perform such simple tasks as dressing and washing. Death usually occurs 5-15 years after diagnosis, often due to a secondary condition, like pneumonia or other infections.

Diagnosis and treatment

The clinical diagnosis of AD is mainly exclusion-based, ruling out other conditions as causes of the dementia, such as infection or stroke. Neuropsychological tests and physical examinations are used in combination with an evaluation of the medical history of the patient. Different brain scans as computed tomography, magnetic resonance imaging, positron emission tomography and single photon emission computed tomography can be helpful tools in the diagnostic process. Several sets of criteria have been developed for an AD diagnosis, for example the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM IV) and the International Classification of Disease 10th revision (ICD-10). Another criteria is the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) where the diagnosis is divided into three different levels of disease; possible, probable and definite AD. A definite AD diagnosis can, however, not be made without examination of *post mortem* brain wherein certain age-dependent neuropathological criteria need to be fulfilled. The criterion is a certain frequency of extracellular plaques and intracellular neurofibrillary tangles in defined brain regions.

Synaptic loss and neuronal atrophy in AD leads to neurotransmitter deficiencies, especially in the areas linked to AD pathology (e.g. neocortex, hippocampus, amygdala and nucleus basalis). In particular, the cholinergic projection neurons in the basal forebrain show atrophy and cell loss in AD *post mortem* brain[6]. The activity of choline acetyltransferase, the rate-limiting enzyme in acetylcholine synthesis, is severely reduced in afflicted regions of the AD brain. Cholinergic innervations of the hippocampus and

the cerebral cortex are associated with memory functions. Based on these findings, treatment of AD has been focused on enhancing acetylcholine levels and the function of remaining cholinergic afferents by using acetylcholinesterase inhibitors. For some patients these drugs slow down the decline of cognitive function, but the effects are modest and often transient since the therapy does not target the underlying neurodegeneration. Recently an N-methyl-D-aspartate (NMDA) receptor antagonist, Memantine was launched for treatment of AD. It is thought that the release and uptake of the neurotransmitter glutamate is dysfunctional in AD, leading to an elevated neuronal influx of calcium triggering downstream neurotoxic pathways. Memantine is thought to decrease this calcium influx and thereby prevent ongoing neurodegeneration. Taken together, the available drugs for AD treatment usually improve memory and cognitive function for only a short period, 1-2 years. Nevertheless, there is no cure of the disease today, although a lot of hope and effort aim toward the development of a vaccine for AD. The discovery that immunization of transgenic mouse models of AD with the 42 amino acid amyloid-β (Aβ42) peptide both prevents plaque formation and reduces already existing senile plaques was a milestone finding in the field[7].

Genetics

It was shown in the late 1960s that patients with Down's syndrome, who carries an extra copy of chromosome 21 almost invariably develop dementia and a neuropathology essentially identical to AD pathology[8]. This was the first indication that chromosome 21 has an important role in AD. In 1984, the amino acid sequence of Aβ was revealed through purification of the amyloid component of cerebrovascular amyloid angiopathy (CAA) in AD brain[9] and Down's syndrome brain[10]. One year later, Aβ42 was purified and characterized from plaque cores of AD brain and aged individuals with Down's syndrome[11]. These discoveries made it possible to eventually identify and clone the gene encoding amyloid precursor protein (APP) on chromosome 21[12-15]. The interest in APP as a possible "gene for AD" increased markedly after the cloning and localization of APP on chromosome 21.

Although most cases of AD are sporadic it was early described that inheritance plays an important role in AD pathogenesis. A number of families have been described where AD is inherited in an autosomal dominant fashion, often with an early onset of disease before 65 years of age[16, 17]. The APP gene was the first gene to be linked to early-onset AD[18]. Today, mutations leading to autosomal dominant AD have been identified in the APP gene, and in the Presenilin 1 and 2 genes (PS1 and PS2) on chromosome 14 and 1 respectively (for updated list of AD mutations see website www.alzforum.org/res/com/mut/default.asp). However, the majority of AD cases are

sporadic, without known genetic cause, particularly those with a late onset. The genes involved in late-onset AD have been more difficult to identify. Among the susceptibility genes reported to date, the apolipoprotein E (ApoE) gene is the most important. This gene was identified as a major susceptibility gene for late-onset AD, with the ε4 allele being a risk factor and ε2 a protective factor for developing the disease[19, 20]. Several other genes have been suggested as candidate genes for AD, for example α-2-macroglobulin[21], angiotensin converting enzyme[22] and α1-antichymotypsin[23]. Chromosome 10 has lately been reported by many to show genetic linkage to AD[24]. Of special interest is the gene for insulin degrading enzyme (IDE), which has been found to be involved with Aβ degradation and clearance[25]. However, ApoE is the only well-replicated, and thus proven, susceptibility gene for AD.

Amyloid precursor protein

Cellular biology of APP

APP is a type I transmembrane protein with a long extracellular amino terminal and a short cytoplasmic tail. Due to alternative splicing, eight different isoforms of APP exist, of which three predominates in the brain (APP_{695}, APP_{751} and APP_{770})[26]. Neurons mainly produce APP_{695}, which is present in the dendrites, cell bodies and axons of neurons, whereas non-neuronal cells express APP_{751} and APP_{770}. The main difference between these variants, except being produced by different cell-types, is that the longer variants (e.g. APP_{751} and APP_{770}) contain a Kunitz-protease inhibitor domain. This indicates a possible function of APP, or a soluble APP fragment, as a protease inhibitor. Apart from this assumption, little is known about the function of APP, although it has been suggested to work as a receptor[27], to stimulate neurite outgrowth[28] and/or to have adhesive properties[29, 30].

APP processing

APP is synthesized in the endoplasmic reticulum (ER) and matured by post translational modifications in the Golgi, where it is tyrosine-sulfated and O- and N-glycosylated[31]. APP is transported from the cell body to the axon by fast anterograde transport[32]. Both during and after the trafficking through the secretory pathway, APP can undergo proteolytic cleavage by different secretases via two different pathways (Fig. 2). α-secretase processing occurs at or close to the plasma membrane[33-35] and precludes Aβ formation, as this enzymatic activity cleaves within the Aβ domain of APP, between amino acid 16 and 17 of the Aβ sequence. This releases a large N-terminal

fragment, αAPPs, and a membrane-retained C-terminal fragment, C83. Further cleavage of C83 by the γ-secretase complex in the endocytic pathway yields the non-amyloidogenic peptide p3[36], hence this is called the non-amyloidogenic pathway. This is the predominant pathway of APP processing[37, 38], especially in peripheral cells where α-secretase cleavage is more common than in neuronal cells[39, 40]. Cell surface APP that escapes α-secretase cleavage is internalized into endosomes where it can be processed by β-secretase at the N-terminus of the Aβ domain, releasing a soluble N-terminal fragment and a membrane-bound C-terminal fragment, C99. Subsequent cleavage of C99 by γ-secretase generates Aβ peptides, mainly in the Golgi/trans-Golgi network and ER but also to some extent at the plasma membrane and in endosomes[41-44]. Aβ production has been suggested to occur mainly in so called lipid rafts, special assemblies of sphingolipids and cholesterol within the membrane[45, 46].

