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# Modeling Amyloid- $\beta$ Pathology in Alzheimer's Disease Using the Arctic Mutation

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#### **Abstract**

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The Arctic mutation in the Amyloid- $\beta$  ( $A\beta$ ) domain of the Amyloid- $\beta$  precursor protein (APP) causes Alzheimer's disease (AD) and confers unique biochemical characteristics to  $A\beta$  peptides. The aims of this thesis were to evaluate a transgenic model with the Arctic mutation, and to use it to gain new insights into the mechanisms of early (pre-plaque) and late-stage  $A\beta$  pathogenesis in AD. The Arctic mutation made  $A\beta$  more prone to aggregate, to accumulate in intracellular compartments and to form extracellular plaques when the models tg-ArcSwe and tg-Swe were compared. By inhibiting APP processing genetically or pharmacologically, the intraneuronal granular immunoreactivity with antibodies binding the  $A\beta$  domain was shown to largely represent  $A\beta$ , and not APP or APP-fragments. At two months of age, the intracellularly accumulated  $A\beta$  decreased rapidly, likely because it was still accessible to intracellular clearance. Extracellular  $A\beta$  deposits emerged at 5-6 months of age and the amyloid fibril structure was more compact than in tg-Swe. Moreover,  $A\beta$  deposits in tg-ArcSwe were more resistant to chemical extraction than those of established models carrying the Swedish APP mutation only, e.g. tg-Swe mice. The stability of deposits better reflects the biochemistry of senile plaques in AD. Thus, the tg-ArcSwe model may better predict the outcome of clinical trials, particularly therapies designed to enhance clearance of  $A\beta$  aggregates and deposits. Postmortem brain of Arctic mutation carriers contained extensive parenchymal plaque pathology. Differential immunostaining patterns with C- and N-terminal  $A\beta$  antibodies revealed a subset of plaques that were unique to the brains of Arctic mutation carriers.  $A\beta$  deposits in the cerebral vessel walls were congophilic and mainly composed of full-length  $A\beta$ . In contrast, N-terminally truncated  $A\beta$  was more prominent in the parenchymal plaques, all of which essentially lacked amyloid cores. A heterogeneous assembly of mutant and wild-type  $A\beta$  was shown to favor the formation of diffuse deposits in bitransgenic mice, and such mechanisms may at least partly explain observations of plaques lacking amyloid cores in postmortem Arctic mutant brain. In the bitransgenic mice, a low level of Arctic  $A\beta$  was sufficient to facilitate aggregation of wild-type  $A\beta$ . This observation, but also our findings of differences in amyloid fibril structure in tg-ArcSwe and tg-Swe, further highlights similarities between AD and prion disorders in which PrP<sup>sc</sup> refolds PrP<sup>c</sup> and facilitates fibril formation.

*Keywords:* Alzheimer's disease, Amyloid, Amyloid- $\beta$ , intraneuronal, transgenic mice, immunohistochemistry, ELISA

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# List of Papers

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

- I **Philipson O**, Lannfelt L, Nilsson LNG. (2009). Genetic and pharmacological evidence of intraneuronal A $\beta$  accumulation in APP transgenic mice. *FEBS Letters*, 583:3021-3026
- II **Philipson O**, Hammarström P, Nilsson KPR, Portelius E, Olofsson T, Ingelsson M, Hyman BT, Blennow K, Lannfelt L, Kalimo H, Nilsson LNG. (2009). A highly insoluble state of A $\beta$  similar to that of Alzheimer's disease brain is found in Arctic APP transgenic mice. *Neurobiology of Aging*, 30:1393-1405
- III **Philipson O**, Lord A, Lalowski M, Soliymani R, Thyberg J, Bogdanovic N, Tjernberg LO, Ingelsson M, Lannfelt L, Kalimo H and Nilsson LNG. Biochemical and morphological analyses of A $\beta$  deposits in postmortem brain of Arctic APP mutation carriers. *Manuscript*
- IV Lord A\*, **Philipson O\***, Klingstedt T, Nilsson KPR, Hammarström P, Lannfelt L, Nilsson LNG. Arctic A $\beta$  selectively increases diffuse deposition of wild-type A $\beta$  in APP transgenic mice with the Swedish mutation. *Manuscript*

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# Abbreviations

APP	Amyloid- $\beta$ precursor protein
A $\beta$	Amyloid- $\beta$
AD	Alzheimer's disease
AICD	APP intracellular domain
Apo E	Apolipoprotein E
$\alpha$ APPs	Soluble $\alpha$ -secretase cleaved APP fragments
$\beta$ APPs	Soluble $\beta$ -secretase cleaved APP fragments
BACE	$\beta$ -site APP cleaving enzyme
CAA	Cerebral amyloid angiopathy
CWP	Cotton wool plaque
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FA	Formic acid
FAD	Familial Alzheimer's disease
GSI	$\gamma$ -secretase inhibitors
H&E	Hematoxylin & Eosin
IHC	Immunohistochemistry
MCI	Mild cognitive impairment
MS	Mass spectrometry
MVB	Multivesicular bodies
mAb	Monoclonal antibody
MMSE	Mini-Mental State Examination
NFT	Neurofibrillary tangle
NP	Neuritic plaque
PBS	Phosphate buffered saline
PHF	Paired helical filament
PrP	Prion protein
PS	Presenilin
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
TBS	Tris buffered saline
tg	Transgenic
wt	Wild-type

# Introduction

## Protein folding, misfolding and amyloid formation

Amino acid residues linked together by peptide bonds make up the protein sequence. There are 20 naturally occurring amino acids, and the unique combination of these is referred to as the primary structure of the protein. The sequence of amino acids and the environmental conditions direct protein folding. There are different secondary structural elements,  $\alpha$ -helices and  $\beta$ -strands and loop regions. Structural rearrangements and complex assembly generates the three-dimensional conformation, the functional form of the protein.

Protein folding depends on energy and it has been suggested that a protein folds correctly in the search for the lowest possible energy state [1]. It is thought that this is achieved by using a local energy minima or funnel along the route from the random coil to the fully folded protein [2]. Chaperones, a diverse group of proteins assist in and prevent erroneous folding by keeping newly synthesised proteins in a favourable conformation. This process prevents unwanted protein aggregation.

If the protein fails to fold correctly, there are cellular mechanisms detecting and removing unfolded or misfolded proteins, i.e. the ubiquitin-proteasome system [3]. Alternatively, aggregates can undergo microtubule-mediated transport to a cytoplasmic site near the centrosome, where the misfolded proteins form an aggresome. The cell can also sequester and dispose parts of the cytoplasm in a membrane structure, the autophagosome, in a process called macroautophagy [4, 5].

If these mechanisms fail, proteins may undergo further conformational changes and self-associate into assemblies of misfolded proteins. Proteins can then form stable aggregates with a  $\beta$ -sheet conformation, a structure called amyloid. The  $\beta$ -sheet with the constituent  $\beta$ -strands is arranged parallel or anti-parallel running perpendicular to the axis of the fibril. These insoluble structures are generally highly protease-resistant and there are limited cellular pathways for their degradation. Amyloid fibrils are 7-12 nm in diameter [6] and normally composed of 2-6 fibrillar subunits arranged in parallel, the protofilaments. A number of factors determine the propensity of a protein for amyloid fibril formation e.g. molecular charge, hydrophobicity, conformational stability, solubility of the folded conformation and secondary structure propensity [7]. Amyloid is a generic term, which is defined by certain criteria. It is formed *in vivo* (*in vitro* derivatives should be referred to as

amyloid-like fibrils), X-ray diffraction reveals a cross beta sheet structure and Congo red stained fibrils display birefringence under polarized light [8]. The amyloid fibril is typically unbranched when viewed in electron microscope. In humans, there are almost 30 known amyloid forming peptides, and yet others form amyloid-like fibrils under suitable conditions [9, 10]. A large number of diseases, described as protein misfolding disorders are associated with amyloidosis.

Misfolded aggregated proteins deposited in tissues is a common feature of several neurodegenerative disorders, including amyloid- $\beta$  ( $A\beta$ ) in Alzheimer's disease (AD), superoxide dismutase-1 in familial amyotrophic lateral sclerosis and the prion protein ( $PrP^{Sc}$ ) in Creutzfeldt-Jakob's disease.

## Alzheimer's disease

### Clinical symptoms, diagnosis and current treatments

AD, the most common age-related neurodegenerative disorder, is believed to affect as many as 20-30 million people worldwide [11]. With increasing life expectancy, the worldwide prevalence is expected to triple within the next 50 years [12]. The clinical symptoms and neuropathology characterizing the disease was first described by the German physician, Alois Alzheimer, at a scientific meeting in Tübingen 1906. The patient, a woman who died in her fifties, suffered from severe memory loss, disorientation and hallucinations.

AD has an insidious onset and a slow but irreversible progress where the end stage is often characterized by a complete loss of independence. Death is usually caused by secondary infection and typically occurs 5-15 years after onset of disease symptoms [11]. Early symptoms of AD are impaired acquisition and retention of memories, visuospatial deficits, difficulty handling complex tasks and a loss of language skills. Increased memory loss in combination with personality changes often contributes to a descending performance in social life, sometimes accompanied by depression.

For the clinical diagnosis of AD, one needs to take into account the medical history and to exclude alternative causes of cognitive decline and dementia. The DSM-IV [13] and ICD-10 criteria [14], proclaim a trustworthy AD diagnosis of patients presenting with persistent (>6 months) and progressive memory loss and other cognitive deficits resulting in impaired social and functional activities of daily life. A common test to evaluate cognitive status is the Mini-Mental State Examination (MMSE). In addition, functional and structural brain imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), position emission tomography (PET) [15] and single photon emission computed tomography are valuable in the differential diagnosis of AD e.g. to distinguish AD from dementia caused by stroke, tumor and subdural hematoma.

Laboratory tests are also valuable in the differential diagnosis. A high level of total tau and/or phosphorylated tau (pTau) and a low level of the 42 amino acid long A $\beta$  (A $\beta$ 42) in the cerebrospinal fluid (CSF) is suggestive of AD [16, 17]. However, a neuropathological examination has to be carried out for a definite diagnosis of AD. The validity of the clinical assessments of patients having probable or possible AD can reach 85-90% when the brain is neuropathologically examined [18]. Above 65 years of age, the prevalence and incidence of dementia increases twofold every succeeding five years, with an estimated prevalence of ~20% of AD among the population over the age of 85 [19].

Today only symptomatic treatment, which helps some patients to temporarily maintain their cognitive abilities, is available. However it does not affect the underlying disease process, synaptic loss and neuronal atrophy leading to neurotransmitter loss and cognitive deficits. The cholinergic neurons projecting from the basal forebrain to the hippocampus and the cerebral cortex degenerate early in the disease process. These pathways are important for memory functions; consequently cholinergic substitution therapy for AD, which can alleviate cognitive symptoms of mild-to-moderate AD, was developed. These drugs (e.g. Aricept<sup>®</sup>) inhibit acetylcholinesterase, which degrades acetylcholine at the synaptic cleft [20]. The release and uptake of the neurotransmitter glutamate is also deemed to be dysfunctional in AD. Memantine, an uncompetitive N-methyl D-aspartate (NMDA) receptor antagonist, is thought to prevent glutamate-induced neurotoxicity by inhibiting overactive NMDA receptors and thereby reducing excessive calcium influx. It is approved for treatment of moderate-to-severe AD [21].

Since current treatments do not target the underlying neurodegenerative processes it is of great importance to develop therapeutic approaches that target molecular pathways involved in AD pathogenesis [22]. There is also a need for better differential diagnosis in the early stages of the disease and objective disease monitoring with biomarkers. It would lead to more valuable clinical trials and in the future, it could help to enable therapeutic prevention.

