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The Amyloid Beta Peptide: A Chemist's Perspective
Role in Alzheimer's and Fibrillization

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1. INTRODUCTION

This review is concerned with the role of fibrillization of the amyloid β (Aβ) peptide in Alzheimer's disease (AD). The perspective is that of a physical chemist and one aim is to introduce relevant key findings on physico-chemical properties. However, in addition, key aspects of the biology and biochemistry associated with the role of Aβ in AD are also summarized (more detailed reviews of these aspects can be found elsewhere),[1] as are developments in potential therapies and biomarkers.

The aggregation of the amyloid β peptide into oligomers or fibrils is now implicated as a key process associated with progression of AD.[2] This is the focus of the current review. Whilst the protein tau has an important role in AD progression, its processing occurs downstream of Aβ accumulation.[3] A marked decrease or absence of tau expression appears to reduce the neurotoxic effects of Aβ.[4] Proteins including NAC (non-beta-amyloid component) are also co-deposited along with Aβ in plaques.[5] NAC comprises residues 61-95 of α-synuclein, which is involved in amyloidoses with Lewy bodies such as Parkinson's disease. These topics are not discussed further herein, with the exception of a brief discussion (Section 2.8) of the interaction between Aβ and tau.

Due to the very large number of papers on Aβ, this review cannot be exhaustive in the space available. We have attempted to focus on key papers, and work that illustrates the main features of the subjects in the following sections. We have attempted to review work by many groups who have made important contributions. As there are also a large number of previous reviews on the topic of

Aβ aggregation and its relationship to neurodegenerative disease, we can also only cite a number of the key earlier reviews here.[1,6]

AD is the most common cause of dementia (representing around 50-80% of all cases[7]) with an estimated 18 million people worldwide currently affected by the condition (according to the World Health Organization).[8] Its incidence increases dramatically with age, and the number of people with dementia is set to double in the next twenty years.[7] The annual cost of dementia in the UK is estimated at £23 billion per annum including care and healthcare costs and lost productivity, which equates to £28k per patient.[9] Alzheimer's disease accounts for about 70% of all late-onset dementia cases.[10] Most cases occur relatively late in life, although around 5% occurs in patients under 60 years old. These cases are termed early onset familial Alzheimer's disease (FAD). Genetic mutations have been linked to these conditions as discussed in section 2.1 below. In AD, neurodegeneration is estimated to start 10-30 years before clinical symptoms are detected.[2a,11]

Intense research activity is focussed on the development of treatments for Alzheimer's disease (AD) as discussed in Section 3. Several existing treatments can manage the condition but they do not arrest or reverse the progression of AD, i.e. there is no cure. A healthy diet and exercise may contribute to reduced AD risk as might enhanced mental activity and social engagement.[12] Calorie restriction and intermittent fasting also ameliorate age-related behavioural deficits in transgenic mice.[13] Further discussion of these epidemiological studies is outside the scope of the present review. A number of strategies to treat the condition are actively being pursued by research teams in academia and the pharmaceutical industry.[12a,14] These

include (i) development of γ-secretase inhibitors (γ-secretase is an enzyme involved in cleavage of amyloid β (Aβ) peptides from the Amyloid Precursor Protein), (ii) passive immunization based on Aβ antibodies (iii) inhibition of aggregation of oligomers. These are discussed further in Section 3.

Susceptibility to AD increases with aging, as indicated by large population screening studies and studies using monkeys[15] and transgenic mice.[15] Much research has focussed on early-onset AD for which genetic markers and the role of Aβ are readily identified.[2c] Table 1 shows characteristics of early-onset AD (EOAD). It is responsible for ~2% of cases and can occur as early as 30 years of age.[2c] Late-onset AD (LOAD) is the more common variant that causes the majority of the cases of age-dependent dementia. Age is the single biggest known risk factor, with the incidence of the disease increasing from approximately one in ten of those over 65, doubling roughly every five years to affect approximately half of individuals over 85.[2c,8,16] Susceptibility to LOAD also seems to have a genetic basis, although a single genetic determinant does not exist – several genes associated with susceptibility to the condition are known (as discussed further in Section 2.1) and a combination of genes may also be involved. The progression of AD is similar for EOAD and LOAD and is arbitrarily divided into early/mild, moderate and severe cases.

Oxidative stress may play an important role in the age-dependent susceptibility to AD.[17] Oxidative stress involves the production of free radicals (especially hydroxyl radicals) in the presence of metal ions, which can influence metabolism, and also promote Aβ aggregation, the latter subject being discussed further in section 4.8. The free radicals can cause increased lipid peroxidation, and the formation of associated

byproducts, as well as protein and DNA oxidation in the AD brain. Diminished mitochondrial energy metabolism may play a role in AD pathogenesis, due at least in part to reduced cyclooxygenase (COX) activity (section 2.1).[17] Excitotoxicity is the overstimulation of *N*-methyl-D-aspartate (NMDA) or 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) receptors (NMDARs and AMPARs respectively) by glutamate or aspartate, leading to neuronal hyperexcitability and death. It can also generate excess reactive oxygen species (ROS). The role of oxidative stress is evidenced by the presence of protein glycation end products in Aβ aggregates, as well as an increase in the number of activated microglial cells (section 2.3).[17] The inflammation that results from oxidative stress as well as Aβ- (and tau-) induced neurodegeneration has an important role in AD pathology, as reviewed by the neuroinflammation working group.[18]

Table 1. Characteristics of Early-Onset AD.[2c]

Gene	**Age of onset, years**	**Aβ phenotype**
APP trisomy 21	50s	Total Aβ production increased
APP mutations	50s	Total Aβ production increased Aβ42/Aβ40 ratio increased
APP triplication of APP gene	50s	Total Aβ production increased
Presenilin 1	40s and 50s	Aβ42/Aβ40 ratio increased
Presenilin 2	50s	Aβ42/Aβ40 ratio increased

Diagnosis of AD is usually through cognitive testing methods detailed elsewhere,[2c] supported by scanning techniques such as magnetic resonance imaging (MRI). However, research into biomarkers is a very active and promising field (section 2.4). Prior to development of AD, patients may suffer mild cognitive impairment (MCI), and around 40-60% of patients with this condition develop AD within five years.[19]

Factors that can be used to track the risk of progression of AD have recently been reviewed, leading to guidelines for the preclinical assessment of the condition.[20] The pathogenic process leading to AD may start many years (a period of approximately one decade has been identified[20]) before obvious symptoms are noted. The development of biomarkers at an early stage of disease progression would be extremely beneficial.

The insulin/insulin growth factor (IGF) signalling pathway influences ageing and AD progression, as discussed further in section 2.1. Genes undergoing age-related changes in expression have been identified, as have markers of mitochondrial dysfunction in response to oxidative stress.[21] Another vital regulator of the aging process is autophagy (degradation of intracellular components through lysosomes). Increased autophagy extends lifespan due to reduced insulin-like signalling and it may be stimulated by calorie restriction. Reduced autophagy leads to neurodegeneration, accompanied by the accumulation of ubiquitinylated protein aggregates.[21] This can occur during normal ageing, but reaches pathological levels in neurodegenerative disorders such as AD. Proteasome dysfunction leads to increased levels of ubiquitinylated protein and to memory deficits in transgenic mice.[22]

The pathology of AD comprises neuritic amyloid plaques and neurofibrillary tangles in the hippocampus, amygdala and association neocortex. Diagnosis with 100% accuracy can only be achieved *post mortem*, however diagnosis with 95% accuracy is possible in living patients using a combination of tools including cognitive testing, brain imaging and analysis of family health history.[12a]

The Aβ hypothesis (Fig.1) implicates Aβ as a key causative agent of AD. Controversies surrounding the Aβ hypothesis, including the apparently paradoxical presence of Aβ deposits in the brains of people not suffering from dementia, and the cause/effect nature of Aβ deposition, have been discussed.[3,6e,23] However, these deposits are diffuse and have none of the characteristic surrounding neuritic and glial cytopathology found in mature neuritic plaques.[6e,24] Biochemical assays such as ELISA and Western blotting indicate that levels of soluble Aβ correlate better with the presence and extent of cognitive defects than simple plaque counts.[25]

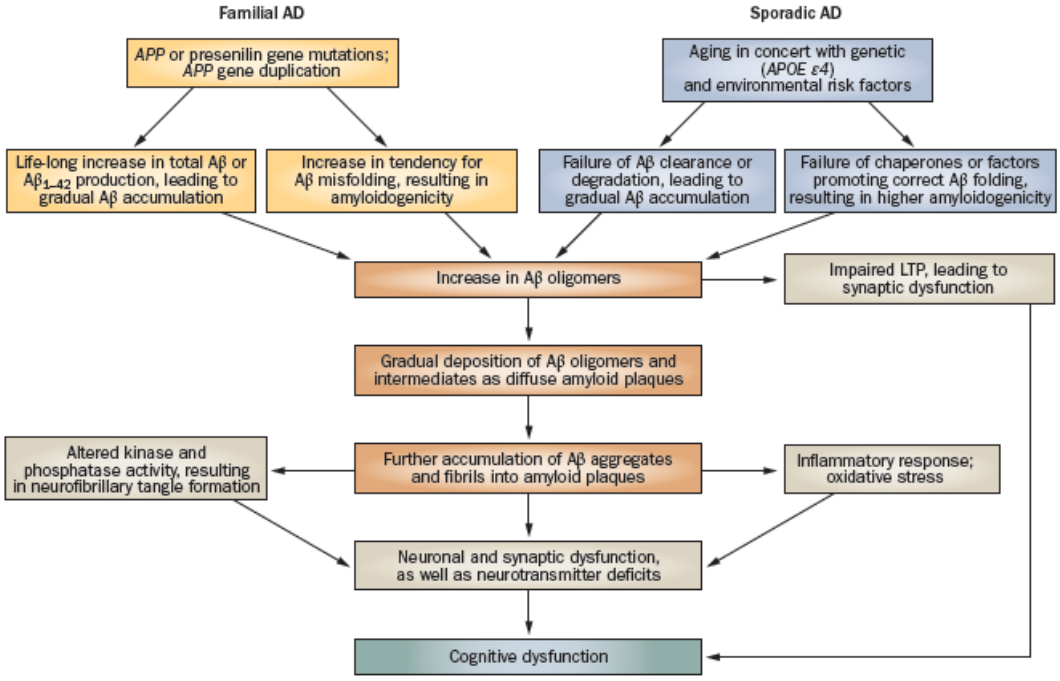

Fig.1. Amyloid cascade hypothesis of AD.[26] Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neurology 6*, 131, copyright 2010.

The term amyloid refers to protein deposits resembling those first observed for starch (amyloid originally meaning starch-like). It is now specifically associated with proteins and peptides adopting fibrils based on the cross-β structure in which the peptide backbone is orthogonal to the fibril axis.[27] The β-sheets form fibrils, which have an internal structure such as parallel protofilaments, and the fibrils themselves can further aggregate into larger fibres or bundles (which often comprise twisted fibrils).[6b,27d] Figure 2 shows representative fibril morphologies for Aβ peptides, the fibril morphology depends on preparation conditions, and fibril polymorphism is also observed and examples of other fibril structures are shown in section 4.1.

(a) (b)

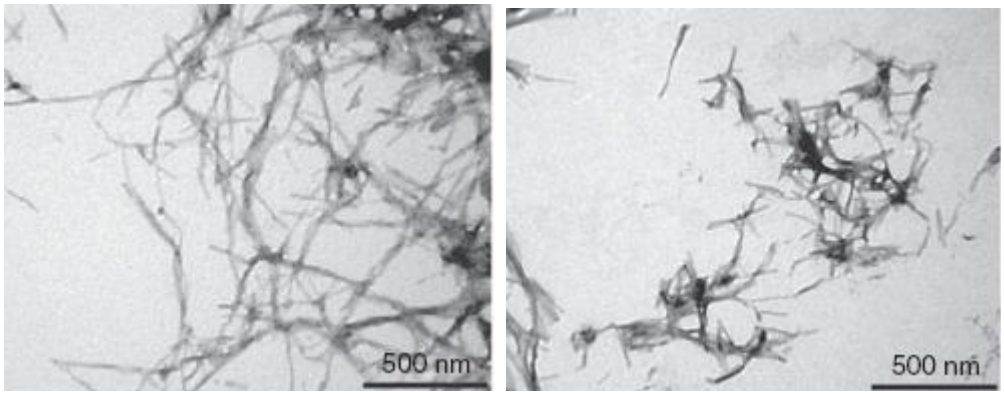

Fig.2. "Typical" Aβ fibril morphology by TEM, obtained from incubated 50 μM solutions of (a) Aβ40, (b) Aβ42.[28]

Figure 3 shows a timeline of some of the principal discoveries in AD research, [3,29] also the subject of other historical overviews.[2a,6e,30] AD is named after Alois Alzheimer who first described the condition now named after him in 1906.[2a,31] It is generally acknowledged that the first paper to identify Aβ in association with neuropathology was by Glenner and Wong in 1984,[32] who identified a 4 kDa major

component of Aβ extracted from the blood vessels of a patient with Down's syndrome. By middle age, the brains of Down's syndrome patients inevitably display the neuropathological features of AD, i.e. deposition of Aβ plaques and AD-type brain lesions, although mental retardation from birth is due to other causes. Glenner and Wong were also able to sequence the first 28 amino acids of Aβ. In the late 1980's, several different groups were able to use Glenner and Wong's Aβ sequence to clone the gene encoding amyloid precursor protein (APP) and map it to chromosome 21.[10,33] Chromosome 21 is duplicated in Down's syndrome, hence the correlation with Aβ deposition which occurs early in this condition.

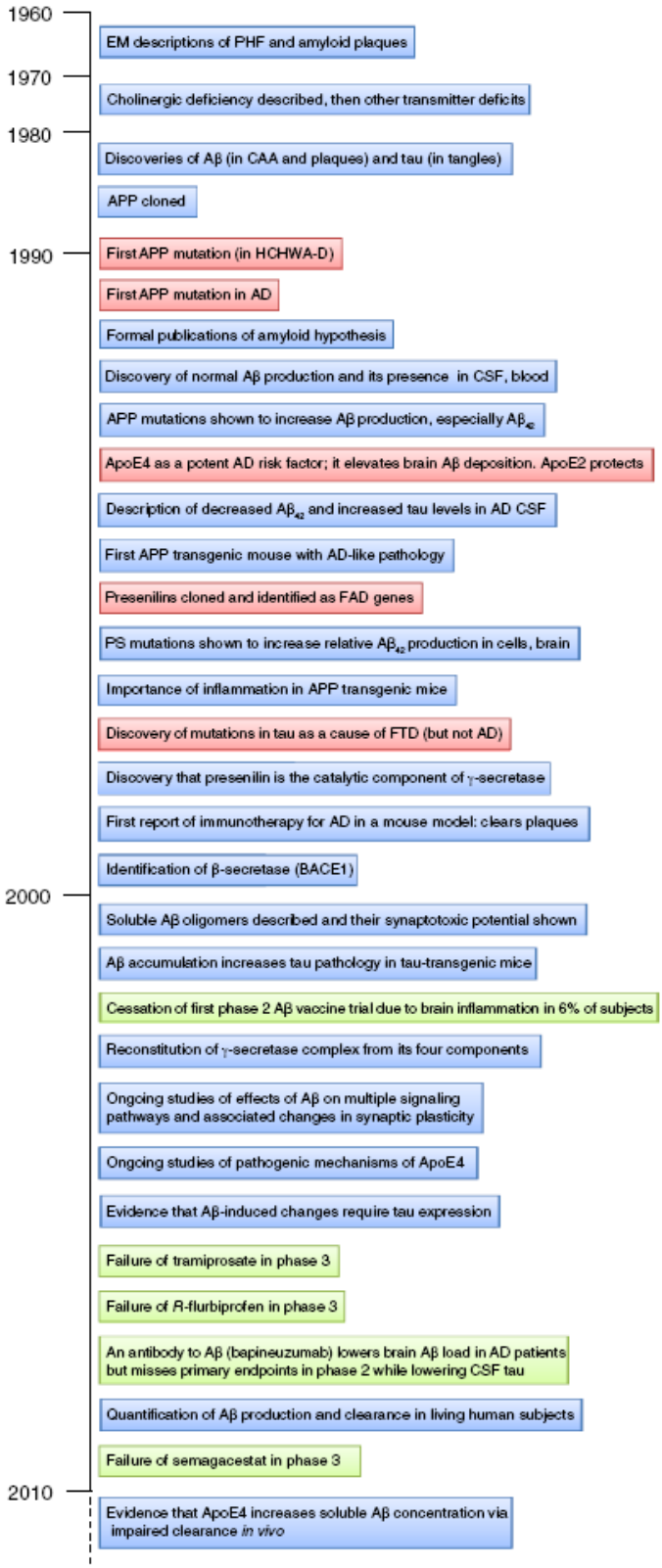

Fig.3. Timeline of selected AD discoveries including those associated with the Aβ hypothesis.[3] Reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine 17*, 1060, copyright 2011.

There are two principal variants of the amyloid peptide in humans – Aβ40 and Aβ42 (this notation will be used consistently for the whole peptide with the number of residues indicated, for fragments the sequence will be indicated).[1a] The former is more abundant, however Aβ42 forms fibrils more rapidly.[1a,6d,34] Aβ43 is also observed[35] as are peptides truncated at the C terminus[36] such as Aβ39.[37] N-terminal truncated peptides are also detected.[36,38] Tryptic degradation of Aβ from AD patient brains also revealed heterogeneous fragments from the Aβ(1-5) and Aβ(6-16) domains.[36]

It is now thought that oligomers formed in the initial self-assembly process are the toxic agents.[2a,2d,39] This is discussed in more detail in Section 2.6. Oligomerization of Aβ occurs intracellularly, as revealed by *in vivo* experiments on human cerebrospinal fluid which yielded SDS-stable dimers of Aβ.[40] Incubation did not lead to the production of extracellular oligomers. However, oligomers were detected in neural and non-neural cell lines. The importance of intracellular Aβ production and its relationship to extracellular production, and re-uptake has been discussed.[41] Whether intra- and inter- cellular pools of Aβ are distinct or related has been the subject of studies with contradictory conclusions. However, it does appear that extracellular Aβ may originate from intraneuronal sources, and a dynamic equilibrium may exist between these pools.[41] Since Aβ is produced via cleavage of APP in membranes (Section 2.2.3) its sites of production include the plasma membrane, but also within the cell in the Golgi and endoplasmic reticulum, as well as endosomes and lysozomes.[41]

There is a relationship between the incidence of AD and cerebral ischemia (reduction in blood supply), eg. following a stroke or other cerebrovascular or cardiovascular condition.[42] Oxidative stress, eg. hypoxia (oxygen deprivation) or ischemia may cause an increase in Aβ levels in the brain due to an upregulation of APP processing.[43] A correlation between serial brain interstitial fluid (ISF) concentration and neurological status (after acute injury) has been noted, Aβ concentration increasing with improved neurological status.[44]

Amyloid β is produced by proteolytic cleavage of APP, a transmembrane protein discussed further in Section 2.2.3.[2a] The peptide N terminus is created by cleavage by β-secretase in the extracellular domain of APP, and the C-terminus results from intramembrane cleavage by γ-secretase. A third enzyme, α-secretase cleaves between amino acids 16 and 17 in Aβ, thus hindering fibrillization. The cleavage by γ-secretase is presenilin-dependent.[45] As discussed further in Section 2.2.3, γ-secretase is a protein complex involving presenilins, nicastrin, APH-1 and PEN-2, all of which are required for γ-secretase function.[46] Selkoe thoroughly reviews the historical literature concerning the relationship between the presenilins and γ-secretase.[1a]

Whilst there have been numerous reviews on the amyloid hypothesis of AD, there are few up-to-date reviews that also discuss the biophysical aspects of Aβ self-assembly and its influence on AD. The present review aims to provide a unified view of the biological, neurochemical and biophysical aspects of Aβ aggregation and its relationship to AD. In addition, this review provides a current overview of developments in potential therapeutic strategies.

This review is organized as follows. The first section concerns the features of AD and the properties of Aβ and APP *in vivo*, including biological and neurochemical characteristics. The development of biomarkers is also considered. This is followed by discussion of therapeutic compounds. The final sections are focussed on different aspects of the biophysical properties of Aβ.