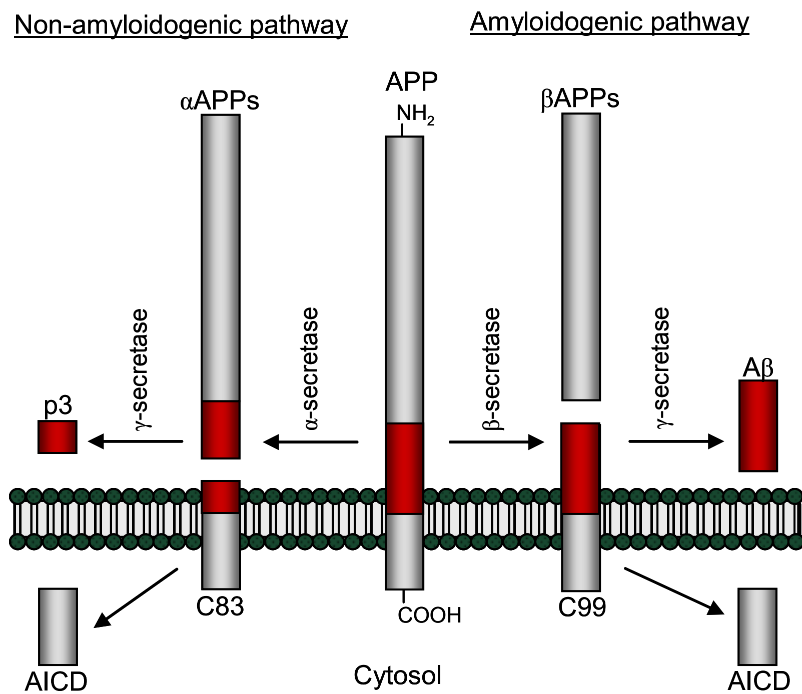

Figure 2. Proteolytic processing of APP occurs via two alternative pathways; the non-amyloidogenic pathway via α-secretase or the amyloidogenic pathway via β-secretase. Further processing by γ-secretase leads to the production of p3 or Aβ.

α-secretase

α-secretase has been identified as a zinc metalloprotease and several members of the ADAM (a disintegrin and metalloprotease) family have been reported to have α-secretase activity. ADAM17/TACE[47], ADAM9[48] and ADAM10[34] all fulfill the criteria required for α-secretase. Although cells have a certain level of α-secretase activity, proteolysis can be increased by the direct activation of protein kinase C with phorbol esters[49, 50]. Likewise, indirect activation of protein kinase C through signal transduction pathways, such as by muscarinic receptor agonists, can reduce Aβ production through enhanced α-secretase activity[51]. α-secretase acts primarily on the plasma membrane and in the late secretory pathway[33-35], mainly in membrane regions with low cholesterol content[52]. Apart from excluding Aβ production, this cleavage releases αAPPs which is thought to have several beneficial effects, for example to stimulate neurite outgrowth and to have trophic activity[53, 54].

β-secretase

The enzyme responsible for β-secretase cleavage belongs to the pepsin family of aspartyl proteases. Two homologs of beta-site APP-cleaving enzyme (BACE) have been identified, BACE1[55-58] and BACE2[59, 60], both having an acidic pH optimum[36, 63]. BACE is mainly localized to the Golgi and endosomes[55], especially in cholesterol rich regions (e.g. lipid rafts or detergent-resistant membranes)[44, 45, 61, 62]. In the brain, BACE1 is predominant, while the expression of BACE2 is low. BACE cleavage of APP produces the C-terminal APP fragments C99 and C89, which following γ-secretase cleavage generates Aβ peptides starting either at Asp^1 or at Glu^{11}. The β-secretase site between amino acid 10 and 11, which is species specific, is usually called β-prime (β')[64]. The physiological functions of BACE1, has since its discovery, been unclear. However, a recent report suggests an essential role for BACE1 for myelination and correct bundling of axons by Schwann cells[65].

γ-secretase

γ-secretase, a membrane protein complex, is an aspartyl protease consisting of at least four proteins[66, 67]. Expression of presenilin (PS)[68, 69], nicastrin[70, 71], anterior pharynx defective-1 (APH-1)[72] and presenilin enhancer-2 (PEN-2)[73] have been shown to be sufficient to generate γ-secretase activity in yeast[74]. Thus, these four proteins are the minimum requirement for proteolytic activity. γ-secretase shows little sequence specificity for APP processing, generating various Aβ peptides differing in length. It also cleaves APP at the ε-cleavage site[75], located between amino acid 49 and 50 (Aβ numbering), and thereby generates the APP intracellular domain (AICD)[76, 77]. AICD has been shown to bind Fe65 and translocate to the nucleus where it is suggested

to be involved in transcriptional activation[78]. γ-secretase is also responsible for the cleavage of the cell surface receptor Notch-1[79], which releases the Notch intracellular domain (NICD), and possibly for cleaving several other transmembrane proteins (for review see[80]). Similar to AICD, NICD translocates to the nucleus where it is involved in transcriptional regulation. The resemblance of PS1 knockout phenotype in mice with a notch phenotype is perhaps the best demonstration of PS1 being involved in Notch processing[81]. γ-secretase activity has been reported in the ER, late-Golgi/trans-Golgi network, endosomes and plasma membrane[41-44]. Similar to β-secretase, γ-secretase activity has been associated with lipid rafts[46].

APP mutations

Although the majority of AD cases are sporadic there are many pathogenic mutations identified within the APP gene[82] (for updated list of AD mutations see www.alzforum.org/res/com/mut/default.asp). All mutations reported so far are located at, or close to, the cleavage sites, thereby affecting APP processing (Fig. 3). These lead either to an increased production of total Aβ, as in the case of the Swedish mutation[83-86] (K670N/M671L), or to a switch towards an increased production of the more fibrillogenic Aβ42 peptide[87], which is true for the APP mutations near the γ-secretase site and also for mutations in the presenillin genes (PS1 and PS2)[88, 89]. APP mutations linked to disease have also been found within the Aβ domain, in the vicinity of the cleavage site for α-secretase. Carriers of these intra-Aβ mutations; the Flemish[90] (A692G), Dutch[91, 92] (E693Q), Italian[93] (E693K) and Iowa[94] (D694N), display cerebral amyloid angiopathy (CAA) and hemorrhagic stroke, alone or in combination with progressive dementia.

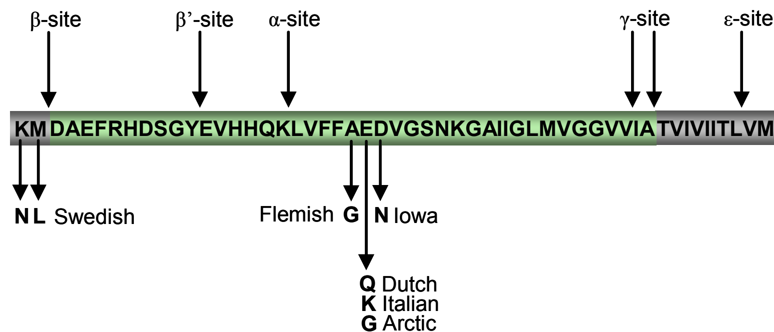

Figure 3. The Aβ domain (in green) of the APP protein is depicted with cleavage sites and pathogenic mutations relevant for this thesis.

The Arctic APP mutation

One of the intra-Aβ mutations is the Arctic mutation (APP E693G) where a glutamic acid at position 22 in the Aβ sequence is replaced by glycine[95, 96]. Contrary to other mutations within the Aβ domain carriers of the Arctic mutation present clinical symptoms of early-onset AD, for instance an insidious onset of cognitive decline without any signs of stroke or vascular lesions. Neuropathologically, carriers of the Arctic mutation display tangles and amyloid plaques lacking the characteristic dense core.