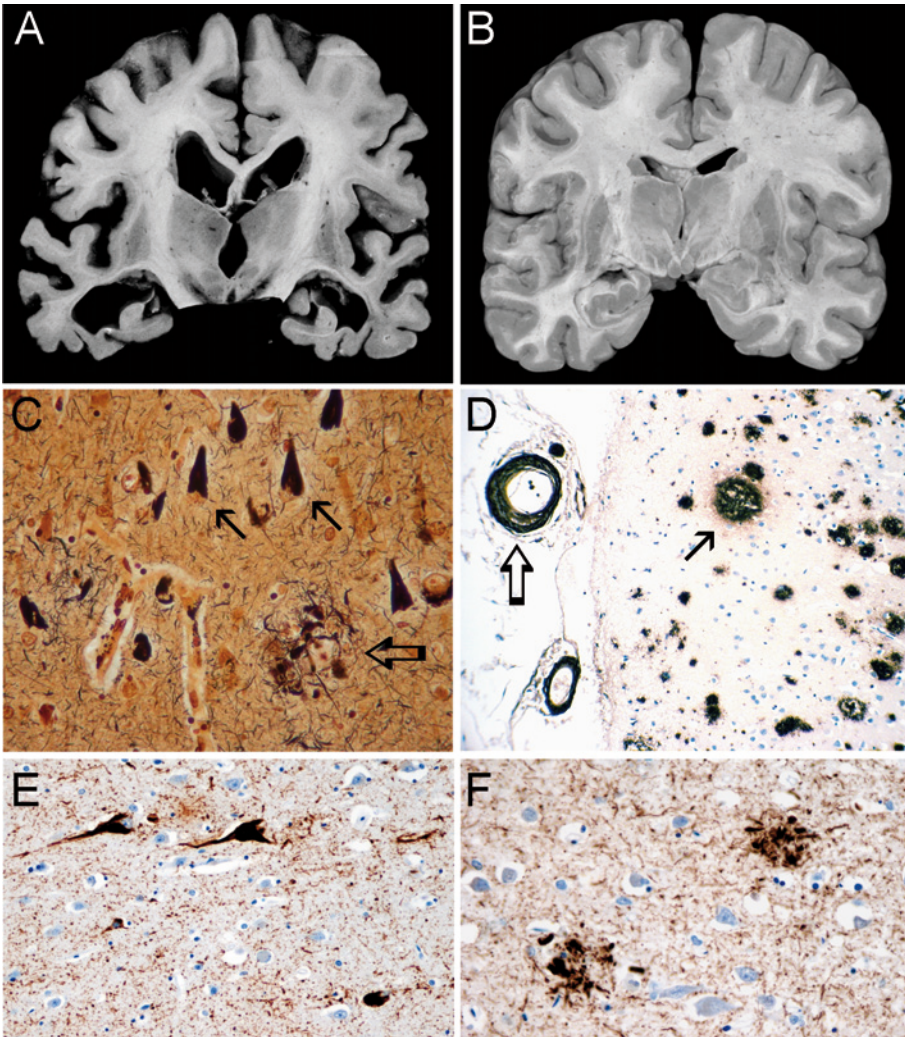
## Neuropathology

The brain of an AD patient typically shows gross atrophy of the hippocampus, the parietal and temporal lobes of the cerebral cortex and enlarged ventricles [23] as illustrated in *Figure 1A-B*. AD is also characterized by reduction in synaptic density and loss of neurons e.g. in the hippocampal formation [24]. The neuritic plaque (NP) of A $\beta$  and the neurofibrillary tangle (NFT) are the two neuropathological hallmarks of AD. In the 1960s, with the advent of electron microscopy, Michael Kidd and Robert Terry described the ultrastructure of filaments in NFT in AD brain

[25], and later the microtubule protein tau was shown to be the main constituent [26]. In the mid 1980s, amyloid- $\beta$  ( $A\beta$ ) was purified and partially sequenced from angiopathic meningeal vessels of AD brains and identified as the main component of the vascular [27] and soon thereafter, parenchymal amyloid [28] *Figure 1C-F*.

NFTs mainly consist of paired helical filaments with hyperphosphorylated forms of the microtubule-associated protein tau, as their main constituent. Tau protein regulates the assembly and stability of microtubules, an essential part of the cytoskeleton, which is vital to vesicle transport. In AD brain, the tau protein in intracellular tangles is abnormally phosphorylated and dissociated from the microtubules. An increased soluble pool of tau undergoing conformational changes is likely an important early step in the assembly of tau filaments [29].

A definite postmortem diagnosis of AD is dependent on the presence of NP and NFT, their frequency and location in the brain and the age of the patient. From the early guidelines regarding the hallmarks of AD pathology [30], more refined criteria for scoring NP [31] and staging NFT/neuropil thread pathology [32] were established and became widely accepted. Later, these criteria were combined in NIA-RI recommendation [33], which took into account the distribution and extent of both hallmarks (NP and NFT/neuropil threads). Based on the presence of the two pathological hallmarks, the likelihood of dementia being a result of AD is staged as low, intermediate or high. Instead of chemical staining procedures (Thioflavin S and Silver stains), current recommendations emphasize the need for molecular classification with immunohistochemistry and assessment of regional distribution of the pathologies. The AD related  $A\beta$  pathology phases 1-5 relate to the spread of  $A\beta$ -pathology throughout the brain [34] with deposits first being found in the neocortex and only in the latest phase in the cerebellum. In general, the severity of hyperphosphorylated tau pathology (Braak stages I to VI) is based on density and regional distribution of AT8-positive neurofibrillary threads in hippocampus (Braak I-III), temporal cortex (Braak IV) and occipital cortex (Braak V-VI) [35, 36].



*Figure 1.* Neuropathological changes in a sporadic AD brain. Macroscopic changes of AD brain (A) as compared to a normal brain (B). The AD brain is severely degenerated with enlarged ventricles, widened sulci and an atrophied hippocampus. The two neuropathological hallmarks of AD, neuritic plaques (NPs) (open arrow) and neurofibrillary tangles (NFTs, closed arrow) are visualized by a silver staining technique in the entorhinal cortex (C). Parenchymal, extracellular deposits (closed arrows) and cerebral amyloid angiopathy (open arrow) are stained for amyloid- $\beta$  (D). Hyperphosphorylated tau in neurofibrillary threads and NFT are stained with the AT8-antibody (E) and dystrophic neurites clustered within and around amyloid plaques (F). Images are kindly provided by Professor Hannu Kalimo.

## Genetics and risk factors

### *Early-onset Alzheimer's disease*

In some families, AD has been described with an autosomal dominant pattern of inheritance, often with an early age of onset (<65 years) [37]. Despite the difference in age of onset, the clinical picture and the neuropathology is generally similar to sporadic AD. This is important since it implies that studies of FAD can help us understand the molecular mechanisms of sporadic AD. Several missense mutations in APP and the presenilin genes (PS1 and PS2) that are inherited in an autosomal dominant mode have been identified. Almost all of these mutations have a complete penetrance. Most APP mutations are located in or around the A $\beta$  domain and alter proteolytic processing of APP [38]. Missense mutations close to the C-terminus of the A $\beta$  domain increase the production of A $\beta$ 42 or the ratio of A $\beta$ 42/A $\beta$ 40, while a mutation close to the N-terminus, the Swedish (Swe) mutation, increases the levels of both A $\beta$ 42 and A $\beta$ 40 [39]. The Swedish mutation (APP K670N, M671L) is a double mutation at codons 670 and 671 caused by two base pair substitutions [40].

The APP gene is located on chromosome 21 and Down's syndrome patients with trisomy chromosome 21, almost invariably develop dementia. This is most likely due to the gene dosage effect with enhanced A $\beta$  production, since the neuropathology is highly similar to AD. APP gene duplications have also been found in families with a variant of AD [41].

Mutations within the A $\beta$ -domain of APP result in different clinical and pathologic phenotypes. Several pathogenic intra-A $\beta$  mutations have been identified, all positioned near the central hydrophobic cluster in position 21-23 in the A $\beta$  sequence. The Arctic (Arc) mutation (APP E693G) identified in a family from northern Sweden, is located at position 22 in the A $\beta$  sequence, where glutamic acid is substituted for a glycine. It drastically alters the kinetics of A $\beta$  aggregation and promotes A $\beta$  protofibril and fibril formation *in vitro*. Individuals in the family with the Arctic mutation have a disease onset ranging from 52 to 62 years and at postmortem examination, the brains show severe parenchymal A $\beta$  deposition and cerebral angiopathy in the absence of hemorrhages [42].

Despite the importance of tau and the formation of NFTs in AD, mutations in the tau gene have not been associated with AD. Instead such genetic lesions causes frontotemporal lobar degeneration [43], indicating that altered tau metabolism (and likely tangle formation) is sufficient to generate neurodegeneration and dementia.

### *Late-onset Alzheimer's disease*

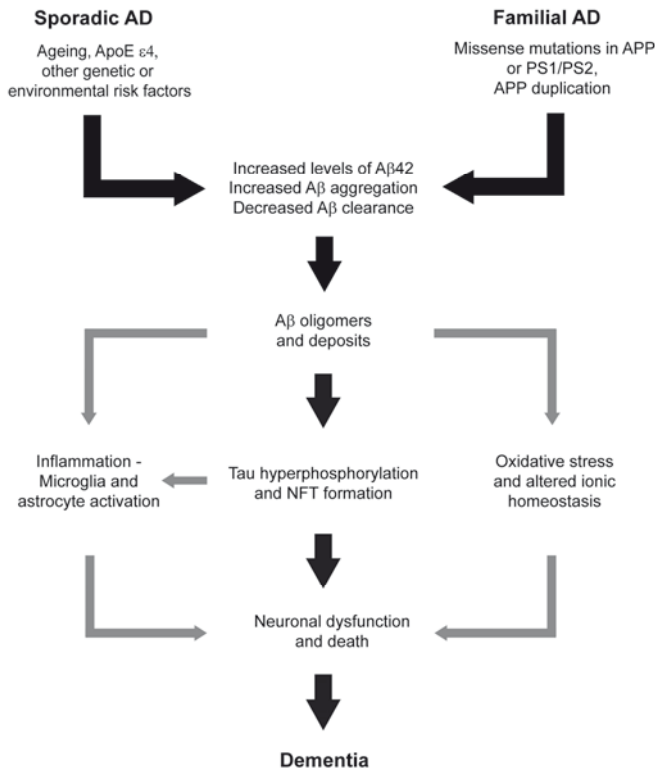
Late-onset AD (representing more than 95% of total AD cases) affects individuals above 65 years of age and is commonly perceived as sporadic in its origin. Some of the late onset cases have a family history of AD [44], and based on twin studies an almost 80% heritability of AD has been estimated [45]. Age is the main risk factor for AD. Females are overrepresented among AD patients, more than would be anticipated by increased life expectancy among women [46]. The only well established genetic risk factor for developing late onset AD is apolipoprotein E (ApoE). Three variants of ApoE exist ( $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ ). The  $\epsilon 4$  allele is associated with increased risk for developing AD [47] and the  $\epsilon 2$  allele confers some protection. The ApoE gene is located on chromosome 19q and generates a 35 kDa plasma protein with important functions in cholesterol transport, metabolism and storage [48]. Some studies show that ApoE affects the process of A $\beta$  fibrillization [49, 50]. Other non-A $\beta$  components, e.g. heparan sulfate (a glycosaminoglycan) has been found codeposited in plaques of AD [51] and likely play a role in the A $\beta$  fibrillogenesis, as shown *in vitro* [52]. Recently three new risk factor genes, clusterin (Apo J), PICALM and complement receptor 1 (CR1) [53, 54] were identified. Their impact on disease susceptibility is much less than that of the ApoE  $\epsilon 4$  allele.

### The amyloid cascade hypothesis

The *amyloid cascade hypothesis* states that the accumulation of A $\beta$  is the primary pathogenic process which instigates all other abnormal processes in AD brain. Formation of NFT, cell loss, inflammation and neurotransmission deficiencies are considered downstream events contributing to neuronal dysfunction and dementia [20, 55]. However, the severity of dementia equates better to the number and location of NFTs than to the extent of senile plaque deposition [56], a finding that is inconsistent with the initial *amyloid cascade hypothesis* [57]. The relationship between NP and the NFT remains poorly understood. Transgenic mice expressing mutant APP and tau suffer from more severe tau pathology than mice expressing only mutant tau. In contrast, the amount of deposited A $\beta$  is comparable in transgenic mice expressing both mutant APP and tau to those expressing only mutant APP [58]. This reinforces the view that NFT formation is a downstream event in AD pathogenesis and that it is facilitated by A $\beta$  aggregation. Furthermore, removal of A $\beta$  with immunotherapy clears early tau pathology, and when ceased, A $\beta$  pathology reemerges before tau pathology in transgenic mice [59].

Soluble A $\beta$  assemblies, more recently discovered in AD brain, [60] correlate better with AD symptoms than A $\beta$  plaque load [61], suggesting that these and not fibrillar amyloid deposits are chiefly responsible for neurodegeneration [62, 63]. Soluble A $\beta$  species have been shown to disrupt

learning, memory and synaptic functions in mice and rats [64, 65]. Moreover, studies in APP transgenic mice suggest that dysfunctions due to A $\beta$  do not relate to the frequency of amyloid plaques [66].



*Figure 2.* AD pathogenesis according to the amyloid cascade hypothesis. Altered metabolism (production and clearance) of A $\beta$ , in particular aggregation-prone A $\beta$ 42, initiates the pathological process. In general, in familial AD, A $\beta$  production or aggregation is increased. A $\beta$  aggregates are thought to trigger tau accumulation and NFT formation, oxidative stress and inflammation, but may also directly inhibit long-term potentiation and synaptic function. These processes initially impair neuronal and synaptic function, and later lead to cell loss and dementia. The main pathogenic pathways of AD are illustrated with black arrows, whereas pathways thought to have less impact are shown with thinner grey arrows. Figure reproduced from [67].

## Processing of the Amyloid- $\beta$ Precursor Protein

A $\beta$  peptides are produced during normal cell metabolism [68]. A $\beta$  is proteolytically cleaved from APP, a type I transmembrane protein expressed in many tissues. There are three major isoforms of APP with a similar A $\beta$  domain; the 695 (predominantly expressed in neurons), 751 and 770 amino

acid isoforms (expressed mainly in non-neuronal cells) [69]. The complete function of APP is not fully understood, but several possible functions have been ascribed to APP holoproteins and/or their major secreted derivatives (APPs). APP knockout mice demonstrate reduced long term potentiation (LTP), loss of synaptic markers and modest cognitive impairment, indicating a central role for APP in normal neuronal development/survival and synaptic function [70]. Based on cell culture studies, soluble APPs appear to act as autocrine factors and APP as a receptor to stimulate neuritic outgrowth [71].

