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Targeting early stages of Alzheimer's disease in a transgenic model

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Abstract

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The Arctic mutation causes early-onset Alzheimer's disease (AD), and makes amyloid- β (A β) peptides more prone to form A β protofibrils. The aims of this thesis were to investigate the mechanisms of the Arctic mutation *in vivo*, and to use transgenic models to determine the role of early intermediates of A β aggregation, like protofibrils, in the pathogenesis. In addition, we aimed to evaluate protofibrils as a therapeutic target.

Transgenic models with Arctic and Swedish mutations (tg-ArcSwe), and with the Swedish mutation alone (tg-Swe) were created. The Arctic mutation favored amyloidogenic processing of amyloid- β precursor protein (APP) in transgenic mice and cultured cells. The observed shift in the subcellular location and processing of APP led to increased production of intracellular A β *in vitro*, and also partly explained the early accumulation of intraneuronal A β in tg-ArcSwe mice. The intraneuronal A β in combination with enhanced levels of protofibrils appeared long before extracellular plaques emerged. Elevated protofibril levels were associated with intraneuronal A β and linked to spatial learning deficits in young mice, suggesting that protofibrils cause AD-related cognitive deficits. The Arctic mutation also enhanced senile plaque pathology in aged tg-ArcSwe mice, and the accelerated plaque deposition was accompanied by decreased intraneuronal A β . This suggests a dynamic equilibrium between the early accumulation of intraneuronal A β and the later senile plaque pathology.

A β protofibrils were evaluated as a therapeutic target in tg-ArcSwe mice with passive immunization using a protofibril-selective antibody. This treatment cleared protofibrils without removing senile plaques. However, plaque formation was prevented if treatment began early, indicating that protofibrils are intermediate species of A β fibrillization *in vivo*. Targeting senile plaques with immunotherapy requires early diagnosis and intervention, whereas protofibrils can be specifically cleared from brain despite substantial AD-like deposition of insoluble A β . The early and persistent presence of protofibrils throughout A β amyloidosis makes them a promising target for future diagnostic and therapeutic strategies in AD.

Keywords: Alzheimer's disease, transgenic mice, amyloid- β , protofibrils, intraneuronal, cognition, immunotherapy, antibody, Arctic mutation, amyloid precursor protein

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List of papers

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

- I **Lord A**, Kalimo H, Eckman C, Zhang X-Q, Lannfelt L and Nilsson LNG. (2006) The Arctic Alzheimer mutation facilitates early intraneuronal A β aggregation and senile plaque formation in transgenic mice. *Neurobiology of Aging*, 27:67-77
- II Sahlin C, **Lord A**, Magnusson K, Englund H, Almeida CG, Greengard P, Nyberg F, Gouras GK, Lannfelt L and Nilsson LNG. (2007) The Arctic Alzheimer mutation favors intracellular A β production by making APP less available to α -secretase. *Journal of Neurochemistry*, 101:854-862
- III **Lord A**, Englund H, Söderberg L, Tucker S, Clausen F, Hillered L, Gordon M, Morgan D, Lannfelt L, Ekholm Pettersson F and Nilsson LNG. (2009) Amyloid- β protofibril levels correlate with spatial learning in Arctic Alzheimer's disease transgenic mice. *FEBS Journal*, 276:995-1006
- IV **Lord A**, Gumucio A, Englund H, Sehlin D, Sundquist V, Söderberg L, Möller C, Gellerfors P, Lannfelt L, Ekholm Pettersson F and Nilsson LNG. An antibody against Amyloid- β protofibrils mediates their clearance and prevents amyloid formation in a mouse model of Alzheimer's disease. *Submitted manuscript*

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Abbreviations

A β	amyloid- β
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
ADDLs	amyloid- β derived diffusible ligands
AICD	APP intracellular domain
ApoE	apolipoprotein E
APP	amyloid- β precursor protein
Arc	Arctic APP mutation (E693G)
BACE	β -site APP cleaving enzyme
BBB	blood-brain-barrier
CAA	cerebral amyloid angiopathy
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DS	Down's syndrome
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
FAD	familial Alzheimer's disease
FcR	Fc-receptor
IgG	immunoglobulin G
IHC	immunohistochemistry
KI	knock-in
KO	knock-out

LMW	low molecular weight
LTP	long-term potentiation
mAb	monoclonal antibody
MWM	Morris water maze
NMDA	N-methyl D-aspartate
NFT	neurofibrillary tangles
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PrP	prion protein
PS	presenilin
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
Swe	Swedish APP mutation (K670N, M671L)
TBS	tris buffered saline
tg-ArcSwe	transgenic mice with Arctic and Swedish mutations
ThT	thioflavin T
wt	wild-type

Introduction

Alzheimer's disease

History

It is now more than a century since this mind deteriorating disease was first described. The German physician Alois Alzheimer met his patient, a 51-year-old woman by the name Auguste Deter, in the year 1901 when she was admitted to Frankfurt's Hospital for the Mentally Ill and Epileptics. She displayed memory impairments that rapidly worsened, aphasia, paranoia, disorientation, psychosocial incompetence; and stated with her own words "I have lost myself" [1]. In 1903 Dr. Alzheimer moved to Munich, but continued to follow the case of Auguste D. until her death in 1906. When Alzheimer studied her brain at autopsy, using recently developed silver staining techniques, he found remarkable neuropathological lesions, later referred to as tangles and plaques. He presented his observations at a meeting in Tübingen later that year and published them in 1907 [2]. A few years later this type of dementia was referred to as Alzheimer's disease.

Clinical picture, diagnosis and current therapeutics

Alzheimer's disease (AD) has an insidious onset and a progressive course characterized by memory dysfunction but also other cognitive disturbances, including problems recalling familiar names and objects, changed behavior and/or impaired semantic abilities. Early symptoms are typically loss of short-term memory and impaired visuospatial orientation. As the disease progresses there is a general decline of multiple cognitive functions that interfere with activities of daily living and make patients become very dependent on caregivers. Patients suffering from AD usually die from secondary infections, e.g. pneumonia or urinary tract infections, 5-15 years after disease onset.

The clinical diagnosis of AD is based on the patient's medical history and a neurological assessment in combination with neuropsychiatric testing of the patient's cognitive functions. There are several tests to evaluate cognition, but the most commonly used test in AD diagnosis is the mini mental state examination (MMSE). It is essential to exclude alternative and treatable causes of cognitive decline, e.g. stroke, infection or depression. Neuroimag-

ing techniques, that can depict cerebral atrophy with a temporoparietal pattern of distribution, are useful for differential diagnosis and to distinguish from other causes of dementia, for instance subdural hematoma, cerebral infarcts and brain tumor. Hypoperfusion of temporal and parietal lobes detected by single photon emission computed tomography (SPECT) is suggestive of AD. Enlargement of cortical sulci and ventricles, with temporal and hippocampal atrophy, is often seen in the brain at early stages of AD by magnetic resonance imaging (MRI). However, these deteriorations partially overlap with normal ageing and other dementias. Imaging of glucose metabolism and amyloid deposition in the brain, by positron emission tomography (PET), is also a valuable diagnostic technique. In addition, assessing levels of certain biomarkers¹ in cerebrospinal fluid (CSF) following lumbar puncture could be indicative of AD. Definite diagnosis of AD cannot be made until a neuropathological *post mortem* exam of the brain has been carried out [3]. The criteria that need to be fulfilled are presence of extracellular neuritic plaques and intracellular neurofibrillary tangles (NFT), present to a far greater extent than could be expected for the given age.

Alzheimer's disease is today the major cause of dementia in the elderly population and accounts for roughly 60-70% of all dementia cases [4, 5]. The estimated prevalence of dementia shows an exponential increase with ageing, from around 1% in age groups 60-64 years up to 24-33% in those over 85 years [6]. Due to altered demography and a predicted increase in life expectancy, the prevalence of dementia will double every 20 years [6], making this a major health problem. Current pharmacological treatments for AD are purely symptomatic and do not affect the disease process itself. The drugs donepezil, galantamine and rivastigmine are all acetylcholinesterase inhibitors that increase levels of the neurotransmitter acetylcholine at synaptic clefts in the brain, helping some patients with mild to moderate AD gain a slight but transient cognitive improvement [7]. The N-methyl D-aspartate (NMDA) receptor antagonist memantine, which is believed to prevent glutamate induced excitotoxicity, is also registered as a symptomatic drug for AD. Brief beneficial effects of memantine have been observed in some patients with moderate to severe disease [8]. Hence, it is necessary to develop and evaluate new therapeutics that target molecular pathways involved in disease development and progression. There is also a need for earlier diagnosis, since future therapeutics will likely target early stages of the disease, before neurodegeneration is widespread. Better monitoring of disease progression, by new imaging techniques and/or biomarkers, is central to determine efficacy of disease-modifying therapeutics.

¹ High tau/phospho-tau and low A β 42 levels combined. Tau and A β will be discussed later.

Neuropathology

The distinct neuropathological features of the AD brain are illustrated in *Figure 1*. Cortical atrophy with enlargement of the ventricles and widened sulci are most obvious at the macroscopic level. The atrophy is severe in the temporal and parietal lobes, but the frontal cortex is also affected. At the microscopic level the disease is characterized by two histopathological hallmarks: neuritic (or senile) plaques and neurofibrillary tangles (NFT). The distribution of plaques and tangles is somewhat different and NFT are analyzed for frequency and anatomical distribution according to Braak staging criteria [9]. Nonetheless, both plaques and tangles are predominantly located in temporal and parietal parts of the neocortex, hippocampus and amygdala, areas of the brain which also present with atrophy and neuronal loss [10].



Figure 1. Macroscopic changes are illustrated by comparing coronal sections from a normal brain (A) and an AD brain (B). Cortical atrophy with reduced brain volume and wider sulci, enlargement of ventricles and hippocampal degeneration are apparent in the AD brain (B). At the microscopic level the two neuropathological hallmarks of AD are detected here with silver staining (C), NFT (marked by arrows, \rightarrow) and a neuritic plaque (marked by an arrowhead, $<$). The pictures were kindly provided by Professor Hannu Kalimo.

Senile plaques are extracellular deposits containing aggregated, fibrillar protein bundles. These stain positive for Congo red, a dye with affinity for β -sheet containing structures known as amyloid, present in a wide range of disorders termed amyloidoses [11]. In 1984 the amyloid- β ($A\beta$) peptide was purified and partially sequenced from amyloid deposited in cerebral vessel walls of AD brains by Glenner and Wong [12] and one year later the $A\beta_{42}$ ² peptide was described as the major subunit of senile plaques [13]. Multiple isoforms of $A\beta$ peptides are produced in many different cell types throughout the body. The most common species secreted in CSF are $A\beta_{38}$ (~15%), $A\beta_{40}$ (main species) and $A\beta_{42}$ (~10%) consisting of 38, 40 and 42 amino acids respectively [14]. $A\beta_{40}$ is the most common peptide in amyloid of cerebral vessels, referred to as cerebral amyloid angiopathy (CAA), whereas

² $A\beta_{42}$ (or $A\beta_{1-42}$) refers to the 42 amino acid long variant of $A\beta$.

A β 42 is more common in parenchymal senile plaques [15, 16]. Senile plaques are called neuritic plaques when surrounded by dystrophic neurites and reactive glial cells. In contrast, diffuse plaques, which are nonfibrillar plaques lacking an amyloid core, are not accompanied by such tissue responses [17]. Diffuse plaques, which can be detected with immunohistochemistry (IHC) using antibodies against the A β peptide, are also found in brain regions not functionally affected by the disease, e.g. the cerebellum [18]. Parenchymal plaques and CAA are visualized in *Figure 2*.

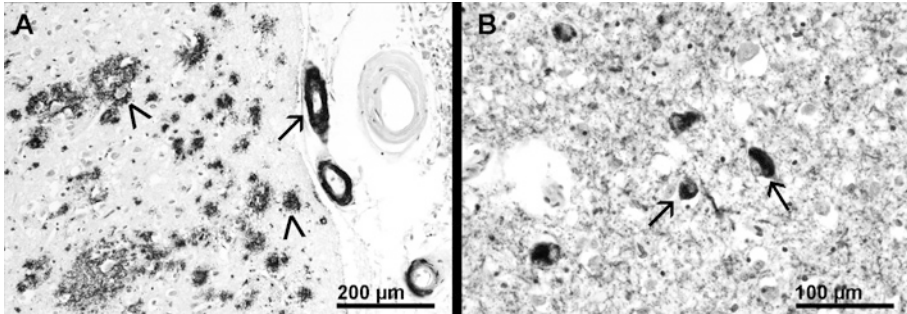


Figure 2. (A) Parenchymal plaques (<) and cerebral amyloid angiopathy (>) are visualized with anti-A β immunohistochemistry. This antibody recognizes an epitope within the A β peptide, thereby staining both A β 40 and A β 42. (B) Hyperphosphorylated tau within neurons (>) can be stained with the AT8 antibody. Pictures were kindly provided by Professor Hannu Kalimo.