As opposed to other pathogenic AD-mutations, the Arctic mutation displays reduced extracellular Aβ levels, both *in vivo* in plasma from mutation carriers and *in vitro* in culture media from transfected cells[96]. The Arctic Aβ1-40 peptide has been shown to aggregate faster than Aβ1-40 wt[97] and to have unique aggregation properties, forming soluble aggregation intermediates, protofibrils, more rapidly and in larger quantities than Aβ1-40 wt[96]. Arctic Aβ1-42 aggregates faster than Aβ1-42 wild type and assembles into protofibrils and fibrils more rapidly than wild type Aβ[98]. Transgenic mice with the Arctic mutation demonstrate early intraneuronal Aβ aggregation that precedes plaque formation[99, 100]. In addition, the inclusion of the Arctic mutation in transgenic mice leads to a more rapid and abundant amyloid plaque deposition[99, 101]. Arctic Aβ1-40 has been demonstrated to be more resistant to both IDE and neprilysin, the two major enzymes involved in Aβ catabolism[102, 103]. It has further been shown that neuroblastoma cells harbouring the Arctic mutation have a decreased viability and enhanced sensitivity to toxic stress[104]. Taken together, the Arctic mutation results in several characteristic changes which could contribute to AD pathogenesis.

Amyloid-β peptide

Generation and clearance

Initially, Aβ was thought to exist only as constituents of insoluble amyloid deposits in AD brain[38]. Soon it was shown that Aβ was produced also under normal metabolic conditions[105-107], but if Aβ has a physiological role it remains to be identified. Aβ peptides of various length are generated from APP following proteolytic cleavage by β- and γ-secretase, and the most commonly produced forms are the 38-, 40- and 42- amino acid long variants[108, 109], where Aβ42 is the most amyloidogenic form[87] and also the variant of Aβ that is linked to the disease[89]. Most of the Aβ produced is secreted from the cell, although some Aβ remains within the cell, especially Aβ generated in the ER/intermediate compartment[110, 111]. Studies have shown that the ratio of Aβ40 and Aβ42 is different in lysates (3:1) and cell media (20:1),

with Aβ40 being more prevalent in secreted material[112]. Only 5-10% of secreted Aβ is Aβ42[113], instead Aβ42 seems to be retained within the cells where it oligomerizes and accumulates as an insoluble pool[41, 42, 111, 114, 115].

In general, Aβ peptides are thought to be cleared from the brain by three different mechanisms: (1) enzyme mediated degradation, (2) transport to the vascular system or (3) receptor mediated endocytosis followed by endosomal-lysosomal proteolysis[116]. However, the primary mechanism of Aβ clearance is thought to be degradation by extracellular peptidases[116]. The major enzymes involved in Aβ catabolism are IDE[25] and neprilysin[116], although other candidates as endothelin-converting enzyme[117] and angiotensin-converting enzyme[118] have also been shown to degrade Aβ.

Oligomerization and fibrillization

Aβ is prone to self-aggregate and form Aβ fibrils, particularly the C-terminal extended variants e.g. Aβx-42[87]. Aβ fibrillogenesis is a nucleation-dependent process. Once a nucleus has formed, further addition of monomers is thermodynamically favorable[119], leading to the formation of oligomers and ultimately fibers. During amyloidogenesis, Aβ undergoes a conformational shift from being mainly α-helical and random coil to a β-sheet structure[120]. Hydrophobic regions in the Aβ sequence, e.g. residues 17-21 and the C-terminus from residue 28, have a high propensity to form a β-sheet structure. Amino acids 5, 10 and 13 in the human Aβ sequence have also been suggested to be essential for Aβ fibrillogenesis, as Aβ aggregates have not been reported in rodents where these amino acids differ from human Aβ[121-123]. Electron microscopy of Aβ fibrils has shown that they are straight, unbranched and with a diameter of 7-12 nm. As all amyloid, Aβ fibrils are stained, due to their β-sheet structure, with the dyes Congo red[124] and Thioflavine[125]. These dyes are commonly used for identification and staining of amyloid. Since amyloid plaques are extracellular lesions it was first thought that Aβ aggregation takes place outside the cell. However, several studies have shown intracellular Aβ accumulations suggesting that aggregation begins already within the cell[41, 99, 100, 111, 114, 126, 127].

During *in vitro* Aβ fibrillogenesis different metastable intermediates are generated ranging from 8 kDa to over 100 kDa, e.g. low molecular weight-Aβ (LMW-Aβ)[128], Aβ-derived diffusible ligands (ADDLs)[129], Aβ globulomers[130], Aβ*56[131], Aβ oligomers[132], Aβ spheroids[133], protofibrillar pores[134] and protofibrils[135, 136] (Fig. 4). The smaller Aβ oligomers have a globular structure[129, 133] whereas protofibrils show curved, flexible fibers 5-200 nm in length and with a diameter of 6-10 nm[135, 136]. All these Aβ oligomers differ from mature fibers in that they are soluble and, unlike fibrils, do not pellet upon centrifugation. Several studies have demonstrated that Aβ oligomers are neurotoxic[137-139], able to inhibit long term potentiation[128, 129] and disrupt cognitive function[140]. It has therefore been argued that Aβ fibrils might not

be the most significant Aβ variant for AD pathogenesis[141]. For this reason it is of high importance to learn more about soluble Aβ oligomers as targets for AD therapy.

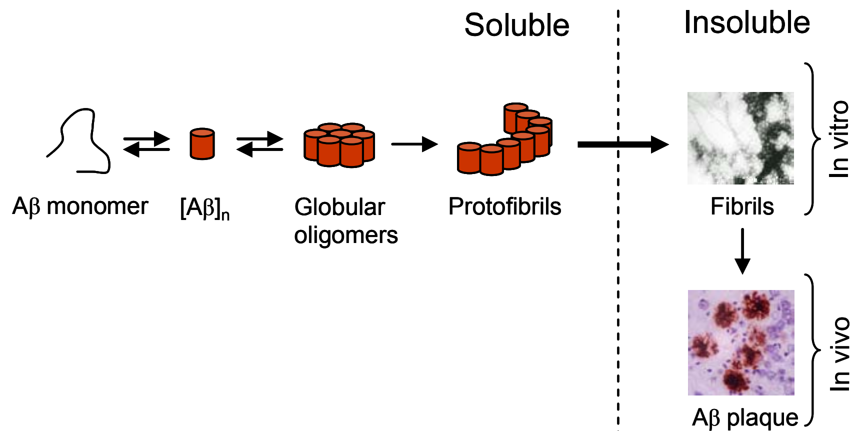

Figure 4. Schematic picture of Aβ aggregation. Aβ fibrillization is a multistep reaction generating different intermediates as the conformation of Aβ is shifted from being mostly α-helical and random coil to a β-sheet structure. Pictures from Ann-Sofi Johansson and Paul O'Callaghan.