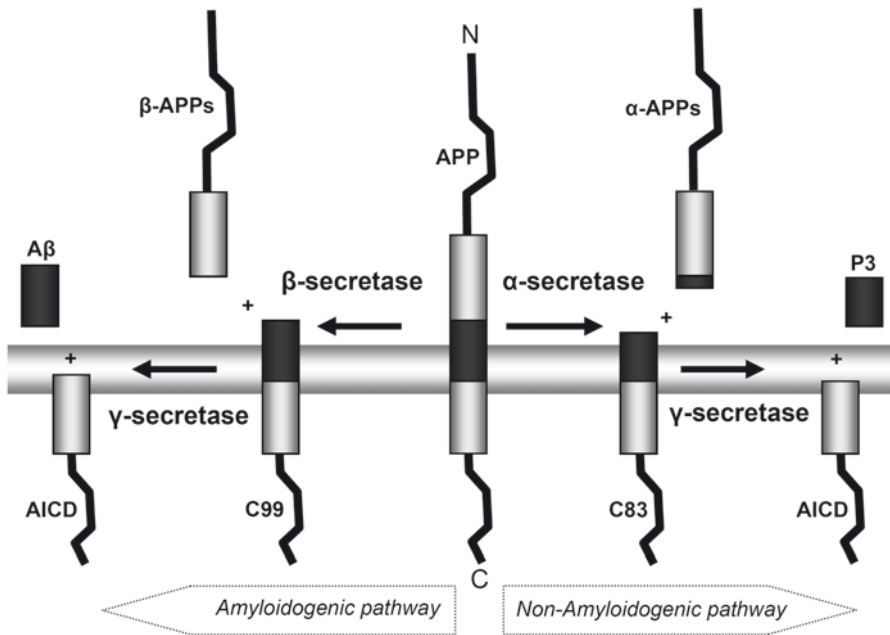
APP has a short half-life and is metabolized rapidly by proteases through two pathways (*Figure 3*). These are called the amyloidogenic pathway, which generates A $\beta$  peptides, and the non-amyloidogenic pathway [72]. The protein can undergo proteolytic cleavage by  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretase activity. In the non-amyloidogenic pathway,  $\alpha$ -secretase enzymes cleave within the A $\beta$ -domain between amino acids 16 and 17 favouring the production of a long, soluble N-terminal protein,  $\alpha$ APP and a shorter C-terminal fragment C83. In the amyloidogenic pathway a membrane-anchored aspartyl protease called  $\beta$ -site APP-cleaving enzyme (BACE-1) cleaves at amino acids 1 and 11 ( $\beta$  and  $\beta'$ ), N-terminal to the  $\alpha$ -cleavage site, and generates smaller ectodomain derivatives,  $\beta$ -APPs and C99 [73].  $\gamma$ -secretase is an intramembrane multi-subunit aspartyl protease consisting of at least four different proteins, of which presenilin-1 or -2 (PS1 and PS2) constitute the active cleavage site, while nicastrin, Aph-1 and Pen-2 are needed for proteolytic activity and specificity [74]. Further cleavage of C83 by  $\gamma$ -secretase results in the non-amyloidogenic peptide-fragments, whereas cleavage of C99 by  $\gamma$ -secretase generates various A $\beta$ -isoforms. In contrast to A $\beta$ , at least one P3-fragment (A $\beta$ 17-40) is a nontoxic peptide that aggregates, but does not form amyloid fibrils. Its physiological function is currently unknown [75]. The A $\beta$  domain of APP is evolutionarily highly conserved and A $\beta$  peptides are produced during normal metabolism and could have physiological functions [68]. Most A $\beta$  (80-90%) found in plaques in sporadic AD brain are peptides extending to position 42 in the amino acid sequence (A $\beta$ x-42) [76, 77], whereas the ratio A $\beta$ x-42/A $\beta$ x-40 is lower in cerebral amyloid angiopathy (CAA) [78]. A $\beta$  in human brain is largely posttranslationally modified with N-terminal truncation, isomerization, racemization, pyroglutamyl formation and oxidation [79].

### *Inhibition of APP processing*

To use BACE-1 and  $\gamma$ -secretase as drug targets for inhibition of APP processing is an intricate task. BACE-1 has a large substrate-binding site, making small nonpeptidic molecules ineffective in selectively blocking the cleavage site. Larger inhibitors inefficiently cross the blood-brain barrier, the plasma and endosomal membranes, thus it is difficult to synthesize compounds that reach the sites within neurons where BACE-1 is active at a sufficiently high concentration. Moreover, since BACE-1 activity is essential

for axon myelination during development [80] a selective inhibitor could cause serious adverse side-effects [81].

Many orally bioavailable  $\gamma$ -secretase inhibitors (GSIs) are toxic since they also cleave other transmembrane proteins e.g. Notch (1-4), E-cadherin and the ErbB4 receptor [for review see [81]]. Notch, which is cleaved by  $\gamma$ -secretase, regulates tissue development. Inhibition of Notch signaling has been shown to cause developmental defects of the skeleton and the brain that resemble phenotypes seen in PS-1 knockout mice [82, 83]. Furthermore, gastrointestinal, skin and immune system abnormalities have been reported following chronic treatment with non-selective GSIs [84, 85]. To overcome some of the adverse toxic effects of GSIs, the pharmaceutical industry has developed “notch-sparing” inhibitors that lowers A $\beta$  levels with reduced Notch-related side effects [86]. Some GSIs have been reported to act on the ATP site of the  $\gamma$ -secretase complex [87], others on the substrate docking site. Recently the discovery of  $\gamma$ -secretase complex heterogeneity due to different Aph1 isoforms has generated new hope that  $\gamma$ -secretase inhibitors that selectively target A $\beta$  production can be developed [88]. Other substances developed with the intent to reduce target-based toxicity are  $\gamma$ -secretase modulators. They change, but do not inhibit, APP processing by generating more A $\beta$ <sub>x-38</sub> and less A $\beta$ <sub>x-42</sub> [89] with minimal impact on other substrates for  $\gamma$ -secretase. Based on photo-crosslinking experiments it has been suggested that  $\gamma$ -secretase modulators alter enzymatic activity by binding to the N-terminal transmembrane domain of APP [90].



*Figure 3.* Processing of the amyloid- $\beta$  precursor protein (APP). The amyloidogenic pathway, with  $\beta$ - and  $\gamma$ -secretase cleavage, generates potentially neurotoxic A $\beta$  peptides, whereas the non-amyloidogenic pathway, with  $\alpha$ - and  $\gamma$ -secretase cleavage, generates the non-toxic P3 peptide fragments.

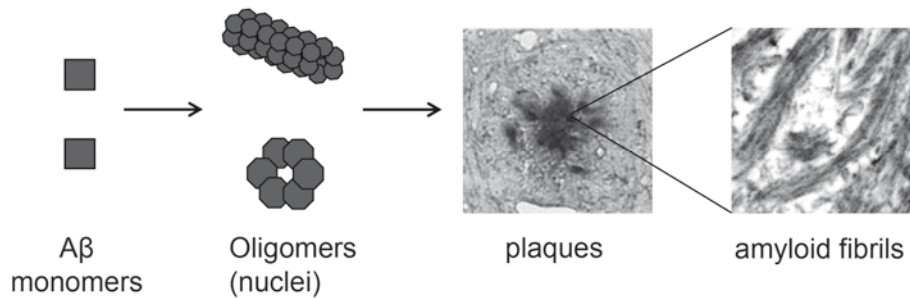
### Mechanisms of A $\beta$ aggregation and amyloid fibril formation

A $\beta$  is continuously degraded and cleared from the brain and periphery and the half-life is partly determined by the properties of different A $\beta$  species. The primary mechanism of A $\beta$  degradation is thought to be mediated by extracellular peptidases. Neprilysin (NEP) [91] and insulin-degrading enzyme (IDE) are two among several enzymes able to degrade A $\beta$ . If A $\beta$  is poorly cleared or produced in high amounts (especially A $\beta$ <sub>x-42</sub> in FAD) it tends to aggregate and form insoluble fibrillar deposits in brain.

The aggregation of A $\beta$  depends on the chemical and structural properties conferred by the amino acid sequence. Two hydrophobic regions in A $\beta$  are critical for structure and aggregation: the C-terminus (residues 29-40/42), which in the precursor protein is contained within the lipid bilayer, and the central region (KLVFF, 17-21) [92]. *In vitro*, a change in  $\alpha$ -helix content occurs in oligomers and small protofibrils prior to the appearance of larger protofibrils and fibrils in which  $\beta$ -sheet dominates. The propensity for this structural shift is largely governed by the two hydrophobic regions [93, 94]. Wild-type A $\beta$ <sub>1-42</sub> displays faster aggregation and transition from random coil via  $\alpha$ -helix to  $\beta$ -sheet than A $\beta$ <sub>1-40</sub> [94]. The mechanisms of A $\beta$  fibril formation are not fully understood. Contemporary models suggest that

fibrillization is a nucleation-dependent process with a lag phase and an elongation phase. During lag phase, nuclei (“seeds”) are formed through slow unfavorable reactions. In a nucleation dependent reaction, the seed is not formed until the monomer concentration exceeds a certain level known as the critical concentration, below which the soluble monomer is the predominant species [94].

Several different soluble A $\beta$  species formed *in vitro* have been reported in the literature. Protofibrils [93-95] and various low molecular weight-A $\beta$  species [62] have been well described. Other A $\beta$  intermediates include globulomers [96], A $\beta$ \*56 [97] and A $\beta$  derived diffusible ligands (ADDLs) [98]. These species are referred to as soluble A $\beta$  species and are defined by size or other unique characteristics.



*Figure 4.* Schematic illustration of senile plaque formation. Monomeric A $\beta$  peptides undergo conformational changes, aggregates into oligomers, and are deposited as structural arranged amyloid fibrils, the main constituents of senile plaques.

## Location of A $\beta$ production and intracellular aggregation

APP is located to several cellular compartments and the vast majority of A $\beta$  produced by cultured cells is secreted, suggesting that A $\beta$  is predominantly produced along the secretory pathway [99] e.g. trans-golgi network, in secretory vesicles or at the plasma membrane. The importance of the endocytic pathway in APP processing has been shown in APP expressing cell lines [100, 101] and recently in transgenic mice [102]. A $\beta$  might also be generated at other locations such as the endoplasmic reticulum and mitochondrial membranes. One should be aware that APP processing has mainly been studied in cells of non-neuronal origin (e.g. HEK293 or CHO), and quite seldom in the brain or even in primary neuronal cell cultures.

There is evidence from staining with A $\beta$  antibodies that intracellular A $\beta$  is an early event in the pathogenesis predating plaque formation in AD and Down’s syndrome [103, 104]. In patients with mild cognitive impairment (MCI) intraneuronal A $\beta$  immunoreactivity is seen in regions that are prone to develop extracellular plaques in later stages of AD pathogenesis. Studies in

transgenic mice reinforce this by showing that intraneuronal staining with A $\beta$  antibodies decrease as extracellular plaques accumulate [105-109].

An emerging literature on transgenic models suggests that A $\beta$  might accumulate inside neurons or be reinternalized after secretion [110]. In transgenic mice, the intraneuronal staining is mainly located to multivesicular bodies (MVB) [111, 112]. It has been suggested that this inhibits its sorting via the ubiquitin-proteasome system [113] and directly blocks the proteasome leading to accumulation of tau protein [59] and diminished enzymatic activity in the mitochondrial respiratory pathway [114]. Taken together, intraneuronal A $\beta$  may have a role in the early stages of pathogenesis and possibly it may also link A $\beta$  and tau pathologies.

The *Arctic APP* mutation leads to severe A $\beta$  deposition in the human brain, and clinical symptoms typical of AD, which are not seen with most other APP mutations inside the A $\beta$  domain [42, 115]. In a transfected cell-line with the Arctic mutation, the total secretion of A $\beta$  decreases, but the relative production of A $\beta$ 40 and A $\beta$ 42 to the P3 peptide-fragments increases [115, 116]. By combining the Swedish double mutation with the Arctic mutation in a transgenic mouse, strong intraneuronal A $\beta$  aggregation associated with an early onset of parenchymal amyloid deposition was demonstrated in the tg-ArcSwe model [106]. A $\beta$  immunoreactivity inside neurons in hippocampus and cerebral cortex is stronger in tg-ArcSwe than in tg-Swe mice. Moreover, the frequency and size of intraneuronal granular staining increases in an age-dependent manner until age of onset of extracellular A $\beta$  deposits (Lord et al., 2006).

## Formation of extracellular deposits

Fibrils of A $\beta$  are linear and insoluble, composed of several intertwined filaments of variable length. The precise mechanisms by which amyloid fibrils are formed and their relationship to the overall neurodegenerative processes in AD are not yet clear, but direct involvement and incorporation of protofibrils and low molecular weight (LMW) A $\beta$  to fibril ends has been suggested [95, 117]. Other

Extracellular plaques in AD brain have different morphologies, including non-fibrillar and loose forms of plaques (e.g. diffuse deposits and cotton wool plaques) and neuritic plaques. Consistent with the ideas that aggregation follows a nucleation-dependent process, recent longitudinal *in vivo* multi-photon imaging studies of transgenic mice demonstrated that individual plaque cores form within 1-2 days [118] or over a period of weeks before reaching a mature size [119]. Alternatively, one might hypothesize that diffuse plaques represent an immature form that can later transform into a neuritic plaque with surrounding cytopathology. This hypothesis arose from findings of diffuse deposits in areas not associated with the cognitive deficits

in AD, such as cerebellum and striatum. Diffuse deposits were also found in non-demented individuals, in e.g. association areas of the cerebral cortex. Moreover, diffuse deposits, but not neuritic core plaques, were found in brain of young Down's syndrome patients [120].