NFT are intracellular deposits containing hyperphosphorylated and aggregated forms of tau [19]. Tau is a microtubule-associated protein in the central nervous system (CNS) and its normal function is to assemble and stabilize microtubules, a function that depends on the phosphorylation state of the protein. It is presumed that tau hyperphosphorylation results in its detachment from microtubules, free tau then polymerizes and forms paired helical filaments. These abnormal fibers, the main component of NFT, occupy the cytoplasm of neurons and are associated with neuronal degeneration [20]. Intraneuronal hyperphosphorylated tau is demonstrated in *Figure 2*.

Genetics and risk factors

AD is genetically complex and based on age of onset, traditionally subdivided into early-onset (< 65 years) and late-onset (> 65 years) AD.

Early-onset Alzheimer's disease

Identified forms of familial AD (FAD) account for less than 2% of AD cases [21], and show an autosomal dominant mode of inheritance with almost

complete penetrance. These familial forms of AD, where onset of disease is before the age of 65, are referred to as early-onset AD. The amyloid- β precursor protein (APP) gene, located on chromosome 21, was the first to be associated with early-onset AD [22], but already in the late 1960's patients with Down's syndrome (DS) were described to develop AD pathology very early in life [23]. DS patients carry a trisomy of chromosome 21, leading to an increased APP gene dosage and AD neuropathology. Recently, it was also shown that duplication of the APP locus leads to FAD [24]. Although the first mutation causing FAD was identified in the APP gene, the majority of the FAD mutations are found in the presenilin (PS) genes, PS1 and PS2. All FAD mutations have been shown to somehow affect the metabolism of A β . They do so by either enhancing steady state levels of A β , like the Swedish APP mutation (K670N/M671L) [25], or by affecting APP processing³ causing overproduction of A β 42, like the PS and many APP mutations do [26]. The clinical picture and neuropathological phenotypes are quite similar for both familial and sporadic forms of AD. This is important since it implies that FAD pathology is representative of sporadic disease and could help us model the molecular mechanisms of AD.

Late-onset Alzheimer's disease

The major risk factor for developing AD, apart from increasing age, is inheritance of the ϵ 4 allele of the apolipoprotein E (ApoE) gene. The ϵ 4 allele approximately triples the risk of developing AD and reduces the age of onset [27, 28]. Brain ApoE, primarily synthesized by astrocytes, is a component of lipoprotein micelles and has a role in mediating transport and redistribution of cholesterol. ApoE binds to A β and likely functions as a chaperone molecule to influence A β brain metabolism, deposition and clearance. The pathogenic mechanism of ApoE in AD is not fully understood, but the ϵ 4 allele is suggested to result in decreased receptor-mediated clearance of A β across the blood-brain-barrier (BBB) [29]. Other studies link the ϵ 4 allele to increased A β fibrillogenesis [30].

A large number of other disease-related loci and candidate genes have been proposed, but associations have not been verified, indicating that each unknown gene only modestly impacts on disease pathogenesis. In most cases AD is referred to as idiopathic, or sporadic, since no direct cause can be found. Numerous environmental factors are suggested to increase the risk for sporadic AD. Head trauma, low education or low cognitive reserve capacity, female gender, hypertension, cardiovascular disease and a high-cholesterol diet are likely risk factors [31]. Dietary intake of unsaturated fatty acids and antioxidants as well as moderate alcohol consumption could reduce the risk, but findings are somewhat inconsistent [32].

³ APP processing refers to the enzymatic cleavage of APP where one potential by-product is A β .

The amyloid hypothesis

More than a decade ago Hardy and Higgins presented the amyloid cascade hypothesis (*Figure 3*) which states that the A β deposition is the main causative event in AD, and that all other neuropathological features emanate from this [33, 34]. The hypothesis was based on early knowledge of APP processing, gained from cloning of the APP gene, the fact that DS patients developed AD and the identifications of a few APP mutations in and around the A β domain in families with early-onset AD. Subsequent discoveries of PS gene mutations; the ability to measure A β 42/A β 40 with enzyme-linked immunosorbent assay (ELISA) in plasma; and the finding of enhanced A β 42 as a common pathogenic mechanism of FAD strengthened the amyloid hypothesis.

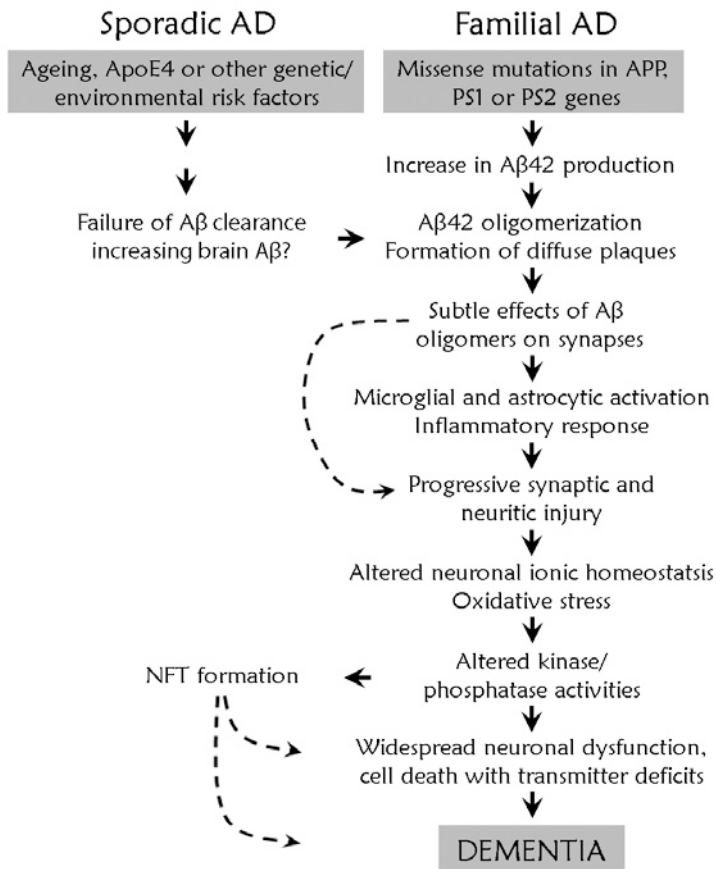


Figure 3. According to the amyloid hypothesis, A β accumulation in the brain triggers AD pathogenic processes eventually leading to neuronal death and/or dysfunction, NFT formation and symptoms of cognitive decline. The accumulation and A β oligomerization result from an imbalance of A β production and clearance [34].

APP and PS mutations, which increase A β production, result in A β plaque and NFT pathology. However, pathogenic tau gene mutations, which result in frontotemporal lobar degeneration, only present with NFT pathology [35]. Therefore, in AD the NFT pathology is likely the consequence of an imbalance in A β metabolism and plaque deposition. Tau pathology is accelerated in transgenic mice, expressing both mutant tau and APP, whereas the amyloid plaque deposition is unchanged [36], which further verifies A β as a driving force in NFT formation and neuronal degeneration. Moreover, A β immunotherapy rapidly cleared intraneuronal A β and later on removed tau pathology in a transgenic mouse, again linking A β and tau [37]. Hence, NFT formation is believed to be a downstream event in AD pathogenesis that is caused or at least facilitated by the A β pathology. A major concern with the amyloid hypothesis has been that the number and location of plaques do not correlate with the severity of dementia [38]. In fact, soluble A β is a much better determinant of the degree of neurodegeneration and appears to correlate well with cognitive decline [39, 40]. This has resulted in a recent shift in focus towards soluble A β oligomers because they, rather than fibrils, are thought to cause synaptic dysfunction and to interfere with mechanisms of learning and memory [41, 42].

APP and its processing

A β is produced during normal cell metabolism [43, 44] by enzymatic cleavage of APP, a type 1 transmembrane protein that is ubiquitously expressed. There are three major isoforms of APP, 695, 751 or 770 amino acid residues long, but the transmembrane and A β domains are the same for all isoforms. APP₆₉₅ is predominantly expressed in neurons while APP₇₅₁ and APP₇₇₀ are expressed in non-neuronal cells [45, 46]. Several functions for APP have been proposed in cell contact, growth and development. For instance APP is needed for correct neuronal precursor migration during brain development [47]. APP knock-out (APP-KO) mice are viable, probably because homologous amyloid precursor-like proteins (APLPs) compensate, although they are somewhat smaller in size and display motor deficits [48]. Soluble fragments of APP cleavage may also have neurotrophic properties and enhance synaptic plasticity and memory [49, 50]. Recently, a physiological role for A β was suggested based on data showing that A β stimulates synaptic plasticity and memory at low concentrations [51]. Others have reported that the production of monomeric A β could be enhanced by synaptic activity [52, 53] and that A β also could affect synaptic activity in a negative feedback loop [54].

APP is translocated to the endoplasmic reticulum by its signal peptide sequence, it then matures along the secretory pathway where it undergoes post-translational modification in the Golgi network before transported in secretory vesicles to the cell membrane [55]. During its transport to or at the cell membrane, APP may be sequentially cleaved by different combinations of

enzymes (known as secretases), and A β is one by-product of this proteolytic processing. In addition, endocytosis of APP also plays a major role in A β production and secretion [56, 57]. Cleavage of APP by α -secretase, between residues 16 and 17 in the A β domain, releases α APPs and leaves a C-terminal fragment (C83) in the membrane. Alternatively, β -secretase cleaves APP into β APPs and leaves another C-terminal fragment (C99) in the membrane (*Figure 4*). APP can also be cleaved by β -secretase at the β' site located after position 10 in the A β peptide, resulting in the formation of C89. C-terminal membrane fragments (C83 and C99) are then cleaved by γ -secretase, producing p3 and A β respectively as well as the APP intracellular domain (AICD). The smaller peptide p3 lacks the N-terminal part of A β and is unable to form amyloid fibrils [58]. Therefore processing which produces p3 is known as the non-amyloidogenic pathway, while A β production is referred to the amyloidogenic pathway (*Figure 4*).

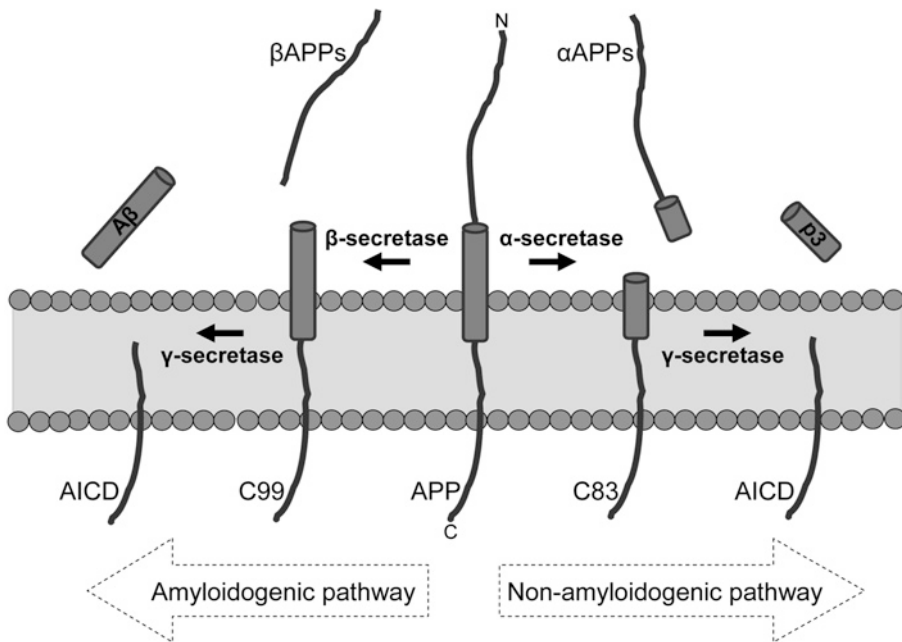


Figure 4. APP proteolysis: “amyloidogenic” with β - and γ -secretase cleavage and “non-amyloidogenic” with α - and γ -secretase cleavage.