2. BIOLOGICAL, BIOCHEMICAL AND NEUROCHEMICAL CHARACTERISTICS OF AD, INVOLVING Aβ AND APP

2.1 Genetic Markers for AD

2.1.1 Genetic Risk Factors

Several genes have been linked to late-onset Alzheimer's disease, most importantly the gene encoding apolipoprotein E (*APOE*)[47], especially the ε4 allele.[47a,47b,47d,48] This is believed to cause the aggressive form of AD with earlier onset.[3,49] APOE (and related APOC1) diagnostic testing systems have even reached the market although some products have been withdrawn due to IP issues.[50] The compound rosiglitazone may ameliorate neuronal dendritic spine loss caused by ApoE-ε4, and thus improve cognition in AD patients.[51]

Mutations in the genes for *APP*,[52] presenilin 1 and 2 (*PSEN1* or *PS1* and *PSEN2* or *PS2*)[1a,53] have a role in hereditary forms of AD.[54] Dominantly inherited forms represent only 1-3% of the total number of cases of AD, most of which are sporadic.[2a,11,54] Mutations in *PS1* and *PS2* potentially account for a large fraction of early-onset cases of familial AD.[1a,55] Mutations in the presenilins cause an increase in Aβ42 in AD patients[1b,55a,56] and also transfected cell lines and transgenic animals

expressing mutant forms of *PS1* or *PS2*.[57] and this occurs selectively for Aβ42/43 over Aβ40.[56-57] Certain presenilin mutations can disrupt the leakage of calcium (see the discussion of the calcium channel hypothesis in section 2.7) induced by presenilins from the endoplasmic reticulum leading to supranormal release and dyshomeostatis.[58] This has been investigated in electrophysiology experiments.[55b,58-59] Missense mutations in PS1 are associated with early and aggressive forms of AD,[1a] Aβ42 plaques being observed as early as 3-4 months.[60] The G209V, A260V and E280A presenilin A mutations lead to substantial overexpression of Aβ42 in the brains of FAD patients.[61] Mutations in *PSEN1* are also associated with *acne inversa* although a correlation between this condition and AD has not been noted.[62]

The gene encoding ApoE was the first confirmed susceptibility locus for sporadic late onset AD, and its alleles have been widely studied. A recent study using microdialysis in a PDAPP/TRE mouse model (to be discussed shortly) indicates that different isoforms of the gene differentially regulate Aβ clearance from the brain.[63] A genome-wide association study identified three other loci, within *CLU* (which codes for apolipoprotein J, ApoJ or clusterin),[54,64] within *CR1* (complement receptor 1)[54] or within *PICALM*.[64] Single nucleotide polymorphs (SNPs) at these loci (as well as *APOE*) were associated with AD risk. ApoE and CLU are the most abundantly expressed apolipoproteins in the central nervous system.[54] Earlier work had shown that ApoJ is over-expressed in individuals with AD and is present in CSF[65] and amyloid plaques.[66] Clusterin binds soluble Aβ to form complexes (especially with the more toxic Aβ42 peptide) which can cross the blood-brain barrier.[67] It promotes amyloid plaque formation and is critical for toxicity towards neurons.[68] Bell *et al*. performed studies on the clearance of radiolabelled (^{125}I) Aβ using a mouse model

and found that Aβ42 is cleared more slowly than Aβ40 and that Aβ, ApoE and ApoJ (detected using human-specific ELISAs) are cleared from the brain by different transport mechanisms.[67b]

A study using a yeast model has identified genetic factors influencing Aβ toxicity, including *PICALM* but also other previously unidentified genes associated with protein trafficking, stress and metabolism.[69] This work also confirmed the effect of PICALM on Aβ toxicity using a *C. elegans* model and also using rat cortical neurons.[69] In terms of mechanisms, the authors suggested that Aβ affects the endocytic trafficking of a plasma membrane receptor. Another genome-wide survey revealed an association between late-onset AD in carriers of the *APOE*-ε4 allele and SNPs from the *GRB*-associated binding protein 2 (*GAB2*) gene.[70] ApoD has also been associated with AD, this lipoprotein circulates as a components of serum high density lipoproteins (HDL) and may be involved in cholesterol transport.[71] Apolipoprotein D is involved in lifespan extension in *Drosophila*, conferring resistance to oxidative stress, and its expression is induced in the AD brain.[71]

Another gene that was identified as a risk for AD is *CALHM1* (denoting calcium homeostasis modulator 1).[72] The CALHM1 protein is localized in the cell membrane, and increased expression leads to enhanced calcium levels within the cytoplasm. Single nucleotide polymorphism of the gene leads to changes in AD susceptibility, specifically a P86L substitution leads to increased Aβ levels. However, these findings have been challenged – Bertram *et al*. also examined several family-based datasets and number of prior genome-wide association study (GWAS) datasets[70,73] and found

no correlation between *CALHM1* and AD.[74] However, the authors of the original study dispute this analysis.[75]

The role of the Orphan G protein-coupled receptor (GPR3) as a modulator of Aβ production has recently been identified.[76] The GPR3 gene has been mapped to a candidate AD linkage region in one chromosome as part of a large-scale genome screen using an NIMH (National Institute for Mental Health) sample.[77] GPR3 expression leads to an increase in production of the γ-secretase complex, and its cell surface localization, in the absence of an effect on Notch processing (discussed further in Section 3.2.1).[76] Notch proteins are transmembrane proteins involved in development and signaling pathways and a key challenge in the development of effective γ-secretase inhibitors is to avoid side effects caused by interference with these pathways. GPR3 was found to be highly expressed in areas of the normal brain implicated in AD and is elevated in the sporadic AD brain. It thus represents a potential target for therapeutic treatment.

Other proteins associated with Aβ production or APP processing include the serotonin receptors[78] and the prostaglandin EP2 receptor.[79] Prostaglandin E_2 is produced during inflammation due to activity by cytosolic phospholipase A_2 (PLA2) or cyclooxygenase 2 (COX-2). [79] The latter enzyme is upregulated in AD brain frontal cortex and synthetic Aβ peptides induce COX-2 expression in SH-SY5Y neuroblastoma cells *in vitro*.[80] COX-2 is involved in the inflammatory response and is the target of NSAIDS (non-steroidal anti-inflammatory drugs). A population-based study pointed to the elevation of serum levels of pregnancy zone protein (PZP) in pre-symptomatic AD, compared to controls.[81]

Genome-wide studies of genes associated with aging indicate that the insulin/IGF-1 signalling pathway may be involved in Alzheimer's. Reduced signalling causes decreased AD pathology in mice[82] while paradoxically increased signalling may also be neuroprotective.[21]

2.1.2 Transgenic Mice

Many studies use the PDAPP mouse, also known as PDGF-hAPP (from platelet-derived growth factor) mouse, which overexpresses mutant human APP (V717F mutant) under control of mouse regulatory elements and leads to Aβ plaque deposition.[15,83] The PDAPP/TRE model expresses human ApoE. The TgCRND8 murine model of AD expresses a doubly mutant (K670N/M671L and V717F) human APP695 transgene.[84] Tg2576 APP mice expressing the Swedish FAD variant of human APP_{695} (section 2.2.3)[85] which leads to a selective increase in Aβ42/43 production have also been used in Aβ immunization experiments.[86] Tg2576 mice develop memory deficits due to the extracellular accumulation of specific Aβ oligomeric species, i.e. dodecamers.[87] A doubly mutant transgenic mouse including the APP(Swe) and mutant PS1 (M146L) has been developed and exhibited a large selective enhancement of Aβ42 and plaque deposition.[60] The 3xTg model in a triply transgenic mouse contains PS1(M146V), APP(Swe), and tau(P301L) transgenes[88] and this has been used to investigate the interplay of Aβ and tau (neurofibrillary tangles) pathologies (section 2.8). Contrary to doubly transgenic mice lacking the APP transgene, deposition of plaques and synaptic dysfunction (LTP deficits) are observed with the 3xTg model.[88] The APP23 mouse overproduces Aβ40 and the APPPS1 mouse overexpresses Aβ42.[89]

2.2 Aβ *in vivo*

2.2.1 Characteristics of Aβ in vivo

Evidence that the Aβ42 form of Aβ is the variant preferentially implicated in AD comes from several sources. Studies of the kinetics of aggregation (through turbidity measurements) indicate that Aβ42 nucleates more rapidly and is more fibrillogenic than Aβ40.[34,90] The toxicity of Aβ42 is much greater than Aβ40[91] due to its greater tendency to fibrillise. Some mutations in APP in cultured cells (discussed in section 2.2.2) lead to increased levels of Aβ42, whereas wildtype APP predominantly releases Aβ40.[92] It is also found that Aβ42 is the principal component of diffuse Aβ plaques and plaques generated from APP mutants[93] and in homogenized brain tissue,[94] and that early and selective deposition of Aβ42 is observed in the brains of AD patients (shorter peptides with different N termini are also found).[36,38] Despite its lower toxicity, Aβ40 is actually produced by a factor of ten times more than Aβ42, by γ-secretase cleavage.[41]

Whilst Aβ is generally associated with disease, a functional role for the peptide has also been suggested. Tanzi and coworkers have demonstrated that it is an antimicrobial peptide, i.e. that it is involved in immune reactions.[95] Antimicrobial activity of Aβ40 and Aβ42 has been demonstrated against eight common microorganisms including *E. coli* and *S. aureus*. This activity can be blocked by immunodepletion of AD brain homogenates with anti-Aβ antibodies.[96] Temporal lobe tissue from AD patients showed higher antimicrobial activity than material from the

brains of age-matched non-AD subjects. Balin's group have suggested that Aβ may be part of the control mechanism following infection by *C. pneumoniae*,[97] and that it can mediate infection of cells with this bacterium.[98] Smith and coworkers argue that Aβ production is a host response to an underlying condition that develops with age.[99] However, this is becoming an increasingly contrarian viewpoint in view of the mass of data implicating Aβ as the causative agent. The ε4 allele of the *APOE* gene, a marker for EOAD (section 2.1.1) may have a beneficial role in enhanced cognitive skills.[100]

The fraction of different variants of Aβ has been investigated. Based on analysis of cell lysates and also tissue from mouse brain, Aβ40 has been found to constitute approximately 90% of the secreted Aβ and Aβ42 comprises ~10%,[57b-d] although somewhat lower[57c] and much higher[57e] fractions have also been reported. Aβ42 is the most commonly found variant in human CSF.

Since APP is expressed in most peripheral cells, Aβ is present in plasma in addition to CSF. The level of Aβ40 in plasma is generally under 200 pM, and of Aβ42(3) is under 60 pM, although both are elevated in patients with *PS1* or *PS2* mutations or in patients with presymptomatic or symptomatic APP patients.[56] The physiological concentration of Aβ (in AD patients) in human CSF has been reported by several groups. An Aβ concentration of less than 500 pg/ml (0.1 nM) is indicative that Aβ is accumulating in the brain and not circulating in the CSF. Using an ELISA assay, Mehta *et al*. reported for Aβ40 c = 30 nM.[101] and for Aβ42 c = 8 pM.[101] On the other hand, Ida *et al*. using a Western blot assay reported a lower c = 6 nM for Aβ40 but a

much higher c = 60 pM for Aβ(1-42).[37] Similar values for Aβ42 for AD patients are reported by Motter *et al*.[102] Values in plasma are also available.[56,101] The CSF concentration of Aβ40 for AD patients is not significantly different to that for nondemented control patients, however the concentration of Aβ42 is lower for AD patients.[37,101-102] This is another evidence for Aβ42 as the disease-related species. Peripheral adminstration of monoclonal antibodies leads to a rapid increase in plasma Aβ.[103] The physiological concentration of Aβ (variant not defined) in normal human CSF is around 1- 2 nM according to ref.[104] whereas a value of 3-8 nM is cited elsewhere.[1a,102] According to Podlisny *et al*. the physiological concentration of Aβ40 is 0.25 – 2.5 nM,[105] in agreement with the value c = 0.6 nM reported by other groups.[106] There is no correlation between plasma Aβ40 and Aβ40 load (in the range 0-40% for 46 nM Aβ40 in PDAPP mouse plasma) in the absence of anti-Aβ antibodies.[103] The concentration of Aβ in serial brain interstitial fluid has also been reported.[44] Ida *et al*. also detected the presence of N-terminally truncated Aβ species in CSF and plasma.[37]

The production of Aβ40 and Aβ42 in the human central nervous system (CNS) does not appear to be different for AD patients compared to control, however the rate of clearance is significantly reduced for AD patients.[107] Production of Aβ is discussed in the following section. Low-density lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE) are involved in receptor-mediated flux of Aβ across the BBB as part of the clearance mechanism.[108] Clearance of Aβ from the brain to the periphery appears to be mediated by LRP while RAGE is implicated in Aβ efflux back into the CNS (Fig.4).[108b]

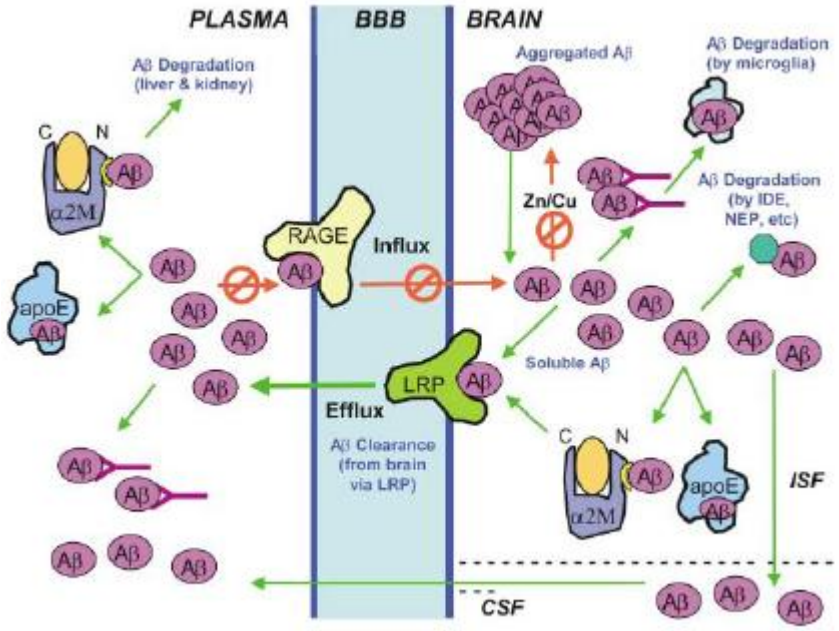

Fig.4. Clearance mechanisms of Aβ involving LRP and RAGE.[108b] Blocking interaction of Aβ with zinc and copper may clear Aβ. Soluble Aβ can be removed via enzymatic degradation (via peptidases such as insulin degrading enzyme IDE or neprilysin NEP, and subsequent degradation by activated microglia) or receptor-mediated clearance. LRP receptor-mediated clearance can occur by direct binding or initial binding to the LRP ligands/Aβ chaperones ApoE and α2M which can deliver Aβ to peripheral sites of degradation (liver or kidney). These chaperones can also deliver Aβ across the BBB into the brain. This process can also occur via the RAGE receptor. Green arrows show pathways that might be pharmacologically relevant. Reprinted from Tanzi, R. E. *et al*., *Neuron* **2004**, *43*, 605, Copyright 2004, with permission from Elsevier

2.2.2. Modelling AD and Aβ Deposition in Other Organisms

AD can be modeled using a variety of organisms including fruit flies (*Drosophila melanogaster*),[109] nematode worms (*Caenorhabditis elegans*),[69,110] potatoes,[111] and yeast (*Saccharomyces cerevisae*).[69,112] *C. elegans* has attracted particular attention as a model to study the function of presenilins, especially in relation to Notch (Section 3.3) since there are great similarities between the *sel-12* gene of *C. elegans* and presenilin genes.[1a,113] *Drosophila melanogaster* has been engineered to express both wild type human and arctic mutant Aβ42.[109d] Yeast exhibits α-secretase activity on APP.[112] The later study showed that yeast can be used to model links between Aβ, endocytosis and human AD risk factors.[69] Aβ(M1-40) and Aβ(M1-42) i.e. with an N terminal methionine substitution, can be expressed in *E. coli*.[28,114] The fibrils formed by the recombinant peptides are indistinguishable than those from chemically synthesized peptides.

2.2.3 APP and the Production of Aβ

APP belongs to the family of type 1 transmembrane glycoproteins.[115] It has been hypothesized to act as a vesicular receptor for the motor protein kinesin-I.[109b] The production of Aβ in the amyloidogenic pathway involves the sequential cleavage of APP by β-secretase and γ-secretase (Fig.5). The enzyme β-secretase is an integral membrane aspartyl protease encoded by the β-site APP-cleaving enzyme 1 gene (*BACE1*)[1a,1b,116] while γ-secretase is a membrane-bound protease complex consisting of at least four components including the presenilins (PS1 and PS), nicastrin, and the genes *APH-1* and *PEN-2*.[110b,117] The γ-secretase complex may also function as an aspartyl protease.[1a,117b] It has been proposed that Aβ up-regulates its own production by increasing BACE1 expression,[118] possibly involving oxidative stress.[119]

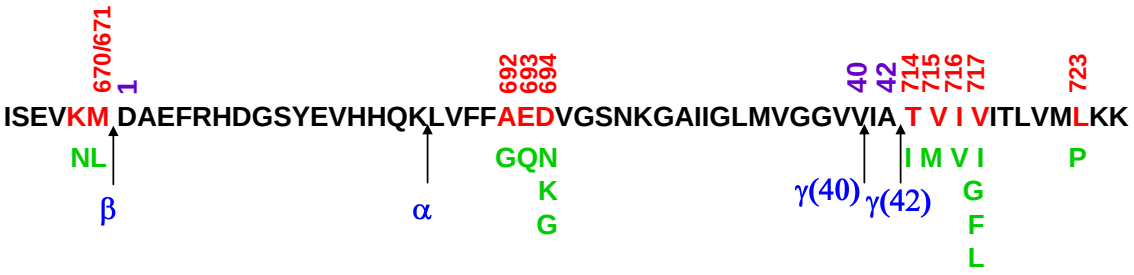

Fig.5. Mutations in APP associated with Aβ production and expression and AD. Redrawn from ref. [1a] Secretase enzymes are indicated in blue, with cleavage sites arrowed. APP sequence numbers are shown in red, Aβ in purple. Mutants are indicated with green letters.

In the non-amyloidogenic pathway, a third enzyme, α-secretase cleaves between amino acids 16 and 17 in Aβ, thus hindering fibrillization of the full peptide. The α-secretases belong to the "A Disintegrin and Metalloprotease" (ADAMs) family of zinc metalloproteinases.[120]

Recent work suggests that in late onset Alzheimer's disease, Aβ accumulation occurs intracellularly in late endosomes where enzymes β-secretase and γ-secretase cleave Aβ, the latter in a presinilin-dependent fashion.[121] The intracellular sites of Aβ production occur where APP is located, as shown in Fig.6.[41] The gene involved in APP recycling in endosomes has been identified, and is termed *SORL1* and the associated protein is SORLA or LR11. Normally the protein product of the gene directs APP into recycling endosomes (retromer recycling endosomes, Fig.6), however mutations produce a decrease in protein product which leads to the pathway

where Aβ production via fragment C99 is increased by enzymes in the late endosomes.[121] It has been reported that the proteolytic processing of Aβ is regulated by glycogen synthase kinase-3 isozymes,[46] however this has been disputed.[122]

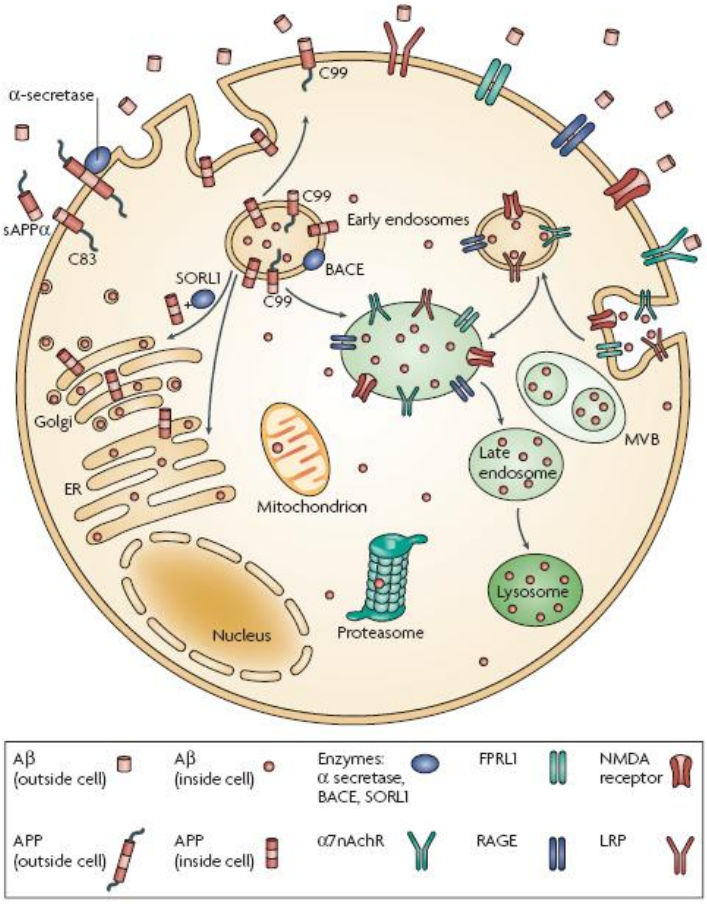

Fig.6. Sites of intracellular Aβ production.[41] Aβ is produced within the endoplasmic reticulum (ER) and Golgi system and secreted. APP is localized in the plasma membrane, where it is cleaved by α-secretase, releasing soluble APPs into the extracellular space and leaving an 83-amino acid fragment known as C83 within the membrane. Unprocessed APP can be internalized into early endosomes. In the presence of SORL1, APP is recycled back to the Golgi in retromer endosomes. Early endosomes contain BACE1 which cleaves APP to produce a 99 residue fragment C99, retained within the membrane. C99 can be shuttled back to the ER to be

processed into Aβ by γ-secretase in the ER, shuttled back to the plasma membrane where the γ-secretase complex is also found, or processed to Aβ within the endosome/lysosome system. Extracellular Aβ (i.e. previously secreted Aβ) can bind to cell surface receptors (including RAGE, LRP, FPRL1, NMDA receptors and α7-nAChR) and the receptor-Aβ complex can be internalized into early endosomes [FPRL1 denotes FMLP-receptor-like protein, NMDA denotes *N*-methyl-D-aspartate and α7-nAChR the α7 nicotinic acetylcholine receptor]. Intracellular accumulation of Aβ mainly occurs in the multivesicular body (MVB) and lysosome, but also in the mitochondria, ER, Golgi and cytosol, where it can influence proteasome function. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neuroscience 8*, 499, copyright 2010.

In addition to the known isoforms of Aβ, i.e. Aβ42, Aβ40 and Aβ38, shorter fragments have been identified in CSF.[120b] This suggested a different APP processing pathway involving concerted cleavage of APP by α- and β-secretases.