Cotton wool plaques (CWPs) are found among some presenilin-1 mutation carriers, but also in sporadic AD. They are mainly composed of A $\beta$  ending at amino acid 42, they lack an amyloid core and there is little or no neuritic dystrophy [121]. Neuritic plaques with amyloid cores are surrounded by dystrophic neurites (from terminal axons), microglia and reactive astrocytes, as opposed to diffuse plaques [122]. Dystrophic neurites are often dilated and ultrastructurally abnormal with enlarged lysosomes, increased numbers of mitochondria and paired helical filaments (the latter generally indistinguishable from those that comprise the neurofibrillary tangles). Usually, the microglia is found adjacent to and within the neuritic plaque core, whereas astrocytes often surround the plaque, having processes extending toward the amyloid core.

The formation of a neuritic plaque is proposed to be caused by amyloid that attracts microglia, which releases molecules toxic to nearby dendrites and axons, causing them to enlarge and degenerate [122]. It has also been suggested that activated microglia clear amyloid deposits [123], are nidi in the initiation of amyloid fibril formation [124], but also restrict plaque size [125]. There are several recent reports with essentially contradicting results regarding the impact of activated microglia surrounding A $\beta$  deposits. In a longitudinal *in vivo* study on transgenic mice, the interaction between newly formed plaque and microglia was observed. It demonstrated that cored plaques form at sites not previously occupied by microglia cells and that the microglial response is unable to clear the plaques, but possibly restrict their size [118]. However, in a related study, using a less invasive transcranial window, microglia and astrocytic activation was essentially absent [119]. Additionally, a recent study reported an unchanged progression of plaque development and formation of surrounding dystrophic neurites following an almost complete microglial ablation from the brain [126].

In addition to being deposited in plaques, A $\beta$  is pathologically located in the vasculature resulting in CAA. It is neither exclusive for AD pathogenesis [127] nor a criterion for the neuropathological diagnosis. Neuron-derived A $\beta$  is thought to be secreted and transported in the interstitial fluid (ISF) along basement membranes of capillaries and arteries. The malfunctional clearance of A $\beta$  along perivascular drainage pathways and subsequent deposition in the cerebrovasculature can lead to rupture of cerebral arteries, causing hemorrhage and stroke [128, 129]. Prominent amyloid angiopathy with A $\beta$  deposition in the vasculature is associated with certain mutations in APP [130, 131]. Studies in transgenic mice suggest that the ratio of two A $\beta$  isoforms, A $\beta$ 42/A $\beta$ 40 determine if A $\beta$  deposits will mainly form in the vasculature or in the parenchyma [132, 133].

## Experimental models of Alzheimer's disease

For many reasons the possibilities of studying pathogenic processes in AD brain are rather limited. Disease models are therefore essential to research and to the evaluation of drugs e.g. by using genetic, pharmacological and invasive approaches. Using any experimental model there is a risk that results may be irrelevant to the situation in the AD brain. If so, they will not contribute to increased knowledge of the pathogenic processes. Cell culture models or other *in vitro* models are often simplistic, but potentially allow detailed knowledge about biological pathways in pathogenesis to be gained. Experiments in such models are also rapid and cost-efficient. In animal models, one can study pathogenic processes directly in a complex biological system, for example the brain.

Non-transgenic animal models are to some extent used in the field of AD research. Current symptomatic drugs for AD were selected based on their ability to improve cognition in animals with cholinergic dysfunctions similar to AD, e.g. animals with excitotoxic lesions in the basal forebrain or with scopolamine-induced amnesia. However the character of these model is acute and their etiological relevance is low [134]. Furthermore, none of the non-transgenic rodent models show any of the neuropathological characteristics of AD.

Infusion or injection of A $\beta$  has been used as an acute model to investigate the synaptotoxic and cognitive effects of various A $\beta$  species. Several studies have received significant attention [64, 65, 135], but the acute nature of these models requires attention to methodological detail, which makes them difficult to standardize. Chronic intracerebroventricular infusion of lipopolysaccharide in rats has been used to induce neuroinflammation, another characteristic of AD. It leads to spatial memory deficits, hippocampal neurodegeneration, macrophage recruitment and increased glial activation [136]. Senescence-accelerated mice, e.g. SAM-P8 mice [137] are one of few nontransgenic options to study AD-like abnormalities in rodents. Age-related increases in A $\beta$  levels and neuropathology with diffuse A $\beta$ -immunoreactive deposits have been reported in SAM-P8 mice. An unmistakably difference to humans, is the rodent amino acid sequence that differs at position 5, 10 and 13.

Primate research is restricted due to ethical concerns, the availability of animals, high maintenance costs and long life expectancy. Primates are nonetheless appealing as AD models due to their close evolutionary relatedness to humans. Early studies in which various antibodies against A $\beta$  in amyloid, but not paired helical filaments, stained deposits in AD and primate brain reinforced this notion [138]. Comprehensive AD neuropathology, including ultrastructural evidence of NFT, was recently demonstrated in an aged chimpanzee [139]. The increased interest in nonhuman primate models is partly due to the unexpected adverse effects of

AN1792, an active vaccination trial with A $\beta$  fibrils. Available transgenic models predicted efficacy, clearance of A $\beta$  deposits, but failed to predict meningoencephalitis as a potential side effect in patients.

## Transgenic models

APP transgenic mice are complex and allow studies to be pursued in the brain and most importantly enable efficacy and safety studies of drug candidates. Transgenic mice models of AD have been developed to study the relationship between the production and deposition of A $\beta$ , onset of dementia and synaptic dysfunction and neuronal cell death and to evaluate modulator genes. An ideal animal model should demonstrate the complete neuropathology of AD and the main clinical phenotypes e.g. progressive cognitive dysfunctions. Therapeutic studies with such models should permit accurate predictions regarding the outcome of clinical trials.

The generation of transgenic mouse models with mutations in the APP gene has enabled major advances in pathogenic understanding. The models show several features of AD, including amyloid plaques with dystrophic neurites and synaptic dysfunction, but most of them fail to develop extensive neuronal death or atrophy. Minor neuronal loss is difficult to detect with stereology, but with *in vivo* two-photon microscopy the fate of single neurons can be tracked [140].

The pathogenic mechanisms of mutant APP, PS-1 or PS-2 genes have been further explained through transgenic experiments. Numerous groups have created transgenic mice models with various features of AD using both cDNA- and genomic based approaches. Some commonly used mice models in AD research are listed in Table 1.

The PDAPP mouse was the first APP transgenic model, which convincingly showed A $\beta$  plaque pathology [141]. Tg2576 [142] and APP23 [143] are other well-characterized and frequently used APP transgenic models to study A $\beta$ -amyloidosis. They all mimic a subset of AD characteristics including diffuse and congophilic parenchymal A $\beta$  deposits, CAA and to some extent synaptic dysfunction. However, there are significant differences in the character and location of the deposits that reflect the specific APP mutation expressed in each line and alterations in A $\beta$ 42:A $\beta$ 40 ratio, and the choice of promoter. In Tg2576, the Swedish double mutation increases the production of both A $\beta$ 40 and A $\beta$ 42, and both dense cored plaques and to some extent diffuse deposits are present [142]. In contrast, the APPV717F mutation in PDAPP mice selectively increases A $\beta$ 42, and this is reflected by a greater proportion of A $\beta$ 42 in plaques [141]. In PDAPP, the pathology is more clearly initiated in the hippocampus. The extent of CAA also differs between models [144] with an abundance of CAA in APP23 and Tg2576, while these lesions are scarce in the brains of PDAPP mice. Moreover, steady state levels of plasma A $\beta$  vary profoundly

between animal models, even with the same mutation and similar age-of-onset e.g. APP23 and Tg2576.

As a result, it is clear that the promoter (i.e. expression level), the peptide composition e.g. A $\beta$ 42:A $\beta$ 40 ratios and the presence of mutant A $\beta$  peptides contribute significantly to steady state levels of A $\beta$  and to the overall pattern of amyloid deposition in the models.

Table 1. Neuropathologic features of some important transgenic models in Alzheimer's disease research

Name	CNS specific expression	Age of plaque onset (mo)	Intraneuronal A $\beta$ accumulation	Neuritic plaques	CAA	Diffuse plaques	Neuronal loss	References
PDAPP	+++	6-8	nr	+	+	+++	-	[141]
Tg2576	+	9-11	+	+++	+	+	-	[142]
APP23	+++	6	nr	+++	+	+	+	[143]
PSAPP	+	6	nr	+++	+	+++	-	[145]
Tg-CRND8	+	3	nr	+++	+	+	-	[146]
APP/PS1KI	+++	2-3	+++	+++	nr	+	+	[112]
APP-London	+++	>12	nr	+++	+	+++	-	[147]
APP-Dutch	+++	22-25	+	-	+++	+	-	[148]
APP <sub>arc</sub>	+++	>12	-	+	+	+	-	[149]
Tg-ArcSwe	+++	6	+++	+++	+	+	-	[106]
Bri-wtA $\beta$ 42A	+	3	nr	+++	+	+++	-	[150]
3xTg-AD	+++	6	+++	+++	+	+++	+	[151]
5xFAD	+++	2	+++	+++	nr	+	+	[108]

Phenotype: **nr**, not reported; -, not detected; +, detectable; **+++**, extensive

Tau mutations associated with NFT in frontotemporal lobar degeneration have been used in transgenic model. Mice expressing mutant tau have been crossed with APP transgenic mice resulting in bigenic mice with amyloid plaques, NFT and neuronal loss, but only to a limited extent in brain regions normally affected in AD [58]. In the bigenic mice, the tau pathology was increased whereas A $\beta$  pathology was unchanged, which emphasize tau pathology being a downstream event in AD pathogenesis. In a transgenic

mouse model expressing wild-type human Tau (3R0N isoform), Swedish and London APP mutation and a PS (M146L) mutation, did develop somadendritic accumulation of tau and cytoskeletal changes like in mice carrying only wild-type human Tau [152]. However, none of the models developed NFT, possibly indicating that a high level of human A $\beta$  in a mouse model is not sufficient to generate NFT formation out of wild-type human tau. For an updated review on transgenic models of AD, see reference [153].

# Aim of the study

The overall aim of this thesis was to investigate the role of the Arctic APP mutation in early and late stages of A $\beta$  pathology, and to use the Arctic mutation to gain new insights that could be of general importance to pathogenic processes of A $\beta$  accumulation in Alzheimer's disease.

## Specific aims

- **Paper I:** To determine if the intraneuronal immunostaining in APP transgenic mouse brain with antibodies recognizing the A $\beta$  domain of APP represents A $\beta$  that has accumulated inside neurons.
- **Paper II:** To investigate the effect of the Arctic APP mutation on late stage A $\beta$  pathology in a transgenic mouse model.
- **Paper III:** To examine and compare A $\beta$  related neuropathology in postmortem brain of patients with the Arctic APP mutation to that of other APP or PS mutation carriers and to sporadic AD brain.
- **Paper IV:** To determine if and how a low level of Arctic A $\beta$  can alter the A $\beta$  related neuropathology in a mouse model producing an abundance of wild-type A $\beta$ .

## Experimental methods

The main experimental procedures are described, but are more thoroughly outlined in the articles. Methodological considerations are further discussed for each study.

### *Generation of transgenic mice*

The transgenic construct used in this study contains the murine Thy-1 expression vector and human APP cDNAs. Two human APP cDNA clones were generated and used, one with the Swedish double mutation alone (K670M, N671L), another also including the Arctic mutation (E693G). The inserts were preceded by a modified Kozak sequence (that enhances translation) and inserted in a vector cassette containing the murine Thy-1 promoter. The DNA constructs were linearized with NotI to remove backbone vector sequences. The linearized DNA construct was separated and purified, microinjected into pronuclear oocytes of (C57BL/6-CBA-F1) mice and implanted in pseudopregnant foster mothers at the two-cell stage. Several founder lines were developed expressing APP at different levels compared to endogenous murine APP (Table 2).

Table 2. Main characteristics of the different APP-transgenic lines

mouse	Line	APP expression <sup>1</sup>	Age onset of plaques
tg-Swe	A	7-fold APP <sub>Swe</sub>	12 mth
tg-Swe	D	3-fold APP <sub>Swe</sub>	18-21 mth
tg-ArcSwe	B	3-fold APP <sub>ArcSwe</sub>	5-6 mth
tg-ArcSwe	D	1-fold APP <sub>ArcSwe</sub>	not at 24 mth
tg-ArcSwe x tg-Swe	bitransgenic	1-fold APP <sub>ArcSwe</sub> + 7-fold APP <sub>Swe</sub>	12 mth

<sup>1</sup>APP expression compared to endogenous murine APP

### *Histochemistry*

Standard histological and immunohistochemical protocols were used. To increase the sensitivity of the immunohistochemical detection of extracellular deposits with A $\beta$  antibodies, several antigen retrieval steps were conducted. In general, sections were treated in pre-heated citrate buffer followed by formic acid which was found to be appropriate for detection of intracellular and extracellular A $\beta$  deposits, a finding also later observed by others [154].

### *Enzyme-linked immunosorbent assay (ELISA)*

Several sandwich ELISAs, all based on capture and detection antibodies with epitopes in the A $\beta$  domain have been used to quantify A $\beta$ . In order to minimize the influence of APP or C99 fragments (highly prevalent in certain compartments), the capture antibodies were specific for neoepitopes only accessible after proteolysis of APP ( $\beta$ - or  $\gamma$ -secretase processed).

### *SDS-PAGE and Urea-based SDS-PAGE*

In standard SDS-PAGE, proteins are separated under denaturing conditions according to their mass. SDS binds evenly to the amino acids generating a total charge equivalent to the mass of the protein. An electric field across the gel allows negatively charged proteins to migrate according to their constant charge to mass ratio. The use of standard gel electrophoretic techniques for determining molecular size is mainly restricted to large proteins. Small peptides (<10 kDa) often do not obey the general correlation between electrophoretic mobility and mass [155], especially peptides like A $\beta$  with stretches of hydrophobic amino acid residues and low axial ratios. Even though the conditions in the standard SDS-PAGE system are denaturing, preformed A $\beta$  aggregates can be SDS-stable [156, 157].