The A β peptides found in brain vary in length but A β ₄₀ and A β ₄₂ are the most common [59] and the 42 residues long variant is more amyloidogenic [60]. Although most A β is secreted, recent evidence of an intracellular A β pool has been reported [61-63]. The γ -secretase activity is the product of an intramembranous multimeric protein complex. This complex contains nicastrin, Aph-1, Pen-2 and PS1 or PS2, which forms the active site [64-66]. Metalloproteases from “a disintegrin and metalloprotease” (ADAM) family are

likely involved in α -secretase cleavage, e.g. ADAM-9, ADAM-10 and ADAM-17 [67]. Most β -secretase activity is derived from a transmembrane aspartic protease termed β -site APP cleaving enzyme (BACE1) [68, 69].

A β aggregation

Under normal conditions A β peptides can be degraded and cleared from the brain. Neprilysin (NEP) and insulin-degrading enzyme (IDE) are both capable of degrading A β [70, 71]. A β can also be cleared from the brain via BBB transport and the low-density lipoprotein receptor-related protein (LRP) has been demonstrated to be involved in such receptor-mediated efflux [72]. However, if A β , especially A β 42, is produced in sufficiently high amounts (as in the case of FAD) or is poorly cleared, it tends to aggregate and form the insoluble fibrils found in senile plaques of AD brain. *In vitro* experiments suggest that the hydrophobic residues 17-21 of A β and the C-terminal part beginning at residue 28, are primarily responsible for the peptides propensity to self-aggregate and form β -sheet structures [73]. The formation of amyloid fibrils is a nucleation dependent process, starting with a lag phase when a nucleus is formed. This step is rate-limiting, and once the nucleus has formed, at a critical concentration of monomers, it acts as a seed to which additional A β monomers can rapidly be added [74].

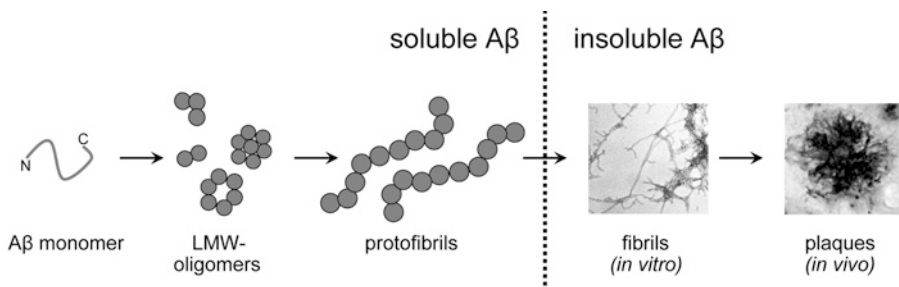


Figure 5. A schematic illustration of A β aggregation. Conformational changes in A β peptides, from α -helical and random coil to a β -pleated sheet structure, will enhance the likelihood that A β monomers begin to form soluble oligomers. Smaller oligomers, often globular in structure, grow and form large curved structures called protofibrils. Eventually insoluble fibrils are generated, the main constituents of senile plaques in AD brains.

Oligomer formation

The aggregation of A β monomers into fibrils occurs via intermediate assembly forms, illustrated in *Figure 5*. The literature on A β assemblies is vast and a number of oligomeric A β species have been described; e.g. low molecular weight (LMW) A β (e.g. dimers/trimers) [75-77], dodecamers (A β 56*) [78], amyloid- β derived diffusible ligands (ADDLs) [79, 80], globulomers [81,

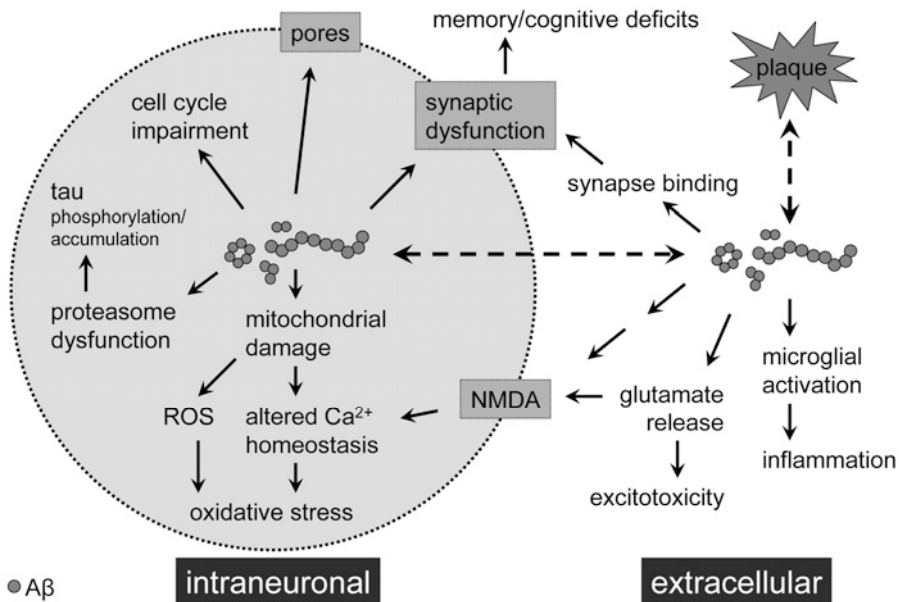
82] and protofibrils [83-85]. They differ in size and conformation, but all are soluble and remain in solution even after high-speed centrifugation. Oligomers have been demonstrated to be neurotoxic, induce synaptic dysfunction and inhibit cognition and long term potentiation (LTP) in the hippocampus [76, 78, 80, 82, 85-88]. LTP is an electrophysiological phenomenon of relevance to enhanced synaptic strength that is thought to underlie mechanisms of learning and memory. ADDLs have been detected in brains from AD patients with antibodies against synthetic A β oligomers [79]. In addition, LMW oligomers were recently extracted from AD brains and, in contrast to plaque-associated A β , found to inhibit LTP and impair synaptic function [89]. Some of the suggested pathogenic mechanisms induced by A β oligomers are presented in *Figure 6*.

The Arctic APP mutation

The Arctic APP mutation (E693G), found in a Swedish family in 2001, supports the theory that A β protofibrils are important species in AD pathogenesis [90]. Clinical and neuropathological features of mutation-carriers were indicative of early-onset AD [91]. The mutation is located within the A β sequence resulting in a substitution of a glutamic acid for glycine at position 22 leading to an increased propensity for the A β peptides to aggregate and form protofibrils *in vitro* [90, 92]. While other FAD mutations increase A β and/or A β 42 in plasma and transfected cell culture models, the Arctic mutation was surprisingly shown to reduce A β in plasma of Arctic mutation carriers and Arctic cell models [90, 93]. Moreover, the protofibrillogenic phenotype of Arc-A β increased its ability to inhibit LTP [94].

Intraneuronal accumulation

An age-dependent intraneuronal accumulation of A β , predating plaques, has been reported in the brains of AD patients [95], DS patients [96] and transgenic mice [97]. These data imply that intraneuronal A β is an early feature of AD pathogenesis and could be of importance to neurodegeneration. The theory is supported by the fact that A β is to a great extent generated within neurons [62] and oligomerized at intracellular locations [77, 98]. A β 42 also accumulates intracellularly after internalization by endocytosis [99] and internalized A β may induce endosomal and lysosomal membrane damage [100]. Moreover, A β 42 is found in multivesicular bodies and the accumulation is associated with abnormal synaptic morphology [101]. Intraneuronal A β , produced within the cell or taken up, could have several pathological effects that disturb neuronal function (*Figure 6*). For instance intraneuronal oligomers of A β have been demonstrated to disrupt the normal function of the proteasome leading to an increase in tau accumulation [102]. Intraneuronal A β also triggers mitochondrial dysfunction eventually causing free radical formation and oxidative damage [103].



● Aβ **intraneuronal** **extracellular**

Figure 6. Suggested pathological mechanisms whereby Aβ causes neuronal dysfunctions and death. Aβ could cause mitochondrial damage by binding to Aβ binding alcohol dehydrogenase and thereby increase free radical formation such as ROS (reactive oxygen species), this leads to oxidative damage and apoptosis [103, 104]. Intraneuronal oligomers are reported to impair the proteasome function, inhibit degradation of ubiquitinated proteins and eventually lead to accumulation of tau [102]. Furthermore, Aβ oligomers have been found to induce re-entry of neurons to the cell cycle [105], which could play a major role in the early neurodegenerative process [106]. Annular protofibrils may increase membrane permeability through the formation of pore-like structures, causing cell dysfunction [107]. Intraneuronal Aβ is associated with synaptic pathology *in vivo* [101], but extracellular Aβ oligomers are also able to cause synaptic damage. For instance ADDLs were demonstrated to bind to neurons and promote a decrease in memory-related receptors and cause synaptic deterioration [108]. Aβ could potentiate glutamate release [109] and thereby mediate NMDA receptor signaling and excitotoxicity. LMW oligomers, but not monomers, were found to induce synaptic loss by disturbing NMDA receptor signaling [87]. Activation of complement, astrocytes and microglia leading to the production of proinflammatory cytokines, nitric oxide and ROS are all parts of the innate immune response in AD brain. These processes could cause or at least aggravate neurotoxicity and neurodegeneration. Aβ oligomers, but not monomers, were demonstrated to activate microglia and switch them to a more cytotoxic phenotype [110]. The dashed arrows indicate the exchange of Aβ species between intracellular and extracellular pools.

Interestingly, intraneuronal accumulation of Aβ was linked to impaired memory retention in transgenic mice [111]. To establish if intraneuronal Aβ plays a significant role in AD pathogenesis it is important to consider the relationship between extracellular and intracellular Aβ. It has been suggested that these two pools are related and that extracellular Aβ originates from

intraneuronal pools [112]. Internalization of A β has been reported to occur via the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) and LRP, but the receptor for advanced glycation end products (RAGE) and the NMDA receptor could also contribute to intracellular uptake [113-116]. A β accumulated in an intracellular location may be secreted [76] or alternatively released following neuronal lysis [117].

Pathogenic mechanisms

Neurovascular dysfunction, cell cycle abnormalities, inflammatory response, oxidative stress and mitochondrial dysfunction are all events reported to occur in AD brains [118-122]. Each mechanism could be triggered by abnormal A β and tau accumulation and contribute to the loss and/or dysfunction of neurons and synapses leading to dementia. Although many disease-causing mechanisms have been suggested, and some are depicted in *Figure 6*, it is still not fully known what molecular pathways are driving the neurodegenerative process; why only certain neurons die; and what causes dementia.

Animal models of Alzheimer's disease

Simple *in vitro* models, like test tube or cell culture systems, can answer questions regarding specific biological pathways and improve understanding of mechanisms. However, animal models of human diseases are needed to enable an understanding of pathogenesis and for the development and pre-clinical evaluation of new therapeutics. An animal model can be defined as a living organism that is used for the study of certain biologic phenomena to gain knowledge about them in another species, e.g. humans. All experimental models carry the risk of being oversimplified and/or incomplete, and potentially generating results that lack relevance to pathogenic processes of human disease. Animal models allow for pathogenic pathways, or certain aspects of them, to be studied directly in a complex biological system such as the brain. An ideal animal model should imitate all the features of the disease, but in reality a model often produces one or few of the characteristics. The concept of validity is often used to estimate the relevance of an animal model [123]. Face validity means that the animal model bears similarity to the human disease which is immediately evident. Predictive validity means that the model's response to a treatment should extend to that seen in the clinic. Lastly, construct validity means that the model reconstructs and therefore mimics the human disease. It should also theoretically provide insight to disease etiology.

There are a number of nontransgenic animal models used in AD research. For instance, dogs develop diffuse A β plaque pathology, cortical neurodegeneration and cognitive symptoms [124]. Also, non-human primates (e.g.

rhesus monkeys) have been used in preclinical immunotherapeutic studies due to their naturally occurring A β plaque pathology and an immune system that is very similar to humans [125]. Still, it is unrealistic that these species could completely replace the more common rodent models in pharmacological studies where quite modest effects are expected and the number of animals therefore needs to be high. Long life cycles of large mammals and the lengthy time for these animals to develop pathology are other practical limitations. None of the nontransgenic rodent models show any of the characteristic neuropathological hallmarks of AD, but they could help us understand more general mechanisms of cognitive deficits and drug action. For instance aged animals have been used to study memory deficits during normal aging [126]. Single or chronic intracerebral injections of A β in rats could help to understand A β toxicity and accompanying cognitive alteration and also be used to evaluate A β as a drug target [127]. By administering an inflammatory stimulus, it was possible to induce neurodegenerative processes and components of chronic inflammation that are seen in AD pathology [128]. CNS-specific lesions, such as lesions of cholinergic neurons, have also been used to understand some of the mechanisms underlying cognitive deficits [129]. The most obvious problem here is of course the lack of native A β deposition in rodents, probably the consequence of differences in the A β sequence where rodent A β differs from human at amino acids 5, 10 and 13.