The amyloid cascade hypothesis

The "amyloid cascade hypothesis" was proposed shortly after the initial genetic discoveries of APP mutations leading to familial AD[142]. The model states that abnormal aggregation of Aβ is initiating AD pathology and that neurofibrillary tangles, cell loss, inflammation, neurotransmitter deficiencies, and dementia follow as a direct result of this process. For several years it was debated whether tangles or plaques initiated the pathological process. However, important findings in 2001 showed evidence that the formation of tangles was accelerated by Aβ, as transgenic mice expressing both mutant tau and mutant APP had more tangles than transgenic mice expressing mutant tau alone[143] and further, tangle formation was enhanced in tau transgenic mice injected with fibrillar Aβ42[144]. Genetic findings have today linked APP, PS1, PS2 and ApoE to AD pathology. Cell biological and transgenic studies have shown that pathogenic variants of these four AD associated proteins result in; increased Aβ production[83-86], increased Aβ42 levels[88, 89], highly fibrillogenic Aβ variants[96, 136, 145], enhanced Aβ fibrillization[146], altered APP processing[147] and decreased Aβ clearance[148]. These findings have

strengthened the amyloid cascade hypothesis immensely and it is today the most favored model to explain the pathogenic events leading to AD. Although plaque burden has been shown to correlate with degree of dementia in some studies[149, 150], there are studies that have been unable to find such a correlation[151]. Therefore it has lately been debated whether or not AD pathology is the result of the insoluble Aβ in the plaques, since it has been shown that soluble Aβ, defined as non-fibrillar Aβ, e.g. monomers and oligomers, correlates better than amyloid deposits to the degree of dementia[152].

Lipids in Alzheimer's disease

Fatty acids

Around 50% of the dry weight of the brain is lipids, mostly polyunsaturated fatty acids[153]. The omega-3 (ω-3) polyunsaturated fatty acid docosahexaenoic acid (DHA) is one of the major fatty acids in the brain, and it is essential for the development of brain and vision[154]. The main source of DHA is fatty fish, but DHA can also be synthesized endogenously, by astrocytes[155], from its precursors α-linolenic acid and eicosapentaenoic acid (EPA).

Several epidemiological studies have implicated a role of fatty acids in AD pathogenesis, where a dietary intake of ω-3 has been correlated with a reduced risk of developing AD[156-158]. In addition, it has been found that AD patients have a reduced level of ω-3 in the hippocampus[159]. Animal studies have supported the positive effects of dietary DHA by demonstrating improved learning ability in AD rats[160]. Moreover, AD transgenic mice given DHA-enriched diets showed a reduced plaque burden and levels of insoluble Aβ[161]. DHA has also been demonstrated to protect against dendritic pathology in transgenic mice[162] and suggested to affect APP processing driving it towards the non-amyloidogenic pathway[161]. A hypothesis for this effect could be that supplementary DHA changes the membrane fluidity in favour for α-secretase cleavage.

Cholesterol

The only proven genetic risk factor for late-onset AD is the ε4 variant of the ApoE gene which involves a dose-dependent risk for developing the disease[19, 20]. As ApoE polymorphism is known to have an important impact on cholesterol metabolism it has been speculated that cholesterol might be involved in the pathogenesis of AD. Epidemiological studies suggest that high serum cholesterol during midlife is a risk factor for AD[163, 164] and pharmacoepidemiological studies suggest that statins, inhibitors of choleterol

synthesis[165], protects against AD[166, 167]. Lowering cholesterol with statins, markedly decrease Aβ levels[168-170]. Further, it has been shown that high levels of plasma cholesterol, associated with the ApoE ε4 variant, can contribute to the pathogenesis of AD[171, 172]. However, there are also studies demonstrating that increased dietary cholesterol lowers levels of secreted Aβ in brain[147] and that loss of membrane cholesterol contributes to excessive amyloidogenesis in AD[62]. Probably, there is a delicate balance in cholesterol levels, where an increased or decreased cholesterol level can disturb this balance and affect different pathological events such as AD.

There are several models for how ApoE and cholesterol affects AD pathogenesis. One hypothesis is that the binding of ApoE to Aβ directly promotes Aβ aggregation, supported by studies showing facilitated Aβ fibril formation in AD transgenic mice expressing the ApoE ε4 variant[146]. Another speculation is that increased cellular cholesterol might regulate APP processing and lead to increased Aβ production[173, 174] or that low cholesterol could stimulate the α-secretase candidate ADAM10 stimulating the non-amyloidogenic pathway[52], as this cleavage mainly takes place in membrane regions low in cholesterol, whereas Aβ generation has been suggested to dependent on lipid rafts with high levels of cholesterol[44-46]. Cholesterol could therefore be involved in regulating the access of α- and β-secretase to APP.

Aim of the study

The overall aim of this study was to investigate the molecular mechanisms of the Arctic APP mutation in order to understand its pathological characteristics in Alzheimer's disease. The specific aims for each paper were:

Paper I	To investigate how the Arctic mutation affects α- and β-secretase processing of APP.
Paper II	To determine in detail the mechanism whereby the Arctic mutation affects APP processing and Aβ production.
Paper III	To examine if reduced Aβ levels by the Arctic mutation was due to inefficient detection of Aβ oligomers by ELISA.
Paper IV	To study how the polyunsaturated fatty acid docosahexaenoic acid (DHA) effects processing of Arctic APP.

Results and Discussion

The Arctic mutation favors the amyloidogenic pathway

The Arctic APP (E693G) mutation was previously found to decrease levels of Aβ40 and Aβ42 in plasma from mutation carriers and Aβ42 in culture media from cells transfected with the Arctic mutation[96]. These results were surprising, since it was the first description of an APP mutation that led to clinically diagnosed AD and reduced Aβ levels. Instead of increased Aβ, studies suggested that the protofibrillogenic nature of the Arctic peptide was the underlying pathogenic characteristic of the mutation[96-98].

To understand the primary mechanism of reduced Aβ levels with the Arctic mutation the processing of Arctic APP was investigated in **paper I** and **II**. For this purpose a hybrid APP cDNA construct was developed in **paper I** containing both the Arctic and the Swedish APP mutations (K670N/M671L), named $APP_{Arc\text{-}Swe}$. Since the Swedish mutation is known to increase Aβ production[83-86], this construct was developed to increase cellular production of Arctic Aβ and to facilitate detection of alterations in APP processing. Aβ levels in $APP_{Arc\text{-}Swe}$ cells, and in cells containing the Swedish mutation together with another intra-Aβ mutation ($APP_{Swe\text{-}Dutch}$, $APP_{Swe\text{-}Italian}$ and $APP_{Swe\text{-}Flemish}$), were compared with Aβ levels in APP_{Swe} cells. Aβ enzyme-linked immunosorbent assay (ELISA) measurements on culture media from these double mutant cells showed the same effect from the intra-Aβ mutation in comparison with control as shown earlier in single mutant cells[96, 175, 176]. This demonstrates that the $APP_{Arc\text{-}Swe}$ construct can be used for studies of the Arctic mutation.

To analyze the effects of the Arctic mutation on APP processing, the Aβ/p3 ratio was studied by Western blot in culture media from $APP_{Arc\text{-}Swe}$ cells in **paper I**. Both Aβ40/$p3_{40}$ and Aβ42/$p3_{42}$ were increased compared to APP_{Swe} control cells, indicating that the Arctic mutation reduced α-secretase cleavage and/or an increased β-secretase cleavage. These results were supported by analysis of N- and C-terminal APP fragments, studied in $APP_{Arc\text{-}Swe}$ cells in **paper I** and **II,** and in $APP_{Arc\text{-}Swe}$ transgenic mice in **paper II**. Levels of secreted α-secretase product, αAPPs, was significantly reduced, whereas the level of secreted β-secretase product, βAPPs, was increased, both *in vitro* and *in vivo*. Further, the β-secretase product C99 was

increased both *in vitro* and *in vivo*, whereas the α-secretase product C83 only was reduced *in vitro* but remained unchanged *in vivo*. This could illustrate that the metabolism of C83 and C99 is not the same *in vitro* as *in vivo*, which is supported by the findings of others[177]. Taken together, these results demonstrate that the Arctic mutation favors β-secretase cleavage and an amyloidogenic processing of APP.