It has recently been proposed that rather than Aβ, another APP fragment may be involved in AD (possibly along with Aβ).[123] The N-APP extracellular N-terminal fragment is adjacent to Aβ and is also cleaved by BACE. It triggers the cell death cascade by binding to a neuronal receptor called DR6 (death receptor 6), which is highly expressed in regions of the human brain most affected by AD, in the presence of caspase 6.[123]

Fig.5 summarizes the mutations in APP linked to AD. It is notable that these mutations are located just outside the cleavage sites of β- and γ-secretase beyond the N- and C-termini of Aβ respectively, as well as close to the α-secretase cleavage site within Aβ. Mutations within Aβ are expected to enhance the aggregation properties, and this has been shown for the E693Q mutation (Dutch-type) (Table 2, section 2.2.4). The Flemish A692G mutation leads to a mixture of Aβ plaque and tangle formation as well as microvascular β-amyloidosis and cerebral hemorrhage due to cerebral amyloid angiopathy.[124] This mutation also enhances the proportion of APP cleaved by the β-secretase homologue BACE-2.[1a,125]

APP comprises a group of ubiquitously expressed polypeptides migrating between 110 and 135 kDa on electrophoretic gels.[1a,126] The heterogeneity arises since there are three main isoforms of human APP with 695, 751 and 770 residues, and additionally due to post-translational modifications including *N*- and *O*-glycosylation, phosphorylation and sulfation. APP forms containing 751 and 770 residues are widely expressed both in neuronal and non-neuronal cells throughout the body whereas the 695-residue form is expressed more highly in neurons, and occurs at very low abundance in other cells.[1a] The 751 and 770 isoforms contain a KPI (Kunitz-type Protease Inhibitor) domain (Fig.7) and are thus able to inhibit serine proteases such as trypsin and α-chymotrypsin.[127]

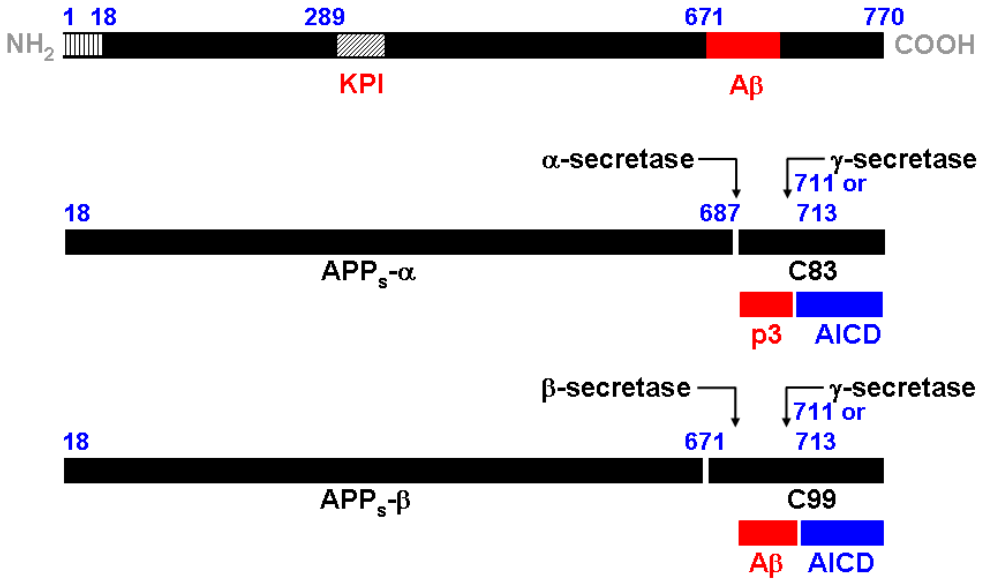

Fig.7. Processing routes of APP to produce Aβ and other peptide fragments (C83, C99 and p3) as well as the soluble APP_s ectodomain fragments. Redrawn, based on schematics by Selkoe.[1a,1b] TM denotes transmembrane domain, KPI denotes Kunitz-type inhibitor domain which is a spliced exon of 56 amino acids inserted at residue 289. Cleavage of both C83 and C99 C-terminal fragments by γ-secretase releases the β-amyloid precursor protein intracellular domain (AICD) into the cytoplasm. A 17-residue single peptide is indicated at the N terminus.

Table 2 presents common mutations in APP. The E693Q mutation was the first to be associated with disease, hereditary cerebral haemorrhage with amyloidosis, Dutch type (HCHWA-D).[128] The A692G Flemish mutation is associated with cerebral haemorrhage with amyloidosis (CHWA).[128] A mutation in APP K670M671 → N670L671 has been associated with the so-called Swedish FAD.[129] The London

variant involves V717I. [52,128,130] Detailed information on mutations in presenilins is provided elsewhere.[10,58a]

Table 2. Effect on Aβ of APP mutations. Adapted from refs.[10,131]

Name/FAD variant	Mutation	Effect on APP	**Effect on Aβ**
APP-717 (London)	V717F/G/I	Differential γ-secretase cut	**Aβ42:Aβ40 ratio increased**
APP-670/671 (Swedish)	K670N and M671L	Increased β-secretase cut	**Increased Aβ40 and Aβ42 in plasma**
APP-692 (Flemish)	A692G	Decreased α-secretase cut?	**Decreased Aβ40 and Aβ42 in media, decreased Aβ aggregation, Aβ42:Aβ40 ratio increased**
APP-693 (Dutch)	E693Q	Unclear	**Decreased Aβ42 in media, increased Aβ aggregation, Aβ42:Aβ40 ratio decreased**
APP-693 (Arctic)	E693G[131]	Unclear	**Decreased Aβ40 and Aβ42 in plasma, Aβ42:Aβ40 ratio decreased**
APP-693 (Italian)	E693K[131]	Unclear	**Decreased Aβ42 in media, Aβ42:Aβ40 ratio decreased**
APP-694 (Iowa)	D694N	Unclear	**Enhanced fibrillization of Aβ40[132]**
PS1-FAD mutations	M139I, H163A, and others[55a]	Differential γ-secretase cut	**Aβ42:Aβ40 ratio increased**
PS2-FAD mutations		Differential γ-secretase cut	**Aβ42:Aβ40 ratio increased**
Trisomy 21 (Down's syndrome)		Increased APP production	**Aβ40 and Aβ42 increased**
Apolipoprotein E4		**Competes for LDL receptor-related protein (LRP)**	**Increased Aβ aggregation**

Overexpression of the mutant V717F leads to neuronal cell death, and damage to synapse termini, well before the development of amyloid plaques.[15] A double mutant transgenic mouse model also expressing the Swedish FAD mutant led to synapse transmission deficits even in young mice without amyloid plaques. These observations suggest that neurotoxicity of Aβ is independent of plaque formation, consistent with the neurotoxic agent being other species such as oligomers as discussed in section 2.6.1.[15] A mutation M67I is found to eliminate production of Aβ42.[133] High levels of Aβ42 result in age-dependent formation of amyloid plaque in FAD-mutant hAPP mice, but not in wild-type hAPP mice.[133] There is no correlation between synapse damage and hAPP levels or plaque load, although there is an inverse correlation with Aβ levels. This points to the neurotoxicity of Aβ even in the absence of plaques.[133]

Aβ is produced from APP via cleavage by the secretase enzymes. APP is a transmembrane protein that is postranslationally modified through the secretory pathway. The first proteolytic cleavage identified is that by α-secretase, occurring 12 amino acids towards the N terminus from the transmembrane domain (Fig.7).[1a] This cleavage produces the large soluble α-APPs peptide into the extracellular space, along with release of the 83-residue C-terminal fragment (CTF) in the membrane. Some APP molecules not subjected to α-secretase cleavage can be cleaved by β-secretase releasing the slightly smaller β-APPs ectodomain derivative and retaining a 99-residue CTF (C99) in the residue of the membrane. The β-secretase cleavage can be followed by γ-secretase activity to produce Aβ, or alternatively sequential action of α- and γ-secretases (the latter acting on C83) produces the p3 peptide fragment (Fig.7).[1a] The presenilin/γ-secretase complex can cleave at other sites ε and ζ in the

transmembrane domain followed by the final cut at the γ-cleavage C-terminal site to produce Aβ38, Aβ40 or Aβ42.[6e]

2.2.4 Aβ Mutations

Strong evidence for the Aβ hypothesis comes from genetic analyses of FAD (Section 2.2.3), since most mutations in the genes for APP, and *PS1* and *PS2* genes appear to cause accumulation of the Aβ42 form.

The following mutations of Aβ are derived from those for APP (section 2.2): [91a,134] A21G Flemish, E22K Italian, E22Q Dutch, E22G arctic, D23N Iowa.

Fig.5 illustrates the location of these mutants, along with the correspondence to the APP sequence. Most mutations occur close to the β-secretase cleavage site, increasing cellular production of Aβ40 and Aβ42, or just after the γ-secretase cleavage which selectively increases production of the more toxic Aβ42.

A systematic investigation of the aggregation tendency of all 798 single-point mutations of Aβ42 was carried out using the Zyggregator algorithm (Section 4.5) to quantify aggregation propensity.[109g] Seventeen mutants were then expressed in *Drosophila melanogaster* and properties including *in vivo* toxicity (survival time) and relative locomotor ability were correlated to the aggregation propensity. Mutants involving E22G (alone, or with one other residue substitution, except I31E/E22G) are found to be most highly pathogenic.[109g] This is consistent with the observed higher rate of oligomerization and fibrillization of the arctic E22G variant compared to the wild-type peptide.[135]

2.3 Neuronal Toxicity of Aβ

At least for human neurons, intracellular Aβ42 is neurotoxic.[91b] There is still some controversy about the precise location of Aβ aggregation *in vivo*,[1a] although the endoplasmic reticulum and Golgi have been identified (see Fig.6).[41] A review discusses the intra-cellular production of Aβ.[41] A novel super-resolution fluorescence imaging technique has been used to probe Aβ42 fibrillization within HeLa cells (differences in fibril morphology *in vivo* and *in vitro* were also noted).[136]

Aβ42 is selectively intracellularly cytotoxic to human neurons, and not to other cell lines.[137] Aβ42 but not Aβ40, Aβ(42-1) or Aβ(40-1) is toxic to human neurons.[137] The proaptotic proteins Bax and p53 are implicated in this intracellular toxicity.[137-138] Disturbances in the cell division cycle may influence apoptosis in AD and this has been related to processing of APP and cyclin-dependent phosphorylation of tau.[138-139]

Long-term potentiation (LTP) is a long-lasting enhancement of synaptic efficacy after brief high frequency stimulation. It has been widely used as a model of synaptic plasticity. Aβ42 and Aβ40 are known to disrupt LTP in neurons.[140] Neurons from transgenic mice expressing genes encoding mutant APP[15,88,133,141] or presenilin linked to FAD[1a,1b,55a,56] exhibit damage to synapses and dendritic spine loss. Aβ is implicated in these defects because γ-secretase inhibition ameliorates some indicators of synapse damage.[141f,142] Synthetic Aβ42 mediates long-term depression (LTD) in an NMDAR-dependent manner *in vivo* [143] as does Aβ secreted by neurons that overexpress APP.[144] Deposition of amyloid plaques and deficits in LTP are observed together, although

spine loss and decrease in pre-synaptic terminal density is observed before plaque deposition probably pointing to the influence of oligomeric or pre-fibrillar Aβ on neurotoxicity. [15,141d] This is also supported by the fact that cognitive loss preceeds the observation of amyloid plaques.[88,141a-c,145] Later work specifically implicated soluble oligomeric Aβ in synaptoxicity and inhibition of LTP.[140b,146] (This is also discussed in section 2.6.1).

Loss of glutamate receptors such as those for AMPA and NMDA caused by Aβ is implicated in synaptic depression and dendritic spine loss.[142,146d,147] Aβ is known to inhibit LTP,[148] as do Aβ fragments such as Aβ(25-35).[149] The blockage is mediated by stimulation of certain kinases.[150] Aβ-induced pathology may progress in a neurotransmitter-specific manner with different susceptibility for cholinergic, gluataminergic and GABAergic transmission (GABA = γ-amino butyric acid).[151] Aβ(25-35) was used to investigate the effect of NMDA and GABA receptor antagonists.[152]

Aβ binds to the α7 nicotinic acetylcholine receptor, nAChR, in neurons.[153] This can result in receptor internalization and hence re-uptake of extracellular Aβ.[41] Since α7 nAChR is co-localized with Aβ in plaques and Aβ disrupts calcium activation and acetylcholine release at the receptor, this interaction may be important in AD pathophysiology.[153a] Aβ blocks the response of these nicotinic receptors, at least at high concentration[154] (at low concentration Aβ42 seems to activate the α7 nAChR, although there is still controversy concerning this).[155] Thus, stimulating nicotinic receptors (eg. with nicotine) protects neurons against Aβ toxicity.[154a,156]

Neuroprotection can also be achieved using α7-receptor agonists.[154a,155a] However, long-term use of nicotinic agonists may induce desensitization of nicotinic receptors.[157] This led to the proposed use of allosteric modulators which bind to a site on nAChR distinct from that of the natural acetylcholine binding site.[157] The specific sequence Aβ(12–28) was implicated in the inhibition of nicotinic currents.[158]

GABA receptors are also potential targets to treat AD. Activation of GABA receptors increases neuronal vulnerability to toxic damage by Aβ.[159] This can be prevented by taurine (2-aminoethanesulfonic acid, related to tramiprosate, Fig.13) or GABA itself, or $GABA_A$ receptor agonists.[152,159]

The role of microglia in the deposition of Aβ plaques (Fig.8) has been examined. Microglia are support cells involved in inflammation that surround senile plaques. Their role is not completely clear,[160] since it has been proposed that they can clear amyloid deposits or alternatively may contribute towards their deposition (and particularly associated inflammation). Reactive microglia associated with Aβ plaques are involved in inflammation in AD. Fibrillar Aβ initiates a tyrosine kinase-based response in mouse microglia (and a human cell line) resulting in production of neurotoxic secretory species, proinflammatory cytokines and reactive oxygen species.[160b] The cytokine TNF-α generated by monocytes and microglia is responsible for most of the Aβ-induced neurotoxicity.[160b] Cytokine TGF-β1 is also involved in the response to injury and has been found in the CNS of AD patients. It has been shown that TGF-β1 induceds Aβ deposition using a mouse model expressing this cytokine from astrocytes.[161]

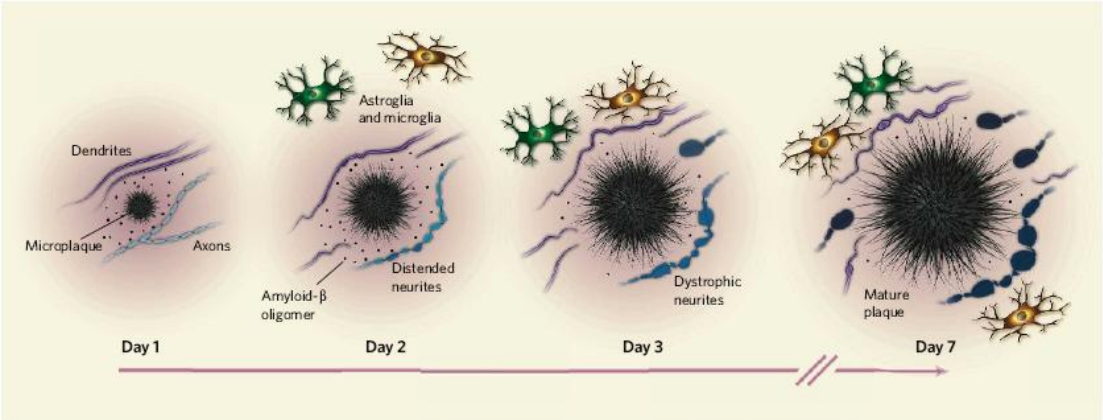

Fig.8. Mechanism of plaque formation and associated migration of glial cells, based on work by Meyer-Luehmann *et al*.[162] Reprinted by permission from Macmillan Publishers Ltd: *Nature 451*, 499, copyright 2008.

Microinjection of fibrillar, but not soluble Aβ, in the brains of aged rhesus monkeys leads to microglial proliferation as well as loss of neurons and tau phosphorylation.[160a] Fibrillar Aβ at plaque-equivalent concentration was found not to be toxic in the brain of young rhesus monkeys, pointing to the role of aging in promoting susceptibility towards Aβ neurotoxicity.[160a] Microglia may enhance the toxicity of Aβ by releasing glutamate through the cysteine-glutamate transporter system x_c^-, and the neurotoxicity can be eliminated via inhibition of NMDA receptors or system x_c^-.[160c] Microglial secretion of ApoE was found to exert a neuroprotective effect.[160c] On the other hand, Nagele *et al*. investigated the role of microglia in Aβ plaque formation and found that they can facilitate the conversion of soluble and oligomeric Aβ into fibrillar form, and that microglia do not remove Aβ from plaques.[163] These authors also highlight the role of astrocytes in accumulating Aβ-positive material as part of their role in debris clearance in response to localized neurodegeneration. It is also suggested that Aβ fibrillization can occur within the surface plasma membrane of microglia.[163] In

contrast to the findings of Nagele *et al*., Simard *et al.* showed using a transgenic mouse model that bone-marrow derived cells (which can cross the BBB) that differentiate into microglia are able to eliminate amyloid deposits by cell-specific phagocytosis.[164] Using multiphoton laser confocal microscopy, Meyer-Luehmann *et al*. showed that microglia are activated within 1-2 days of the appearance of a new plaque and that micro-plaques are rapidly formed that eventually develop into mature plaques (Fig.8).[160d] Accumulation of microglia during inflammation can be mediated via Chemokine (C-C motif) receptor 2 (CCR2)-signaling and thus CCR2 and its main ligand CCL2 (MCP-1) might also be involved in the altered metabolism of Aβ underlying Alzheimer's disease (AD).[165] Cannabinoids may also have a role in neuroprotection by blocking microglial activation.[166] Senile plaques express cannabinoid receptors CB_1 and CB_2 together with markers of microglial activation and a synthetic cannabinoid was shown to prevent Aβ-induced microglial activation.[166]

The zinc metalloprotease insulin-degrading enzyme (IDE, insulysin) is central to the turnover of insulin and degrades Aβ in the mammalian brain.[108,167] IDE forms a stable complex with Aβ40 and with Aβ(17-27).[168] IDE actually forms a complex with Aβ monomer and not oligomers and so is not able to inhibit oligomer-induced loss of LTP.[146a] Monomeric but not aggregated Aβ was able to associate irreversibly with IDE via the substrate binding site of the protease.[168] The phosphorylation of Aβ at serine residue S8 reduces its clearance via IDE and angiotensin-converting enzyme (ACE).[169] The other major endopeptidase involved in Aβ clearance is the zinc metalloprotease neprilysin (NEP),[170] although other proteases capable of degrading Aβ have been investigated.[108b,167] Aggregation-mediated Aβ42 toxicity is decreased

when aging is slowed (in a *C. elegans* model) by decreasing insulin growth factor-1-like signaling (IIS), pointing to a link between the aging process and aggregation-induced neurodegeneration[110c] On the other hand, the transcription factors DAF-16 and HSF-1 which express numerous chaperones (Section 3.2.2) regulate Aβ aggregation and disaggregation activities respectively to promote cellular survival in response to toxic aggregation events (Fig. 9),[110c] and may be the target for therapeutics. ACE plays an important role in blood pressure and body fluid regulation and sodium homeostatis. It is associated with AD in the Japanese population.[171] ACE is found to inhibit Aβ aggregation and can degrade it by cleavage at N7-S8.[171b]

Figure 9 shows schematically proposed pathways for *in vivo* aggregation of Aβ42, relevant to age-related proteotoxicity.[110c] The IIS pathway is regulated by the receptor DAF-2 (inhibition of DAF-2 expression extends the lifespan of *C. elegans* worms). The transcription factors heat shock factor (HSF-1) and DAF-16 regulate opposing disaggregation and aggregation processes. The preferred mechanism whereby toxic aggregates are rapidly degraded (5-II) is positively regulated by HSF-1 (stage 5-A) and negatively regulated by DAF-2 (stage 5-C). When the HSF-1-regulated dissaggregation mechanism is overloaded, a second comes into play (5-III). This produces less toxic higher M_w aggregates. This is positively regulated by DAF-16 (stage 5-B) and negatively by DAF-2 (stage 5-D). The high M_w aggregates can be eliminated by several methods indicated in the scheme.

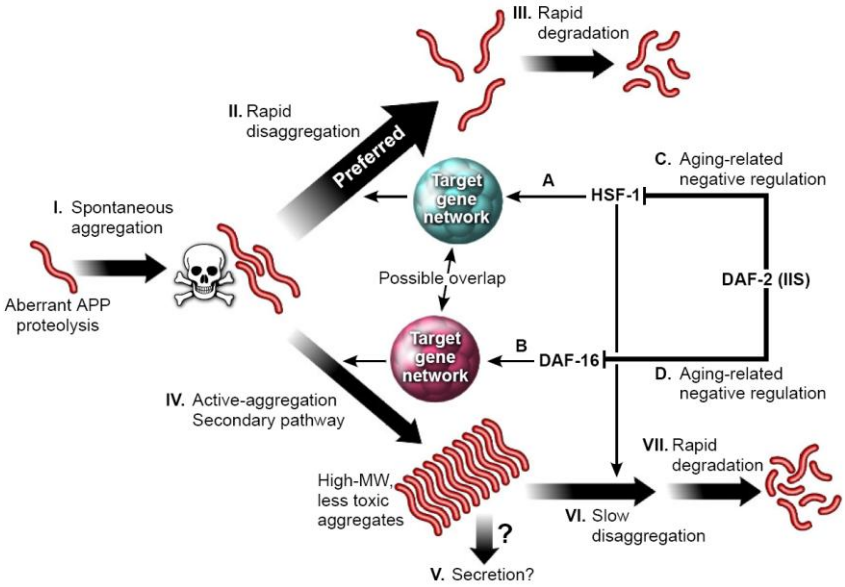

Fig.9. Pathways of regulation of fibrillization in age-onset Aβ proteolysis.[110c]

Adapted from Cohen, E. *et al*., *Science* **2006**, *313*, 1604. Courtesy of Ehud Cohen.