Transgenic models

Several genetic techniques, reviewed in [130], have been developed to generate animal models that can be used to gain better insights of normal and pathological processes occurring in humans. Almost all current transgenic models are mouse models due to available experimental techniques, although rat and pig transgenics have been developed [131, 132]. Transgenic animals are created by microinjection of a transgene, wild-type (wt) or mutated, into one of the pronuclei of a fertilized oocyte. The transgene is incorporated into the genome and the insertion occurs somewhat randomly, although there are particular hot spots for these events. The gene expression is determined by number of copies introduced, integration site and selected promoter. Addition of one or several genes by pronuclear oocyte injection can be applied to investigate gain-of-function traits; to determine if a disease-related gene can mimic or modify a disease phenotype; and for studies of the relationship between different disease-associated genes.

In knock-out (KO) mice an endogenous gene is deleted or mutated to model loss-of-function traits. The site-specific targeting of a certain gene is based on homologous recombination and carried out in embryonic stem cells, which are later injected into blastocysts and implanted into the oviducts of pseudopregnant females. The KO technique can be used to knock down a specific gene on one or both alleles or to replace the endogenous

gene with its human homologue. Such knock-out and knock-in mice are at present frequently utilized to study the biological functions of certain genes and to model disease phenotypes. In addition, more recent techniques using small interfering RNA (siRNA) and viral vectors provide increased possibilities for sophisticated *in vivo* manipulation.

AD transgenic models

Development of transgenic technologies together with the identification of FAD associated genes has revolutionized the AD research field and a wide number of transgenic models have been developed and used to gain better understanding of AD. In the mid-90's the first transgenic AD model based on a FAD mutation was developed, the PDAPP mouse [133], and in the following years two other transgenic mouse models were described, tg2576 [134] and APP23 [135]. These all express a human APP transgene and they have been extensively used as models of A β amyloidosis in AD research. PDAPP mice carry a human APP minigene with the Indiana APP mutation (APPV717F) driven by the platelet-derived growth factor (PDGF) promoter, while tg2576 and APP23 both express human APP as a single isoform with the Swedish mutation driven by the hamster prion protein (PrP) and murine Thy-1 promoter respectively. These three models all mimic a subset of AD characteristics including A β deposition, congophilic senile plaques, CAA, synaptic dysfunction and inflammation (Table 1). The discovery of the PS genes and the understanding of FAD-associated PS mutations' influence on APP processing led to the production of PS transgenic mice and the subsequent double-cross models APP/PS [136-139]. The major disadvantages of the mentioned APP and APP/PS models are their lack of NFT formation and the sparse evidence of neurodegeneration.

Tau transgenic mice crossed with APP transgenic mice resulted in amyloid deposition, tau phosphorylation, NFT formation and neuronal loss [36, 140]. However, the tangles and neuronal loss occurred only to a minor extent in AD relevant brain regions such as hippocampus and cortex. A problem partly overcome recently in a triple-transgenic mouse, 3xTg-AD [141, 142], and in a transgenic mouse model, rTg3696AB, where expression of mutated APP and tau driven by the CaMKII promoter can be regulated [143]. Although main neuropathological features of AD including amyloid plaques, NFT and neuronal loss are present in relevant brain regions, it is important to bear in mind that there are always limitations when rodents are used to model human disorders. These models mimic important neuropathological features of the human disease (Table 1), but the underlying causes differ. A high artificial expression of a transgene is often needed to drive the pathology within the relatively short life span of a mouse.

Table 1. Summary of common transgenic models of AD.

Name	Transgene	Promoter	Pathology						References	
			Plaques	Gliosis	P-tau	NFT	Neuronal loss	Cognitive deficits		
PDAPP	APP minigene (V717F)	PDGF	+	+	+	-	-	+	6-8	[133, 144-146]
Tg2576	APP ₆₉₅ cDNA (Swe)	hamster PrP	+	+	+	-	-	+	9-11	[134, 147]
APP23	APP ₇₅₁ cDNA (Swe)	murine Thy1	+	+	+	-	+	+	6	[135, 148, 149]
CRND8	APP ₆₉₅ cDNA (Swe + V717F)	PrP	+	+	?	-	?	+	3	[150]
PSAPP	Tg2576 + PS1 (M146L)	PrP + PDGF	+	+	+	-	-	+	6	[139, 151]
APP/PS1KI	APP ^{Swe,V717I} + PS1 knock-in (M233T, L235P)	Thy1 + endogenous PS1	+	+	+	-	+	+	2	[137, 152, 153]
JNPL3	4R tau (P301L)	PrP	-	+	+	+	+	+	5	[154]
TAPP	Tg2576 + JNPL3	PrP + PrP	+	+	+	+	+	?	6	[36]
3xTg-AD	APP ₆₉₅ (Swe) + 4R tau (P301L) + PS1 (M146L)	Thy1.2 + endogenous PS1	+	+	+	+	+	+	3	[111, 141]

Measuring cognition

Although several transgenic animal models develop neuropathology that resembles AD, it is important to investigate the function of these changes. The models ought to display relevant cognitive impairments in order to be of value for assessing the human disease. Behavioral phenotypes and etiology are also important when the clinical significance of potential therapeutics is to be assessed. There are a number of ways to test behavior in mice and some relevant tests for AD-related research are briefly discussed here. To investigate cognition in rodents and make conclusions that apply for the human condition is problematic. There are large inter-species discrepancies and therefore it is important that the cognitive pathway subjected to memory testing is highly conserved. The cognitive function studied should also be associated with a defined neuroanatomic structure affected in AD. For instance, patients with AD display a significant atrophy of the hippocampus along with spatial disorientation [155]. Spatial navigation is highly conserved in mammals and dependent on the hippocampus [156], and many cognitive tasks applied in AD research are based on spatial memory, e.g.

Morris water maze (MWM) and radial arm maze. However, other brain regions and functions are also affected in AD and this needs to be considered when investigating cognition.

MWM is the most common test to study hippocampal-dependent spatial learning in rodents. It was described in the 80's by Richard Morris [157] and incorporates the use of a water-filled pool with a submerged platform at a constant position and external visual cues around the test arena to help animals locate the hidden platform. Mice are trained over several days and the time or distance to reach the platform is used as a measure of spatial learning. In a probe trial the platform is removed and memory can be tested by evaluating the time an animal searches the area where the platform was previously located. An alternative is the radial arm maze, which depends on both spatial and working memory. It consists of several arms radiating from a central arena; one "correct" arm contains a concealed food reward. Number of correct and incorrect arm entries can therefore be scored instead of time, as for MWM. The need to food deprive mice in order to motivate them is a drawback as it also increases stress levels. A wet version of this test is the radial arm water maze [158, 159], with the advantage that water, a good motivational drive, replaces food deprivation.

A more general characterization of the behavior of a transgenic model could be valuable since certain phenotypes, e.g. motor impairments, could influence interpretations of a cognitive test. The open field test is an easy way to evaluate general activity levels, gross locomotor deficits and exploration in mice. Elevated plus maze and light-dark box are commonly used anxiety tests based on the mouse's inherent preference for dark protected spaces and dislike of light unprotected areas [160]. Sensorimotor deficits can be detected with a rota-rod test [161] and exploratory activity with a holeboard. Another test measuring both exploratory and cognitive behavior is object recognition, where rodents' preference for novel objects is taken advantage of. Memory impairments, claimed to be caused by dysfunction of the temporal cortex, can be detected in this task by measuring relative preference for a novel object when presented with a familiar object [162]. Fear conditioning is a type of associative learning based on functions of the amygdala and hippocampus. Mice are trained to associate a conditioned stimulus (e.g. a tone) to an unconditioned stimulus (e.g. a foot-shock) and animals develop a fear response to the conditioned stimulus presenting as freezing and increased heartbeat. Contextual fear conditioning is when the unconditioned stimulus is associated with the training context, to incorporate a spatial memory component to the test [163].

Future treatment strategies of Alzheimer's disease

Present treatments of AD are only symptomatic and do not affect the molecular pathways causing the disease. It has therefore become of utmost importance to develop new disease-modifying treatments. Since considerable evidence suggests that A β plays a central role in AD pathogenesis, most novel intervention strategies aim to facilitate the clearance of A β or inhibit its production and aggregation. A number of new drug candidates are currently in clinical trials. Inhibitors of β - and γ -secretases that reduce the synthesis of A β are promising for future drug development. However, inhibitors of these enzymes could cause adverse effects due to interference with other normal secretase functions. For instance, the β -secretase BACE1 is important for the myelination of axons [164] and γ -secretase regulates the Notch signaling pathway [165]. Different metal chelators reduced A β deposition in transgenic mice by binding zinc and copper ions, which are known to induce A β fibrillization [166]. Also, drugs that affect tau and its phosphorylation are under investigation. Here, tau kinases GSK-3 β and CDK5 have been the favorite targets.

Immunotherapy

A β immunotherapy is perhaps the most promising preventive and disease-modifying treatment strategy for AD. In 1999, Schenk and co-workers demonstrated that active vaccination with fibrillized A β 42 in PDAPP transgenic mice prevented younger mice from developing the A β related pathology and reduced progression of amyloid deposition in older animals [167]. The results were reproduced in other APP transgenic models, and the reductions in amyloid deposition were also accompanied by the prevention of age-dependent behavioral impairment and memory loss [158, 168]. The marked effects facilitated a rapid initiation of human clinical trials based on active immunization with A β fibrils (AN1792). Phase II trials were halted due to meningoencephalitis, an adverse side effect in a subgroup of patients [169-171]. However, the AN1792 trials generated valuable clinical experiences with reports suggestive of A β plaque clearance in brains of vaccinated patients and evidence of slower cognitive decline in those patients with high titers of antibodies against A β fibrils [172-174]. More recently, in a study of the long-term effects of the AN1792 vaccine there was no evidence of delayed disease progression reported, despite high anti-A β antibody titres and clearance of amyloid plaques [175]. These negative findings could perhaps be explained, at least partly, by an inefficient clearance of soluble oligomeric A β species.

Passive immunization

Passive immunization, i.e. to directly administer A β specific antibodies, was introduced as an alternative immunotherapeutic approach; since experiments early on in transgenic mice suggested that the effects of active vaccination were mediated by antibodies [176]. Passive administration of anti-A β antibodies is likely a safer alternative compared to active vaccination, since it minimizes the risk of a proinflammatory and unfavorable T-cell response [177]. It also permits better dosage control and can be halted if side effects should occur. Dosage control is particularly important in the case of AD vaccination, as elderly individuals are less capable of generating a sufficient humoral immune response to new antigens, partly due to defects in naïve CD4⁺ T cells [178]. Encouragingly, anti-A β antibodies were effective in both reducing A β pathology and improving memory in different transgenic models [176, 179-181] and several clinical trials with different anti-A β antibodies are currently ongoing. This illustrates how preclinical evaluation of an intervention strategy in transgenic models can have rapid clinical implications. Proposed mechanisms of antibody-mediated clearance of A β [182] are illustrated in *Figure 7*.

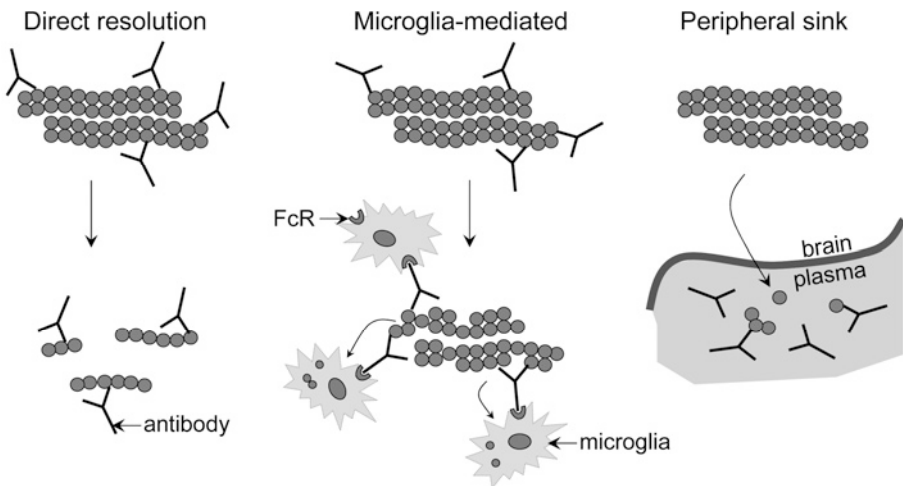


Figure 7. Suggested mechanisms of anti-A β antibodies (adopted from Weiner and Frenkel, 2006, Nat. Rev. Immunol. [182]). An A β specific antibody may reduce A β deposition in different ways: (1) Direct resolution – dissolution of fibrils or neutralization of A β oligomers by direct binding. Antibodies could also bind and sterically hinder further fibrillization. (2) Microglia-mediated – Fc-receptor (FcR) mediated phagocytosis of A β . (3) Peripheral sink – the antibodies create an efflux of A β from brain by binding and sequestering circulating A β in plasma. The transgenic model and antibody used influence the outcome, but it is likely that to varying extent all of these hypotheses play a role in A β clearance.