When urea, a potent chaotropic agent, is added to the gel system, A $\beta$  aggregates are dissociated and aggregation is inhibited, but the charge to mass ratio is altered and thereby the migration speed. Urea (6-8 M) decreases the affinity of SDS for proteins and consequently only the most hydrophobic amino acids are able to bind SDS [157, 158]. For example, even though A $\beta$ 1-42 and A $\beta$ 1-40 have equal intrinsic net charges and differ by only two non-polar amino acids, A $\beta$ 1-42 migrates faster [159]. The hydrophobicity and consequently the net negative charge have higher impact on the migration than the total number of amino acids. The relative electrophoretic mobilities of different A $\beta$  isoforms can at least theoretically be calculated from the consensus hydrophobicity indices [160, 161]. Details on methodological consideration, e.g. separation of C- and N-terminally truncated A $\beta$  isoforms, is discussed in the section relating to paper III.

### *Mass spectrometric analyses*

Mass spectrometry (MS) is a technique for identifying molecules according to their molecular mass. The principle of MS is the ionization of molecules generating charged molecules or molecule fragments that can be identified and analyzed by their specific mass-to-charge ratios (m/z). There are several techniques for ionization, but matrix-assisted laser desorption/ ionization (MALDI) is mostly used for proteomic analyses [162]. In MALDI, the sample to be analyzed is normally mixed with a light absorbing matrix that is excited by the laser resulting in an ionized sample, which is accelerated by an electric field and detected by the mass analyzer. Time-of-flight (TOF) is

one of the most frequently used analysis parameters and it detects the elapsed flight time of the different ions. MALDI Imaging mass spectrometry (MALDI-IMS) is a novel method for the spatial analysis of biomolecules in tissue or cells. Similar to the way that an image from an optical scanner consists of pixels, each pixel of mass spectrometric image represents one mass spectrum. An advantage of the MALDI-IMS method over MALDI-TOF is spatial resolution of molecules and their subsequent allocation to histoanatomical structures of the whole tissue section [163].

# Present investigations

## Paper I

### Inhibition of APP processing diminishes punctate intracellular immunostaining with A $\beta$ antibodies in transgenic mice

Although intraneuronal immunoreactivity has been demonstrated with A $\beta$ /APP antibodies (having epitopes in the A $\beta$  domain of APP) in brains of MCI, Alzheimer's disease, Down's syndrome and in APP transgenic mice, there is still widespread concern regarding the true identity of the intracellular immunosignals. Is it really A $\beta$  or simply antibodies cross-reacting with APP or an APP-fragment? In the initial description of the tg-ArcSwe mouse model, granular intraneuronal inclusions were demonstrated by immunohistochemistry with different antibodies. The frequency and intensity increased with age and predated senile plaque formation. Similar immunoreactivity, although less frequent and intense, was shown in a mouse model with only the Swedish APP mutation [106].

The aim of this study was to assess if A $\beta$  was the main component of the granular inclusions in the tg-ArcSwe and if so to investigate if they were likely to be intermediates of A $\beta$  fibrillization.

## Methodological considerations

### *Immunohistochemical detection of intraneuronal A $\beta$*

An immunosignal depends on specificity of the antibody and on the conditions of the tissue. Duration and type of tissue fixation, embedding, pre-treatment for antigen-retrieval are crucial steps that will influence the outcome. The ability to determine if an immunohistochemical signal in postmortem human brain is specific, i.e. if it detects the intended target is further complicated by agonal state, postmortem interval and the lack of an adequate negative control. Transgenic models carry the advantage that tissues from animals with a single genetic difference can be prepared in parallel and thus serve as excellent negative controls.

In the literature, protocols used to study intraneuronal A $\beta$  are variable particularly regarding antigen retrieval. Heat treatment has been shown to enhance the staining compared to none or enzymatic pretreatments [164]. Formic acid (FA) covalently modifies and solubilizes A $\beta$  by formate

esterification of serine residues [165] and is customly used for improved immunostaining of extracellular plaques [166]. However, there is little consensus regarding the use of FA for detection of intraneuronal A $\beta$ , with reports of low effect [167] or even adverse effect [168]. In our studies and in a recent report [154], a combination of heat and FA pretreatment intensified the intraneuronal immunostaining.

Antibodies raised against the A $\beta$  domain often bind APP fragments and holo-APP, which are much more abundant than A $\beta$  in the cell. Antibodies against neopeptides generated after APP processing (the free C- and N-terminal ends of the A $\beta$  domain) should not detect APP. However, while antibodies directed against the free N-terminus (Asp1) of A $\beta$  (e.g. antibodies mAb82E1 and 3D6) should not cross-react with full-length APP, they do detect  $\beta$ -cleaved APP (C99) [169].

## Experimental setup

In the first part of this study we used an inhibitor to reduce the  $\gamma$ -secretase cleavage of APP in young tg-ArcSwe mice following acute treatment. The aim was to rapidly lower the production of A $\beta$  monomers in young mice, expecting three possible outcomes:

- A diminished intraneuronal immunostaining following treatment would suggest that the aggregates are dynamic and largely composed of A $\beta$  having low stability and a rapid turnover.
- Unchanged levels of intraneuronal immunostaining after treatment would be expected if intraneuronal aggregates were mainly APP.
- An increase in punctate intraneuronal immunostaining following treatment would be expected if intraneuronal aggregates were mainly composed of  $\beta$ -secretase cleaved C-terminal fragments. C99 is the precursor of A $\beta$  and known to rapidly accumulate in response to  $\gamma$ -secretase inhibitor treatment and are found in PS-1 knockout mice.

Thus by combining biochemical analyses of APP, APP-cleaved fragments e.g. C99 and A $\beta$  with histological measures of intraneuronal A $\beta$ -immunoreactivity it should be possible to determine if the intraneuronal staining represents mainly APP, APP-fragments or A $\beta$ .

To complement this experiment, we crossed tg-ArcSwe mice with mice lacking murine  $\beta$ -site APP-cleaving enzyme 1 (BACE-1). If the punctate intraneuronal immunostaining in young mice was due to APP, the A $\beta$  fragment P3 or unrelated artifacts we would expect to see a similar pattern of intraneuronal immunostaining in tg-ArcSwe mice with and without BACE-1. In contrast, if the staining was due to accumulated A $\beta$  inside neurons, there

should be no intraneuronal immunoreactivity with A $\beta$  antibodies, and brain tissue extracts of tg-ArcSwe BACE-KO should be devoid of A $\beta$ .

## Results and discussion

Following administration of  $\gamma$ -secretase inhibitor to tg-ArcSwe mice by oral gavage, the  $\beta$ -cleaved C-terminal APP-fragment (C99) was redistributed and quantitatively increased compared to vehicle treated mice (using western blot and immunohistochemistry). This was likely due to an accumulation of C99 at the nerve terminals, the site where APP is mainly processed [102, 170, 171]. An increase in C99 following  $\gamma$ -secretase inhibition has previously been reported in transgenic mice [172, 173]. The increase in C99 levels accompanied reduced levels of A $\beta$  and diminished immunohistochemical staining with antibodies specific for the neoepitope after proteolysis (mAb82E1). The reduction in immunohistochemical staining was similar to the reduction of A $\beta$  in the SDS-soluble pool, ~30% (intracellular and extracellular A $\beta$ ) as measured by ELISA. A more marked reduction of A $\beta$  (50-65%) in the TBS pool (presumably mainly extracellular soluble A $\beta$ ) indicated that A $\beta$  in the intracellular pool had a prolonged half-life as compared to A $\beta$  in the extracellular pool. It suggested that the punctate immunostaining was an aggregated form of A $\beta$ .

In both the tg-Swe model (tg-Swe line A, also used in **paper II**) and the Tg2576 model, intraneuronal A $\beta$ -immunoreactivity has been shown, but is less frequent and intense in tg-Swe compared to tg-ArcSwe mice [106]. The  $\gamma$ -secretase inhibitor which we used [BMS-299897 [174]] has previously efficiently inhibited APP processing in transgenic mice with a maximum reduction of A $\beta$  (80% reduced A $\beta$ 40-level compared to controls) in brain, plasma and CSF at 4-6 h after a single oral administration at 10 mg/kg [175, 176]. The half-life of A $\beta$ 40 was 0.63 h with BMS-299897 in young brains of Tg2576 mice [175] and 2.5 h in a study with a different setup [177].

The lower effect of the  $\gamma$ -secretase inhibitor (25-30% reduction of biochemical and immunohistochemical levels of A $\beta$ ) in tg-ArcSwe was likely partially due to larger and more stable intracellular inclusions of Arctic A $\beta$  in the tg-ArcSwe model compared to inclusions of wtA $\beta$  in Tg2576 mice. **In paper II** we showed that extracellular Arctic A $\beta$  deposits were more resistant to detergent dissociation than wild-type A $\beta$  deposits in the tg-Swe mice. Our findings suggest that this is also true for intraneuronal pre-amyloid aggregates in the tg-ArcSwe model.

Although the brain tissue was mechanically homogenized and extensively sonicated in SDS, there might still be stable aggregates which partition to the pellet upon centrifugation under the conditions used. However, when brains of 4 months old (pre-plaque) mice were sequentially extracted in TBS, SDS and FA (**paper II**), we did not detect any SDS-insoluble A $\beta$ . Consequently,

the use of 2% SDS in this study recovers essentially all A $\beta$  in the brain of 2-months-old mice.

We can not exclude the possibility of some C-terminal ends of aggregates were masked and are less available for the A $\beta$  antibodies used in the ELISA, causing underestimated A $\beta$  levels in these samples, as has previously been demonstrated [178]. A $\beta$ 40 in tg-ArcSwe shown in this study (and similarly for tg-Swe in **paper IV**) is somewhat more abundant than A $\beta$ 42 in the 2-months-old mice, which markedly differs to 16-months-old mice in which A $\beta$ 40 is roughly 20 times more abundant (**paper II**). This emphasizes the impact of the Arctic mutation to differentially promote accumulation of A $\beta$  with shorter C-terminal during aggregation and formation of extracellular plaques.

Intraneuronal inclusions were more readily stained with N-terminal A $\beta$  antibodies than antibodies directed to the C-terminal epitope in A $\beta$ . There are several possible explanations for this observation. In general, C-terminally selective antibodies are optimal in detection of monomers, but have lower affinity for molecular aggregates of A $\beta$  [179]. Moreover, in **paper II** we showed that brain homogenates from old plaque-depositing tg-ArcSwe mice demonstrated peaks on mass spectrometry corresponding to A $\beta$ 1-37/38/39 in addition to A $\beta$ 1-40/42. In contrast to A $\beta$ 40/42 antibodies, N-terminal A $\beta$  antibodies on immunohistochemistry will readily detect these shorter A $\beta$  isoforms. It is also likely that the hydrophilic N-terminus of A $\beta$  is exposed and the C-terminus is hidden when A $\beta$  adapts a suggested micelle-like conformation intracellularly [111]. Moreover, as previously described, when N-terminal A $\beta$  antibodies are used some immunoreactivity could possibly be due to co-aggregated C99 and thus increased immunoreactivity. Since C99 levels increase concurrently as A $\beta$  levels decrease when  $\gamma$ -secretase is inhibited, C99 can only be a minor component of the punctate immunostaining in the tg-ArcSwe model.

Intracellular A $\beta$  immunoreactive inclusions have recently attracted much attention with the emergence of new transgenic models with distinct intraneuronal A $\beta$  immunostaining, such as the 3xFAD mouse model harboring APP, tau and PS1 mutations demonstrating synaptic physiological and behavioral abnormalities months before the appearance of extracellular deposits [151, 180]. In previous studies [181] by us as well as in a very similar model, both behavior abnormalities and extensive intraneuronal immunoreactivity was detected before the onset of plaque deposition [105]. The present study is therefore of great importance when the impact of A $\beta$ -lowering treatments on early pathology is evaluated. However, the conformational state of the aggregates, the mechanisms of inclusion formation and how they are released from the cell is not fully understood. Intracellular aggregates do not normally stain with amyloid dyes; however in the 5xFAD model intraneuronal A $\beta$ 42 positive inclusions were positive for Thioflavine S and this was associated with neuronal death [108].

In conclusion, with two independent non-invasive approaches targeting APP processing, we demonstrated with independent biochemical and immunohistochemical techniques that the punctate intraneuronal inclusions observed in the tg-ArcSwe model stained with A $\beta$ -domain antibodies were mainly composed of A $\beta$ .