2.4 Biomarkers for AD

Biomarkers for AD are expected to improve the accuracy of diagnosis and to assist in differentiation of cases involving changes in Aβ metabolism. Biomarkers can also be used to investigate the influence of drugs on Aβ production (theranostics) along with safety monitoring, eg. of inflammatory responses in the case of adverse effects.[26] A major public-private partnership initiative funded by the NIH, non-profit AD research organizations and major international pharma companies is the Alzheimer's Disease Neuroimaging Initiative (ADNI), which aims to identify biomarkers in volunteer patients.[172]

Different biomarkers may be appropriate during the progression of neurodegeneration towards AD leading to a dynamic model for applicable biomarkers as shown in Fig. 10.[173]

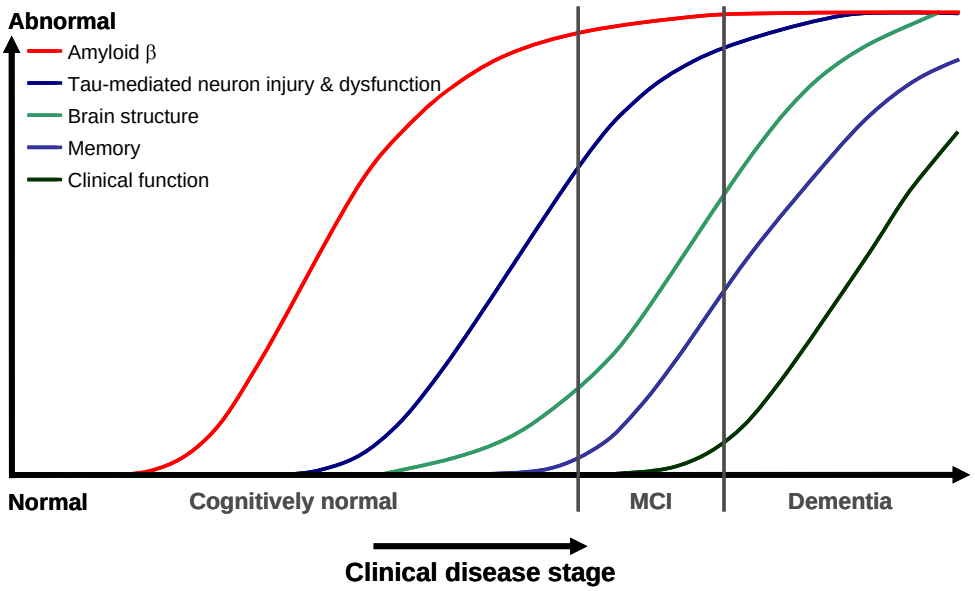

Fig.10. Dynamic events as a basis for use of biomarkers.[173] Aβ is identified by CSF Aβ42 or PET amyloid imaging. Tau-mediated neuronal injury and dysfunction is identified by CSF tau among other indicators. Brain structure is probed using structural MRI. MCI = mild cognitive impairment. [173] Reprinted from Jack, C. R. *et al.*, *The Lancet Neurology*, **2010**, *9*, 119. Copyright 2010, with permission from Elsevier.

Research on biomarkers in CSF and plasma has recently been reviewed.[26,173-174] The only established biomarkers are three that can be assayed in CSF: Aβ42, total tau (t-tau) and p-tau (phosphorylated tau, at position threonine 181 or threonine 231).[19,175] A combined analysis of two or more these biomarkers accurately diagnoses AD more accurately than a single one.[26] The combination of these three analytes has high

predictive value for prodromal (early symptomatic) AD.[26] Reduced CSF Aβ42 (and Aβ40[176]) levels in cognitively normal elderly people who later developed AD have been observed from population-based studies[176-177] and clinical studies.[178] No changes were observed in CSF t-tau or p-tau.[176-177] Biomarkers for tau phosphorylation appear specific to AD in contrast to changes in total tau and Aβ42 which are found in patients with other neurodegenerative diseases.[26] An analysis of multiple study populations to examine potential CSF biomarkers for AD revealed that these three biomarkers can be used as diagnostics to predict incipient AD in patients with mild cognitive impairment.[19,175,179] A total of eighteen signalling proteins have been identified in plasma that can predict for AD, the study being based on plasma samples from individuals with presymptomatic to late-stage Alzheimer's disease and from controls.[180]

Due to the need for invasive treatment (lumbar puncture) associated with measurements using CSF, reliable biomarkers in blood are also sought. It has been suggested that Aβ(1-42) plasma levels are not a sensitive and specific indicator for early diagnosis.[26,174b] This was ascribed to (i) the fact that plasma Aβ is derived from peripheral tissues and not the brain, (ii) variations in Aβ levels due to time-dependent fluctuations, (iii) binding of Aβ to other proteins, (iv) the influence of medications and (v) the involvement of APP and Aβ40 in platelet aggregation.[26,174b] However, it has been reported that a reduction in plasma Aβ42/Aβ40 ratio is associated with cognitive decline over 9-10 years.[181] Tau-related enzymes have been studied as potential blood biomarkers, including kinases involved in tau hyperphosphorylation.[174b] However, it has been concluded that currently tau-related

biomarkers are not reliable diagnostics.[174b] Several other potential blood biomarkers are discussed by Blennow *et al*.[26]

Antibodies associated raised against Aβ (discussed further in Section 2.5) may be useful biomarkers.[172a] A recent study reports the development of immunoglobulin G biomarkers for AD via a screening study using synthetic oligomeric peptoids to capture antibodies.[182]

Biomarkers associated with inflammation have also been investigated, in particular proinflammatory cytokines.[174b] Other disease-related biomarkers include ubiquitin and biomarkers related to cellular senescence such as p53 conformational state or telomere shortening.[174b] Finally, there are biomarkers associated with cerebrovascular damage.[174b] Other candidate CSF biomarkers include BACE, APP isoforms, truncated Aβ isoforms, Aβ oligomers, endogenous Aβ antibodies and neuronal and synaptic markers.[26,174a] In particular, Aβ oligomers are promising biomarkers, however low CSF concentrations make sensitive detection a challenge.[174a] Recently, the (small cytokine) chemokine (C-C motif) receptor 2 (CCR2) has been proposed as a CSF biomarker.[165]

Methods to identify biomarkers for AD diagnosis based on different ELISA assays, mass spectrometry, DNA and gene chips etc are also discussed elsewhere.[174b]

Imaging methods such as MRI (magnetic resonance imaging), fMRI (functional MRI) and PET (positron emission tomography) to diagnose AD are reviewed elsewhere.[173-174] The use of Aβ ligands for PET imaging has attracted attention, notably Pittsburgh

compound B (PIB, Fig.11b). This benzothiazole is derived from the well-known amyloid binding compound thioflavin T (Fig.11a) and it enables direct visualization of fibrillar Aβ load in the brain of living patients.[183] Another widely used PET reagent is FDG, [^{18}F]-2-deoxy-D-glucose, which is sensitive to neuronal glucose metabolism.[174a,183d] Investigation of changes in the retina related to neurodegeneration, i.e. the monitoring of nerve cell death using *in* vivo cell marker methods, has been proposed as a method to screen for AD.[184] Indeed, Aβ is deposited also in the retina.

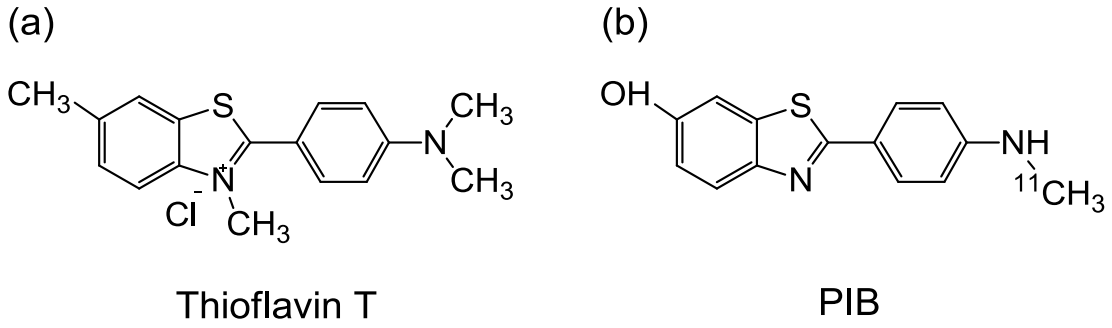

Fig.11. (a) Thioflavin T, (b) Pittsburgh-compound B (PIB) used as a tracer in PET imaging.

The SERPIN (serine protease inhibitor) α_1-antichymotrypsin has been shown to be a biomarker for Alzheimer's, and is present in CSF.[185] This is probably due to the role of oxidative stress and inflammation in Alzheimer's, specifically in the overproduction of secretase,[118-119] which in turn is correlated with Aβ load.[186]

2.5. Antibodies to Aβ and Sequences Therein

Table 3 lists antibodies raised against Aβ and Aβ sequences. These are widely used in studies to identify particular Aβ species produced *in vivo*.

Table3. Commonly used monoclonal Aβ antibodies. Developed from ref.[40]

Name	**Epitope**	**Ref.**
2G3	Aβ(33-40) Aβ(31-40) Specific for Aβ40	161 187 188
3D6	Aβ(1-5)	161,187
4G8	Aβ(17-24) Aβ(17-28)	87,189 24
6C6	Aβ(1-16)	106a,190
6E10	Aβ(1-16) Aβ(1-17) Aβ(4-9) Aβ(4-13)	24,189c 189a 191 168,192
8F5	Aβ42 globulomers (docademeric oligomers)	193
10D5	Aβ(1-28) epitope Aβ (x-12)	161
14C2	Aβ(33-40)	190b
14C12	Aβ(13-28)	190b
21F12	Aβ(33-42) Aβ42 specific	187 188b
266	Aβ "central domain" Aβ(13-28)	103,106a,188a,189b 106a,190a

Several polyclonal antibodies are also used, as listed for instance by Walsh *et al.*[40]

An antibody, now known as A11, that recognises an epitope that is displayed specifically by soluble oligomers of many polypeptides has been identified.[194] The antibodies were raised in rabbits to an oligomeric model antigen comprising gold nanoparticles grafted with Aβ40. This antibody inhibits the toxicity of Aβ40 and Aβ42 oligomers.[194a] This antibody also recognises oligomers from a range of other proteins and peptides.[194a] Recognition was not observed for low M_w- or fibrillar- Aβ

species. This indicates that the antibody recognises a common epitope in soluble oligomers. An antibody termed 8F5 is raised specifically against so-called Aβ42 globulomers (docademeric oligomers).[193] An antibody, distinct from A11, that recognizes on-pathway oligomers (i.e. pre-fibrillar oligomers) called OC has also been reported.[194b,194c] An antibody against Aβ(4-10) (FRHDSGY) inhibits Aβ fibrillization and cytotoxicity, without generating an inflammatory response.[195] Similarly, an antibody against Aβ(1-11) prevents aggregation of Aβ42 and causes disaggregation of preformed Aβ42 fibrils.[196] The binding of fluorescently labeled antibody 6E10 to amyloid deposits within the TgCRND8 mouse brain has been observed, up to 2.5 mm away from the site of injection.[197]

A number of monoclonal antibodies raised against oligomers and fibrils have been identified, which have activity against binding of Aβ to cells and reactive oxygen species generation.[198] Antibodies that recognize the N or C terminal residues of Aβ42 have been used in immunological studies using soluble dimers.[199] A solution NMR structure of the complex formed between Aβ40 and an affibody protein $Z_{A\beta3}$ indicates that this affinity ligand protein stabilizes the β-sheet structure, and the hairpin observed in Aβ40 (section 4.1) is retained.[200] Coexpression of $Z_{A\beta3}$ with Aβ40 and Aβ42 can be used to produce both isoforms recombinantly.[201]

2.6 Oligomers

2.6.1 Toxicity of Oligomers

It is now thought that oligomers formed in the initial self-assembly process of Aβ are the toxic agents.[2a,2d,6d,39,91,104,145a,194a,202] Evidence for this comes from several

experiments on disease related and non-disease related proteins. *In vivo* and cell culture experiments showed that Aβ42 oligomers, formed under conditions that inhibited fibril formation, were neurotoxic.[91a,145a,202a] Synthetic Aβ oligomers inhibit long term potentiation of neuronal cells, as discussed in more detail in section 2.3. It is possible to obtain naturally excreted SDS-stable oligomeric forms of Aβ and extract them from the conditioned medium of 7PA2 Chinese Hamster Ovary cells.[40,105,146a,146d,189b] The oligomers are produced soon after generation of human Aβ in intracellular vesicles in CHO cells which express an APP isoform. The intercellular production of oligomers, principally dimers, was inferred from previous studies using primary human neurons.[40] The secreted oligomers (predominantly dimers and trimers in the conditioned medium, i.e. released from the microsomes) disrupt the LTP of rat hippocampal neurons[146a,146c,146d] as discussed further in section 2.3. These oligomeric forms of Aβ were also shown to disrupt the learning behaviour of rats.[104] The soluble oligomers induce tau hyperphosphorylation leading to disruption of the microtubule skeleton and neuritic degeneration.[199]

Further evidence in support of the toxic oligomer hypothesis comes from the observation that molecules that stabilize fibrils by accelerating Aβ fibril formation leads to a loss of inhibition of LTP by Aβ oligomers.[191] An orcein-related small molecule O4 was found to bind to hydrophobic residues in Aβ and to promote the formation of β-sheet rich fibrils whilst decreasing the concentration of oligomers.[191]

Anti-Aβ antibodies isolated from immunoglobulin strongly disrupt fibrillization.[203] Experiments using polyclonal antibodies indicate that they suppress the toxicity of soluble oligomers whereas there is no antibody response to mature fibrils.[194a] This has

been proposed as a route to vaccination using Aβ42 oligomers.[204] Experiments on Aβ, α-synuclin and transthyretin suggest that cytoxicity shares a common cause not related to the specific sequence.[39a,39c] Recent *in vivo* studies using a mouse model suggest that specific soluble Aβ multimeric species are associated with memory loss in Alzheimers, specifically dodacameric 56 kDa species.[87]

Inhibition of γ-secretase can prevent oligomer formation and restore LTP of rat neurons.[146a] Two γ-secretase inhibitors (flurbiprofen and semagacestat, Fig.13) recently reached, but failed, phase 3 trials. Possible reasons for the failure of these trials that do not necessarily invalidate the Aβ hypothesis have been discussed.[3,49] The γ-secretase inhibitor semagacestat developed by Eli Lilly[205] also failed phase 3 trials due to low potency and signs of apparent Notch toxicity including gastrointestinal symptoms and skin cancer.[3,49]

Aβ oligomers adversely affect synapse function.[140b,206] This leads to the damage to neuropili believed to underlie AD.[206] Aβ oligomers may inhibit long-term potentiation and facilitate long-term depression,[147b] depending on the extent of change in the calcium ion concentration, although there is still controversy around this issue. This has been associated with the synaptic removal of AMPA receptors (AMPARs).[140b] Soluble oligomers, whether naturally secreted or prepared from synthetic Aβ, inhibit hippocampal long-term potentiation,[146a,207] due to removal of AMPA receptors[140b] and disruption of neuronal glutamate uptake.[147b] They also cause a rapid decrease in membrane expression of memory-related receptors such as NMDA and EphB2.[206]

The importance of "gatekeeper residues" that cap aggregation prone sequences in natural proteins and help to hinder aggregation into fibrils has been highlighted.[202c] There is clearly scope for evolutionary pressure to ensure that proteins contain residues that hinder aggregation and/or promote folding into the native state.[202c,208] Specific residues that oppose aggregation were analysed using a computer algorithm that analyses sequence aggregation propensity, including those in Aβ as discussed further in Section 4.5.[202c]

Several characteristics of the AD phenotype can only be replicated using oligomers, including synaptic loss, hippocampal synaptic plasticity, microgliosis and tau hyperphosphorylation.[3,49] The presence of oligomers trapped within plaques, points to the dynamic equilibrium that may exist between these species. Oligomers are formed intracellularly in human neurons.[40] They appear mainly in the form of dimers.[40] Oligomers influence synaptic plasticity and impair LTP in brain tissue.[6e,140b,146,188b] The molecular conformation of a highly synaptotoxic Aβ oligomer structure has recently been eludicated using ssNMR.[209] This study revealed that the oligomer formation is controlled by an N-terminal β-strand.

Stable SDS-resistant oligomers have been detected in normal and AD brain.[210] Oligomers comprising dimers and trimers were detected in tissue extracted from AD brain,[210b] and AFM revealed that these structures comprise 3-4 nm diameter ellipsoids. Aβ-Derived Diffusible Ligands (ADDLs) formed by Aβ42 have been imaged by liquid state AFM.[211] AFM has also been used to compare the formation of oligomer-like species and protofibrils by Aβ40 and Aβ42.[212] AFM has also been used

to image the oligomer-induced formation of membrane pores, as discussed further in section 2.7.

2.6.2 Types of Oligomers

Various types of oligomeric species have been identified, such as protofibrils, paranuclei, globulomers or so-called Aβ-Derived Diffusible Ligands (ADDLs),[146d] especially in the early literature which is not reviewed in detail here. Distinctions between these species have been summarized.[1c,6e,213] ADDLs are larger (4-6 nm diameter) structures than "low *n*" (where *n* is the number of associated monomers) oligomers or globulomers.[213] ADDLs are thought to comprise mixtures of monomer and heterogeneous higher *n* oligomers.[1c] The observation of these oligomeric structures depends on how synthetic Aβ is prepared and incubated. More recently, as discussed below, protocols to prepare oligomers from synthetic Aβ in a controlled and reproducible manner, or in the secreted medium of a rodent cell line (7PA2), have been described.[146d] The natural Aβ oligomers are resistant to SDS and insulin-degrading enzyme (IDE) which can only digest monomeric Aβ.[6e] All soluble oligomers display a common structure against antibodies raised against them (A11 antibody, section 2.5).[194a]

Stable *globul*ar olig*omers* termed globulomers can be prepared by careful preparation methods starting from Aβ monomers. Different groups report various protocols to prepare stable oligomers.[193,214] Using synthetic Aβ, Kayed and coworkers prepare oligomers by controlled evaporation of HFIP which is used to disperse Aβ into monomeric form[194a,215] followed by redispersion in water, or dissolving the peptide in NaOH and diluting this stock solution in a PBS solution containing sodium

azide.[215b,216] Chromy *et al*. reported a related method to prepare stable oligomers from synthetic peptides.[214] As mentioned in Section 2.6.1, the 7PA2 CHO cell line expressing mutant V717F APP has been developed to secrete Aβ in oligomeric form.[189b,213] The oligomers produced by Chromy *et al.* are neurotoxic[214] and block LTP.[193] Electrophoresis in denaturing gels revealed a spectrum of oligomers including dimers, trimers, tetramers, pentamers, and higher order oligomers up to 24-mers.[217]

Globulomers can be prepared by incubation in the presence of SDS or fatty acids. [193,218] These oligomers appear to be dodecameric species with a mass of 60 kDa. They raise oligomer-specific antibodies 8F5 (cf. Section 2.5).[193] They could be related to the brain-derived soluble Aβ*56 dodecamers.[6e,207] Solution NMR has been used to characterize Aβ(M1-42) globulomers indicating a mixed parallel and antiparallel configuration [219] and a dimeric state. To confirm that the globulomers (oligomers) comprise repeats of the dimer, a mutant peptide with L17C, L34C substitution to enable disulfide crosslinking was prepared. This was found to bind to anti-oligomer antibodies with the same affinity as the WT peptide.[219] The mixed β-sheet configuration is in contrast to fibrils which contain only parallel β-sheets (Section 4.1). Aβ oligomers can be used to cross-seed tau oligomers.[215b]

Electrospray mass spectrometry has been used to probe oligomeric states, in particular via analysis of arrival time distributions which can distinguish distinct species with the same charge/mass ratio due to differences in cross-sections of the ions generated.[217] This technique reveals that unfiltered solutions of Aβ42 contain monomers and large oligomers.[217] Filtration can be used to isolate smaller oligomers – dimers up to dodecamers, the latter being proposed as the species that initiate

fibrillization.[217] Later, the same ion mobility mass spectrometry method was applied to investigate oligomer formation by Aβ42 with a comparative study to Aβ40.[220] For Aβ42, oligomers up to dodecamers were observed whereas for Aβ40 only oligomers up to tetramers were found. The authors proposed different mechanisms of fibril nucleation based on these observations (Fig.12).[220] These distinct aggregation mechanisms were supported by earlier conclusions from experiments using photo-induced cross-linking of unmodified proteins (PICUP)[221] to cross-link oligomers which were analysed using a variety of sizing techniques.[221a] These studies showed that the formation of monomers up to tetramers only are observed for Aβ40, whereas pentamer/hexamer paranuclei are formed preferentially by Aβ42.

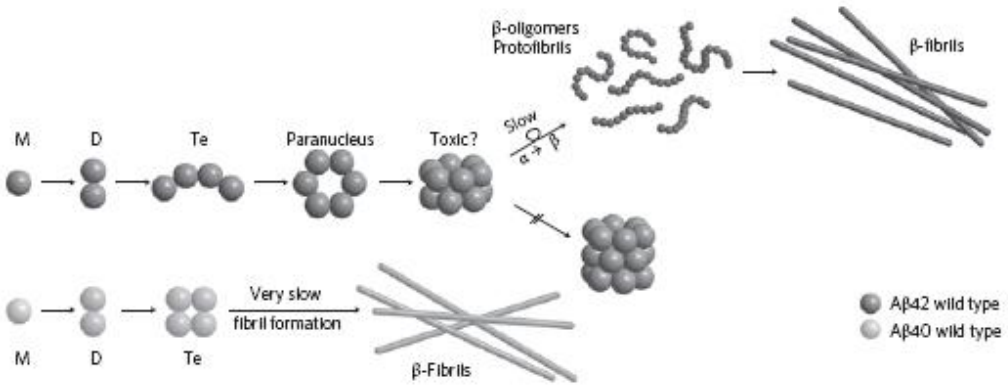

Fig.12. Distinct mechanisms of aggregation of Aβ42 and Aβ40 proposed by Bernstein *et al*.[220] M denotes monomer, D dimer, Te tetramer and the initial toxic species for Aβ42 is proposed to be the dodecamer whilst the planar hexamer serves as a paranucleus. Reprinted by permission from Macmillan Publishers Ltd: *Nature Chemistry 1*, 326, copyright 2009.