Aims

The overall aim of this thesis was to understand how the Arctic mutation causes Alzheimer's disease; to use it to investigate early stages of amyloid formation in an *in vivo* situation; and to target A β protofibrils with immunotherapy as a novel treatment strategy.

Specific aims

- Paper I: To investigate the pathogenic mechanism of the Arctic mutation *in vivo* and to establish a transgenic model with enhanced soluble A β aggregates in the brain.
- Paper II: To investigate the influence of the Arctic mutation on APP processing and the generation of A β .
- Paper III: To investigate the influence of A β protofibrils on cognitive functions and how they relate to amyloid pathology.
- Paper IV: To target A β protofibrils with a conformation-selective antibody *in vivo* and investigate its mechanism of action.

Present investigations

Paper I

The Arctic mutation in an *in vivo* model

The creation of an *in vivo* model with high levels of soluble A β intermediates is an important tool for determining the role of these species in AD pathology. Our hypothesis when generating transgenic mice with the Arctic and Swedish mutations (tg-ArcSwe) is illustrated in *Figure 8*. As the Arctic mutation, located within the A β sequence, results in an enhanced propensity for the peptide to aggregate; it was proposed to cause AD pathology through enhanced protofibril formation [90]. By combining the Swedish mutation [25], known to increase total A β levels, with the Arctic mutation in a transgenic mouse, the hypothesized outcome was enhanced levels of the more protofibrillogenic Arctic A β . Such a model could help to elucidate regulatory mechanisms and functional implications of the early stages in AD amyloidosis.

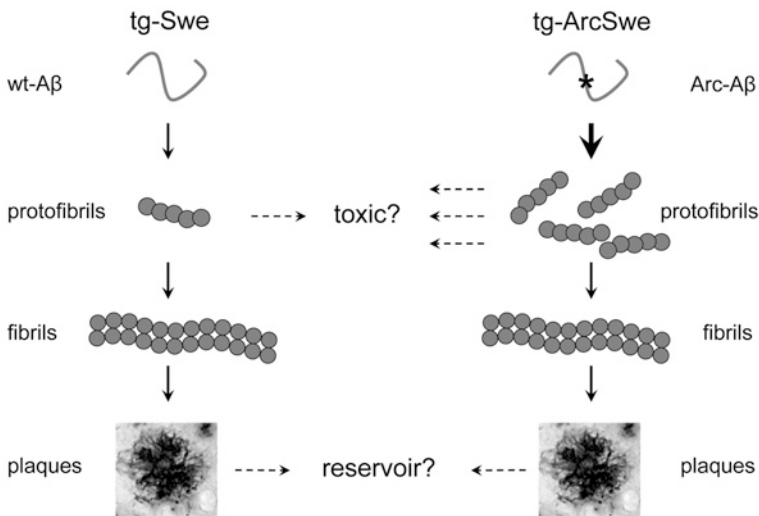


Figure 8. A schematic drawing of the hypothesis that led us to develop transgenic mice with both the Arctic and Swedish mutations. By combining the Arctic mutation, which is known to generate high levels of A β protofibrils *in vitro*, and the Swedish mutation, which is known to markedly enhance A β production, we maximized the chances for soluble A β intermediates to accumulate in the brain.

Methodological considerations

Generation of transgenic mice

The transgenic constructs used contained human APP cDNA carrying either the Swedish mutation alone or the Swedish and Arctic mutations together. The cDNA was inserted into a murine Thy-1 expression vector cassette. Similar vectors have previously been used in AD transgenic models [135, 183], and the Thy-1 promoter is known to give almost purely neuronal transgene expression. The DNA construct was microinjected into one of the pronuclei of a fertilized mouse oocyte. Injected DNA integrates into the mouse genome and as many as 100 copies can be incorporated. Injected embryos at the two-cell stage were then implanted in a pseudopregnant mouse. During optimal conditions approximately 10-30 percent of the offspring will obtain the transgene. Tail-biopsies were taken from offspring, which were on a mixed CBA/C57Bl/6 background, genomic DNA was purified and genotypes determined by PCR screening. Two primer pairs were originally used, one at the junction of the Thy-1 promoter and the APP gene and another pair around the stop codon of APP translation. These initial experiments insured that vital parts of the transgene had incorporated into the mouse genome. APP protein synthesis was then evaluated with western blot to determine human APP protein expression in the brain. Transgene-positive offspring were crossed with nontransgenic littermates for breeding of founder lines.

IHC and anti-A β antibodies

Standard protocols for IHC were used. However, brain tissue sections were pretreated in concentrated formic acid (FA) to increase signals from A β epitopes within aggregated structures and to eliminate signals from APP. Antibodies used for the characterization of intraneuronal A β are described in Figure 9.

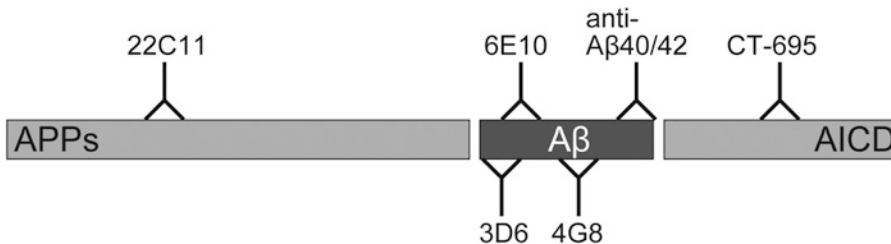


Figure 9. The following APP/A β antibodies were used for the characterization of intraneuronal A β found in tg-ArcSwe mice. 22C11 binds to the N-terminal of APP recognizing full-length APP, α APPs and β APPs. CT-695 recognizes the C-terminal of APP and binds full-length APP and C-terminal fragments (i.e. C83 and C99). A β antibodies have the following epitopes; 3D6 binds to the neopeptide-specific sequence A β 1-5, 6E10 binds to A β 3-8, 4G8 binds to A β 17-24, and anti-A β 40/42 are end-specific for C-terminal neopeptides of A β .

Biochemical measurements of A β

To measure steady state levels of A β in the transgenics, mouse brains were homogenized in a three-step procedure: first, carbonate extraction; second, SDS-detergent extraction and third, FA extraction. This enabled ELISA-measurements of soluble, SDS-soluble (membrane bound) and insoluble pools of A β respectively. A β 1-40 and A β 1-42 ELISA measurements were performed on the different extraction pools.

Results and discussion

Several lines of transgenic mice expressing human APP were developed; the most promising lines displayed threefold overexpression (tg-ArcSwe and tg-Swe) and one tg-Swe line showed as much as sevenfold overexpression of human APP. The expression was restricted to neuronal tissue and appeared to be strongest in hippocampus and neocortex, i.e. brain regions affected in AD patients. The most prominent phenotype in tg-ArcSwe mice was an early appearance of intraneuronal A β . The intense A β immunostaining within the neurons was granular and detection depended on FA pretreatment, indicative of A β aggregates with strong intramolecular interactions. The number of A β aggregates increased with age and predated plaque formation, an observation leading us to believe that intraneuronal A β was an intermediate species in the aggregation process. In fact, the idea that amyloid starts to deposit within neurons in AD brains was originally suggested in 1985 [184]. This hypothesis was neglected for a long time, mainly because the focus had instead been on the secretion and extracellular A β accumulation. In order to exclude the possibility that intraneuronal A β is a phenotype that depends on the Arctic mutation only, we investigated tg-Swe with sevenfold APP overexpression. At seven months of age, prior to plaque onset in this model, a similar intraneuronal A β staining was then noticed, albeit to a lesser extent.

Notably, the intraneuronal A β was negative for thioflavine S, ApoE and ubiquitin, all markers of fibrillar A β deposits. The finding of non-fibrillar A β aggregates within neurons agrees with a study by Langui and co-workers [185]. The intracellular A β aggregates were best detected by N-terminal A β antibodies, leading us to speculate that the aggregates were of a micelle structure, with exposed N-terminal epitopes and concealment of hydrophobic C-terminal parts. Another possible explanation for different antibody reactivity could be the presence of C99 fragments that cannot be detected with A β 40 and A β 42 specific antibodies. Results in a recent study from our group indicate that A β 38 is one of the major A β species in tg-ArcSwe mice [186], which could also help explain the modest binding of C-terminal A β antibodies.

As the steady state levels of A β in young tg-ArcSwe did not greatly differ from tg-Swe (both lines had the same overexpression of human APP) we believed that the accumulation of intraneuronal A β was facilitated by the

protofibrillogenic character of Arc-A β , and not an overall increase in A β levels. However, this suggestion was based on ELISA-measurements using A β 40 and A β 42 C-terminal specific A β antibodies, thereby precluding detection of other C-terminal truncated A β peptides, such as A β 38. Another problem with these A β ELISAs is the fact that aggregates of A β , which probably hide their hydrophobic C-terminal epitopes, could lead to underestimated A β levels. This was confirmed as A β levels in tg-ArcSwe mice decreased with a non-denaturing ELISA (adopting A β 40 and A β 42 specific antibodies), but increased when we used a denaturing SDS-PAGE and Western blot [187]. In a recent study, enhanced levels of soluble A β protofibrils were detected in tg-ArcSwe compared to tg-Swe mice [188]. Taken together the data indicate that the tg-ArcSwe model expressed high levels of soluble A β aggregates, leading to underestimated A β levels when measured with ELISA based on C-terminal A β antibodies. Early intraneuronal accumulation of A β in tg-ArcSwe mice may therefore be explained by both increased total A β levels and an enhanced property of Arc-A β to aggregate.

The intraneuronal immunoreactivity declined with increased plaque formation, a finding also reported in another mouse model [189] and in DS patients [96]. Extracellular amyloid deposition in tg-ArcSwe mice started at roughly six months of age and was strongly accelerated as compared to tg-Swe. Similar results were also found in another study where the Arctic mutation was used in transgenic mice [190]. In tg-ArcSwe, the plaques were surrounded by reactive astrocytes and dystrophic neurites that were positive for phosphorylated tau, and almost all plaques bore an amyloid core. In DS and AD brain most of the parenchymal plaques begins with and mainly consists of A β 42 peptides with A β 40 adding on at later stages [191, 192]. However, in our tg-ArcSwe model most of the core plaques were immunoreactive for A β 40 suggesting that the highly fibrillogenic property of Arc-A β 40 is enough to generate amyloid core plaques. The study implies that A β starts to self-associate within neurons and once released from neurons (or perhaps after cell lysis) the A β aggregates can act as nuclei of A β fibrillization. Hence, the tg-ArcSwe mouse is a good model to increase the knowledge of intraneuronal A β and early pathogenic stages of A β aggregation and fibrillization.

Paper II

The Arctic mutation triggers intracellular production

As FAD linked mutations increase A β levels or the A β 42/A β 40 ratio, it was a bit surprising that the initial mechanistic studies of the Arctic mutation demonstrated reduced levels of A β in plasma of mutation-carriers. Instead the mutation directly affects A β aggregation, with Arc-A β peptides having

an enhanced propensity to form A β protofibrils [90]. However, it was later shown that the Arctic mutation also interferes with APP processing *in vitro* [93]. The objective of this study was to determine if the Arctic mutation also affects APP processing *in vivo* and to elucidate the mechanism thereof. Furthermore, the study aimed to investigate how changes in APP processing relate to intracellular A β accumulation in the transgenic model, tg-ArcSwe.

Methodological considerations

Different proteolytic APP fragments in the brains of tg-ArcSwe and tg-Swe mice (with the same threefold overexpression of human APP) were compared to estimate the influence of the Arctic mutation on APP processing *in vivo*. Moreover, we detected A β in cell culture models carrying either APP-wt or APP-Arc constructs, enabling us to observe effects of the Arctic mutation independent of the Swedish mutation. Steady state levels of different APP fragments were measured in young mice, prior to A β deposition.