## Paper II

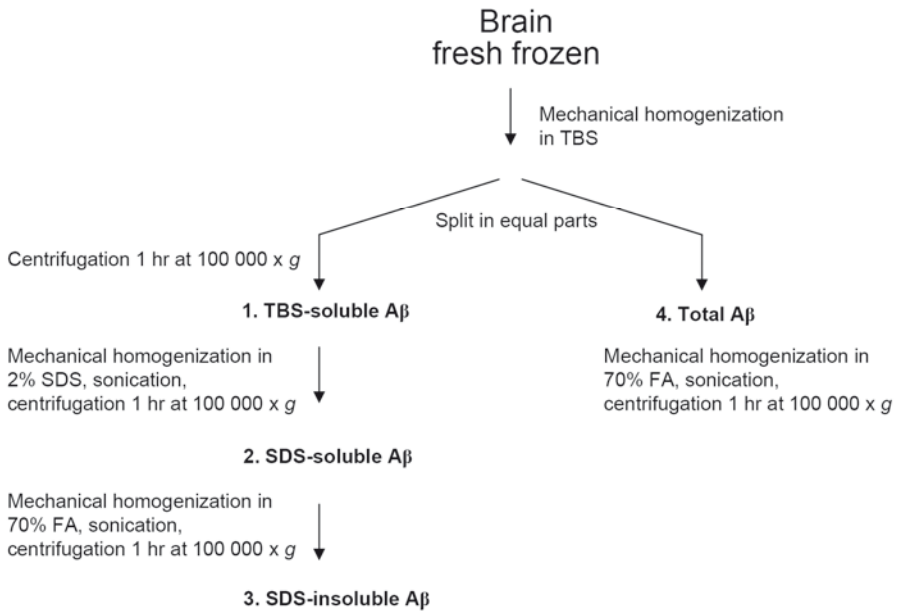
### The Arctic APP mutation alters senile plaque formation in a transgenic model of Alzheimer's disease

The Arctic mutation, situated in the A $\beta$  domain, increases the propensity for A $\beta$  peptides to aggregate and form amyloid fibrils. Initial analyses revealed a unique phenotype of tg-ArcSwe mice with distinct punctate immunostaining of intracellular A $\beta$  [106]. We considered it of interest to investigate if and how the Arctic mutation would influence late-stage A $\beta$  pathology at advanced ages.

### Methodological considerations

#### *Sequential extraction of A $\beta$ from brain tissue*

One way to study different types of amyloid aggregates is to compare their solubility. In transgenic models, A $\beta$  plaques have been reported to be highly soluble in SDS [107], and this in contrast to A $\beta$  in AD brain in which the plaques do not dissolve unless formic acid or other strong solvents are used [28]. We considered it of interest to study A $\beta$  in transgenic mouse brain and humans in terms of the solubility in a three-step extraction procedure (*Figure 5*). To selectively measure non-deposited A $\beta$  and A $\beta$  loosely attached to the plaques, fresh frozen brain tissue was homogenized in Tris-buffered saline (TBS) and following centrifugation the supernatant represented TBS-soluble A $\beta$  (fraction 1). The TBS-insoluble pellet resulting from the previous centrifugation was reextracted in 2% SDS, sonicated and following centrifugation the supernatant was referred to as the SDS-soluble fraction (fraction 2). The remaining pelleted material was finally extracted in FA, solubilizing essentially all SDS-insoluble material. This final fraction was entitled SDS-insoluble (fraction 3). To directly recover essentially all A $\beta$  in the brain, tissue was also directly extracted in FA, sonicated and centrifuged, and this pool was referred to as total A $\beta$  (fraction 4).



*Figure 5.* The sequential extraction protocol used for brain tissues. Fractions 1-4 represent the supernatant after each centrifugation. The homogenization procedures include 10 strokes of a tissue grinder followed by 30 s of sonication on ice.

#### *Labeling of A $\beta$ deposits with a conformational sensitive probe*

Polythiophene acetic acid (PTAA), is a luminescent conjugated polymer (LCP) [182, 183], which can be used for visualization and differentiation of deposits with altered fibrillar arrangements. In contrast to sterically more restricted amyloidotropic dyes, like Congo red and Thioflavin, the thiophene backbone of LCPs is flexible. Depending upon the structure of the amyloid fibril, the conformation of the PTAA backbone and the stacking of PTAA molecules is altered. This will result in different spectral characteristics effectively creating an optical fingerprint of the fibril structure. For example, PTAA bound to distinct amyloid fibrils of A $\beta$ 1-42 generates a fluorescence shift toward longer wavelengths as compared to A $\beta$ 1-42 arranged in more disordered fibrils [184].

#### *Removal of A $\beta$ from brain by passive immunization*

At least three different mechanisms are suggested for A $\beta$  clearance following treatment with A $\beta$  antibodies. Two of the mechanisms, microglial mediated [123] and catalytic dissolution [185], assumes that a sufficient amount of antibody enters the central nervous system. In the third mechanism, the peripheral sink hypothesis, antibodies are supposed to act outside the blood brain barrier (BBB) [186]. It is thought that antibodies bind to A $\beta$  in the blood vessels, shifting the distribution of A $\beta$  between the brain

and the peripheral circulatory system and thereby causing a net efflux of A $\beta$  from the central nervous system to plasma where it is degraded. A 10<sup>3</sup>-fold increase in the plasma A $\beta$  levels was observed in APP transgenic mice after peripheral administration of an antibody directed against the central domain of A $\beta$  [186]. Also arguing in favor of the sink hypothesis are findings that plasma A $\beta$  levels 24 hrs after antibody administration correlate with A $\beta$  burden in brain [187] and CSF-A $\beta$  levels rapidly increase and thereafter decrease shortly after antibody administration.

Based on the outcome of the studies by DeMattos, we administered a single intraperitoneal dose of 10 mg/kg antibody to the mice. The aged mice were perfused and the brains and plasma were collected 24 h after antibody administration. The A $\beta$  levels in plasma collected 5 min prior injection and at 24 h after injection were compared between the two transgenic models, tg-ArcSwe and tg-Swe. In this setup, we assumed that only a low level of antibody enters the CNS following a peripheral administration [188]. The outcome of the suggested experiment can however be influenced by other effects. Antibodies administered in the periphery will prolong the half-life of A $\beta$ , and the metabolism and the relative ratio of free and bound Arctic and wild-type A $\beta$  to antibodies and other proteins in plasma might differ.

## Results and discussion

By comparing the neuropathology of tg-ArcSwe and tg-Swe mice, we showed that the inclusion of the Arctic mutation resulted in a more rapid and abundant formation of extracellular A $\beta$  deposits having a patchy structure, possibly caused by fibrils forming at multiple closely adjacent locations.

In an attempt to reveal possible effects of the Arctic mutation on plaque formation, brain tissue was sequentially extracted in TBS, SDS and formic acid (*Figure 5*). A small but measurable pool defined as soluble A $\beta$  was then detected in brains of young tg-ArcSwe mice. Extracellular deposited A $\beta$  was mainly SDS-insoluble in tg-ArcSwe mice and formic acid was needed to extract the bulk of deposited A $\beta$ . These findings contrasted to those in age-matched tg-Swe mice in which A $\beta$  was largely SDS-soluble [189, 190]. Interestingly, the solubility pattern of A $\beta$  deposits in tg-ArcSwe was similar to AD brain although the composition of A $\beta$  peptides differed, with mainly A $\beta$ 38 and A $\beta$ 40 in tg-ArcSwe mice, and mostly A $\beta$ 42 in sporadic AD brain. This might be due to the Swedish mutation and/or Arctic mutation, which differentially promote accumulation of C-terminally shorter A $\beta$  species.

Tg-ArcSwe and tg-Swe mice have different age of onset of amyloid deposition, and plaques in tg-ArcSwe are possibly present for a longer period potentially resulting in a more condensed structure. However, the insoluble state of Arctic A $\beta$  in tg-ArcSwe was determined early on and did not gradually increase with time. The fraction of SDS-insoluble A $\beta$  was even greater in A $\beta$  deposits early on compared to deposits in aged mice;

suggesting that juvenile A $\beta$  deposits with an amyloid core were structurally more stable and that with age A $\beta$  mainly adhered to existing plaque cores creating a less stable outer part. Moreover, reduced increase in plasma A $\beta$  was observed at 24 h following acute administration of an A $\beta$  antibody in tg-ArcSwe as compared to tg-Swe, results that might imply diminished brain to plasma A $\beta$  efflux, but could also be due to differences in peripheral A $\beta$  metabolism. Together, the findings suggest that the Arctic mutation creates highly stable A $\beta$  aggregates through intermolecular interactions that are much stronger than for wtA $\beta$ .

In tg-ArcSwe, A $\beta$  plaques displayed a patchy morphology with bundles of A $\beta$  fibrils. The plaques varied little in size and structure as demonstrated by Congo red, a luminescent conjugated polymer (LCP) amyloid ligand (PTAA) and electron microscopy. In contrast, tg-Swe presented with large circular amyloid cores with varied size, and PTAA staining indicated that these had a less compact structure. Since Arctic A $\beta$  fibrils possibly had an altered peptide composition, the changed composition of the deposits resulted in a weaker staining with modified Bielschowsky's silver stain.

APP transgenic mice have been criticized for being too easy to cure [107, 191]. An abundance of strategies that are not linked to A $\beta$  metabolism have been reported to reduce A $\beta$  burden [191]. It could be due to poor experimental design, insufficient power i.e. chance or publication bias, with studies demonstrating efficacy being far more likely to be published [153]. Still it could also suggest that the predictive validity of the models is poor. The tg-ArcSwe model mimics some of the aspects of A $\beta$  deposits in AD brain, such as their insoluble nature, although the ultrastructure of the amyloid deposits is not that of classic senile plaques. The tg-ArcSwe model may serve as a valuable complement to existing APP transgenic models to better predict the outcome of clinical trials, particularly in therapies designed to enhance clearance or catabolism of A $\beta$  aggregates [22].

## Paper III

### Atypical A $\beta$ deposition in brain of Arctic APP mutation carriers

The Arctic mutation leads to early cognitive alterations with mean age of onset of AD in the fifth decade of life, without any signs of stroke or other cerebrovascular events [42]. In this study, mutation carriers having a similar clinical picture, were comprehensively evaluated. The neuropathology in the autopsied brain has previously only been sparsely described with reported ring-like plaques using C-terminal A $\beta$  antibodies. Fresh frozen tissue from another individual became available and made it possible to further analyze and compare neuropathology with sporadic AD and PS1- $\Delta$ 9 mutation brains with both biochemical and histological techniques.

### Methodological considerations

#### *Urea-SDS PAGE separation of C- and N-terminal truncated A $\beta$*

In Urea-SDS PAGE, only the most hydrophobic amino acid residues are SDS-bound and the proteins are separated according to their hydrophobicity. More hydrophobic residues are located at the C-terminus of A $\beta$ , and C-terminally truncated A $\beta$  migrates slower than A $\beta$  that is truncated at the more hydrophilic N-terminus, allowing different A $\beta$  peptides to be separated [157]. In the human brain, a spectrum of different A $\beta$  isoforms may migrate at the same speed, making the separation of a heterogenous mixture of A $\beta$  isoforms challenging. In addition to truncated A $\beta$ , other post-translational modifications of A $\beta$  (e.g. isomerization, racemization, pyroglutamylation) may influence the migration in the gel. When proteins are transferred to nitrocellulose membrane and immunodetected using western blot, it is possible to overcome some of these problems by using different A $\beta$ -antibodies.

#### *Analyses of C- and N-terminal truncated A $\beta$ with ELISA*

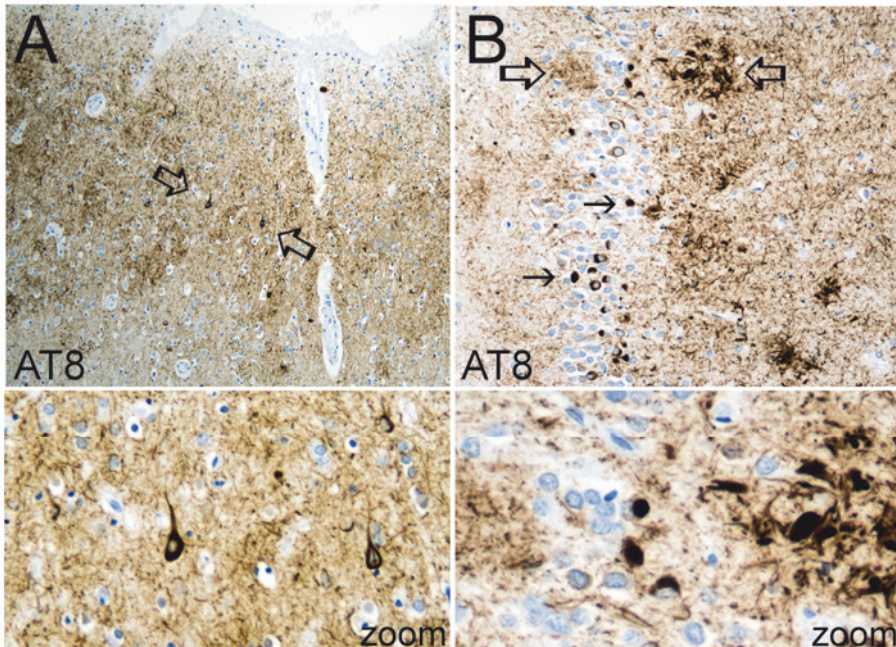
The different sandwich ELISAs used are all based on the combination of two A $\beta$  antibodies having epitopes in different parts of the A $\beta$  domain. To measure mainly full-length A $\beta$  (A $\beta$ 1-40 or A $\beta$ 1-42), polyclonal antibodies specifically recognizing neoepitopes created when APP has been cleaved at amino acid 40 or 42 in the A $\beta$  domain were used as capturing antibodies. The wells were coated with these antibodies and the antibody 6E10 (epitope A $\beta$ 3-8) was used as detection antibody. For measuring total A $\beta$ , a combination of 4G8 (epitope A $\beta$ 18-22) and mAb27 [181] was used for detection. The mAb27 antibody has a conformation-dependent epitope in the mid-domain of the Arctic A $\beta$  peptide. There are no commercially available antibodies that are suited for a total measurement of N-terminally truncated A $\beta$  species in a heterogeneous mixture of peptides with and without the

Arctic mutation (at amino acid 22). The 4G8 has high affinity for wtA $\beta$ , but lower affinity for Arctic A $\beta$ . In order to measure total A $\beta$  levels, we calibrated the ELISA system by mixing the two detection antibodies, 4G8 and mAb27 in a ratio where recombinant wild-type A $\beta$  and Arctic A $\beta$  were equally well detected.