Arctic APP is less available to α-secretase

The mechanism whereby the Arctic mutation affects APP processing and Aβ production were studied in **paper II**. Two assumptions were tested; (1) the Arctic mutation is situated close to the α-secretase cleavage and the single amino acid substitution could impair the enzymatic cleavage of α-secretase itself (similar to the Flemish mutation[34]) or, (2) the Arctic mutation might affect the transport and/or localization of APP altering the availability of Arctic APP to different secretases.

To challenge our first assumption we tested the ability of ADAM10, one of the major α-secretase candidates[34], to cleave synthetic Arctic Aβ fragments. We found that ADAM10 cleaved Arctic and wild type Aβ (10-24) fragments with the same efficiency across a broad range of substrate concentrations suggesting that the Arctic amino acid substitution does not impair the epitope for α-secretase cleavage of APP. These results were in agreement with a previous finding where α-secretase was shown to cleave APP at a specific distance from the membrane rather than at a specific sequence of the substrate[35]. However, the possibility that the Arctic amino acid substitution alters secondary structures important for α-secretase processing remains unsolved. In addition, the ability of other α-secretase candidates (e.g. ADAM17/TACE[47], ADAM9[48]) to cleave Arctic APP could still be impaired and should be tested before a more general conclusion can be made.

To test our second assumption we studied levels of cell surface APP in $APP_{Arc\text{-}Swe}$ cells. A significant reduction of this pool of APP was found, indicating decreased availability at the cell surface where α-secretase cleavage mainly occurs[33-35]. APP with the Swedish mutation has however been shown to be cleaved by β-secretase mainly in secretory vesicles whereas β-secretase cleavage of wild type APP occurs during endocytosis[178]. Therefore, levels of cell surface APP were analyzed also in APP_{Arc} cells to ensure that reduced APP at the cell membrane was not a result of the Swedish mutation. Levels of cell surface APP were found to be reduced in this Arctic cell model as well. Overall, these experiments indicate that the transport of Arctic APP to the cell surface is either decreased or its removal from the cell membrane by endocytosis is increased. As a result of reduced cell surface APP, less APP is available for α-secretase cleavage thereby shifting APP processing towards the amyloidogenic pathway.

Increased Aβ by the Arctic mutation with IP/Western

An increased β-secretase cleavage of Arctic APP, demonstrated in **paper I** and **II**, suggests increased Aβ production. However, reduced plasma Aβ levels in mutation carriers as well as reduced Aβ42 in culture media from APP_{Arc} cells have previously been reported[96]. This was verified in **paper I** analyzing Aβ levels in culture media from $APP_{Arc\text{-}Swe}$ cells. Interestingly, the Aβ ELISA in **paper I** revealed reduced levels of both Aβ40 and Aβ42 in culture media from $APP_{Arc\text{-}Swe}$ cells, and a slightly decreased Aβ42/Aβ40 ratio, whereas earlier studies of APP_{Arc} cells only showed reduced Aβ42[96] and a clear reduction of the Aβ42/Aβ40 ratio. Importantly, these results indicate that the combination of the Arctic and the Swedish mutations was necessary to be able to detect the Aβ40 reduction, as this decrease was smaller than the reduction of Aβ42 and therefore more difficult to detect.

One suggestion to explain reduced levels of extracellular Arctic Aβ is that the aggregation of Arctic Aβ could begin already within the cell, increasing the intracellular pool. An increased amount of protofibrils, formed and retained within the cell, might reduce the extracellular Aβ level. To test this hypothesis, levels of both extra- and intracellular Aβ were analyzed in $APP_{Arc\text{-}Swe}$ cells in **paper II**. Because most of the Aβ produced is secreted from the cell, intracellular Aβ is more difficult to detect and therefore more sensitive techniques are needed. For this reason, Aβ levels in $APP_{Arc\text{-}Swe}$ cells were analyzed by immunoprecipitation (IP)/Western blot in **paper II**, as opposed to ELISA which was used in **paper I**. To our surprise, IP/Western blot analysis in **paper II** showed a three-fold increase of Aβ in culture media from $APP_{Arc\text{-}Swe}$ cells whereas the intracellular Aβ level was increased almost six-fold, in comparison with APP_{Swe}. Opposite to earlier findings, these data indicated increased Aβ production by the Arctic mutation and also an intracellular accumulation of Arctic Aβ, in agreement with *in vivo* studies[99, 100]. To verify these results, extracellular Aβ was measured also in APP_{Arc} cells using Western blot in **paper II**. This was done to ensure that the increased Aβ levels in $APP_{Arc\text{-}Swe}$ cells found with IP/Western blot was not an artifact of the combined usage of the two mutations. These experiments further demonstrated increased extracellular Aβ levels in APP_{Arc} supporting data from $APP_{Arc\text{-}Swe}$ cells. Unfortunately, intracellular Aβ levels were below the detection limit and could not be analyzed in APP_{Arc} cells, illustrating the necessity of using the Swedish APP mutation as a signal enhancer for analyzing the effects of the Arctic mutation. In summary, IP/Western blot data from **paper II** revealed increased Aβ levels suggesting increased Aβ production in Arctic cells, in contrast with the results found in **paper I** using ELISA.

Aβ oligomerization affects Aβ ELISA measurements

In **paper III** we examined if the divergent Aβ results obtained with ELISA (**paper I**) and IP/Western blot (**paper II**) were due to inefficient detection of Arctic Aβ oligomers by ELISA. Based on the protofibrillogenic nature of the Arctic mutation[96-98] our assumption was that Arctic protofibrils might hide epitopes for the antibodies in the ELISA resulting in falsely low Aβ levels.

Aβ levels in $APP_{Arc\text{-}Swe}$ culture media and in brain tissue from $APP_{Arc\text{-}Swe}$ transgenic mice were measured by ELISA and Western blot in parallel in **paper III**. Remarkably, analyzing the very same sample with different techniques gave opposite results. The ELISA demonstrated reduced levels of both Aβ1-40 and Aβ1-42 (in accordance with **paper I**), whereas Western blot analyses instead revealed an increased total Aβ level (in accordance with **paper II**). These results strengthened our hypothesis that Arctic Aβ oligomers were inefficiently measured by ELISA resulting in falsely low Aβ levels, as observed in **paper I**. To investigate if this was true for wild type Aβ oligomers as well, synthetic Aβ1-42 wild type was incubated at 37ºC for up to 23h to generate protofibrils. Aβ in the exact same sample were then analyzed with size exclusion chromatography (SEC), ELISA and Western blot in parallel. The Aβ1-42 level was reduced over time with ELISA, as the peptide oligomerized into protofibrils, whereas the Aβ level remained essentially unchanged when assayed with Western blot (Fig. 5). This clearly demonstrates that Aβ oligomers are not measured efficiently by ELISA leading to falsely low Aβ levels.