Western blot

SDS-PAGE separates denatured proteins from a sample based on their charge to mass ratio, resulting in a relationship between molecular weight and distance migrated in an electric field. After transferring proteins to a nitrocellulose (or nylon) membrane proteins/peptides of interest can be detected with antibodies, specific for certain epitopes. APP is detected as a ~100 kDa large band with either antibody 6E10 or CT-695, C-terminal fragments (C83, C89 and C99) as ~9-12 kDa bands with the CT-695 antibody and A β as a ~4 kDa band with the 6E10 antibody. β APPs was recognized with the Sw192 antibody, specific for β APPs carrying the Swedish mutation [193]. For protein bands of ~100 kDa, 6E10 detects both full-length APP and α APPs, whereas CT-695 only detects full-length APP. As full-length APP and α APPs did not separate sufficiently to yield two distinct bands, the difference between 6E10 and CT-695 immunostaining was therefore used as an estimate of α APPs levels. Epitopes of 6E10 and CT-695 are illustrated in *Figure 9*.

Results and discussion

Initially levels of N-terminal APP fragments were measured in brain and the results showed a shift toward proamyloidogenic APP processing in tg-ArcSwe mice in comparison with tg-Swe mice, confirming and extending previous observations in cell cultures [93]. Next, C-terminal APP fragments were analyzed, and increased levels of C99 were found *in vivo* and *in vitro*, whereas C83 levels were only reduced *in vitro*. This *in vivo* increase of C99 and C89 levels with static C83 levels, has been described earlier when human APP transgenics were crossed with human BACE overexpressing mice [194], suggesting that the turnover mechanism of C-terminal APP fragments could differ in cell culture and transgenic mouse brain. The altered APP

processing favoring β -secretase cleavage leads to increased steady state levels of A β both at extracellular and intracellular locations (by 240% and 480% respectively), implying enhanced A β production and intracellular A β accumulation. Interestingly these results support the observation of intraneuronal A β accumulation in tg-ArcSwe mice seen in paper I and later verified by another group [195].

We considered several mechanisms that could explain how the Arctic mutation alters APP processing and thereby generates more intracellular A β . One possibility is that Arctic APP is an inferior substrate for α -secretase, indirectly favoring β -secretase cleavage and A β generation through reduced substrate competition [196]. This idea seems plausible as the Arctic mutation is located within the A β sequence not far from the α -secretase cleavage site. Moreover, A β fragments with the Flemish mutation (A692G), located at the adjacent amino acid of the Arctic mutation, reduce the efficiency of ADAM-10 mediated cleavage [197]. To investigate this hypothesis, we developed a cell-free enzymatic assay where various concentrations of Arc-A β and wt-A β fragments (amino acids 10-24) were digested by recombinant ADAM-10, one of the main α -secretase candidates [198]. However, ADAM-10 did not show any preference for the wt-A β substrate, when compared to Arc-A β .

We then speculated that the Arctic mutation could affect APP trafficking, causing altered subcellular location of APP, which could influence its availability to different secretases. BACE1 cleavage mainly occurs in acidified compartments in the trans-golgi and endosomal pathways [68], whereas α -secretase cleavage predominantly takes place at the cell surface [199, 200]. According to our idea, a reduced presence of Arctic APP at the cell surface would reduce α -secretase cleavage and thereby explain the shift towards increased β -secretase cleavage and A β generation. Concordantly, cell-surface biotinylation experiments followed by immunoprecipitation and western blot demonstrated 20% reduction in levels of cell-surface APP in APP-ArcSwe as compared to APP-Swe cells. However, in cellular models, β -secretase cleavage of APP-Swe occurs to a greater extent along the trans-golgi network secretory pathway as opposed to APP-wt where endocytic processing predominates [201]. Although a more recent study in tg2576 mice, carrying the Swedish mutation, implied that the majority of the A β production was dependent on endocytosis-associated mechanisms [57], we still wanted to confirm that the reduction in cell-surface APP by the Arctic mutation did not depend on the Swedish mutation. Therefore, the same experiments were used to compare APP-Arc and APP-wt cells. This comparison gave an even greater (60%) reduction in cell-surface APP in cells harboring the Arctic mutation, demonstrating that the effect of the Arctic mutation on cell-surface APP location did not entirely depend upon the Swedish mutation.

To summarize, we showed that the Arctic mutation leads to reduced presence of APP at the site of α -secretase cleavage, either by decreased cell sur-

face transport or increased endocytosis, thereby affecting APP processing. The shift in proteolytic cleavage leads to increased production of intracellular A β in cell culture experiments, a mechanism that likely contributes to an enhanced intraneuronal A β accumulation in the brains of transgenic mice harboring the Arctic mutation.

Paper III

In vivo function of A β protofibrils

A new method to specifically measure A β protofibrils in biological samples was developed in our group and protofibril levels were found to be increased in the tg-ArcSwe model [188]. If A β protofibrils are central toxic species in AD pathogenesis their elevation in tg-ArcSwe mice would likely have considerable consequences. To investigate the *in vivo* function of protofibrils we quantified them with the novel ELISA and then correlated levels to cognitive abilities. By examining how these soluble A β species quantitatively relate to measures of insoluble A β pathology we also aimed to estimate their likely role in the A β aggregation process.

Methodological considerations

The protofibril ELISA

The protofibril specific sandwich ELISA system [188] employs the monoclonal antibody 158 (mAb158) as both capture and detection antibody (*Figure 10*).

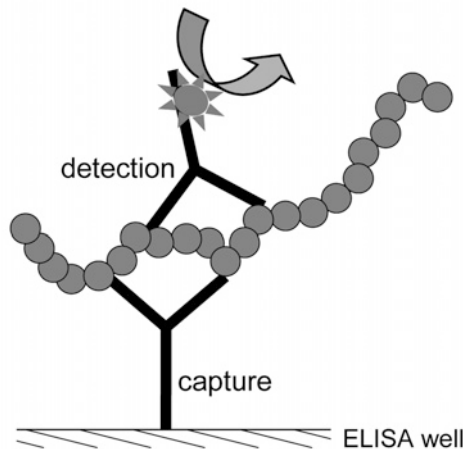


Figure 10. The protofibril sandwich ELISA is based on mAb158, both as capture and detection antibody. The detection antibody is conjugated to an enzyme (directly or indirectly via a biotin-streptavidin step). The enzyme converts a substrate to a colored product which can be spectrophotometrically measured.

This permits the specific measurements of large A β oligomers. The antibody has a protofibril-selective epitope that depends upon the N-terminal part of the A β sequence. It was reported that mAb158 has a 200-fold higher affinity for A β protofibrils than for LMW A β . Although mAb158 does bind A β monomers when they are present in great excess, this system prevents monomer detection, as the sandwich will only be completed when the identical capture and detection antibodies each bind to an individual epitope. Moreover, the protofibril signal was not affected in the ELISA when measured in the presence of a 5×10^5 -fold molar excess of A β 1-16, representing A β monomers [188].

Spatial learning

A MWM task was applied to measure spatial learning and memory, since it relies on functions of the hippocampus which are affected early in AD pathogenesis, as previously mentioned. Tg-ArcSwe mice display early A β pathology as intraneuronal A β accumulation (described in paper I) and elevated A β protofibril levels. Both these characteristics emerge long before the appearance of extracellular plaque pathology. To evaluate the function of early pathology we tested young mice, aged four and eight month-old, in a MWM setting. At this age, mice do not have plaque deposits or are just beginning to develop plaques. Animals were handled and habituated to the new environment prior to the test and once acquisition began they were given five trials a day for four consecutive days. They were allowed to swim for up to 60 seconds and time taken to escape onto the hidden platform was recorded. All swim sessions were monitored by a video camera to allow subsequent analyses of swim paths and speed. The platform was submerged at a constant position throughout the study, but starting points for the mice were varied. Time to reach the platform (escape latency) was recorded and used as a measure of learning. Calculations of improvement in spatial learning (by subtracting the time in the last training trial from the mean escape latency the first day of acquisition) were completed to take initial performance of the individual mice into consideration, as this likely affects how well the animals performed at subsequent days of training.

Seventy-two hours after the last training session mice were tested in a probe trial where the platform had been removed. The animals were allowed to swim for 60 seconds and time spent in a predefined goal area around the previous platform position (twice the area of the platform and 2.5% of the total pool area), and number of platform crossings were used as measures of memory retention. Memory retention is typically assessed 24 hours after last training trial, but this 72 hour protocol has previously been validated by our collaborators [202]. In fact, even longer delays have been used by others [203]. Although memory retention is commonly reported as time spent in the target quadrant, we used time spent in the goal area which is a more demanding measure and perhaps reflects memory retention more accurately [204].

Measuring soluble and insoluble A β

To assay the effect of age on different pools of A β , brains from tg-ArcSwe mice of different ages (2-17 months of age) were homogenized in a two-step procedure: mild extraction in tris buffered saline (TBS) followed by high speed centrifugation and harsh re-extraction of the pellet in FA. This enabled ELISA-measurements of soluble and insoluble pools of A β respectively. To biochemically measure total A β levels, FA-extracted brain homogenates were neutralized in 1M Tris (pH 10) and loaded onto ELISA-plates coated with the 82E1 antibody (neopeptide-specific for A β 1-5). A β peptides with the Arctic mutation were then detected by the mid-region anti-A β antibody mAb27 (characterized in supplementary figure 3). Plaque-associated A β was estimated by visualizing extracellular deposits with immunohistochemical anti-A β staining and mature amyloid plaques with Congo red staining.

Results and discussion

In this study we were able to detect A β protofibrils in 2-month-old tg-ArcSwe, PSAPP and tg2576 mice, but levels were undetectable in nontransgenic mice as well as APP-KO mice. Protofibril levels were approximately three-fold higher in tg-ArcSwe compared to the other two AD mouse models, reinforcing our previous finding of lower protofibril levels in tg-Swe compared to tg-ArcSwe [188]. Yet the data suggested that protofibril formation occurs in other tg-APP models as well and could be of general importance. Interestingly, including the human PS1 with the FAD linked M146L-mutation did not affect protofibril levels, neither in young nor old mice, despite its ability to accelerate plaque pathology. We, and others [89], have used very mild extraction procedures and high speed centrifugation for the brain homogenization. Still the supernatant of brain homogenates could contain small amounts of plaque-derived A β species, detached during homogenization, and generate false positive signals. However, we found significant amounts of protofibrils in CSF from middle-aged tg-ArcSwe mice. The presence of A β protofibrils in this compartment suggests that these species exist as soluble and biological A β assemblies, and are not artifacts from the homogenization procedure.

Protofibril levels remained rather stable with age, although a modest but significant increase was seen between the 10- and 14-month-old mice. This increase did not reflect the overall rise in total A β that appeared once plaque deposition began. Amyloid fibril formation typically involves a rate-limiting lag phase when a nucleus is produced followed by an elongation phase when amyloid fibrils are formed very rapidly. This phenomenon may explain the exponential and age-dependent development of amyloid deposition in APP transgenic mice, and potentially explains the increment in biochemically measured total A β . Although the soluble protofibril pool did not reveal the same exponential increase as seen for levels of insoluble A β and plaque

deposition in aged tg-ArcSwe mice (17-month-old), we saw a closer relationship between protofibrils and plaque pathology in younger mice (10- and 14-month-old). Protofibrils are likely important early in the amyloidogenic process, and high individual levels of protofibrils were associated with more plaque burden. The close relationship between protofibrils and core plaques, detected with Congo red, remained in older mice, but the correlation to immunostained A β plaques disappeared. An increase in plaque size with a relatively constant Congo burden likely explained the accelerated plaque deposition seen between 14- and 17-month-old mice. Therefore soluble A β species, such as protofibrils, are probably added to existing A β deposits resulting in growing plaques. It is unlikely that A β protofibrils form due to detachment from plaques, as this should be reflected by higher A β protofibril levels in aged mice with substantial plaque load.

As the proportion of A β protofibrils was highest in the 4-month-old mice, at a time point when intraneuronal A β peaks (paper I), it was decided to examine cognitive functions at this age. Four and eight month-old mice were combined since the groups were relatively small and they represent a reasonably similar stage of pathology. It is important to emphasize that we first determined that age had no effect on MWM escape latency in a single variance analysis, and we thereafter pooled the age-groups. The similar performances of tg-ArcSwe mice, regardless of age, perhaps reflect that these mice are at a stage when intraneuronal A β and elevated protofibrils are present, but prior to the substantial development of extracellular plaque pathology. A modest effect on learning was seen during the third day of training with longer escape latencies (and swim paths) for transgenic mice as compared to their nontransgenic littermates. On the last day of acquisition the differences were no longer significant, probably due to a ceiling effect where the non-transgenic mice could not improve further. Interestingly, learning improvement inversely correlated with protofibril levels in individual mice. Animals that improved the most in MWM were also the ones that had the lowest levels of protofibrils indicating that early A β pathology, such as protofibrils, could affect cognitive function. However, other A β species might also contribute to, or perhaps explain, the spatial learning deficits. It is quite possible that, in complex samples like brain homogenates, other soluble A β aggregates are detected by the protofibril ELISA. In fact, a synthetic preparation of ADDLs (protocol described in [205]) could be detected with the ELISA. However, the ADDLs protocol generated oligomers of a wide size-range, and when fractionated on a gradient by ultracentrifugation only the large high molecular weight forms of A β oligomers were detected by the protofibril ELISA (Englund, Sehlin *et al.*, unpublished data). Although different forms of large A β oligomers were likely measured in this study, we found it reasonable to use the term protofibrils as the assay is based on mAb158, an antibody, generated from mice immunized with synthetic A β protofibrils and thereafter characterized to be highly protofibril-selective [188].