Single molecule fluorescence methods indicate that in *vitro* Aβ40 forms a heterogeneous distribution of small oligomers (from dimers to 50-mers).[222] The oligomers represent about 1% of the total number of species present, at the concentrations examined (30 nM to 2 μM). Oligomers have been shown, by SAXS, SDS-page and Western blotting, to bind to APP.[223] Dimers of Aβ cause APP to dissociate from the native homodomer conformation into monomers, whereas Aβ oligomers bind to APP but its homodimer structure is preserved.[223]

2.7 Ion Channel Hypothesis

The mechanism of Aβ cytotoxicity may be due to its ability to form membrane pores or channels.[2d,58a,224] This could be due to the exposure of hydrophobic regions in misfolded proteins such as those that form amyloid fibrils.[224b] Positive charge on a peptide that enables interaction with negatively charged lipid membranes may also be important.[224b] According to the channel hypothesis, pore formation is responsible for the neurotoxicity of Aβ. The original work by the group of Arispe *et al*.[225] established that Aβ is capable of forming membrane channels.

The oligomeric form of Aβ is implicated in pore formation.[224g,226] Lambert *et al*. showed that Aβ42 oligomers bind to cell membranes and cause cytotoxicity under conditions in which mature fibrils do not form.[202a] Further support for this is the finding that pore formation is inhibited by Congo red binding,[227] indicating that the Aβ needs to be aggregated into protofilaments/oligomers for this mechanism to be effective. Aβ forms pores in lipid membranes that contain multimers of the protein, as

revealed by AFM.[226a] Evidence for pore formation *in vivo* has also been obtained via TEM on neuronal cell membranes.[228]

Uptake of Ca^{2+} across the ion channels leads to neuronal degeneration in a dose- and time- dependent manner and ultimately cell death.[226a] Changes in the calcium level and the morphology of cultured cells was also found to be sensitive to the aggregation state of Aβ42.[229] Aβ pore formation leading to an increase in intracellular calcium has been linked to depletion of synaptic vesicles and hence blocked neurotransmission.[230] Transient Ca^{2+} currents are observed near Aβ plaques in the brain of transgenic mice suggesting the presence of clusters of "hyperactive" neurons.[231] A correlation between an increase in calcium ion production and a decrease in Aβ production has also been noted in studies on SERCA (sarco ER Ca^{2+} ATPase) which is a calcium channel-forming protein in the endoplasmic reticulum (ER) membrane.[232] Down-regulation of SERCA leads to increased Ca^{2+} and reduced Aβ levels and over-expression leads to increased Aβ production. SERCA activity was also shown to be decreased in fibroblasts lacking the *PS1* and *PS2* presenilin genes.[232]

2.8 Interaction of Aβ with tau

The tau protein is involved in microtubule assembly and stabilization within the cytoskeleton (in particular in F-actin fibrils). Mutations can lead to filamentous deposits which have been observed for several neurodegenerative diseases such as Pick's disease, Parkinsonism-dementia complex of Guam etc.[2a] Filamentous tau deposits are invariably present even in the absence of Aβ deposits and it is not clear in the context of fibril deposition precisely how Aβ and tau interact although there seems to be a synergistic effect which enhances actin bundling and

neurodegeneration.[2a] Neuronal degeneration induced by tau has been studied *in vivo,* although this is outside the scope of the current review. However, tau can influence Aβ-induced neuronal dysfunction, as exemplified by a study using transgenic mice that express APP along with tau.[4a] Synergistic interactions between Aβ, tau and α-synclein can accelerate neuropathology and cognitive decline, as indicated by a study using transgenic mice.[233] These authors note that the aggregation of α-synuclein into Lewy bodies is a pathology associated with up to 50% of AD cases.

A correlation between an increase in CSF tau and ptau-181 and the amount of cortical amyloid has been reported via brain imaging studies using the PIB biomarker (Section 2.4).[234] There is an inverse relationship involving cortical PIB binding, i.e. Aβ42 deposition is inversely related to CSF Aβ42 levels, but this is not true for plasma species.[183c,234]

3. THERAPEUTIC TREATMENTS

3.1 Existing treatments

The only drugs currently available do not cure AD but may delay the development of symptoms. The current standard of care for mild to moderate AD includes treatment with acetylcholinesterase inhibitors to improve cognitive function and memantine, an NMDA antagonist. The acetylcholinesterase inhibitors include galantamine, an alkaloid available commercially as Reminyl (Shire), Razadyne (Janssen), rivastigmine (Exelon, Novartis) and donepezil (Aricept, Pfizer).[8,31,235] The patent on the latter drug expired in 2010,[236] however it has recently been shown that it may also be effective in the treatment of moderate-to-severe AD as well as mild-to-moderate symptoms.[237] The cholinesterase inhibitor tacrine is rarely used due to poor oral bioavailability and

several potential adverse drug reactions.[31] The NMDA receptor antagonist memantine developed by Eli Lilly is also available under trade names including Ebixa (Lundbeck) and Namenda (Forest).[8,31] The development of the market for these drugs (sales forecasts) has been assessed – sales were $4 billion in 2005.[8]

Although developed as an acetylcholinesterase inhibitor, galantamine also acts to inhibit Aβ aggregation.[238] It is also known as an allosteric modulator of nicotinic receptors.[239] Memantine is thought to function therapeutically as an open-channel blocker of NMDA receptors,[240] and also attenuates the ADDL-induced increase in intraneuronal calcium.[241]

3.2 Inhibitors of Fibrillization/Oligomerization

3.2.1 Small Molecules

Since there have been a very large number of papers on small molecule inhibitors of Aβ fibrillization (also γ-secretase inhibitors) we are not able to review all of them. Reviews on this topic are also available.[242] Here, we focus on key classes of compounds and individual compounds that have attracted particular interest, for example moving to advanced stage clinical trials. Other reviews cover many more of the compounds researched to date. The main focus in the following is on Aβ fibrillization inhibitors, as this has been the main focus of small molecule inhibitor approaches.[189c,216,242a,242b,242d] However, some compounds have been developed to inhibit β-secretase or γ-secretase.[242a]

A large number of small molecules have been studied for their ability to influence Aβ aggregation and toxicity.[216,242] Possibly the most high profile work has been on

tramiprosate (Alzhemed, 3-amino-1-propanesulfonic acid, Fig.13) which reached phase III trails, which however were not successful.[3,49] This compound is a glycosaminoglycan (GAG) mimetic (*vide infra*) shown to bind to soluble Aβ40 and Aβ42 and to maintain them in a non-fibrillar form.[242d,243] It also decreases Aβ42-induced neurotoxicity, is able to cross the blood-brain barrier (BBB) and, using a TgCRND8 mouse model, can reduce amyloid plaque and cerebral levels of Aβ40 and Aβ42.[243a,244]

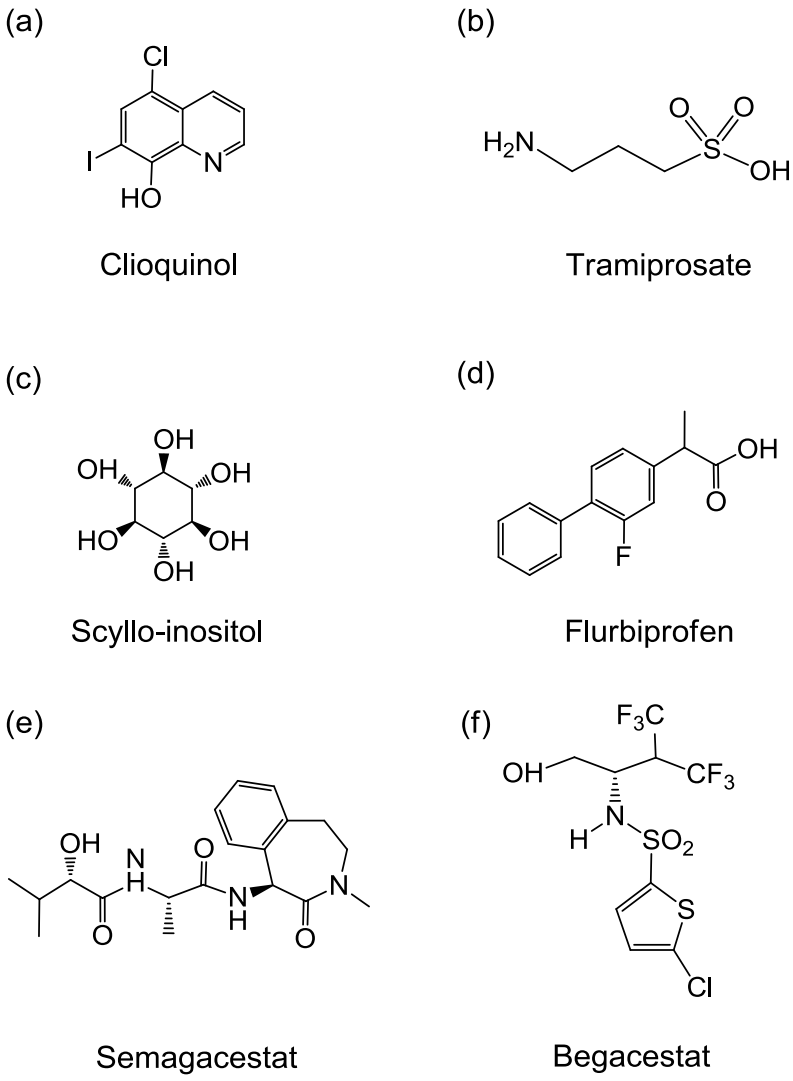

Fig.13. Therapeutic compounds for AD.

A number of dye compounds have been investigated in terms of their effect on Aβ aggregation and neurotoxicity.[196] Congo red (CR), an amyloid-staining dye, has been the subject of several studies in this regard.[245] CR is found to inhibit fibril formation and neurotoxicity towards rat hippocampal cells.[245b] NMR and light scattering suggest that it binds to Aβ40 monomers[245e] and AFM indicates that this ultimately leads to a distinct aggregation pathway.[245f] However, CR is potentially toxic due to the metabolic release of benzidine, and has poor BBB permeability.[245d] CR has been found by Podlisny *et al*. to inhibibit the oligomerisation of Aβ40 using the Aβ expressed by 7PA2 cells in conditioned medium (see Section 2.6.1).[105,189b] In contrast, Knowles and Dobson *et al*. found CR has no effect on Aβ fibrillization.[246] It has been suggested that CR binding arises from a specific conformation of the sulfonate groups in the compound which bind Aβ via electrostatic interactions.[242d] Other sulfonated dyes investigated as Aβ aggregation inhibitors include chrysamine G and thioflavin S.[242d] Methylene blue inhibits Aβ oligomerization by stimulating fibrillization.[247] It has low toxicity and is able to cross the BBB. Wong and coworkers have studied a series of Brilliant blue derivatives, which are food dyes with blood-brain barrier permeability properties.[189c] Reduction in Aβ-induced cytotoxicity due to the formation of off-pathway non-toxic aggregates was noted for some derivatives.

Necula and coworkers investigated a large series of compounds including many dye molecules and classified them according to whether they selectively inhibit the fibrillization or oligomerization of Aβ42, or both.[216] The existence of these different classes of inhibitors might suggest that the pathways of Aβ oligomerization and fibrillization are independent. However, as discussed in section 2.6, whether

oligomers are on- or off-pathway intermediates is still an open question.[248] In a similar analysis of a smaller number of aromatic compounds, including dyes and polyphenols, Ladiwala *et al*.[249] classified the molecules according to whether they (i) remodel soluble oligomers into large non-toxic off-pathway aggregates (some also remodel fibrils), (ii) convert soluble Aβ oligomers into fibrils but are inactive against fibrillar Aβ or (iii) disaggregate soluble oligomers or fibrils into non-toxic low-molecular weight species (Fig.14).

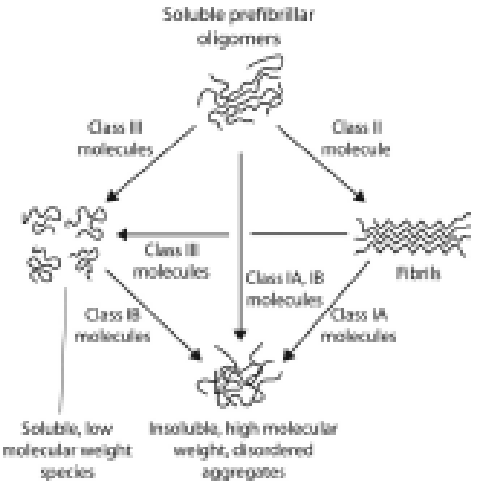

Fig.14. Pathways of aggregation for different classes of small molecule inhibitors of Aβ oligomerization/fibrillization.[249] This research was originally published in Ladiwala, A.R.A. *et al*., *Journal of Biological Chemistry* **2011**, *286*, 3209. © The American Society for Biochemistry and Molecular Biology.

Polyphenols are also able to influence Aβ aggregation. A review provides details of the many compounds investigated in this context[250] and another review describes the associated mechanisms of action with a focus on the antioxidant role of these compounds.[251] Polyphenols that have attracted particular attention such as tannic acid and epigallocatechin gallate (EGCG) have been shown to reduce Aβ cytotoxicity in

cell-based assays,[242a,242d,249,252] and can disassemble mature Aβ42 fibrils[252b] although these compounds are not able to cross the BBB.[189c] Catechins and related polyphenols have also been shown to inhibit Aβ fibrillization, as assayed using ThT fluorescence.[242a,253] It has been noted that competitive binding of the analyte and ThT means that these results need to be treated with caution.[254] Other biologically derived polyphenols including NDGA (nordihydroguaiaretic acid), curcumin and rosmaric acid have also been examined as Aβ aggregation inhibitors, or in terms of disaggregation of pre-formed fibrils.[242a,242d] Research has developed to the stage of *in vivo* studies using Tg2576 mice, with a focus on the pathway of Aβ aggregation, i.e. via oligomers or otherwise.[255] Curcumin can cross the BBB and has been shown to reduce plaque burden using a Tg2576 mouse model.[256] Resveratrol, a polyphenol with antioxidant properties found in wine has been shown to inhibit Aβ42 fibril formation and to reduce cytotoxicity.[257]

Gazit and coworkers have screened a series of small molecule inhibitors of Aβ aggregation that contain aromatic recognition elements as well as β-breaker units.[258] A lead compound NH_2-D-Trp-Aib-OH was identified. This compound is able to inhibit the formation of toxic oligomers and *in vivo* studies using a model mouse (expressing human APP Swedish and London mutations) also indicated improved cognitive function.[258]

Metal ion chelators, in particular of Cu^{2+} and Zn^{2+}, have also been shown to inhibit or reverse aggregation of Aβ *in vitro*.[242a,242d,259] Cherny *et al*. indicate that to efficiently extract Aβ from brain tissue using metal ion chelators including EGTA and ethylene diamine, Ca^{2+} or Mg^{2+} are also required.[259a] Metal ion chelators can be specific to

particular ions, for example clioquinol (Fig.13a), an anti-malerial quinone, selectively binds Zn^{2+} and Cu^{2+} with greater affinity than Ca^{2+} and Mg^{2+} and reduces brain Aβ deposition in an Tg2576 mouse model.[260] Studying Aβ40, Raman *et al*. found that Zn^{2+} and Cu^{2+} but not Fe^{3+} reduce fibril formation, however pre-formed fibrils are stable in the presence of these metal ions. Clioquinol-induced resumption of fibril growth suppressed by Cu^{2+} but not Zn^{2+}, points both to a chelation effect but also a synergistic effect of a Zn^{2+}-clioquinol complex on Aβ40 fibrillization.[259b] A successor to clioquinol is the 8-hydroxyquinoline analogue PBT2 which reached phase II clinical trials, and shows ability to reduced CSF Aβ42 levels as well as cognitive performance.[261] This compound is believed to perform as a superior ionophore than clioquinol, i.e. to more effectively promote the transport of copper and zinc ions across cell membranes.[261b] It is also designed to be easier to synthesize, more soluble and to have increased BBB permeability.[261b] The role of metal ions in the inhibition of Aβ fibrillization or promotion is discussed further in Section 4.8. Inspired by clioquinol, bifunctional compounds that can interact with both metal ions and Aβ have been developed based on pyridine/stilbene derivatives[262] and related pyridinones.[263] These compounds can disaggregate Aβ40. A responsive copper chelator that is released when a pro-chelator is cleaved by β-secretase has been developed and shown to inhibit Cu^{2+}-induced Aβ aggregation.[264] Similarly, a pro-drug compound comprising a glycosylated (glucose-receptor targeting) metal ion chelator (hydroxypridinone) is able to cross the BBB with enzymatic loss of the glucose unit.[265] The compound has an antioxidant property.

Compounds that can block Aβ-induced channel formation in lipid membranes including tromethamine have been investigated via conductance

experiments.[225a,225b,266] Zinc can also block such channels.[229,266b] Arispe *et al*. also developed a peptide-based channel blocker, designed specifically to modulate late Aβ effects on caspase activation and apoptosis.[266b]

Glycosaminoglycans or proteoglycans have been associated with AD since sulfated GAGs such as heparan or chondroitin sulfate are present in neuritic plaques, neurofibrillary tangles and vascular amyloid deposits.[242d,267] Binding of some sulfated GAGs can prevent the proteolytic degradation of fibrillar Aβ. Heparan or heparan sulfate can accelerate the fibrillization of Aβ *in vitro*,[267b] probably due to electrostatic binding to a specific domain in the Aβ(11-28) region.[245a] Chondroitin sulfate also promotes the aggregation of Aβ42 into stable fibrils of reduced toxicity.[268] These studies suggested that inhibitors of this interaction might prove useful as therapeutic agents.[242a] In a related manner, sulfated compounds such as heparin and dextran sulfate can inhibit the interaction of heparan sulfate with Aβ.[269] Some sulfated compounds inhibit Aβ aggregation. Synthetic glycopolymers bearing sulfated saccharide units are also able to suppress the fibrillization of Aβ42, Aβ40 and Aβ(25-35).[270] Simple sugars can either accelerate or inhibit fibrillization.[271] As mentioned above, the GAG-mimetic tramiprosate (Fig.13b) reached phase III clinical trials but was not successful despite reducing CSF Aβ42 levels in mild-to-moderate AD patients.[243b] Reviews that discuss the effect of GAGs on Aβ aggregation are available[267b,267c,272] (the former considers also proteins and lipids). The location and distribution of sulfate groups on the GAG chains may define the interaction with Aβ.[267c,273] Fibril formation of Aβ42 is actually promoted in the presence of GAGs with a suitable spacing of sulfate units, although the cationic polysaccharide chitosan can inhibit such aggregation.[273] Fraser and coworkers showed that in the presence of

low concentrations of sulfate ions, Aβ(11-28), Aβ(13-28), and Aβ(11-25), but not Aβ(15-28) undergo extensive lateral aggregation into "macrofibers".[245a] Ariga *et al*. point to the key role of the Aβ(13-16) HHQK region in the binding process, especially the histidine residues.[274] They also note that low molecular-weight heparins can inhibit fibril formation.

As discussed in the Introduction, oxidative stress plays an important role in AD.[17] The role of antioxidants as novel neuroprotective agents has been reviewed, including polyphenols and other nutraceuticals such as flavonoids and turmeric.[235,251] The effects of a series of flavonoids, catechins and related compounds on Aβ42 fibrillization has been compared and cytotoxicity studies were performed on fibril inhibitors (some fibril promotors were also identified) leading to the identification of promising lead compounds.[275] The structural features of derivatives of the flavonoid fisetin involved in Aβ42 fibril inhibition have been probed.[274] Several studies have suggested that antioxidant vitamins may reduce neurotoxicity, due to their role in alleviating oxidative stress.[242a]

Lipid-based small molecule inhibitors have also been developed, since the presence of lipid membranes may accelerate Aβ fibrillization,[267c] since Aβ deposition is initiated in a plasma membrane-bound form (Section 4.7). A particular focus has been on the interaction of Aβ with phosphatidylinositol since this causes a dramatic increase in fibril growth. This can be inhibited using headgroups from other members of the phosphatidylinositol family.[276] This led to the identification of *scyllo*-inositol (cyclo-hexanehexol, Fig.13c) as lead compound.[277] This compound stabilizes an oligomeric form of Aβ.[277] Administration to TgCRND8 mice prevented Aβ plaque formation,

synaptic toxicity and cognitive deficits.[84b] These effects were seen both in prophylactic and treatment studies.[278] The compound has high availability due to the presence of the inositol transporters at the BBB. This compound has reached phase II trials (as AZD-103, Transition Therapeutics).[279]

The action of other compounds on Aβ aggregation including nicotine, melatonin, rifampicin and tetracyclines has been reviewed.[242a]

Inhibition of Aβ aggregation has been targeted via use of self-recognition elements (SREs). These are molecules based on fragments of the Aβ peptide, which are capable of binding to the corresponding sequence in the native peptide, but are modified so as to disrupt β-sheet fibrillization.[242d] Findeis *et al*. proved that compounds based on a core sequence of the Aβ peptide implicated in fibrillization, Aβ(16-20) (KLVFF), showed promise as SREs.[280] Murphy and co-workers have investigated the effect on Aβ aggregation of compounds based on KLVFF extended at the C-terminus by cationic or anionic residues to give, for example, KLVFFKKKKKK or KLVFFEEEE.[281] Molecules containing three or more lysines in the extension were found to be most effective.[281-282] Modified versions of these compounds have been used to examine the role of surface tension on the kinetics of aggregation of Aβ40.[283] The compounds contained modifications including charge, branching, D/L-isomer substitution and counterion type (motivated by the Hofmeister series) that influence the stabilisation of the protein structure. Gordon and co-workers studied the *N*-methylated compound Aβ(16-20)m and showed that it inhibits fibrillization of Aβ(1-40).[284] *N*-methylation of alternate residues disrupts β-sheet self-assembly due to the presentation on one face of the β-strand of residues incapable of adopting the usual

hydrogen bonding pattern.[284] Doig and co-workers have followed this up and screened a number of *N*-methylated "meptides" based originally on the same sequence, but with all D-amino acids and with modifications of the termini and incorporation of branched residues in certain positions, that are promising as SREs towards targeted aggregation inhibitors, and which also contain elements to improve their pharmacokinetics.[242c,285] A lead compound based on a pentapeptide framework has been shown to have favourable cytotoxicity, and to reverse the inhibition of LTP by Aβ40.[242c] Doig *et al*. have also reviewed other promising compounds.[242b,242c] Austen *et al*. have developed compounds based on KLVFF but with terminal modifications to aid solubility and showed that these were effective inhibitors of toxicity using human neuroblastoma cells.[286] The binding of β-alanine and GABA-modified peptide fragments to amyloid fibrils formed by α-synuclein, Aβ40 and amylin has recently been examined.[287] The binding sequence was based on self-recognition element $V^{77}AQKTV^{82}$ of the full length α-synuclein peptide and shorter sequences therefrom. Pentapeptides containing gamma-amino acids and the KLVFF SRE (or its D-amino acid variant) have also been examined, and a promising compound able to reduce Aβ toxicity, due to inhibition of fibrillization, was identified.[288] Watanabe and coworkers have shown by a binding assay using immobilized KLVFF in the presence of fluorescently-labelled KLVFF in solution that the KLVFF motif is a self-recognition element.[289] Incorporation of the retro-inverse peptide ffvlk into cross-linked PEG networks (as a monomer, dimer or tetramer, all linked to PEG via a cysteine residue) produces gels that can bind Aβ42.[290] It was proposed that these gels could serve as sinks or "detoxification depots" to capture Aβ.