## Results and discussion

We extended the initial morphological studies by a detailed biochemical, light microscopic and ultrastructural evaluation of neuropathological alterations associated with extracellular A $\beta$  deposits in postmortem brain of patients with the Arctic mutation. This specialized study of A $\beta$  pathology was performed in parallel with a broader neuropathologic characterization including secondary pathologies, neuritic and gliotic responses as well as NFT and neuropil thread formation. Moreover, in the other study differences between anatomical regions and among the three individual cases with the Arctic mutation were investigated (Kalimo *et. al. manuscript*).

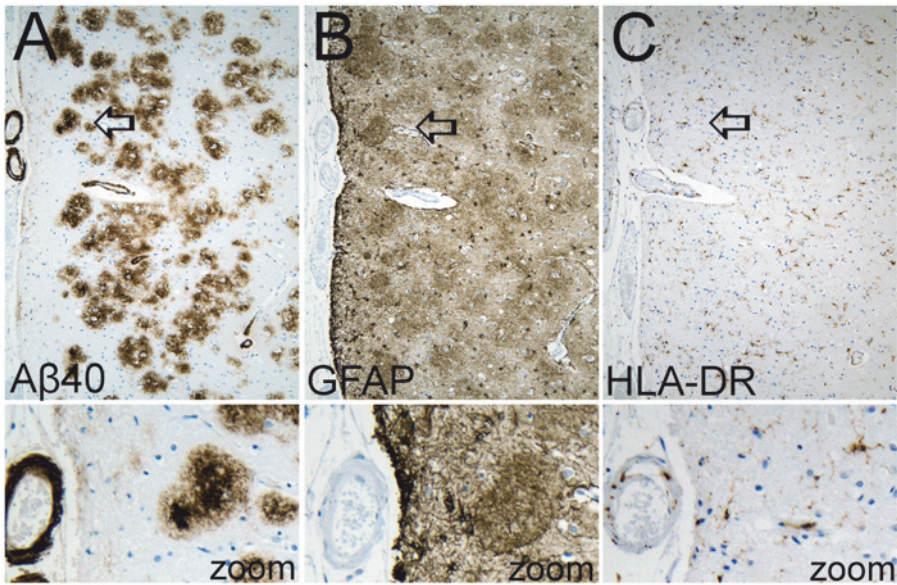
Prevalent neuropil threads (AT8-positive) were found in the occipital cortex, a criterion for Braak V-VI staging [36]. Several hyperphosphorylated tau-immunopositive NFT were seen in cerebral cortex layer III. However, much more pronounced NFT immunoreactivity was found in dentate gyrus of hippocampus (Figure 6A), with dystrophic neurites associated with plaques (Figure 6B).



*Figure 6.* Layer 3 in the occipital cortex of the Arctic AD patient harbors abundant neuropil threads that are immunopositive for hyperphosphorylated tau (AT8-antibody) and neurons (open arrow) with neurofibrillary tangles in their cytoplasm (A). Several neurons (marked with an arrow) in the dentate gyrus contain AT8-immunopositive inclusions in their cytoplasm. In the hilus of dentate gyrus in the hippocampus (CA4) there are abundant neuropil threads and dystrophic neurites associated with neuritic plaques (open arrow) (B). Sections are counterstained with haematoxylin.

The core of the A $\beta$  deposits of tg-ArcSwe was shown in **paper II** to be essentially Congo red positive and this was also seen in tg-Swe mice. In the Arctic brain, cerebral amyloid angiopathy was Congo-positive whereas parenchymal A $\beta$  deposits were negative for Congo red. Superficially they resembled cotton wool plaques, which are found in FAD with presenilin  $\Delta$ 9 mutations [192] and other presenilin-1 mutation carriers [193]. Using Bielschowsky's silver stain, the plaques were moderately stained with accentuation of peripheral parts making the plaques appear ring-like in their structure.

As with cotton wool plaques, deposits in Arctic brain were to a little extent surrounded by a gliotic tissue response and neuritic dystrophy (Figure 7). In tg-Swe (**paper II**) A $\beta$  plaques were large, circular and Congo red positive with radiating A $\beta$  fibrils, associated with more neuritic dystrophy (both dendrites and axon terminals) compared to human brain with the Arctic mutation. The neuritic and gliotic reaction around parenchymal plaques in tg-ArcSwe has shown to be somewhat more pronounced than in tg-Swe (unpublished observations).



*Figure 7.* In the frontal cortex of the Arctic AD (Case 1), an A $\beta$ 40 antibody strongly stains large plaques (A). GFAP staining is highly immunopositive in the immediate vicinity of plaques, but the number of astrocytes is limited and their cell bodies are quite randomly distributed without apparent relationship to the plaques (B). There is neither a significant increase in the number of HLA-DR, DP, DQ positive microglial cells nor an association with plaques (C). The sections are adjacent and counterstained with hematoxylin.

The pattern of immunostaining of plaques varied remarkably depending on the A $\beta$ -epitope recognized by the antibody. Those directed against the N-terminal epitopes (82E1 and 6E10) detected larger plaques often with a darkly stained centre, while the outer parts stained with somewhat varying intensity. A mid-domain A $\beta$  antibody (4G8) stained the entire plaque surface uniformly. With C-terminal antibodies, recognizing aa 40/42 ending A $\beta$ , and Bielchowsky silver staining, plaques appeared ring-like which confirms and extends previous observations [42].

Laser capture micro-dissection and biochemical analyses of the Arctic mutant brain demonstrated that N-terminally truncated Arctic A $\beta$  peptides (A $\beta$ <sub>x</sub>-40 and A $\beta$ <sub>x</sub>-42) were the predominant isoforms in parenchymal plaques, whereas A $\beta$ <sub>x</sub>-40 and often full-length A $\beta$ 1-40 was predominant in cerebral amyloid angiopathy (CAA). The pattern of C-terminally truncated A $\beta$  species of plaques in the Arctic brain differed markedly from sporadic AD brain. Different A $\beta$ <sub>x</sub>-42/A $\beta$ <sub>x</sub>-40 ratios were evident when comparing plaques in postmortem brain with sporadic AD brain, having a predominance of A $\beta$ <sub>x</sub>-42 in parenchymal deposits. This is consistent with previous reports [78, 194, 195]. Similarly to Arctic brain, in CAA of sporadic brain, amino acid 40 ending A $\beta$  was the prominent species; however more N-terminal truncated A $\beta$  was detected.

In particular, one aspect of the ELISA analyses needs to be highlighted. The mAb27 antibody, which we have developed and previously characterized [181], has a conformational epitope in the Arctic A $\beta$  peptide. This could possibly underestimate the levels of Arctic A $\beta$  in the brain.

When Arctic brain tissue was directly extracted in formic acid and separated using urea/SDS-PAGE and Western blot (with 82E1 antibody) mainly Arctic A $\beta$  and to a lesser extent wild-type A $\beta$  was found. However, the data, particularly with the A $\beta$ 40-specific antibody, needs to be interpreted cautiously, since different truncated or posttranslational modified forms of A $\beta$  might migrate similarly (see section ‘Methodological Considerations’). The abundance of N-terminal truncated A $\beta$  contrasts to the tg-ArcSwe model where full-length A $\beta$  peptides beginning at amino acid 1 were common (**paper II**). These marked differences between transgenic mice and humans are likely partly explained by the inclusion of the Swedish mutation in the animal models, and possibly species differences in the amino acid sequence of A $\beta$  and enzymes involved in APP processing. For example, murine BACE-1 cannot cleave transgenic human APP at position 11, while human BACE-1 in human Arctic brain cleaves at this position and generates N-terminally truncated Arctic A $\beta$  [196]. Furthermore, other plaque-associated proteins and species-specific differences e.g. the cellular milieu may affect the fibrillization of Arctic A $\beta$  differently in human brain as compared to mouse brain [197]. We limited our measurements to A $\beta$  ending at amino acid 40 or 42 with ELISA, since they have been reported to be the predominant A $\beta$  species in sporadic AD brain.

The MALDI-TOF MS analyses of the Arctic brain confirmed the result of N-terminal truncation of A $\beta$  and pointed towards a great variety of C- and N-terminal truncated A $\beta$ , with and without the Arctic mutation. It is possible that the heterogeneity of both wild-type and Arctic A $\beta$  alters the fibrillogenesis in human brain. We investigated this by creating a bi-transgenic mouse producing both Arctic and wild-type A $\beta$ , in which A $\beta$ 42 positive diffuse deposits accumulated with age (**paper IV**). Heterologous production of wild-type A $\beta$  and mutant A $\beta$  (APP A693V) were also shown to inhibit fibril formation, and to only cause disease among homozygous carriers [198]. The predominance of N-terminally truncated and modified Arctic A $\beta$ <sub>x-42</sub> and Arctic A $\beta$ <sub>x-40</sub>, which are likely less able to form amyloid fibrils can possibly explain why parenchymal A $\beta$  deposits in human Arctic brain do not contain fibrillar amyloid.

In conclusion, the atypical plaque features of Arctic mutant brain suggest that the amyloid core pathology is not necessary for the macroscopic AD associated atrophy, indicating that other forms of soluble/insoluble A $\beta$  are sufficient to cause neuronal dysfunction and dementia.

## Paper IV

### Arctic A $\beta$ modifies the aggregation pathway of wild-type A $\beta$ *in vitro* and *in vivo*.

The Arctic APP mutation has been shown to generate A $\beta$  peptides that are more prone to form soluble oligomers and amyloid fibrils [115, 199]. Monomeric A $\beta$  is thought to form nuclei in a time-limited reaction, followed by a rapid polymerization process that results in A $\beta$  fibrils. The processes have been frequently investigated *in vitro* [200, 201] but far less is known about A $\beta$  polymerization *in vivo*. It was shown that intracerebral injection of extracts from human AD and transgenic mouse brain can induce amyloidosis in transgenic mice [202, 203]. These findings were reminiscent to those of prion diseases. One of the theories is that dimerization of pathogenic PrP<sup>Sc</sup> and healthy PrP<sup>C</sup> leads to refolding of PrP<sup>C</sup> into PrP<sup>Sc</sup> [204].

The aim of this study was to investigate whether or not a low level of Arctic A $\beta$  could influence the aggregation process of much greater levels of wild-type A $\beta$  in transgenic mice. Crossing the tg-ArcSwe mouse producing low levels of Arctic A $\beta$  (that do not form extracellular A $\beta$  deposits) with a tg-Swe mouse, producing high levels of wild-type A $\beta$ , one could expect either of three possible outcomes of the bitransgenic mouse:

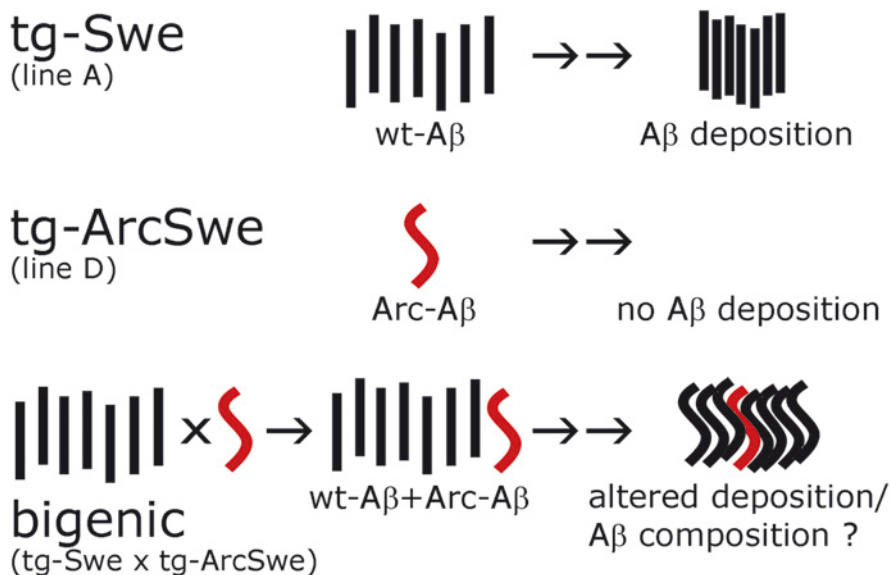
- The processing of APP<sub>ArcSwe</sub> does not interfere with APP<sub>Swe</sub> processing; neither does the aggregation of Arctic A $\beta$  interfere with wild-type A $\beta$  aggregation. Thus, the A $\beta$  related pathology in bitransgenic mice would be identical to that of tg-Swe mouse producing only wild-type A $\beta$ .
- There is an interaction between APP<sub>ArcSwe</sub> and APP<sub>Swe</sub>, e.g. APP dimerization, which alters APP processing. It would lead to changes in the rate of APP processing or A $\beta$  levels and thus increased or decreased A $\beta$  deposition or a redistribution of A $\beta$  deposits in aged bitransgenic mice as compared to tg-Swe.
- A physical interaction between Arctic A $\beta$  and wild-type A $\beta$  alters aggregation and deposition of wild-type A $\beta$ . Possibly Arctic A $\beta$  could reduce the lag-time for nucleus formation and generate a more rapid seed or fibril elongation process of wild-type A $\beta$ . Alternatively, it could interfere and delay the aggregation process.