The different results obtained with Western blot and ELISA were probably primarily due to Western blot being a denaturing method whereas ELISA is a non-denaturing method. Thus, the conditions under which Aβ peptides are measured are very important, since these peptides are highly prone to aggregate. Oligomers of Aβ can mask epitopes for antibodies in the ELISA and lead to an underestimation of the Aβ level, whereas a denaturing method like Western blot will, at least partially, dissolve these oligomers and measure the Aβ level more accurately. Therefore, measuring Aβ levels with ELISA might not give the correct amount of Aβ, unless the sample is pretreated under denaturing conditions before analysis. Apart from the problem with Aβ oligomers in ELISA, Aβ could be bound to other cellular proteins which could interfere with the Aβ-antibody interaction. Binding of synthetic Aβ peptides to several plasma and cerebrospinal fluid (CSF) proteins have been shown to mask epitopes for antibody detection of Aβ[179-181]. Mutations within the Aβ sequence might change the affinity for these interactions, thereby increasing the need of a denaturing environment to dissolve these protein-protein complexes.

In conclusion, oligomerization of Aβ results in an underestimation of Aβ levels with ELISA, most likely due to epitope masking, whereas Western blot analysis measures Aβ oligomers and gives a more accurate result.

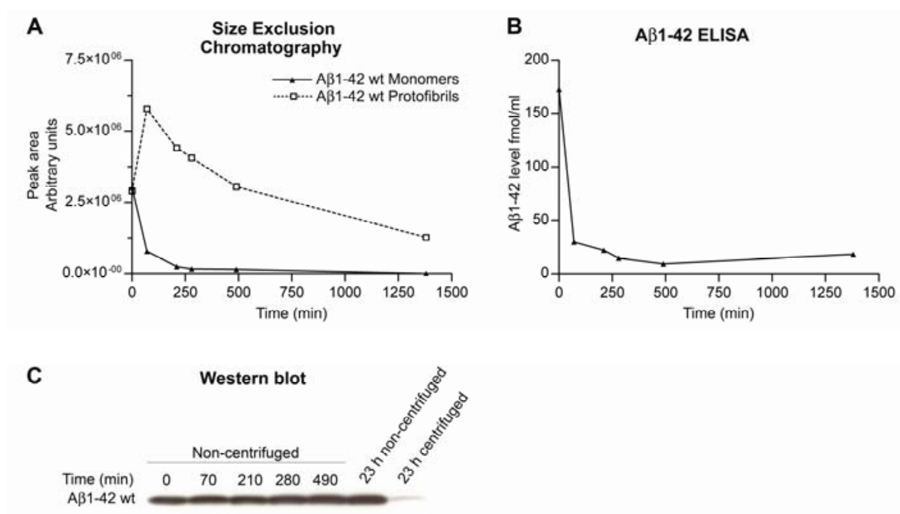

Figure 5. Oligomerization of synthetic Aβ1-42 wt. **(A)** At time zero, approximately 50% of the Aβ1-42 wt peptide has oligomerized into protofibrils, as visualized with size exclusion chromatography (SEC). Over time, the level of monomers declines as more protofibrils are formed. Moreover, after 70 minutes the level of protofibrils starts to decline as fibrillization begins. **(B)** At time zero, ELISA Aβ1-42 wt levels are approximately 50% of the theoretical level, and this level continues to decline in association with protofibril formation. **(C)** Western blot demonstrates unchanged Aβ1-42 wt levels over time, despite the presence of oligomers. Centrifugation of the 23-hour sample before gel loading results in a markedly weaker band, as compared with the sample that was not centrifuged.

DHA stimulates non-amyloidogenic APP processing

Epidemiological studies suggest that increased intake of docosahexaenoic acid (DHA), an omega-3 (ω-3) polyunsaturated fatty acid found for example in fat fish, is protective against the development of AD[156-158]. This is supported by different animal studies demonstrating positive effects of DHA on AD pathology[160-162]. DHA is known to be in equilibrium with the omega-6 (ω-6) polyunsaturated fatty acid arachidonic acid (AA). DHA has been suggested to both decrease the synthesis of AA and physically replace AA in the membrane[182, 183]. An increased intake of DHA therefore leads to a decreased ω-6/ω-3 ratio which is thought to be AD protective[156-158]. The effects of DHA on APP processing has only been briefly studied, indicating some effects on levels of C-terminal APP fragments[161].

The aim of **paper IV** was to study if and how DHA affects APP processing in human neuroblastoma SH-SY5Y cells stably transfected with $APP_{Arc\text{-}Swe}$ or APP_{Swe}, as cellular models for AD. Cells were treated with

100 μM DHA for 11 days after which levels of C- and N-terminal APP fragments were analyzed to evaluate the effects of DHA on α- and β-secretase processing of APP. Previous studies of transgenic mice fed a DHA enriched diet showed reduced ratios of both C83/APP and C99/APP[161]. In **paper IV**, the C83/C99 ratio was increased in DHA treated cells, indicating a shift towards non-amyloidogenic APP processing. We choose to present our data as C83/C99 ratio since this ratio gives a better picture of the relationship between α- and β-secretase cleavage than the CTF/APP ratio. Supporting our data from levels of C-terminal APP fragments, the αAPPs/βAPPs ratio was increased in APP_{Swe} cells. However, no effect was found in $APP_{Arc\text{-}Swe}$ cells treated with DHA, suggesting that the effects of the Arctic mutation itself probably overcame the supposed effects from DHA. It was demonstrated in **paper II** that Arctic cells have less APP at the cell surface. One can therefore speculate that levels of soluble APP fragments might not be as affected in $APP_{Arc\text{-}Swe}$ cells as in APP_{Swe} cells if DHA mainly act at the cell surface. Interestingly, we still found an effect on the C83/C99 ratio in $APP_{Arc\text{-}Swe}$ cells treated with DHA. A simple reason could be that small differences in protein levels are easier to find in lysates as proteins are allowed to accumulate for a longer time, 11 days in this case, whereas the medium is changed every two-three days. In sum, studies of APP processing in DHA administered cells displayed an increased α-secretase and/or decreased β-secretase cleavage, suggesting reduced Aβ production. Indeed, analysis of total Aβ in $APP_{Arc\text{-}Swe}$ and APP_{Swe} cells treated with DHA demonstrated reduced Aβ levels in both culture media and lysates. The specific levels of Aβ38 and Aβ40 were also found to be reduced in both $APP_{Arc\text{-}Swe}$ and APP_{Swe} cells treated with DHA, although only $APP_{Arc\text{-}Swe}$ levels were significantly decreased. These results suggest a protective role for DHA in AD linked to a molecular mechanism, Aβ formation, which likely drives the pathogenesis.

Interestingly levels of APP intracellular domain (AICD), the product generated by γ-secretase cleavage at ε-site of C83 and C99[76], increased significantly upon DHA administration. The γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) was used to verify the identity of this fragment, showing an accumulation of C83 and C99 whereas AICD was decreased. Upon DAPT supplementation the effect of DHA on AICD level was totally abolished. This suggests that the increased AICD level was a result of enhanced γ-secretase activity instead of an effect from increased levels of precursor. The increase of AICD in DHA treated cells was markedly higher in APP_{Swe} than in $APP_{Arc\text{-}Swe}$ cells. This might indicate that AICD is mainly generated from C83, as suggested elsewhere[184], since Arctic cells have less C83 than C99 as found in **paper II**. Studies have shown that AICD can translocate to the nucleus and activate transcription of the neprilysin gene[185], one of the major enzymes involved in Aβ catabolism[116]. However, findings in **paper IV** did not reveal any changes

in levels of neprilysin as a result of increased AICD levels upon DHA treatment. Perhaps there is a rapid degradation of neprilysin, or a longer period of DHA treatment is needed to be able to detect this effect by increased AICD. Nevertheless, our studies suggest that AICD does not affect neprilysin levels, in agreement with Hébert *et* al[186].