Ligands that stabilize the Aβ(13-26) domain in an α-helical conformation (as revealed by NMR, section 4.1) have been studied as a means to reduce aggregation of Aβ40 and Aβ42, and also cytotoxicity.[109e] The inhibitors were based on self-recognition modelling of the Aβ(13-23) region and comprise two peptidomimics and *N*-decanoyl-diethyelenetriamine. Studies using a *Drosophila melanogaster* model (expressing human Aβ42) also suggested reduced neurodegeneration in the presence of the molecules.[109e] Schrader and coworkers have explored the use of functionalized aminopyrazole derivatives in binding to Aβ42.[291] Ligands bearing multiple lysine residues were found to interact with the ladder of stacked E22 residues and to completely dissolve pre-existing fibrils.[291b] These ligands interact through electrostatic and hydrophobic interactions with the KLVFF sequence although lipophilic groups on some of the designed ligands can interact with the nonpolar residues between I31 and V36.[291] This group have also developed lysine-specific molecular tweezers (Fig.15) with aromatic pincers able to inhibit the aggregation and toxicity of Aβ40 and Aβ42.[292] Mihara *et al*. have shown that even dipeptides LF and CF can form mixed fibrils with small amounts of Aβ42, hence "capturing" the peptide.[293]

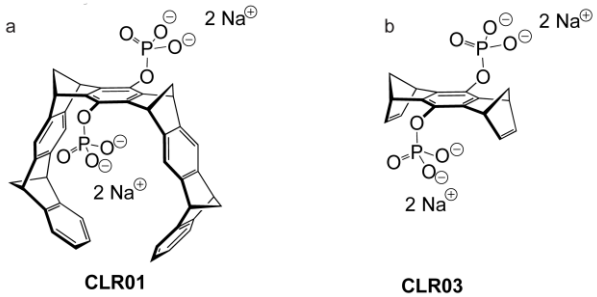

Fig.15. Lysine-specific molecular tweezers CLR01 and CLR03. Reprinted with permission from ref.[292]. Copyright 2011 American Chemical Society.

Statins reduce cholesterol levels in blood and this in turn correlates to Aβ production and aggregation as discussed in Section 4.7. Reduction in cholesterol using lovastatin and methyl-β-cyclodextrin inhibits the production of Aβ in hippocampal neurons without altering APP production.[294]

In a novel approach related to the "toxic oligomer" hypothesis (Section 2.6.1), the action of selective fibrillization accelerators (with an oligomer-reduction activity) has been probed.[191] Compounds related to the dye orcein are shown to accelerate Aβ42 fibril growth, while reducing oligomer formation.

3.2.2 Proteins and Particles

Chaperone proteins have been shown to bind to Aβ and to inhibit fibrillization/ oligomerisation. Several proteins that interact with intracellular Aβ *in vivo* have been identified based on mass spectrometry analysis of human Aβ expressed in *C. elegans*. including the small heat-shock protein αB-crystallin and related proteins such as members of the HSP70 family.[295] αB-crystallin is a chaperone for Aβ, binding to it, and preventing fibril growth.[296] It prevents the spontaneous fibrillization of Aβ42[296] and the Aβ42-seeded growth of Aβ40.[296a] The extracellular chaperone protein clusterin has been shown to sequester oligomers of Aβ40 during aggregation and disaggregation.[101] As discussed above, clusterin (apolipoprotein J) is an identified risk factor in Alzheimer's disease.[54,64] A large-scale screening study has recently identified small molecule proteostasis regulators that induce expression of the chaperone heat shock transcription factor-1 (HSF-1).[297] This may form the basis to

treat a number of protein conformational diseases including AD. The inhibition of fibrillization in the presence of clusterin (Section 2.1.1) has also been examined.[298]

Other proteins and peptides have been shown to bind Aβ and to influence its aggregation. The secretory protein gelsolin[299] and the ganglioside GM1[300] are able to bind Aβ, and it has been proposed that peripheral treatment with these materials can be used to reduce Aβ levels in brain (mouse model).[301] Transthyretin, itself an amyloidogenic protein, and variants, can inhibit the aggregation of Aβ *in vitro* and *in vivo*.[302] The cellular prion protein PrP^C is also able to bind Aβ oligomers at nanomolar affinity, indicating that it may act as a receptor and that it is involved in Aβ-induced synaptic disfunction in the mouse brain.[303] However, these findings have not been replicated by two other groups who found that mice suffered memory deficits even in the absence of prion protein, carefully excluding PrP^C.[146e,146f] Very recent work suggests that cellular PrP^C is essential for oligomeric Aβ-induced cell death, since PrPC antibody blocks Aβ oligomer-induced neurotoxicity and mice expressing PrPC are resistant to Aβ toxicity.[304] It has been proposed that PrP^C may have a physiological role in modulating NMPAR activity, mediated by copper ions, which is disrupted in the presence of Aβ42 (section 4.8).[305]

Polymeric nanoparticles (uncharged acrylamide-based copolymers) inhibit the fibrillization of Aβ40, an observation ascribed to the binding of Aβ (in monomeric or oligomeric form) to the nanoparticles.[114] The binding mainly affects nucleation, and the lag time was found to be strongly influenced by the copolymer composition. The binding is due to a combination of hydrophobicity (controlled via copolymer composition) and hydrogen bonding between polar groups on the polymer and in

Aβ.[114] In the case of cationically (amide) functionalized polystyrene nanoparticles, inhibition of fibrillization is observed for high particle surface area, whereas fibrillization is accelerated for low particle surface areas due to reduction of the lag phase.[306]

Inorganic nanoparticles can function as Aβ fibrillization inhibitors,[307] although this was demonstrated with cytotoxic CdTe nanoparticles. The precise mechanism is unclear. Polyoxometalates which comprise inorganic early transition metal clusters also inhibit the aggregation of Aβ.[308] Surprisingly, organic nanoparticles based on porous silica have been shown to penetrate the brains of fruit flies (*D. melanogaster*), without exhibiting neurotoxic effects and potentially enabling delivery across the BBB.[309] BBB permeability can be modeled using the parallel artificial membrane permeability assay, which measures passive diffusion of small molecule through an artificial lipid membrane.[262,310]

One example of a study using dendrimer molecules employed maltose-functionalized dendrimers to influence the fibrillization of Aβ40.[311] A smaller dendrimer led to fibrillar clumps, sequestering the Aβ and reducing toxicity, whereas a larger one produced amorphous aggregates, toxic to cells.

3.3 Inhibitors of the secretase enzymes

It has proved difficult to identify small molecule inhibitors of β-secretase (BACE1) with favorable pharmacokinetic characteristics.[242a] The crystal structure of β-secretase complexed to an 8-residue peptide inhibitor EVNLAAEF has been reported – the enzyme has the usual binding cleft of an aspartyl protease.[312] A Japanese group have

developed several peptide-based compounds based on phenylnorstatine.[313] A lead compound KMI-429[313b] was explored further *in vivo* using a mouse model.[314] Since the enzyme is membrane bound, an inhibitor has recently been developed by linking a peptide β-secretase inhibitor to a sterol moiety designed to insert in the membrane.[315] Statins may be used to inhibit cholesterol biosynthesis and the expression of BACE and ultimately Aβ production.[316] Statins can inhibit the dimerization of BACE by inhibiting lipidation.[317] This may provide a therapeutic target to reduce Aβ production. The role of protein-protein interactions in the correct assembly of BACE, which is required for Aβ production has been discussed.[317] BACE associates with APP in cholesterol-rich rafts.[318] Knocking out the β-secretase gene *BACE1* causes no adverse phenotype in mice,[319] indicating that β-secretase inhibitors may still provide an attractive target for treatments.

A greater number of compounds have been developed as potential inhibitors of γ-secretase. A complicating factor here is that NOTCH1 and other ligands are also γ-secretase substrates (as well as APP)[242a] and therefore Notch-related side effects may be problematic.[320] However, certain non-steroidal anti-inflammatory drugs (NSAIDs) can modulate γ-secretase cleavage without blocking Notch cleavage.[321] Some NSAIDs can reduce Aβ levels independent of cyclooxygenase (COX enzyme, associated with inflammation) activity.[321a-c] Application of NSAIDs including ibuprofen, flurbiprofen and indomethicin inhibits the release of amyloidogenic Aβ42 from cultured cells.[321a] Flurbiprofen (Fig.13d, in the *R* enantiomeric form, shown to be particularly effective[322]) reached phase III clinical trials, but these were unsuccessful. The target of γ-secretase modulators, such as flurbiprofen and related

compounds, has been located to Aβ(28-36) based on investigation of the localization of fluorescently labeled and biotinylated variants of these NSAIDs.[321e] Some γ-secretase modulators were shown to alter the production of cell-derived Aβ oligomers[321e] while compounds that interact with this region of Aβ act as γ-secretase inhibitors, highlighting the interplay between γ-secretase-influenced Aβ production and Aβ aggregation.[321e] Furthermore, since some NSAIDs bind to to an APP substrate rather than γ-secretase, Notch toxicity may be avoided in this way.[321e]

The γ-secretase inhibitor Semagacestat (Fig.13e) was shown to reduce formation of Aβ in cell assays and also *in vivo* studies.[205,323] Lanz *et al.* show that this compound leads to lowering of Aβ in plasma (in guinea pigs) at low doses, but an elevation of Aβ levels at low concentrations.[323] However, Semagacestat was not successful (due to inferior performance relative to placebo) in phase III clinical trials.

Other strategies have been employed to avoid side effects from Notch impairment. Other than NSAIDs, γ-secretase inhibitors that block Aβ production without influencing Notch activity such as the thiophene sulfonamide Begacestat (Fig.13f) have reached clinical trials, although the mechanisms are unclear.[324] Serneels *et al*. have targeted specific components of the γ-secretase complex (Section 2.2.3), specifically produced by two *APH1* (*APH1A* and *APH1B*) genes.[325] Inactivation of the Alph1B complex decreases Aβ plaque deposition and improves behavioural deficits. The different Alph1 complexes also produce Aβ of different length.[325] As an alternative to targeting γ-secretase, the reduction of γ-secretase-activating protein (GSAP) has been shown to decrease Aβ production in cells and *in vivo* using a mouse

model.[326] The anticancer drug imatinib (Gleevec, Novartis) is able to inhibit Aβ production without affecting Notch cleavage since it prevents interaction of GSAP with the γ-secretase substrate. Thus, GSAP is a potential selective therapeutic target avoiding side effects associated with γ-secretase inhibition.[326] Phiel *et al*. showed that therapeutically relevant doses of lithium chloride, a GSK-3 inhibitor, block the production of Aβ peptides by interfering with APP cleavage at the γ-secretase step, but do not inhibit Notch processing.[46]

3.4. Immunization

In the development of Aβ-based immunotherapies, several strategies have been pursued including passive immunization with monoclonal anti-Aβ antibodies, active immunization with synthetic Aβ42 and active immunization with modified Aβ fragments (Fig.16).[327] Progress with active immunization has been dogged by the failure of phase IIa clinical trials due to the development of meningoencephalitis in several patients (*vide infra*).

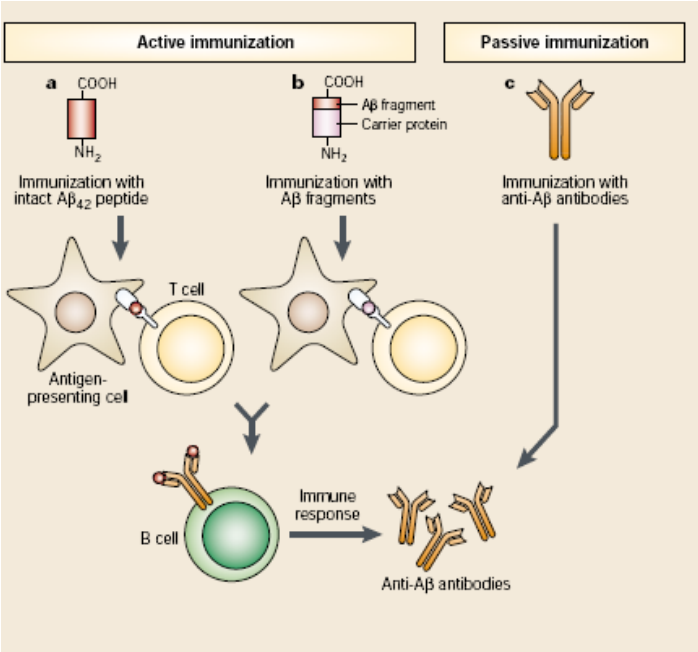

Fig.16. Antibody strategies.[327a] Three different approaches are illustrated. The first (panel **a**) involves immunization with full-length Aβ42. After injection, the peptide is taken up by antigen-presenting cells, and fragments of the peptide are presented to T cells. Subsequently, various B cells that recognize epitopes on Aβ42 are engaged and proliferate. These eventually produce anti-Aβ antibodies. The second active immunization method (panel **b**) involves administration of small fragments of Aβ conjugated to an unrelated carrier protein. This approach is similar to the first with the exception that the T cells are stimulated by the carrier protein rather than the Aβ fragment (which lacks T cell epitopes). This approach yields a strong antibody response to part of the Aβ peptide. The third strategy (panel **c**) is to administer anti-Aβ antibodies directly. This does not require any immunological response from the host and might be useful in individuals in which an immune response is not otherwise produced. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neuroscience 3*, 824, copyright 2002.

Active immunization of transgenic mice with fibrillar Aβ42 leads to a reduction in Aβ deposits in the brain (mouse model) and prevents development of plaque pathology when administered before its formation.[83c,328] The Aβ antibodies generated were reported to reduced Aβ plaque deposition without reducing overall Aβ levels.[84a,329] An improvement in the cognitive performance of mice was also noted. [84a,329] Phase IIa trials pursued by Elan pharmaceuticals, based on this approach (using aggregated Aβ42 termed AN1792 with an immunogenic adjuvant QS-21) were halted when 6% of patients developed meningoencephalitis.[327a,328,330] However, after one year patients producing antibodies that targeted plaques had a lower rate of cognitive decline.[328] In

further follow-up studies it was found that there was a reduction in CSF tau levels following Aβ42 immunization.[331] and a decrease in brain volume.[332] Although clearance of Aβ plaques occurred, this did not prevent neurodegeneration.[333] Using a triply transgenic mouse (3xTg) model it has been shown that Aβ immunotherapy can not only reduce Aβ plaques, but also early hyperphosphorylated tau pathology.[334] Vaccination with soluble oligomers of Aβ42 has also been proposed as a method to produce toxicity-reducing antibodies.[204] Intraperitoneal injection of Aβ-laden brain extracts into the brains of transgenic mice leads to the deposition of amyloid plaques after prolonged incubation time.[335] These observations point to the possible prion-like behaviour of Aβ and the need for great caution (at the very least) in the development of immunization using Aβ directly.

Sigurdsson *et al*. [336] investigated immunization using the modified peptide K_6Aβ(1-30)-NH_2, with an N-terminal hexa-lysine extension to Aβ(1-30), the oligo(lysine) enhancing immunogenicity, this extension following work by Palitto *et al*.[282] (section 3.2.1). Using a transgenic APP mouse model, this peptide was found to reduce the burden of hippocampal and cortical Aβ, and more particularly the amount of soluble Aβ42 in brain tissue.[336]

Passive immunization using antibodies prevents amyloid formation by brain extract from AD patients or APP23 transgenic mice (which over-express Aβ40 which deposits in diffuse and filamentous form), pointing to the suppression of seeding activity.[89] It has been suggested that Aβ disrupts the integrity of the BBB and that this is restored after immunization as the immune system clears Aβ from the brain.[337] The

ability of Aβ40 to cross the BBB and increase its permeability had been observed earlier, using a cell culture model.[338]

Passive immunization using the humanized monoclonal antibody bapineuzumab against Aβ got as far as phase II clinical trials.[339] However, no significant benefit was observed in cognitive performance tests, and in addition some patients exhibited vasogenic edema.[339a] However, this compound continues to be investigated in ongoing phase III trials.[14b,279a,340] as is the related compound solaneuzumab.[14b,279a,341] Passive immunization using monoclonal antibodies (mAbs) has been investigated by Solomon *et al*.[190b,342] They found via *in vitro* studies that selected mAbs prevent the aggregation of Aβ, antibodies recognizing epitopes within Aβ(1-28) and Aβ(8-17).[342] Antibodies raised against Aβ(1-28) can disaggregate Aβ fibrils *in vitro* and reduce the neurotoxic effects of Aβ *in vivo* (MTT cytotoxicity assays using PC12 cells).[190b] Antibodies selectively directed against residues 4–10 of Aβ42, inhibit both Aβ fibrillogenesis and cytotoxicity without eliciting an inflammatory response, in addition they can disaggregate preformed Aβ42 fibrils.[195] De Mattos *et al*. showed that an antibody (m266, Table 3) against Aβ40 is able to bind and completely sequester plasma Aβ.[188a] Peripheral administration leads to a large and rapid increase in plasma Aβ due to a change in Aβ equilibrium in plasma and in CNS. The m266 antibody does not bind to Aβ deposits in the brain.[188a] However, using mouse anti-Aβ IgG_1 antibodies (recognizing Aβ(1-16)) some clearance of compact amyloid deposits is observed after several days, along with microglial activation.[86] The increase in plasma Aβ levels was correlated to Aβ load in the hippocampus and cortex (of PDAPP mice) after immunization.[103] Administration of m266 to PDAPP can improve

cognitive learning, despite no alteration in brain Aβ burden.[343] A complex of m266 and Aβ was found in the plasma and CSF of treated mice. Bard *et al*.[344] noted the presence of anti-Aβ antibodies in the central nervous system after peripheral administration, which were able to bind Aβ plaques and to reduce the pre-existing Aβ burden. The mechanisms of clearance of Aβ *in vivo* by immunotherapy have been investigated, this showed that direct disruption of plaques as well as Fc-dependent phagocytosis was involved.[345]

Immunization using antibodies to human serum amyloid P component leads to the elimination of visceral amyloid desposits, in mice.[346] The plasma glycoprotein human serum amyloid P (SAP) component non-selectively binds all kinds of amyloid deposits.[347] Clinically, it is possible to reduce circulating human SAP using a known SAP-depleting compound, thereby stimulating antibody production. Inhibitors of SAP binding to Aβ fibrils have also been developed, and the lead compound (CPHPC) also reduced levels of circulating human SAP.[346-348] This technology is proceeding to human clinical trials.[349]

3.5 Other Approaches

Other treatment strategies are being pursued, these are discussed elsewhere[8,12a,31] and are not considered further here as they do not involve Aβ. Summaries of compounds in phase I, II and III clinical trials are available.[14,235,279]

Due to a correlation between insulin resistance (section 2.1) and AD, the peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist rosiglitazone has been investigated, [51] and has reached phase III trials.[31] This compound is an insulin sensitizer and

mitochondrial activator, and it activates the PPAR-γ pathway. This increases dendritic spin density and rescues spine loss caused by APOE-ε4.[51] Nerve Growth Factor (NGF) mimics have also reached phase III trials,[31] however since this does not involve Aβ it is outside the scope of the present review. An antihistamine drug Dimebon[350] reached phase III clinical trials for AD which failed due to the absence of a significant effect.[351] The clearance of Aβ is facilitated by ApoE (section 2.1.1). This process is impaired in AD. The compound bexarotene that influences ApoE expression has been shown to enhance clearance of soluble Aβ in an ApoE-dependent manner, also improving cognitive performance in mice.[352]

4. BIOPHYSICAL CHEMISTRY STUDIES OF Aβ AND FRAGMENT PEPTIDES– STRUCTURE AND AGGREGATION

4.1 Aβ Conformation and Structure of Fibrils

Amyloid fibrils contain bundles of β-sheets with backbones orthogonal to the fibre axis, in the so-called "cross-β" structure (Fig.17), and this is observed in fibre X-ray diffraction (XRD) patterns obtained from stalks of Aβ and fragments. [27a,27b,27d,27e,353] The prominent equatorial reflections in Fig.17a (a typical amyloid fibre XRD pattern) arise from the spacing of stacked β-sheets (10-12 Å, the range of values arising from different side chain packing modes) and the meridional reflections arise from the β-strand spacing (4.7-4.8 Å).