## Methodological considerations

### *Aggregation assay using conformational selective probes*

There are a number of compounds with affinity for amyloid ( $\beta$ -sheet). Congo red and Thioflavine are among the most commonly used amyloid dyes. When Thioflavine T (ThT; a benzothiazole) binds to a  $\beta$ -sheet conformation there is a spectral shift such that the dyes excitation and emission wavelength is altered. This allows amyloid fibril formation to be detected and monitored. The excitation maximum is shifted from 342 nm (unbound ThT) to 442 nm ( $\beta$ -sheet bound ThT) with subsequent emission spectra change from 430 nm (unbound ThT) to 482 nm ( $\beta$ -sheet bound ThT) [205]. It has been shown that ThT bound to A $\beta$  oligomers with lower  $\beta$ -sheet content, is less prone to change spectrum [93].

More recently, dyes with other spectral properties upon binding were developed. Luminescent conjugated polythiophenes (LCPs) [182, 183, 206] and luminescent conjugated oligothiophenes (LCOs) [207] have been thoroughly characterized. The LCOs enable staining and analyses of prefibrillar and fibrillar A $\beta$  assemblies [207]. The fluorescence of the LCO, p-FTAA, was used as readout of prefibrillar A $\beta$  and fibrillar aggregates in parallel with Thioflavine T, which detects essentially amyloid fibrils. The molecular probes were added to monomeric A $\beta$  immediately prior to the initiation of the aggregation protocol, instead of adding the probe at specific time-points during aggregation. Variability, a major problem, was thereby reduced. The caveat is that co-aggregation of the probe might influence the aggregation process.



*Figure 8.* Schematic illustration of the experimental design for paper IV. Previous findings that the Arctic mutation promote A $\beta$  aggregation and deposition *in vitro* and *in vivo* led us to suggest that Arctic A $\beta$  could serve as a template for wild-type A $\beta$  aggregation.

## Results and discussion

In the process of developing transgenic models, tg-ArcSwe mice were identified after having screened many founder lines expressing human APP with the Arctic and Swedish mutations (**paper I and paper II**) and Table 2. One of the transgenic founder lines expressed only 1-fold human APP<sub>ArcSwe</sub> and did not develop extracellular deposits during its lifetime. Likewise, many founder lines of tg-Swe (expressing APP with the Swedish mutation) were screened and the line with the highest expression level (7-fold APP) developed extensive extracellular A $\beta$  deposits from 12 months of age (**paper II**). When the low-expressing tg-ArcSwe mice were crossed with the high expressing tg-Swe mice, some of the resulting progeny were bitransgenic (tg-ArcSwe x tg-Swe). These mice developed numerous diffuse A $\beta$  deposits in an age-dependent manner. Thus, the burden of diffuse deposits, but not amyloid plaque burden differed from littermate tg-Swe mice but also from tg-ArcSwe mice with high APP expression. Furthermore, the diffuse deposits of bitransgenic mice were shown to contain mainly wild-type A $\beta$ <sub>42</sub> and to be located in regions both with and without transgene expression. The selective increase of a single type of parenchymal A $\beta$  deposit suggests that different pathways of A $\beta$  aggregation lead to the formation of diffuse and compact A $\beta$  deposits in the brain.

The increase in diffuse A $\beta$  deposition was not due to altered APP processing. Analyses of brain homogenates of young bitransgenic mice with A $\beta$  ELISA did not reveal any changes in A $\beta$  levels between bitransgenic mice and tg-Swe that would not be expected by the additive effects of the two transgenes. Thus, there was a trend toward increased A $\beta$  levels in young bitransgenic mice, as compared to age-matched tg-Swe, which was explained by an additional copy of human APP.

Instead, the altered pathology was most likely due to direct physical interactions between Arctic A $\beta$  and wild-type A $\beta$ . Using aggregation assays, we showed that a mixture of Arctic A $\beta$ 1-42 and wild-type A $\beta$ 1-42 facilitated the formation of prefibrillar assemblies, but inhibited the further elongation of amyloid fibrils. Interestingly, in a very old tg-Swe mouse (~25 months), diffuse deposits in the striatum was seen i.e. reminiscent of that observed in younger bitransgenic mice, indicating that Arctic A $\beta$  strongly accelerated the process of wild-type A $\beta$ 42 aggregation and favoured the formation of diffuse A $\beta$  deposits.

Tg-ArcSwe mice (also used in **paper I and paper II**) express only human APP with the Arctic and Swedish mutation (apart from the endogenously expressed murine APP), whereas patients with the Arctic mutation (**paper III**) are heterozygous for the mutation and consequently produce a mixture of both human wild-type A $\beta$  and Arctic A $\beta$ . The crossed mice in this study express a small fraction of Arctic APP as compared to Swedish APP (1:7 ratio). For future studies, it would be of great interest to cross mice expressing equal levels of Arctic APP and Swedish APP and to find out if the A $\beta$  pathology then more closely resembles that of the Arctic AD brain, with non-amyloidogenic parenchymal plaques and cerebral amyloid angiopathy. However, the conditions in the human brain would still greatly differ to that of mouse brain regarding posttranslational modifications of A $\beta$  (isomerization, racemization, pyroglutamyl formation, and truncations) [194, 197, 208, 209] including cleavage potential of murine BACE-1. Such modified A $\beta$  isoforms were not found in plaques of old tg-ArcSwe mouse brain (**paper II**) and have rarely been reported in other APP transgenic models.

The Arctic mutation generates A $\beta$  that is more resistant to proteolytic degradation [210]. The half-life of Arctic A $\beta$  in the brain could possibly be extended and thereby be even more prone to be deposited in human brain. However, a novel peptidase (PreP peptidasome) localized to the mitochondria has an additional cleavage site on Arctic A $\beta$  as compared to wild-type A $\beta$  *in vitro* [211], but the *in vivo* relevance of the peptidase to A $\beta$  clearance (if any) is unclear.

An almost twofold higher level of protofibrils was detected in a TBS soluble fraction of the bitransgenic mouse brain compared to the tg-Swe. Whether these assemblies are a result of Arctic A $\beta$  itself forming protofibrils or interactions between Arctic A $\beta$  and wild-type A $\beta$  generating a slower

conversion of protofibrils to fibrils is not known. The latter has been shown to be true for aggregation of recombinant A $\beta$  *in vitro* [212].

In conclusion, by developing APP-bitransgenic mice we showed that a low level of Arctic A $\beta$  facilitates the aggregation of wild-type A $\beta$ 1-42 resulting in an abundance of small diffuse deposits. This rather unique observation of a selective increase in only one type of parenchymal A $\beta$  deposits suggests a branched mechanism of extracellular A $\beta$  leading either to diffuse deposits or amyloid deposits. Furthermore, it shows that aggregation of A $\beta$  can be manipulated by a diminutive amount of a heterologous protein/peptide, comparable to the proposed pathogenic mechanism of prion diseases.

## Concluding remarks

During the last 20 years, the understanding of the molecular mechanisms of AD has grown tremendously. Many observations made have been consistent with the amyloid cascade hypothesis or modified versions thereof. The pathogenesis is thought to be initiated by an imbalance between production, and clearance of A $\beta$ , resulting in aggregation. The mutations implicated in the familial forms of AD are present in the genes of the APP substrate and one of the key regulatory enzymes (presenilins) generating A $\beta$ . Apolipoprotein E isoforms, which alter the risk of disease development, regulate A $\beta$  deposition in tissues. Since early-onset AD is clinically and neuropathologically rather similar to sporadic AD, A $\beta$  mismetabolism is likely relevant to the cognitive decline in sporadic AD. The Arctic APP mutation invariably generates clinical features that are indistinguishable from AD, making the Arctic mutation an interesting tool to further study mechanisms of pathogenesis.

In this thesis, the Arctic APP mutation was used in transgenic models to study accumulation of intracellular and extracellular A $\beta$ . The intracellular immunoreactivity with A $\beta$  antibodies was shown to be at least partially reversible in young mice, when inhibited by a  $\gamma$ -secretase inhibitor and absent in APP transgenic mice lacking BACE-1. The study suggests that the pathogenesis begins with accumulation of A $\beta$  inside neurons and with time this leads to a growing number of A $\beta$  assemblies that are finally released into the extracellular space when the neuron is unable to store them.

Intraneuronal aggregates of A $\beta$  have been associated with neuronal cell death in both humans [213] and transgenic models [214]. They have also been found in NFT-containing neurons in humans [215] and could possibly provide an explanation to how A $\beta$  gives rise to intracellular NFT formation and neuronal loss in AD pathogenesis.

There are several issues regarding the transition of intracellular to extracellular A $\beta$  aggregates. What governs the process that leads to the rapid decline in intracellular A $\beta$  aggregates when extracellular plaques start to appear? Is it an active process e.g. altered firing activity of neurons or simply a sink effect? Perhaps the aggregates become unstable due to accumulation and exposure of C-terminal hydrophobic A $\beta$  residues, making the aggregates dissociate and become actively rerouted out of the cell. Since in these models (and in most other APP-transgenic mice), the neurons accumulating these aggregates do not degenerate, this release process is likely actively

regulated by the cell. The Arctic mutation not only influenced intracellular A $\beta$  aggregation, but also extracellular accumulation, with plaques being morphologically different and having a greater stability in tg-ArcSwe than those of tg-Swe mice. The biochemical characterization of amyloid deposits revealed that A $\beta$  in the aged tg-Swe mouse was well recovered in 2% SDS, whereas formic acid was needed to extract the majority of A $\beta$  in aged tg-ArcSwe mouse brain. This feature resembles that of sparingly insoluble A $\beta$  in human AD brain. Together with the results of a more condensed A $\beta$  fibril structure in tg-ArcSwe, as demonstrated with a conformational sensitive probe, our results suggest that Arctic A $\beta$  aggregates differently to that of wild-type A $\beta$ .

Assemblies of A $\beta$  have been suggested to directly or indirectly trigger aggregation of tau and the formation of NFTs. The tg-ArcSwe model has a high level of A $\beta$  protofibrils, an extensive A $\beta$  plaque pathology, but display neither NFTs nor neuronal loss, as would be expected if tau pathology were a downstream process of A $\beta$ . Possibly murine neurons do not have the pathways necessary for A $\beta$ -induced neurotoxicity because they lack processes leading to tau aggregation and NFTs. New discoveries and more sophisticated transgenic models will likely be needed to understand the molecular link between A $\beta$ , tau pathology, synapse dysfunction and neuronal loss.

The substitution of the charged glutamic acid for the uncharged amino acid glycine is expected to confer a more flexible nature to the A $\beta$  peptide [216]. This effect has previously been shown to accelerate the formation of protofibrils *in vitro* [115, 199] and in tg-ArcSwe mice [181, 217]. Postmortem brain from patients carrying the Arctic mutation contains cerebrovascular, but not parenchymal, amyloid. This is in contrast to observations made in tg-ArcSwe mice. The discrepancy between the human and transgenic mouse brain might in part be due to the inclusion of Swedish mutation (and increased BACE cleavage at position 1 in the A $\beta$  domain of APP) in the mouse model, but also post-translational modifications of A $\beta$  that are much less found in transgenic models. Importantly, it shows that the clinical features of AD can develop in the absence of parenchymal amyloid formation. The sheer abundance of A $\beta$  and/or other more soluble forms of A $\beta$  might create neuronal dysfunctions. In tg-ArcSwe, Arctic A $\beta$  is overproduced, whereas in humans with the Arctic mutation both wild-type and Arctic A $\beta$  is being produced. To test if wild-type and Arctic human A $\beta$  interact and alter the aggregation, we generated a bitransgenic mouse model expressing low level of Arctic APP and high level of Swedish APP. In the bitransgenic mouse, an accumulation of A $\beta$ 42 was selectively increased in small non-amyloid deposits in regions where the transgene was expressed, but also in areas essentially devoid of APP-expression. Diffuse A $\beta$  pathology accumulated with age in bitransgenic mice, while the burden of cored plaques remained unchanged as compared to tg-Swe. In the literature,

diffuse plaques are regarded as predecessors of cored plaques, while our findings instead suggest that the mechanism of extracellular A $\beta$  deposition is branched such that either amyloid plaques or diffuse plaques can form directly and independent of each other. Certainly, this does not exclude that some diffuse plaques can mature and give rise to amyloid plaques. These observations may be relevant to our observations of A $\beta$  neuropathology in postmortem brain from patients with the Arctic mutation, demonstrating only non-cored parenchymal deposits. In the future, to achieve an even more relevant model for the human situation, a mouse with APP<sub>Swe</sub> and APP<sub>ArcSwe</sub> expressed at equal levels would be valuable.

The non-invasive introduction of a heterologous protein in this study, facilitating the deposition and spreading of the endogenously produced protein, resemble findings made by invasive approaches. Transmission of exogenous proteins facilitated aggregation and cell-to cell spreading of wild-type  $\alpha$ -synuclein in humans [218, 219] and tau in mice [220]. A $\beta$ -containing extracts from human AD brain was also shown to induce A $\beta$  amyloidosis in transgenic mice [203]. There is so far no evidence that neurodegenerative disorders, other than prion disorders, can spread from one individual to another. Still our findings add to the accumulating evidence that aggregation, deposition and spreading of amyloidogenic proteins can be facilitated by the introduction of a heterologous protein, a process resembling that of prion disorders.

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A handwritten signature in black ink, appearing to be 'Lars', with a long, sweeping horizontal stroke extending to the right.

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