In view of our results, it was tempting to speculate how membrane enrichment with DHA could influence APP processing. We reasoned that a possible mechanism could be altered cholesterol levels, since reduced cholesterol levels have been reported in rats fed with DHA[187, 188]. The connection between cholesterol levels and APP processing is not obvious, but cholesterol lowering drugs have consistently been shown to reduce Aβ levels [170, 189] and increase αAPPs [190], a pattern similar to that observed in this study. Nevertheless, neither free cholesterol nor total cholesterol levels were changed upon DHA treatment in **paper IV**, indicating that the altered APP processing is not an indirect effect related to the membrane content of cholesterol.

In conclusion, findings in **paper IV** demonstrate that DHA administration to cell models of AD stimulates non-amyloidogenic APP processing with reduced Aβ levels as a result, supporting the hypothesis of a protective role for DHA in AD pathogenesis.

General discussion and future perspectives

When first discovered and described, the Arctic APP mutation (E693G) was thought to exhibit its pathogenic characteristics mainly by the high tendency of Arctic Aβ peptides to oligomerize and form protofibrils. It was the first familial mutation leading to AD without increasing Aβ levels, instead levels of Aβ were found to be decreased in plasma from mutation carriers and in cell culture media[96]. In this thesis, the pathogenic mechanisms of the Arctic mutation were further evaluated, specifically addressing if and how the mutation affects APP processing and Aβ levels.

Here, we show evidence of an increased amyloidogenic processing of Arctic APP, resulting in increased steady state levels of Aβ. Interestingly, levels of Aβ were lowered by administration of docosahexaeonic acid (DHA), thereby "rescuing" the cells from the suggested increased Aβ production driven by the Arctic mutation. As opposed to the Swedish APP mutation, where enhanced β-secretase processing results in an increased Aβ generation[83-86], the Arctic mutation might increase Aβ production by reduced α-secretase cleavage. Arctic cells were demonstrated to have less APP at the cell surface, the location where α-secretase processing mainly occurs[33-35], thereby leaving more substrate for β-secretase processing. An altered APP localization with the Arctic mutation also implicates involvement of mechanisms, other than increased Aβ formation, in AD pathogenesis. A reduced level of cell surface APP together with an increased ratio of intracellular:extracellular Aβ, as demonstrated in this thesis, could lead to accumulation of newly formed Aβ peptides in an environment where the oligomerization reaction is more favourable. This suggests an important role for intracellular Aβ accumulation and aggregation in AD pathogenesis, as previously indicated[99, 100]. In the future, it would be interesting to learn more about the cellular distribution and transport of Arctic APP and to study its rate of endocytosis, as possible mechanisms for reduced levels of cell surface Arctic APP. Moreover, the role of DHA and cholesterol in AD pathogenesis is not fully resolved and needs to be further evaluated.

Throughout the work with this thesis there has been a continuous development and refinement of sensitive techniques for Aβ detection and quantification. Initially, Arctic Aβ levels were measured with ELISA, demonstrating reduced levels of Aβ. At that time, results from IP/Western blot analysis of Arctic Aβ were difficult to interpret and further improvements were needed. We were able to increase the sensitivity of Aβ detection,

enabling analysis of Arctic Aβ in both cell culture media and lysates. Unexpectedly, IP/Western blot analysis of Arctic Aβ revealed increased steady state levels at extra- and intracellular locations. This was an essential finding which suggested that the Arctic mutation, similar to other AD-causing mutations, actually might increase Aβ production, despite earlier results demonstrating the contrary. Pulse-chase experiments will be needed to further verify an increased Aβ formation with the Arctic mutation as increased steady state levels of Aβ also can be the result of stabilized Aβ aggregates, which were demonstrated to exist in Arctic samples. Most importantly, the data raised questions regarding the efficiency of the Aβ ELISA. We discovered limitations of ELISA as a tool for measuring total Aβ levels, as Aβ oligomers were shown to be inefficiently measured resulting in an underestimation of total levels of Aβ1-40 and Aβ1-42. Moreover, the results imply that the reduced levels of Aβ observed in plasma from carriers of the Arctic mutation might be a sign of presence of Aβ oligomers. The occurrence of Aβ oligomers in human AD CSF has previously been described[191, 192], and recently enhanced Aβ protofibril levels have been detected in Arctic cell culture media and Arctic transgenic mouse brain using a protofibril specific ELISA (*personal communication Hillevi Englund and Dag Sehlin*). Hopefully, this ELISA will enable us to investigate levels of protofibrils in biological fluids like plasma and CSF from AD-patients and controls, as well as carriers of the Arctic mutation. This will help us address questions about the relevance of Aβ protofibrils in AD pathogenesis. It would also be exciting to study the intracellular pool of Arctic Aβ, to see if protofibrils are generated and are able to accumulate inside the cell, as previously suggested[41, 99, 100, 111, 114, 126, 127]. In that case, perhaps protofibrils could seed further fibril formation and extracellular plaque generation.

In conclusion, the pathogenic mechanisms of the Arctic mutation underlined in this thesis involve effects on both APP processing and Aβ oligomerization. Our data highlight mechanisms, other than increased formation of Aβ peptide monomers through endoproteolysis that could be important also in sporadic Alzheimer's disease.

Experimental approaches

Here, the techniques used are briefly described and discussed in terms of their advantages and limitations. Detailed descriptions of experimental approaches are found in respective papers.

Cellular models of Alzheimer's disease

The experiments were carried out with human embryonic kidney (HEK) 293 cells (**paper I-III**) and human neuroblastoma SH-SY5Y cells (**paper II** and **IV**), both commonly used cellular models in AD research. The major difference between these cells is that HEK293 cells are non-neuronal and therefore process more APP via the non-amyloidogenic pathway[39, 40]. Cells were cultured in DMEM supplemented with 10% fetal calf serum, antibiotics and plasmocin. Transfections were performed with FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions, introducing the pcDNA3 plasmid (Invitrogen) containing the APP^{695} gene, wild type or with different Alzheimer mutations (especially the Arctic mutation), into the cells. HEK293 cells were used for transient transfection whereas SH-SY5Y cells were found to be more suitable for stable transfection.

Transfection is a process for bringing foreign DNA into cells thereafter protein expression can be monitored and analyzed. Both transient and stable transfections have been used in this thesis. Upon transient transfection cells will momentarily express the genes (approximately 24 to 72 h post transfection) in the introduced plasmid since it is not integrated into the host chromosomes and will be lost as cells divide. The advantage of this method is that it is an easy and fast way to express the gene of interest in cells, however since the expression is not permanent one has to replicate the transfection procedure for each study and therefore this method is not suitable for experiments running over long time periods. On the other hand, in stably transfected cells the plasmid is integrated into the host DNA and will become a permanent part of the genetic material of the cell. Stably transfected cells are identified by selection with antibiotics, since only cells with an integrated plasmid will have the gene encoding resistance to the antibiotic. Obtaining stable cells may take as long as 6-8 weeks, but once you have stably transfected cells these will permanently express the gene of interest.