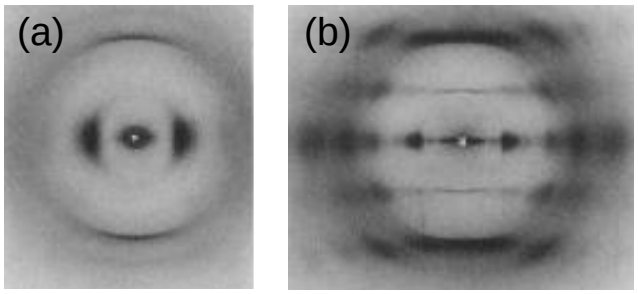

Fig.17. Fibre X-ray diffraction patterns from (a) Aβ40, (b) Aβ(11-28).[353a] Reprinted from Inouye, H. *et al*. *Biophysical Journal* **1993**, *64*, 502, Copyright 1993, with permission from Elsevier

Many studies have analysed the conformational tendencies of Aβ and associated fragments. Early work is reviewed by Serpell[27d] and others.[354] A study using CD and NMR showed that Aβ40 in aqueous buffer solution adopts a predominantly polyproline II conformation at 0 ^{o}C and a random coil state at 60 ^{o}C.[355] The residues in the central hydrophobic region Aβ(12-28) tend to adopt β-strand-like conformations at temperatures below 20 ^{o}C. The thermal PPII – random coil transition was found to be weakly cooperative. The conformation of the N terminal Aβ(1-9) domain was also analysed.[355] CD has also been exploited to examine the secondary structure formation of Aβ(1-28), Aβ(1-39), Aβ42 and Aβ(29-42) in water and TFE or HFIP.[356] In aqueous TFE, Aβ42, Aβ(1-28) and Aβ(1-39) adopt an α-helical structure at low and high pH, but a β-sheet structure at intermediate pH.[356a] Aβ42 and Aβ40 have a net charge of +3 at pH 7.4 and β-sheet formation is possible under these conditions. The solubility of Aβ42 and shorter peptides in aqueous solution was studied as a function of pH and concentration and aggregation was found to be enhanced at low pH.[90a] Murphy and coworkers investigated the aggregation of Aβ(1-28), including the kinetics of aggregation via light scattering.[357]

Much effort has focussed on analysing the conformation of the core hydrophobic domain of Aβ. NMR provides the most detailed conformational information. Benzinger *et al*. applied ^{13}C cross-polarization magic angle spinning solid state NMR to probe the conformation of Aβ(10-35).[358] Multiple quantum analysis using the DRAWS pulse sequence enables the registry of specific labelled residues to be determined. This revealed a parallel β-sheet structure with residues in register. The parallel in-register structure persists despite pH dependent variation in fibril morphology as revealed by electron microscopy.[359] Similar solid state NMR techniques (with constraints from X-ray diffraction and TEM measurements) have indicated a parallel configuration of β-strands of Aβ40, each molecule of which has a β-strand/turn/β-strand arrangement (Fig.18).[360] A similar parallel in-register arrangement was deduced from solid-state NMR data for Aβ42.[359] A turn structure was located in the Aβ(26-29) domain based on proteolysis of disulfide-bridged Aβ(10-43) analogues.[361] A turn was also predicted around residue 26, based on a primary sequence conformation index.[362] This feature was also anticipated based on MD simulations of Aβ(16-35) and Aβ(10-35) that showed turns in the Aβ(24-27) region due to intramolecular D23-K28 salt bridging.[363] In contrast to Aβ40 and Aβ42 which show in-register parallel β-sheets, NMR studies reveal that Iowa mutant D23N Aβ40 can form antiparallel β-sheets.[364]

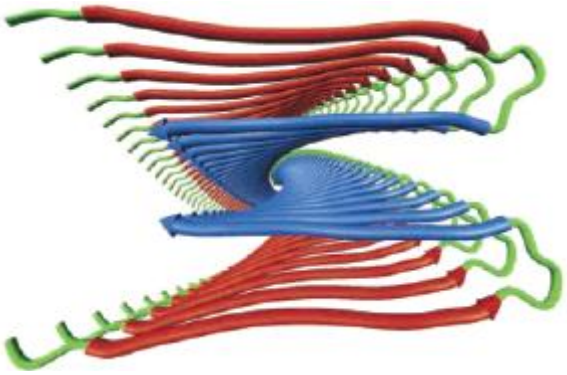

Fig.18. Stacking of Aβ40 into parallel β-sheets according to modelling with constraints from solid state NMR. Reprinted with permission from ref. [360b,360c]. Copyright 2011 American Chemical Society.

Detailed conformations of the β-strand/turn/β-strand U-shaped arrangement have been compared for the Ma-Nussinov[363] models and the NMR derived conformations of Lührs[365] and Tycko.[360b,360c,366] Replica-exchange molecular dynamics (REMD) and discrete molecular dynamics (DMD) simulations[367] have also confirmed these features, as discussed in Section 4.2. Later MD simulations confirmed a turn in the Aβ(23-28) domain.[368] Benzinger *et al*. did not find evidence for a turn structure in the Aβ(25-29) domain in their study of Aβ(10-35),[358b] highlighting the importance of the E22 and V24 residues in salt bridging and hydrophobic interactions respectively. An analysis of fibre X-ray diffraction data from Aβ(11-25) fragments was also consistent with a hairpin turn structure, but in the L17-F20 domain.[353b] However, in a later report from the same group, the unit cell was modelled based on an extended conformation of Aβ(11-25).[369] The β-sheet structure of this peptide has been imaged by cryo-TEM which indicates in-register β-strands.[370]

In contrast to work focussed on the core hydrophobic domain, there have been fewer studies on the conformation of the C terminal region. The hydrophobic C-terminal Aβ(29-42) segment forms a β-sheet structure independent of pH, solvent or temperature[356] pointing to the high aggregation propensity of this domain, in agreement with computer simulations discussed above. The important role of the Aβ(33-35) domain was also shown by Pike *et al*. who performed a thorough study on the secondary structure of variants (amino acid deletions and substitutions) of Aβ(25-35) via CD and on the neurotoxicity of these peptides.[371] These authors also imaged fibril morphology using electron microscopy.[371] Aggregation of β-sheets is found to be maximal at pH 5.4.[356b] A low resolution NMR study indicated a pleated antiparallel β-sheet structure for Aβ(34-42).[372] Hoyer and coworkers reported, on the basis of NMR and computer modelling, a β-hairpin structure for Aβ40 in a complex with a phage-display selected affibody protein.[200] The hairpin comprises residues Aβ(17-36). On the other hand, SDS at pH 7.2 stabilizes an Aβ42 conformation comprising an extended chain (D1-G9), two α-helices (Y10-V24 and K28- A42), and a looped region (G25-S26-N27) as revealed by solution NMR.[373] Helical content of Aβ(12-28) in the K16-V24 domain in presence of SDS is also confirmed by CD.[374] Figure 19 shows the β-strand/turn/β-strand conformation of Aβ(18-42) (residues 1-17 are disordered) within the cross-β fibril structure.

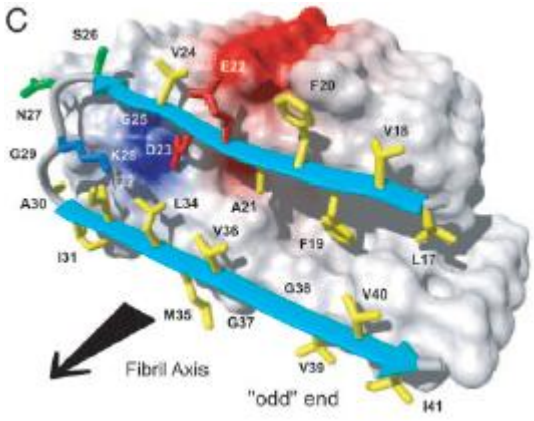

Fig.19. Conformation of Aβ(17-42) as revealed by H/D-exchange NMR.[365] PDB: 2BEG. The hydrophobic, polar, negatively charged and positively charged amino acids are shown in yellow, green, red and blue respectively. Positively and negatively charged surface patches are shown in blue and red, others in white. Copyright 2005 National Academy of Sciences, USA.

Disctinct from longer Aβ fragments (except the Iowa mutant, discussed above), the Aβ(16-22) peptide appears to adopt an antiparallel β-sheet arrangement as indicated by solid state NMR,[375] and isotope-edited FTIR on labelled peptides (combined with ssNMR)[376] and computer simulation.[363]

The crystal structure of several Aβ fragments has recently been reported, specifically Aβ(16-21) (for which three polymorphs were observed), Aβ(27-32), Aβ(29-34), Aβ(30-35), Aβ(35-42) (in two forms), Aβ(35-40) (in two forms) and Aβ(37-42).[377] These peptides all form steric zipper structures, i.e. based on self-complementary pairs of β-sheets. Sequences prone to form steric zipper structures were screened using a 3D-profile self-association energy calculation, which indicated a cluster of aggregation-prone sequences in the C terminal Aβ(30-42) domain.[377] In the first

report by Eisenberg's group on crystal structures of amyloid steric zipper structures Aβ peptides including Aβ(37-42) GGVVIA and Aβ(35-40) MVGGVV were among the peptides studied. [378] GGVVIA belongs to the class of parallel up-down face-to-back β-sheet structures while MVGGVV β-sheets adopt an antiparallel up-down face-to back arrangement.

Based on cryo-TEM images, Aβ40 fibrils are reported to comprise two protofibrils whatever the overall fibril morphology (considerable polymorphism was noted, see also section 4.4).[379] An initial report based on cryo-TEM suggests that Aβ42 forms hollow fibrils in which the hairpin Aβ(17-42) regions fit within the reconstructed density map of the shell.[380] However, a later report from the same group based also on cryo-TEM along with additional mass-per-length measurements from scanning TEM, indicates that Aβ42 forms a single filament structure without a hollow core (Fig.20).[381] The cryo-TEM images published for Aβ(11-25) also do not show a hollow interior.[370] Malinchik *et al*. had earlier proposed a hollow filament structure for Aβ40 on the basis of TEM cross-section images on plastic-embedded samples, as well as analysis of XRD data.[382] Although hollow fibrils were proposed as a common structure for amyloid fibrils,[383] there is in now consensus that this is not the case, and Aβ fibrils are not hollow nanotubes, although these structures can be observed for fragments such as Aβ(16-22) under appropriate conditions (section 4.5). On the other hand, MD simulations constrained by the cryo-TEM density maps for Aβ42, and NMR coordinates based on data from Aβ(17-42) suggest that a hollow core structure might be relevant under physiological and acidic pH conditions.[384]

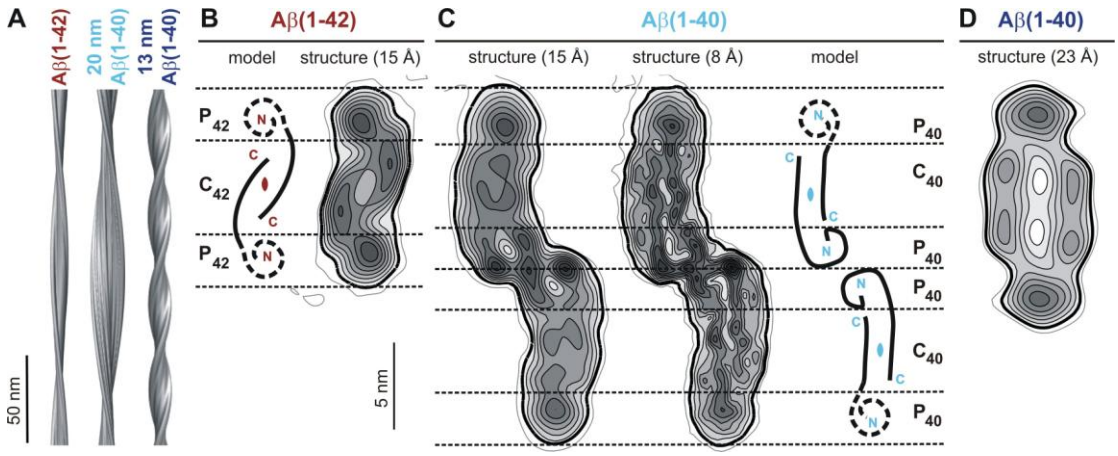

Fig.20. Electron density maps for Aβ40 and Aβ42, reconstructed from cryo-TEM images.[381] Copyright 2009 National Academy of Sciences, USA.

The extent of unfolding of Aβ and ultimately the fibril morphology seem to depend on the level of denaturation.[385] By studying Aβ40 and Aβ42 in which the methionine (residue 35) side chain was in oxidised or reduced state, Hou *et al*. found on the basis of solution NMR, residue-specific interactions in the early stages of aggregation.[386] These authors suggest that both hydrophobic and turn-like structures are required in the first self-assembly steps. The solution structure of the methionine-oxidised form of Aβ40 has also been studied by NMR and CD by Craik and coworkers – random coil structures were observed at pH 4 in water.[387] In SDS solutions, a helical region in the Aβ(16-24) is noted for this peptide.[387] The specific region of Aβ40 involved in contacts between fibrils has been identified by solution-state NMR, and corresponds to Aβ(15-24).[388] The NMR data also show that there is an exchange between a monomeric, soluble state and an oligomeric aggregated state under appropriate (physiological) conditions of salt concentration. The equilibrium can be shifted by varying anionic strength.[388]

4.2 Computer Simulations of Aβ Conformation and Aggregation

This subject has recently been discussed in several reviews, and some aspects are mentioned in the preceeding section.[366,389] Discrete molecular dynamics simulations using a four bead model (corresponding to different peptide sequences) with hydrogen bonding interactions have been used to model the oligomerization of Aβ40 and Aβ42.[390] These simulations uncovered that the most common species are dimers for Aβ40 and pentamers for Aβ42, although oligomers up to 9-mers were found for each. These results may be compared with the experimental data on oligomer size distribution for these two Aβ peptides discussed in Section 2.6.2. Intramolecular contacts were analysed and a turn structure was proposed to stabilize the pentamer structure favoured by Aβ42.[390] REMD has elucidated the conformation of Aβ42 in which loops and turns predominate, although helical regions are found near the C terminus.[391] It was proposed that these helices are involved in the formation of the oligomeric paranucleus revealed by ion mobility mass spectrometry (Section 2.6.2).[391]

The structure of Aβ(17-42) protofilaments in solution has been modeled via MD simulations, along with the influence of mutations at E22 and M35.[392] Steric zipper formation is observed due to favorable C- and N- terminal interface interactions. Double layered models of oligomers were constructed with association via CC or NN interfaces (Fig.21), these being stabilized by salt bridges. The U-shaped β-strand/turn/β-strand conformation (section 4.1) leads to a hydrophobic cavity within the stacked β-sheets.[392] All-atom MD simulations have been performed to elucidate the structural stability and conformational dynamics of Aβ(9-40) for wild type and

mutated sequences.[368] The peptides adopt in-register parallel β-sheets. Salt bridges are formed between N23 and K28, solvated by water molecules, leading to a hydrated channel along the fibril axis.[368] The results were compared to models based on solid state NMR data (section 4.1).[360b,360c]

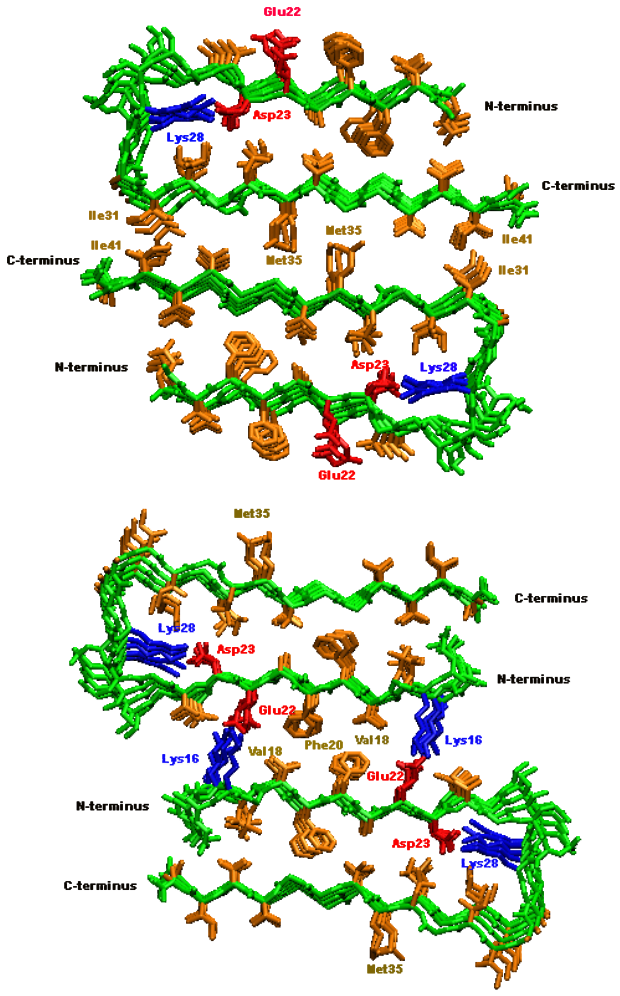

Fig.21. Double layer models used for Aβ(17-42) oligomers.[392] Top- association via C-terminal interfaces; Bottom- association via N-terminal interfaces. Charged residues are shown in red and blue. Reprinted from Zheng, J. *et al*. *Biophysical Journal* **2007**, *93*, 3046, Copyright 2007, with permission from Elsevier.

The oligomerization of Aβ peptide fragments Aβ(16-22) and Aβ(25-35) has been studied by all-atom Monte Carlo simulations.[393] The aggregation of the weakly hydrophobic Aβ(25-35) peptide is driven by the tendency to form hydrogen bonds that stabilize β-sheets, and this peptide aggregates in a single step. The formation of a critical nucleus involving four peptides was noted for this peptide. In contrast, for the more hydrophobic Aβ(16-22) peptide, initial collapse into disordered oligomers was observed. In these oligomers, hydrophobic residues are sequestered from the solvent. The subsequent reorganization of oligomers into β-sheet aggregates involves inter-chain H-bonding interactions and exposure of certain residues to solvent.[393] MD simulations have eludicated the structure of dimers formed by Aβ(25-35) with high β-sheet content aggregates coexisting with less structured dimers.[394] The effect of the self-recognition KLVFF domain and the β-sheet breaker peptide LPFFD on the oligomerization of Aβ(16-22) was examined by all atom MD simulations.[395] The binding domain near the N-terminal involving H13 was identified, and binding energies were calculated. LPFFD as expected has a greater inhibitory effect on aggregation.

DMD simulations using a "united atom" model (specifying all atoms except hydrogens) have been performed to examine the conformation of Aβ(21-30).[367b] A hairpin conformation driven by hydrophobic interactions between V24 and the butyl units of K28, and stabilized by transient salt bridging between E22/N23 and K28, was identified.[367b] REMD simulations have been carried out for the same fragment with similar conclusions.[367c] The significance of the mutations at the E22 residue (Section 2.2.4) in influencing the stability of turn structures, and hence of aggregation into toxic assemblies, was noted.[367c] These observations are consistent with ssNMR

experiments as mentioned above and in the preceeding section. The results of REMD simulations on Aβ(21-30) have been compared to ion mobility mass spectrometry data, in particular the collision cross-section can be modelled, providing information on conformation.[367e] A particular focus was the turn structure and the influence of the E22G, E22Q, E22K and D23N mutations.[367e] The same group also performed all-atom MD simulations in explicit water of the folding of Aβ(21-30) and the Dutch mutant with the E22Q substitution.[367a] Similar conclusions were drawn concerning the presence of loops in the V24-K28 region in the wild-type peptide, although this is not observed for the Dutch mutant sequence. The influence of salt ions on the salt bridging interaction was examined.[367a]

MD simulations have been used to investigate the adsorption of Aβ(17-42) oligomers (from dimers to hexamers) onto self-assembled monolayers (SAMS) with different end-functional groups.[396] The SAMS are proposed to serve as models for cell membranes. The simulations complement experimental studies (CD, AFM, SPR) on Aβ42 by the same group,[397] which shows that fibrillization is accelerated in the presence of SAMs, to the greatest extent for the hydrophobic $–CH_3$ capped SAMS or the cationic $–NH_2$ functionalized SAMS. This observation points to the role of both hydrophobic and electrostatic interactions. The simulations suggest that trimers constitute the smallest nucleus that can seed Aβ polymerization.

4.3 Kinetics and Mechanisms of Fibrillization

4.3.1 Mechanisms

As mentioned in Section 2.2.1, Aβ42 is more aggregation prone and exhibits faster fibril growth than Aβ40. A detailed model for the aggregation into initial aggregates (oligomers, termed "micelles" in the original work) and subsequently fibrils has been developed.[398] This permits nucleation and elongation rate constants to be calculated.

Fibrillization of Aβ appears to occur via a proto- or pre- fibrillar stage.[6d,212,221a,399] These oligomeric species are consumed as fibrillization proceeds.[212,399a] The pre-fibrillar stage has been proposed to correspond to micelle formation. Fibrillization of Aβ40 occurs above a critical concentration which has been described in analogy with a critical micelle concentration (cmc) as shown in Fig.22.[6a,90b,400] Fibrillization can be described using the corresponding one-dimensional model of self-assembly.[401] The initially formed protofibrillar species for several proteins including Aβ40 and Aβ42 are spherical annular-shaped species[212,221a,399b] which may be linked into chains.[221a,399b] The proto-fibril formation process may occur before or during the lag phase.

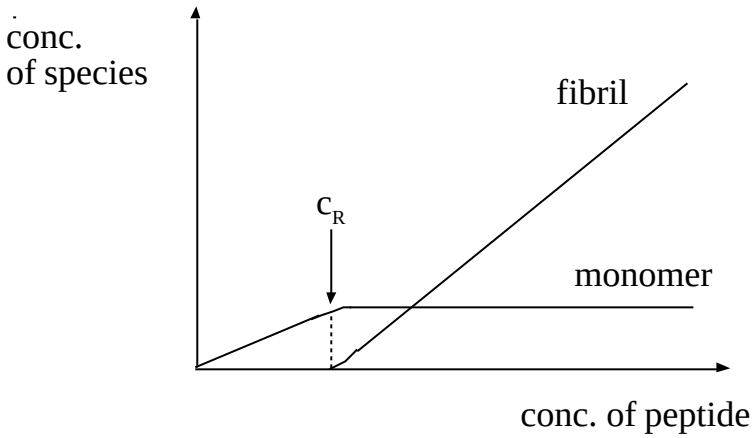

Fig.22. Amount of fibril and monomer as a function of added protein. Fibrils are formed above a critical concentration, c_R. From ref. [402], redrawn from ref. [6a]

Models that extend beyond the Oosawa-Asakura model[403] for one-dimensional self-assembly have been developed to describe more realistically amyloid aggregation kinetics.[404] In particular, the influence of fragmentation, i.e. the generation of secondary nuclei, has been considered. It will be interesting to see this model applied to further analyse the kinetics of Aβ fibril growth.