In addition, the early and prominent intraneuronal A β accumulation in tg-ArcSwe mice could affect functions linked to learning. In fact, intraneuronal A β accumulation seems to be an early pathological event that could promote cognitive deficits in AD transgenic models [111, 152, 195]. We therefore quantified accumulation of intraneuronal A β in the CA1 layer of hippocampus by 6E10-IHC and image analysis. Data, which were not included in paper III, showed that punctate staining within pyramidal neurons of 4 month-old mice correlated with protofibril levels in the same set of mice. Moreover, we observed a trend of intraneuronal A β staining inversely correlating with spatial learning. Though likely connected early in the amyloidogenic process, these two measures of A β pathology are diverging over time with protofibrils being relatively stable (paper III) and intraneuronal A β decreasing as plaque pathology accelerates (paper I). It is of course important to mention that protofibrils are assayed as soluble species in TBS extracts of whole brain hemispheres, whereas CA1 intraneuronal A β accumulation, measured with histological techniques, is probably located within multivesicular bodies and/or other acidified compartments in the pyramidal cells of hippocampus. However, by measuring these early phenotypes of amyloidosis, located at different compartments and probably representing different pools of A β , we wanted to assess the relationship between the two.

Our data suggest that A β protofibrils emerge long before the plaques appear and could lead to cognitive deficits associated with AD pathogenesis. If so, targeting these species could be a promising new therapeutic strategy to improve cognitive functions in AD patients.

Paper IV

Targeting A β protofibrils with immunotherapy

Ever since the initial positive report of A β vaccination ten years ago and the subsequent clinical trials, the research on anti-A β passive immunization has intensified. Oligomeric A β species receive increasing attention in the field and we found a relationship between A β protofibrils and learning disabilities in tg-ArcSwe mice. We therefore set out to target protofibrils selectively with passive immunization using mAb158.

Methodological considerations

Immunizations and antibodies

Two different strategies were evaluated; one where antibodies were given before development of A β plaques (*preventive treatment*) and another where the antibodies were administered after extracellular plaques had appeared (*curative treatment*). The latter should reflect the human situation where patients have substantial amounts of plaques in the brain when diagnosed.

Two different anti-A β antibodies were tested; mAb1C3 (IgG1) had a linear epitope and mAb158 (IgG2a) had a conformation-dependent epitope that was highly selective for A β protofibrils (*Figure 11*). Importantly, both antibodies depend on N-terminal epitopes of A β , and it was thereby possible to investigate the importance of conformation-selectivity. However, the IgG subclass of the two antibodies was different and this could of course lead to some misinterpretations. As a negative control we used the IgG1 antibody Ly-128 which recognizes the p41 flagellin structure of *Borrelia*. To estimate mAb1C3 and mAb158 levels in the brain after treatment we set up a direct ELISA for TBS extracts. Plates were coated with A β 1-42 and antibodies present in brain extracts were captured and subsequently detected by anti-mouse IgG antibodies.

An important aspect to consider is whether mAb158, used for immunization, could end up in the brain and interfere with the A β protofibril ELISA, as the ELISA is based on the same antibody. Immunization antibodies could potentially bind to protofibrils, mask the epitopes and result in an artificially low signal. Therefore we investigated the ability of mAb158 to interfere with the ELISA by mixing different concentrations of mAb158 with protofibrils of known concentrations and incubating these mixtures prior to analysis with the ELISA. Results, presented in supplementary figure 3 in paper IV, indicated that mAb158 concentrations needed to be ~50 ng/ml in order to interfere with the ELISA, concentrations more than ten times higher than antibody levels in TBS brain homogenates of treated mice.

Biochemical and histological methods were used to evaluate the efficacy of antibody treatment. Soluble and insoluble pools of A β were measured with different ELISAs and effect on intraneuronal A β accumulation, plaque pathology and amyloid plaques were evaluated with immunohistochemistry and Congo red staining.

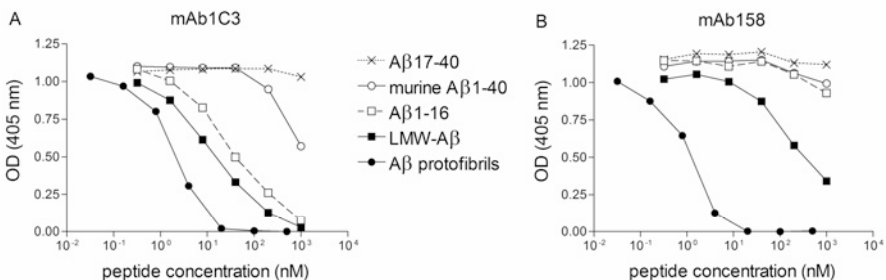


Figure 11. The epitopes of mAb1C3 (A) and mAb158 (B) were investigated with inhibition ELISA (the method is described in [188]). The 1C3 antibody bound to A β 1-16, A β protofibrils, low molecular weight A β and murine A β 1-40 peptides, whereas mAb158 only bound A β protofibrils and low molecular weight A β . Thus mAb1C3 has several alternative and competitive targets that could potentially influence its therapeutic efficacy.

MTT cell toxicity assay

This common assay of cell viability is based on a colorimetric reaction where the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced by living viable cells. The reduction, occurring intracellularly involving pyridine nucleotides [206], yields water and purple formazan crystals that are insoluble in aqueous solutions. The formazan crystals are then dissolved and the purple solution is measured in a spectrophotometer as absorbance at 570 nm. The degree of MTT reduction will reflect the number of viable cells. Protofibril-mediated toxicity was estimated in the MTT assay and the ability of the treatment antibodies to protect cells from toxicity was evaluated and expressed as “protection”. The protective effect was investigated in the presence or absence of non-toxic A β 1-16 in excess, to evaluate how alternative targets of the antibodies could compete with the binding to A β protofibrils in an assay with a biological readout.

Thioflavin-T assay

The β -sheets of amyloid structures are traditionally stained with Congo red, a dye that generates “apple-green/gold” birefringence in polarized light. Amyloid plaques can also be visualized with the fluorescent benzothiazol salt Thioflavin-T (ThT). When ThT binds to amyloid β -sheet structures, such as A β fibrils, the excitation maximum is shifted and enhanced compared to free dye alone [207]. The property of ThT to shift excitation to 450 nm and emission to 482 nm when binding β -sheet structures allows for *in vitro* studies of A β fibril formation kinetics. Oligomers of A β contain β -sheets, though not to the same extent as fibrils, and can therefore also increase the amount of emitted light at 482 nm. Indeed protofibrils increase the ThT signal, but to a lesser extent than fibrils [208]. The ThT assay was used to investigate the ability of different antibodies to affect the A β fibril formation in an attempt to understand the mechanism of anti-A β antibody efficacy, and to compare antibodies.

Results and discussion

In this study we reported that a protofibril-selective antibody could diminish levels of soluble A β protofibrils in young and aged tg-ArcSwe mice. However, plaque pathology was only affected if the treatment started before the onset of plaque deposition. This emphasizes the difficulty of clearing core plaques, such as the ones seen in the tg-ArcSwe model. We previously reported that the senile plaques of tg-ArcSwe mice were highly insoluble and similar to those of AD brains and also harder to clear with acute administration of an anti-A β antibody compared to another model (tg-Swe) with more diffuse plaque pathology [186]. Here we found that mAb158 had a greater potential to cross the BBB than mAb1C3. It is likely that mAb158, by bind-

ing protofibrils in a selective manner, was able to clear these species from the brain and thereby hinder the appearance of extracellular plaques. This strongly suggests that protofibrils are *in vivo* intermediate species of plaque formation. The lack of effect on intraneuronal A β , perhaps explained by the non-existent binding to cell-surface APP and no subsequent cellular uptake [209], stresses the difficulty of accessing an intracellular target with immunotherapy. Intraneuronal A β has been linked to loss of cognition in AD models [111, 152, 195], and therefore clearing these species could be of key importance. See *Figure 12* for a simplified view of our mAb158 efficacy hypothesis.

When examining the *in vitro* properties of mAb158, we found that the antibody-protofibril binding resulted in inhibition of further aggregation and reduced toxicity. In a kinetic experiment where antibody and A β 1-42 was co-incubated for 5 days, the formation of fibrils was essentially blocked. It is possible that further fibrillization of protofibrils is inhibited by the antibody through steric hindrance, explaining some of the *in vivo* efficacy. However, parts of the plaque-preventive effect in tg-ArcSwe mice could also be explained by FcR-mediated phagocytosis of protofibrils leading to clearance of an A β aggregation intermediate, necessary for *in vivo* fibrillization. The *in vitro* protection from protofibril-mediated toxicity was a key finding that could have implications for the *in vivo* situation, but perhaps the antibody's ability to maintain this protective function even in the presence of a major excess of A β monomers is of greatest importance.

A bit surprisingly mAb1C3 had no therapeutic effect, although this antibody was able to inhibit fibril formation *in vitro* and diminish the toxicity induced by protofibrils. However, the antibody had a reduced capacity to protect cells from protofibril-mediated toxicity when an excess of A β monomers were present. Results from the inhibition ELISA also indicated that endogenous APP fragments were alternative targets of mAb1C3. Therefore, the absence of antibodies in the brain after treatment could be due to binding of APP or APP fragments in the periphery, resulting in their degradation during the APP or APP fragment metabolism. This highlights a possible and problematic consequence of alternative targets of an immunization antibody. Due to its highly selectivity towards protofibrils, mAb158 is less likely to bind other targets, i.e. A β monomers. This could be an important aspect, since significant biological functions e.g. roles in neuronal activity, have been proposed for A β [54]. Furthermore, our experiments suggest that despite substantial amounts of insoluble plaques it is feasible to selectively clear soluble A β aggregates from the brain, at conditions reflecting those in AD brain where insoluble A β plaques constitute an overwhelming majority of total A β .

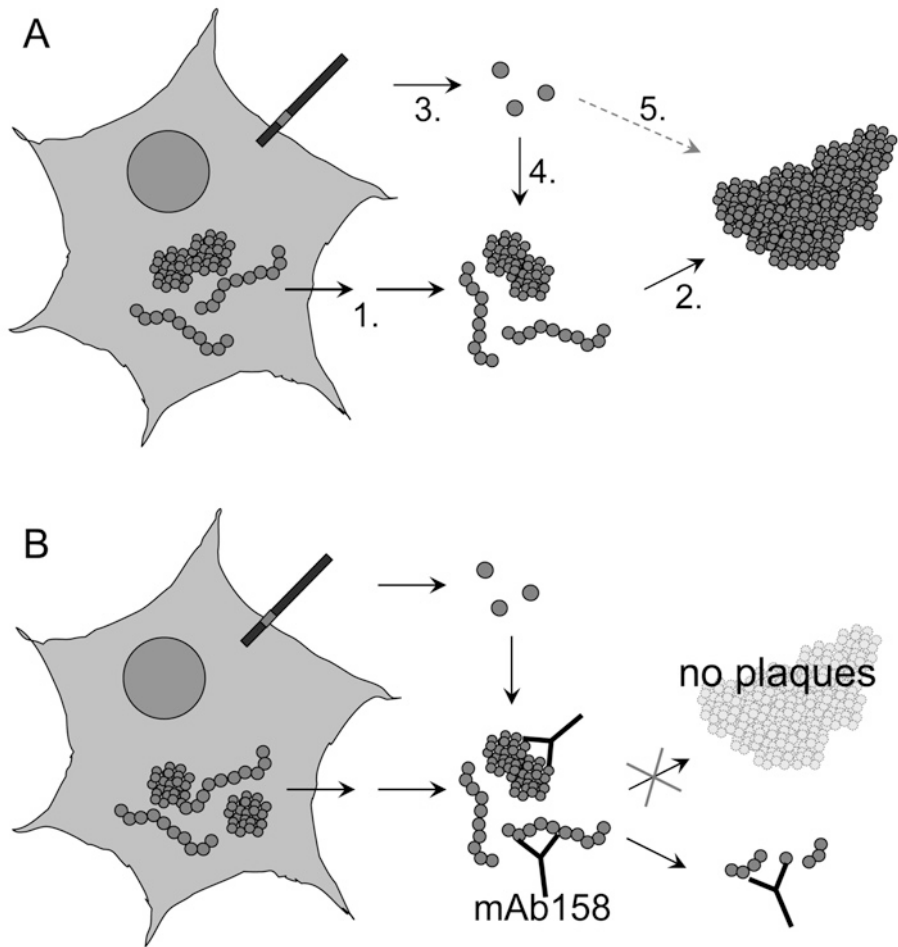


Figure 12. A schematic illustration of mAb158 efficacy in tg-ArcSwe mice. (A) Our data suggest that protofibrils are intermediate species in the *in vivo* formation of plaques. **1.** A β starts to aggregate within the cell and could after cell lysis or exocytosis be released to the extracellular space. **2.** Eventually this leads to the formation of plaques. **3.** A β monomers are also produced by APP cleavage and secreted at the cell surface. **4.** Monomers could form oligomers or add to existing A β protofibrils at extracellular locations. **5.** It is likely that A β monomers add to existing plaques as well. (B) When mAb158 enters the brain the formation of plaques is severely delayed. The binding of antibodies clears protofibrils and prevents plaque pathology, but has little to no effect on A β monomers. In theory, mAb158 could bind to the plaque surface and shift the equilibrium to plaque degradation, but results in the curative treatment study question the importance of this process.