Transgenic models of Alzheimer's disease

A transgenic animal is an animal where the genome has been modified by insertion of foreign DNA. In general, mice are used for this purpose as they are inexpensive, their zygotes are easy to inject and they have a short generation time. Another advantage is that the mouse genome is well defined. Commonly, the foreign DNA is integrated into the host DNA at a random location. Injection of DNA into the fertilized egg is followed by implantation into pseudopregnant foster mothers. The offspring, founders, are screened for the desired gene and, if wanted, these hemizygous founders can be crossed to generate mice homozygous for the transgene.

There are today several established transgenic mouse models for AD, most commonly used is the Tg2576 model which overexpresses human APP with the Swedish double mutation (K670M/N671L). Transgenic mouse models of AD present a number of AD characteristics like amyloid plaques, congophilic amyloid angiopathy, CAA and synapse loss together with cognitive deficits. However, no mouse model fully reflects all characteristics of AD, especially the lack of neurofibrillary tangles and macroscopic atrophy are obvious distinctions from human AD pathology.

Transgenic mice hemizygous for human APP^{695} with the Arctic and Swedish APP mutations ($APP_{Arc\text{-}Swe}$) or with the Swedish mutation alone (APP_{Swe}) were developed as briefly described above (for details see reference[99]). These mice originally had a C57BL/6-CBA background and express the APP gene under the control of the Thy-1 (thymus cell antigen 1) promoter. The benefits of using this promoter are its strong expression with high mRNA levels and its neuronal specificity. Transgenic mice used in **paper II** and **III** had a threefold overexpression of human $APP_{Arc\text{-}Swe}$ or APP_{Swe}, and were 2-3 months old when used for biochemical analysis.

Immunoprecipitation

Immunoprecipitation (IP) can be used to purify and concentrate a specific protein from a mixture of proteins, for example a cell extract, for further detection and quantification. Usually the target protein is metabolically radiolabeled before IP, but this technique can also be used to analyze unlabeled proteins. In general, a primary antibody specific for the target protein is added to the protein mixture generating antigen-antibody complexes. An immobilized antibody binding protein (protein A- or protein G-sepharose) or an immobilized secondary antibody (for example anti-mouse IgG coupled to sepharose or magnetic beads) is then added to physically separate the antigen-antibody complex from the protein mixture. The advantage of IP is that it is a very sensitive technique provided that the primary antibody is specific for the target protein. The antigen-antibody complexes can then be purified

from contaminating proteins and enable sensitive detection of the protein of interest. However, when analyzing very “sticky”, hydrophobic proteins, like Aβ, one can sometimes dilute and loose sample during this process. Possibly this is influenced by adsorption of Aβ to different surfaces, but it can also be a result of problems with antigen-antibody interaction due to Aβ oligomers or interaction of Aβ with other proteins in the mixture. If the antigen-antibody interaction is disturbed the target protein will simply be washed away during the IP procedure resulting in poor yield. In general, quite a substantial amount of the primary antibody is needed to get a good yield from an IP. In the earliest Aβ studies (**paper I** and **II**), IP of Aβ preceded the SDS-PAGE/Western blot, as the SDS-PAGE/Western blot at that time was not sensitive enough for Aβ analysis. However, as our SDS-PAGE/Western blot was optimized, this technique was found to be even better without the preceding IP (**paper II**-**IV**), probably due to poor yield.

Gel electrophoresis/Western blot

The immunoprecipitate is usually analyzed by polyacrylamide gel electrophoresis (PAGE) followed by Western blot, but other techniques can be used as well. In PAGE, proteins are separated according to their net charge, size and shape, when migrating in a gel in response to an electric field. To be able to separate proteins according to their size only, sodium dodecyl sulfate (SDS) is used. SDS is a denaturing detergent which dissolves hydrophobic molecules and makes them linear. In addition, SDS gives the protein a negative charge in proportion to its length allowing proteins to migrate in the gel towards the positive pole with different rates according to their molecular weight. As a result, low molecular weight proteins will move faster in the gel than high molecular weight proteins. Regularly, a reducing agent such as 2-mercaptoethanol is added to reduce disulfide linkages (remove tertiary and quaternary structure) which is not disrupted by SDS alone. Finally, proteins in the gel are transferred by electrophoresis, from the gel to a membrane where target proteins are analyzed by Western blot with specific antibodies together with enzymatic chemiluminescent or fluorescent detection.

The benefit of using SDS-PAGE instead of ELISA is the denaturing condition where under proteins are quantified, which is of high relevance when analyzing highly fibrillogenic proteins such as Aβ, especially Arctic Aβ. Though, the limitation with SDS-PAGE/Western blot is that you only get relative amounts of the proteins instead of absolute levels obtained by for example ELISA. Additionally, one should be aware of that the enzymatic chemiluminescent reaction and the intensity of these bands on the film are not linear. It is therefore important to monitor the enzymatic reaction carefully to be able to stop it before the signal reaches the maximum signal pla-

teau. On the other hand, fluorescent detection, used in **paper IV**, has a more linear dynamic range than chemiluminescent detection.

Enzyme-linked immunosorbent assay

With enzyme-linked immunosorbent assay (ELISA) one can measure quantitative levels of either antibodies ("indirect" ELISA) or antigens ("sandwich" ELISA). In sandwich ELISA (used in **paper I** and **III**) an antigen specific antibody, the "capture antibody", is coupled to a surface. The antigen-containing sample is added after which another antigen specific antibody, the "detection antibody", is applied and binds to the antibody-antigen complex. An "enzyme-linked" secondary antibody, usually anti-mouse/-rabbit IgG, generally coupled to an enzyme, e.g. horse radish peroxidise (HRP), then binds the detection antibody and produces a detectable signal upon addition of substrate.

ELISA is widely used for measuring Aβ in preclinical research, but also in clinical research evaluating Aβ in biological fluids as a potential biomarker of AD. In comparison with SDS-PAGE/Western blot this technique is simple and straightforward to use, the success of the ELISA lies mainly in having good antibodies for your antigen. Another advantage with ELISA is that you can compare many samples and replicates at the same time in the same assay, whereas the number of samples in a SDS-PAGE/Western blot is more limited. However, the major concern with ELISA is that it is non-denaturing. As shown in **paper III**, oligomers of Aβ can mask epitopes for the antibodies in the ELISA leading to falsely low Aβ levels. In addition, binding of Aβ to other proteins in plasma[179, 180] and CSF[181] is also a problem partly solved by instead using denaturing SDS-PAGE/Western blot.

High performance liquid chromatography

High performance liquid chromatography (HPLC) is a column chromatography used to separate, isolate, quantify and/or characterize components of a mixture by different chemical interactions. The substance to be analyzed (the "analyte") is forced under high pressure through a column and the time it takes for the analyte to travel through the column is measured. This "retention time" depends on the distribution ratio of the analyte between the stationary phase in the column and the mobile phase. There are several types of HPLC; for example reverse-phase HPLC (RP-HPLC, **paper II**) and size exclusion HPLC (SEC-HPLC, **paper III**). With RP-HPLC substances are separated according to their hydrophobicity, non-polar molecules have longer retention time than polar molecules in this system. On the other hand,

SEC-HPLC separates particles depending on their size, where small molecules have a longer retention time than large molecules. Since this method is native it is possible to determine the size and separate protein aggregates that are not SDS-stable. The size determination is however not as exact as SDS-PAGE/Western blot, since the folding of these aggregates will partly affect the retention time and proteins might interact with the matrix.

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