General discussion and future perspectives

A central role for A β in AD pathogenesis was suggested more than 15 years ago and today this theory remains widely accepted. Despite ongoing and thorough studies there is still an incomplete understanding as to how A β causes the neurodegeneration and dementia of AD. More and more evidence presents A β oligomers as important pathogenic species. In this thesis the Arctic mutation, which increases A β protofibril levels, was used as an experimental tool to study the role of these intermediates in the AD pathogenesis. *In vitro* studies of the Arctic mutation demonstrated not only protofibril formation, but also altered APP processing resulting in more A β both at intracellular and extracellular locations. This implied that the Arctic mutation not only changed the properties of A β , but also had an impact on production and distribution of A β . The Arctic mutation was further investigated *in vivo* in a novel transgenic model of A β amyloidosis. Here it was used to examine the function of early stages of amyloid pathology, such as protofibrils, and also to evaluate therapeutics, targeting early stage A β pathology. Long before amyloid plaques appeared, the tg-ArcSwe model showed increased formation of A β protofibrils and intraneuronal accumulation of A β , suggesting that these are intermediate species of plaque formation. Since intraneuronal A β peaked before plaque onset and then diminished when plaque pathology accelerated, it is likely that intraneuronal A β represents an early stage of A β accumulation that precedes and perhaps even is a prerequisite for senile plaque formation.

With the intent to evaluate the biological significance of early amyloid pathology in AD, tg-ArcSwe mice were further characterized with respect to cognitive functions. Although spatial learning was impaired to some extent and these deficits were associated with protofibril levels, the model needs to be more extensively investigated in other cognitive and behavioral tasks. It would also be interesting to extensively study cognition in older tg-ArcSwe mice, as amyloid load has also been reported to correlate with cognitive deficits [210, 211]. Another important experiment would be to isolate protofibrils from tg-ArcSwe brain and determine their effect on synaptic plasticity and memory. This could be achieved by investigating their ability to influence LTP in electrophysiological experiments. The demonstration and characterization of these A β species in brain and CSF from AD patients is of course central to determining their ultimate relevance. Since the mAb158

protofibril ELISA only measures larger oligomers, it is possible that A β oligomers of smaller size, present in tg-ArcSwe brain, negatively influence cognition as well. In fact, LMW A β dimers isolated from AD brains caused synaptic and memory impairments, possibly by affecting the NMDA and other glutamate receptors [87, 89]. Events downstream of both A β protofibril formation and intraneuronal A β accumulation could also contribute to learning deficits in tg-ArcSwe mice. Mechanistic studies of cellular effects induced by protofibrils could help expand the understanding of their pathological function.

The ultimate test to explore the biological significance of A β protofibrils would be to clear these species from the brain and to investigate functional effects of such clearance. In an effort to do this we specifically targeted A β protofibrils by passive immunization with mAb158. The treatment was found to be highly selective and lead to the clearance of protofibrils, leaving monomeric A β and insoluble A β pools essentially unchanged. The antibody efficiently reduced protofibrils even when plaque-associated A β was present in a great excess. In mice where the extracellular A β pathology had not yet appeared the treatment prevented formation of senile plaques, indicating that protofibrils are critical intermediate species of *in vivo* fibrillization. Although mAb158 was effective in protecting against protofibril-mediated toxicity in a cell-based model, biological implications of the protofibril-selective clearance need to be further investigated. If the treatment could improve spatial learning performance in tg-ArcSwe mice, and perhaps also ameliorate other AD-related cognitive deficits, this could have implications for future intervention strategies designed to diminish cognitive symptoms of AD patients.

The importance of intraneuronal A β accumulation was also evaluated in this thesis. A β starts to form aggregates within the neurons, an event occurring before the appearance of extracellular pathology, and this process is accelerated by the Arctic mutation. Intraneuronal A β accumulation has been associated with apoptotic cell death and neurodegeneration in AD patients and transgenic mice [212, 213], and proposed to cause cognitive dysfunction in AD mouse models [111, 195]. A more thorough evaluation of secondary molecular events of intraneuronal A β accumulation in tg-ArcSwe mice would be needed. The understanding of such molecular pathways is of major importance for future therapeutic targeting. It could serve as an essential complement to the A β -modifying strategies, since the passive administration of anti-A β antibodies had no impact on the intraneuronal A β pool. The lack of effect on intraneuronal A β could be explained by the inability of mAb158 to bind to the extracellular A β domain of APP, suggested to be required for antibody internalization [209]. However, in this respect mAb158 has a major advantage, since it does not bind to alternative targets and more efficiently

reaches the brain. Binding to alternative targets in the periphery, as in the case of mAb1C3, could lead to metabolism prior to affect and demand a much higher effective dose.

Passive immunization is today receiving a lot of attention as a promising strategy for future treatment of AD. In this thesis work, I have investigated some aspects of this approach. To have an effect on plaque pathology, the treatment needs to be preventive and initiated before symptoms appear. Perhaps antecedent diagnostic biomarkers visualizing early signs of emerging amyloidosis at regular health examinations would be needed to identify patients in which the therapy could diminish and delay amyloid plaque formation. On the other hand, plaque clearance may not be required for the treatment of cognitive symptoms, if the plaques themselves are not causing the cognitive decline. Either way an early diagnosis might still be crucial for successful treatment, since A β oligomers/protofibrils and intraneuronal A β appear long before amyloid pathology. These early changes could result in pathological alterations that are irreversible at a later stage. Despite many suggestions, it is still not completely clarified if and how A β oligomers instigate all other pathogenic hallmarks of the disease, i.e. tau phosphorylation with subsequent tangle formation, microglial response and cell death. It is therefore very important to continue to examine the molecular pathways linking all these abnormalities in order to unravel the mystery of Alzheimer's disease.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom är den vanligaste orsaken till demens och drabbar drygt fem procent av befolkningen över 65 års ålder. Sjukdomen kommer smygande och problem med närminnet är oftast det första tecknet. Förutom minnesproblem drabbas även andra kognitiva funktioner tidigt i sjukdomsförloppet, till exempel språket och förmågor som att orientera sig på kända platser, planera och lösa problem. Symtomen förvärras med tiden alltefter som fler av hjärnans nervceller dör och patienten får så småningom problem med vardagliga sysslor som att laga mat, sköta sin hygien och klä på sig. Detta medför naturligtvis stort lidande för både patienten och de anhöriga. Eftersom sjukdomen är så vanlig medför den också stora socioekonomiska konsekvenser, och situationen förvärras i och med att befolkningen ständigt åldras. Det finns för närvarande inte något botemedel mot Alzheimers sjukdom och det är därför mycket viktigt att forskning inriktas på att förstå vilka mekanismer som ligger bakom sjukdomsförloppet samt att försöka utveckla behandling mot dessa.

Symtomen beror på att nervcellerna i hjärnan slutar att fungera normalt, och att det sker en gradvis förlust av nervceller. Denna nervcellsöd, eller neurodegeneration, sker främst i hjäss- och tinningloberna samt i en struktur kallad hippocampus, regioner som spelar en viktig roll för minnesinlagring. Man vet inte varför nervcellerna dör men man har hittat onormala proteinklumpar i hjärnan hos patienter med Alzheimers sjukdom och man tror att de kan ha stor betydelse. Dessa sjukliga proteinklumpar, även benämnda plack eller senila plack, består huvudsakligen av ett litet protein, eller peptid, kallad amyloid- β ($A\beta$). De senaste årtiondenas forskning har gett oss viktig kunskap om $A\beta$ och vi vet att denna peptid är giftig för nervceller och spelar en central roll då sjukdomen uppstår. $A\beta$ -peptiden har en vattenavstötande del som gör att den börjar klumpa ihop sig (aggregera) och bilda små proteinklumpar ($A\beta$ -aggregat). Om proteinklumparna fortsätter att aggregera bildar de till slut olösliga trådliknande strukturer (fibriller), och dessa fibriller är huvudbeståndsdelarna i senila plack. Den rådande hypotesen bland många forskare idag är att det är mellanstadierna i bildningen av fibriller och plack, alltså de lösliga $A\beta$ -aggregaten, som är mest giftiga för nervceller och de som orsakar den massiva nervcellsöden.

Alzheimers sjukdom uppkommer oftast sporadiskt och man vet då inte varför bara vissa personer drabbas. I sällsynta fall har man emellertid kunnat påvisa att ett visst arvsanlag kan orsaka sjukdomen. Dessa ärftliga fall beror

på mutationer i arvsmassan, bland annat i APP-genen. Exempel på mutationer i denna gen är den svenska mutationen som ökar nivån av A β generellt och den arktiska mutationen som ökar bildningen av stora lösliga A β -aggregat, även kallade protofibriller. För att kunna utvärdera vilken betydelse protofibriller har för sjukdomsutvecklingen, har vi med hjälp av mutationerna utvecklat en ny djurmodell för Alzheimers sjukdom. Djuren har fått den mänskliga APP-genen innehållande både den svenska och den arktiska mutationen tillagd i sin arvs massa, och bildningen av mänskligt A β sker framförallt i de hjärnregioner som drabbas vid Alzheimers sjukdom.

I den här avhandlingen har jag dels studerat hur den arktiska mutationen kan ge upphov till sjukdomen, dels beskrivit den nyutvecklade djurmodellen med avseende på relevant sjukdomsbild i hjärnan, men även försökt att rikta behandling mot de nervcellsgiftiga protofibrillerna. Den arktiska mutationen ledde till ökade nivåer av protofibriller i hjärnan hos djuren men påverkade även produktionen av A β . Genom att specifikt infärge A β i hjärnan kunde vi visualisera och kvantifiera tidiga ansamlingar av A β inne i nervcellerna. Dessa A β -klumpar uppkom mycket tidigt, innan placken bildades, och vi tror därför att de kan vara förstadier till placken. Protofibrillerna verkar också spela en stor roll tidigt i sjukdomsprocessen eftersom de påverkade djurens förmåga till inläring negativt. Detta studerades i en vattenlabyrint genom att låta djuren simma och lära sig att hitta till en plattform dold under vattenytan. Tiden det tog att finna plattformen undersöktes vid upprepade tester och under flera dagar och förbättringen utgjorde ett mått på inläring. Vi såg här att höga nivåer av protofibriller i hjärnan var förenat med en sämre inläring.

I en relativt ny behandlingsstrategi försöker man vaccinera patienter med Alzheimers sjukdom mot A β . Idén bygger på att stimulera kroppens immunförsvar att bilda antikroppar mot A β , för att på så vis kunna hjälpa kroppen att göra sig av med denna skadliga peptid. En amerikansk forskargrupp visade att detta var möjligt i försöksdjur och testade även ett A β -vaccin på patienter i kliniska studier. Studierna fick tyvärr avbrytas då ett antal patienter utvecklade allvarliga biverkningar. För att minska risken för biverkningar, försöker man nu istället ge patienten bioteknologiskt framställda antikroppar mot A β för att på så vis undvika att provocera immunsystemet alltför mycket. Vår idé var att antikroppar mot protofibriller borde testas som behandling eftersom dessa A β -aggregat tros orsaka neurodegeneration och sjukdomssymtom. Genom att framställa antikroppar som är selektiva för protofibriller och sedan använda dem för behandling i vår djurmodell kunde vi hindra att plack uppstod. Resultaten tyder på att protofibrillerna är nödvändiga mellanstadier i fibrillbildningen som kan tas bort specifikt med antikroppar.

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