It is commonly observed that fibrillization occurs after a lag phase, suggesting a nucleation and growth process.[6a,90b,208,385a,400,405] The lag phase can be eliminated by addition of pre-formed aggregates, i.e. by seeding (Fig.23).[6a,90b] The influence of Aβ40 seeds on Aβ42 aggregation and *vice versa* has been examined using immobilized seeds.[406] Oligomeric forms of the peptides were found to be more effective seeds than either monomers or fibrils and Aβ42 monomers aggregated onto Aβ42 fibrils more rapidly than other combinations.

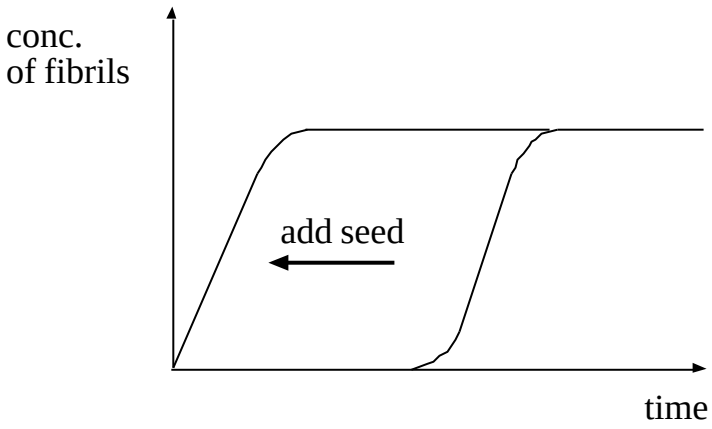

Fig.23. Addition of seed can eliminate the lag time in fibrillization. From ref. [402], redrawn from ref. [6a]

AFM on Aβ40 and Aβ42 provide a picture of the initial formation of protofilaments followed by their replacement with fibrils,[212] this technique being complemented with light scattering, and TEM and SEC analysis of the molecular weight of fractions obtained at different stages of the polymerization process.[399a]

The aggregation of Aβ on planar substrates, hydrophilic mica and hydrophobic graphite, has been investigated. On mica, pseudo-micellar aggregates were noted at low concentration and fibrils at higher concentration. In contrast, on graphite, sheets were observed with a thickness equal to the extended peptide length, oriented along the graphite lattice directions.[212] A later AFM study examined Aβ42 fibrillization and plaque formation, and the interaction between Aβ40 and Aβ42.[406] Deposition was studied on a synthetic template comprising an *N*-hydroxysuccinimide ester-activated solid surface. Aβ42 oligomers were found to be more effective seeds for fibril growth than monomers or mature fibrils.

Lin and coworkers investigated the kinetics and mechanisms of Aβ40 fibrillization under different conditions including variable temperature, ionic strength and pH.[407] The rate of fibrillization was enhanced with increasing temperature or ionic strength. A two-state mechanism of growth was proposed, nucleation being the rate-determining step. The aggregation mechanism was dependent on ionic strength since addition of monomers to fibrils can occur either at the ends (at low ionic strength), or depending on screening of electrostatic interactions at high ionic strength, by lateral aggregation.[407] Linse *et al*. suggest that Aβ42 (D1M mutant) fibrillization occurs via a two-phase state involving soluble Aβ (liquid phase) and aggregated Aβ (solid phase).[408] They used ThT fluorescence and ELISA to monitor fibril growth, and free

Aβ concentration respectively. The free Aβ concentration varied linearly with total Aβ concentration up to 0.2 μM, followed by a decrease to an asymptotic value. This suggests a *cmc* of 0.2 μM in the buffer conditions examined.[408] These authors also point to the need to be extremely careful with the preparation conditions to obtain reproducible fibrillization data on Aβ42 due to the known complications associated with avoiding initial Aβ aggregation (for example, aggregation of Aβ40 even on resin during solid phase synthesis has been observed by SSNMR[409]) as well as nucleation at interfaces (air/water interface of bubbles, surface of vessel).[408] It is already known that very careful protocols have to be followed in studying fibrillization of Aβ42 for example, for example starting from a well-defined state of unaggregated peptide (achieved by initial dissolution in a hydrophobic solvent) and then carefully controlling the addition of water or buffer to a dried film, as discussed in Section 2.6.2.[214] Agitation, e.g. via sonication, is known to have a particularly marked effect on fibril morphology and indeed can be used to create specific polymorphs.[364,410]

Exchange dynamics between monomers and NMR-invisible (dark state) protofibrils of Aβ40 and Aβ42 have been investigated using single residue ^{15}N dark-state exchange saturation transfer NMR.[411] This revealed that the first 8 residues at the N terminus exist in a mobile tethered state while the hydrophobic central residues are either tethered to, or in contact with, the protofibril surface. The C terminal residues display lower affinity for the protofibril surface. The ^{15}N relaxation rates of the C terminus residues are larger for Aβ42 than Aβ40 and this was proposed as an explanation for the higher fibrillization rate of the former peptide.[411]

4.3.2 Kinetics

In the growth state of fibrillization, early work on Aβ40 suggested that the kinetics are first order, i.e. the rate of fibril elongation is proportional to the concentration of monomers.[400,412] This was confirmed via light scattering on Aβ40 in 0.1 M HCl[400] (aggregation kinetics are pH dependent), *in vitro* studies of deposition onto plaques in unfixed Alzheimers disease brain tissue[412a] and ThT fluorescence studies.[412b] However, in the absence of seeds, Aβ aggregation exhibits faster than first order kinetics and an optimal pH of 5 instead of 7 as for templated aggregation.[412a] The temperature dependence of fibril extension for Aβ40 in 0.1 M HCl (determined from size measurements via dynamic light scattering) follows the Arrhenius equation,[413] with an activation energy 96 kJ mol^{-1}, comparable to the value for unfolding of several other peptides.[413] The concentration dependence of fibrillization kinetics was also analysed. Light scattering has also been used to monitor the aggregation of Aβ40 in PBS at different concentrations, and the size and shape of (proto-)fibrils was analysed,[414] and previously, in a similar fashion, this method was applied for Aβ(1-28) [357] and Aβ(1-39).[415]

The rate of fibrillization is strongly influenced by seeding.[90b] Fibril morphology is also influenced by seeding, as revealed by TEM and solid state NMR studies on Aβ40 (see also section 4.4).[410] The kinetics of Aβ fibril growth by covalent attachment of seeds to the surface can be monitored using using quartz crystal microbalance (QCM),[246,416] surface plasmon resonance (SPR)[417] or AFM[418] techniques. Peptide can be immobilized on the QCM crystal via, for example, attachment of the peptide *N*-terminus to a tethered 16-mercaptohexadecanoic acid monolayer (via carbodiimide/*N*-hydroxysuccinimide activation) or biotinylated peptide attached via avidin.[416b] The rate of elongation is observed as a change in resonant frequency of the crystal which

correlates to an increase in surface-bound mass. In this way, monomer addition to Aβ40 fibrils was studied, these were found to elongate in a reversible fashion with a rate that varies with monomer concentration and immobilized seed density.[416b] The growth was consistent with a first-order kinetic model for the single growth phase observed.

Rate constants have been determined for Aβ fibril growth using several methods. Direct comparison between results from these measurements is often not possible, due to differences in the precise definition of the measured quantity. Based on 2D studies using immobilized Aβ40, the following values were reported for the rates of association and dissocation: $k_{ass} = 3.6\times10^{-4}$ μM^{-1} min^{-1}, $k_{dis} = 7.6\times10^{-5}$ min^{-1} and the association constant $K_d = k_{dis}/k_{ass} = 210$ nM.[416b] On the other hand, based on isotope exchange ESI-MS experiments on the dissociation of molecules from fibrils, the following values were determined for Aβ42: $k_{dis} = 0.01$ min^{-1} for Aβ40 and $k_{dis} = 1.67\times10^{-4}$ s^{-1} for Aβ42.[419] Linse *et al*. studied fibrillization of Aβ(M1-40) on polymeric nanoparticles and reported $k_{ass} = 0.13$-0.28 min^{-1}, with a lag time 37-250 min.[114]

Wetzel gives rate constants as shown in Table 4.[417,420] These measurments were based on surface plasmon resonance measurements using immobilized seeds and may not represent bulk values.

Table 4. Rate constants for dissociation and association determined by Wetzel and coworkers[417,420] in the rapid reversible binding of monomer to the fibril in step 1 is follwed by two successive relatively slow processes (steps 2 and 3).

Mechanistic Step	k_{dis} (s^{-1})	k_{ass}
1	8.1×10^{-1}	$6.6\times10^{3}\ M^{-1}s^{-1}$
2	4.4×10^{-3}	$6.4\times10^{-2}\ s^{-1}$
3	4.3×10^{-4}	$4.6\times10^{-3}\ s^{-1}$

Lomakin *et al*. [398] obtained for Aβ40 at pH 2 (0.1M HCl) a fiber nucleation rate k_n = $2.4\times 10^{-6}\ s^{-1}$ and elongation rate $k_e = 90\ M^{-1}\ s^{-1}$. These authors propose mechanisms of fibrillization of Aβ40 under these conditions depending on whether the concentration exceeds the *cmc*, *c** (Section 4.6) or not, as illustrated in Fig.24.[400]

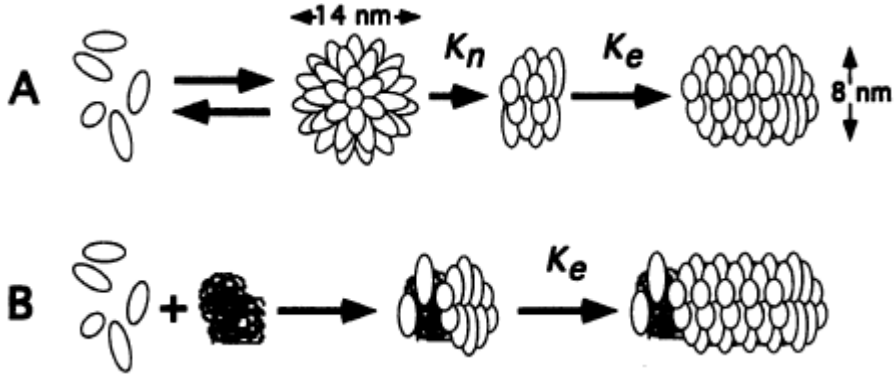

Fig.24. A) Homogeneous nucleation for *c* > *cmc* via formation of initial nuclei (rate constant k_n) followed by extension, rate constant k_e. Initially monomers associate into micelles with R_h = 7 nm, B) Heterogeneous nucleation for *c* < *cmc*, nucleation occurs mainly on seeds not comprising Aβ. Figure from ref.[400] Copyright 1996 National Academy of Sciences, USA.

Inouye and Kirschner[421] analysed the kinetics of Congo red binding to Aβ40 at pH 5.8, based on the measurements by Wood *et al*.[422] Using the Oosawa-Asakura model for one-dimensional self-assembly, they obtained values for the dissociation constant for spontaneous assembly of K_d = 55.1 μM and k_{dis} = 2 h^{-1} (K_d = 53.7 μM and k_{dis} = 5 h^{-1} for seeded assembly) as well as the rate coefficient and proton dissociation constant.[421] The authors note that the kinetics of seeded fibril formation at pH 5.8 are faster than observed by Lomakin *et al*. [400] at pH 2.

4.3.3 Thermodynamics

Wetzel *et al*. have analysed the thermodynamics of amyloid fibrillization.[420] The free energy of fibril elongation of wild type Aβ40 was found to be approximately -37.7 kJ mol^{-1}.[420] This group note that seeded fibril growth can be described as a dissociation/elongation equilibrium. The equilibrium is achieved with a remaining pool of monomer with a concentration of 0.7 – 1 μM (corresponding to the *cmc*).[420] The equilibrium constant K_d was found to be around 0.8 – 1 μM (the value being higher for mutants, and for fibril growth in the presence of ThT). A three step mechanism for elongation was proposed with three different sets of dissocation/association constants as described in the preceeding section.[420] The change in Gibbs energy for a series of mutants of Aβ40 was analysed in a similar fashion.[423] The exchange of Aβ molecules due to fibril association and dissociation has been monitored via hydrogen/deuterium exchange electrospray ionization mass spectrometry.[419] Molecular recycling was found to be much more prevalent for Aβ40 than for Aβ42. The free energy of fibril growth for Aβ40 can been determined from

the critical concentration (Section 4.6) and this leads to ΔG_{el}^{0} = -46.1 kJ mol^{-1},[424] in reasonable agreement with the value from Wetzel's study quoted above. The Wetzel group also studied the effect of Aβ40 alanine mutations on the free energy of elongation.[424] By measuring the amount of soluble monomer, and total protein concentrations at steady state for a series of amyloid forming proteins including Aβ and others, the free energy of elongation was determined and these values may be compared with those for Aβ40.[425] A correlation with the number of residues in the peptide was noted.

4.3.4. Lack of Sequence Specificity in Aβ Aggregation

The cross-β structure (Section 4.1) is a common feature for amyloids formed by many different proteins and peptides, including Aβ.[2d,39b,426] Evidence that formation of amyloid fibrils is a common state for many if not all proteins comes from several types of experiments. First, fibrils can be induced to form by partial denaturing of proteins not involved with any disease[427] or using *de novo* designed peptide fragments (see Section 4.5). Secondly, amyloids can be induced to form by seeding with fibrils of the same, related or unrelated protein,[6a,89,385a,405b,428] a process that may be implicated in the transmission of prion diseases,[6a] although the transmission of spongiform encephalopathies may involve cofactors in addition to prions, the full mechanism being unclear as yet.[429]

A study of mutants of Aβ40 (including Dutch type, Section 2.2.4, but also variants for rodents compared to primates) revealed that N-terminal substitutions that distinguish primate Aβ40 from rodent Aβ40 do not have a significant effect on fibril

morphology.[430] The fibrils formed by the Dutch mutant were found to have enhanced stability at high pH compared to the other variants.

Using fusion constructs with green fluorescent protein (GFP), libraries of mutants of wild-type Aβ42 have been prepared in which hydrophobic residues at the C terminus[431] or 8-12 other residues in the C terminal domain[432] have been substituted with random nonpolar residues. The folding and fluorescence of GFP is prevented by Aβ42, and mutations in Aβ42 that disrupt aggregation then lead to increased fluorescence.[433] It was shown that fibrillization is promoted with hydrophobic residues at positions 41 and 42.[431] and all the hydrophobic mutations (8-12 residues).[432] This implies that generic hydrophobic sequences may be sufficient to promote Aβ42 fibrillization.[432] In related work, Aβ42-GFP fusion constructs with Aβ42 mutations across the whole sequence were expressed and Aβ42 aggregation was probed. This also identified key hydrophobic sequences involved in fibrillization.[433]

4.4 Polymorphism

The morphology of Aβ fibrils can be controlled through the growth conditions, and seeding, leading to distinct polymorphism. This in turn is related to the selection of specific conformations.[389b] An important contribution on the subject of polymorphism was the study by Petkova *et al*.[410] on Aβ40 polymorphism, controlled through application of sonication or not, and imaged by TEM (Fig.25). Solid state NMR was used to identify cross-correlations between different residues, i.e. to probe

conformations in different polymorphs. The cytotoxicity towards rat neurons was also shown to depend on Aβ40 polymorphism. *In vivo*, the phenotype of seeded amyloidosis depends on both the source and the host.[89] This was examined using APP23 and APPPS1 transgenic mice, which overexpress Aβ40 and Aβ42 respectively. It was suggested that the dependence of amyloid morphology on the seed indicates the existence of Aβ polymorphism with associated distinct polymorph biological activity, reminiscent of prion strains.[89]

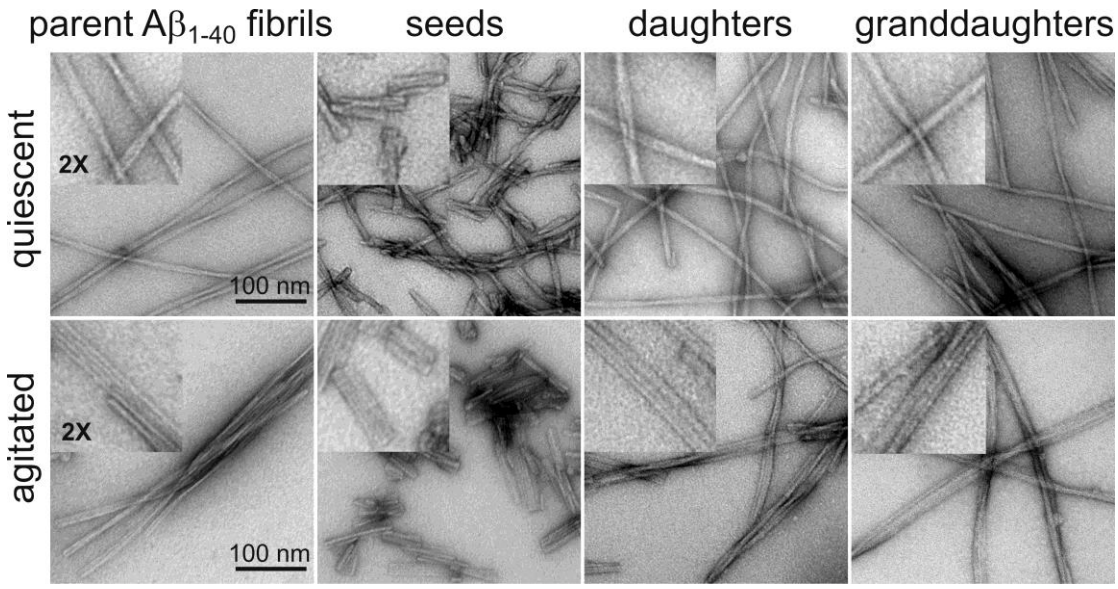

Fig.25. Polymorphism of Aβ40 fibrils.[410] From Petkova, A. T. *et al*., *Science* **2005**, *307*, 262. Reprinted with permission from AAAS.

Tycko's group have recently shown that the polymorphism of Aβ40 (Iowa mutant) can be reduced by repeated seeding (up to nine generations were studied), which can be used to amplify a particular polymorph, even when it is initially a minor component.[364]

Seeds comprising nanotubes (self-assembled in methanol) of the modified peptide AAKLVFF (containing Aβ(16-20), KLVFF) can seed nanotube formation in aqueous solution (conditions that favour non-nanotube fibrillization of unseeded peptide).[434] TEM was complemented by solid-state NMR which probed differences in the strand registry and residue packing in the nanotubes and fibrils. These observations point to the non-equilibrium nature of the seeded polymorphism.

The factors that can influence amyloid fibril polymorphism are complex, and apart from factors such as agitation during sample preparation, the presence of hydrophobic interfaces or the air-water interface can influence fibrillization.[435]

4.5 Fragments

The fibrillization of fragments of Aβ40 and Aβ42 has been extensively investigated. Several early studies are summarized by Teplow[6b] and Serpell.[27d]

Aggregation-prone sequences in Aβ have been identified by computer modelling of measures of β-sheet forming propensity. The TANGO algorithm, based on a statistical mechanical model of protein conformation (based on measures of propensity to form defined secondary structures), predicts a strong tendency for aggregation of Aβ(17-21) and Aβ(31-36), with enhanced aggregation of Aβ42 compared to Aβ40.[436] It can also account for the aggregation propensity of some of the mutant forms of Aβ (Section 2.2.4). The Zyggregator algorithm is based on measures such as hydrophobicity and patterning of hydrophobic residues that lead to a parameter set obtained by screening of sequences in protein databases.[437] It predicts a strong tendency for aggregation of Aβ(15-23) and Aβ(30-42), i.e. similar domains as

indicated by TANGO.[202c,437] These predictions have been compared to results from solid state NMR experiments for both protofibrils and mature fibrils and protofibrils are found generally to have shorter aggregation domains.[438]

Several key regions are associated with aggregation propensity, including the transmembrane hydrophobic domain and the C-terminal hydrophobic domain. In the following, we focus on fibrillization by the shortest core fragment critical to fibril formation which is believed to lie in the Aβ(16-22), KLVFFAE, region. Sequences in this central region of Aβ42 are of great interest because cleavage by the enzyme α-secretase occurs between K and L.[2a] Hilbich *et al.* showed that a region in the hydrophobic core around residues 17 to 20, i.e. LVFF, is crucial for β-sheet formation.[439] They prepared variants of Aβ43, with substitution of various residues 17 to 20, and investigated fibrillization by CD, FTIR and TEM. Substitution with hydrophilic amino acids led to a significant reduction in amyloid formation. Tjernberg *et al*. studied the binding of fragments and variant fragments of Aβ40 to the full peptide.[440] Binding of ^{125}I-labelled Aβ40 was studied by autoradiography. A series of fragments of Aβ40 ranging from 3 to 10 residues was prepared. Only pentapeptides or longer showed significant binding to Aβ40, and fragment Aβ(16-20), i.e. KLVFF, is contained in all strongly binding sequences.[440b] By preparing pentapeptide variants of KLVFF with substituted amino acids, it was found that residues 1, 2 and 5 (K, L, F) are most important for binding of this fragment to Aβ40.[440] A model for the binding of KLVFF to Aβ(13-23) confirmed the importance of these residues in forming an anti-parallel β-sheet. The binding capacity of pentapeptides containing D-amino acids instead of L-amino acids was also studied, since the latter are resistant to proteolysis. Residues 2 and 3 were found to be most critical for binding, D-Lys, D-Phe enhancing

binding.[440b] Findeis *et al*. presented a very thorough study on Aβ-based inhibitors of Aβ fibrillization.[280] This revealed again the importance of the Aβ(16-21) region. A derivative of Aβ(17-21), cholyl-LVFFA-OH was found to be a particularly potent inhibitor of fibrillization, although with limited biochemical stability. The D-amino acid version however was found to be stable in monkey cerebrospinal fluid. Other researchers have explored the role of molecular architecture on the inhibition of fibrillization. Dendritic tetramers containing terminal KLVFF motifs inhibit the aggregation of low molecular weight and protofibrillar Aβ(1-42) into fibrils, substantially more than the linear KLVFF peptide.[441] Recently, bifunctional molecules with terminal KLVFF pentapeptides linked by aminohexanoic acid spacers, have been shown to recognise early Aβ oligomers, manly trimers and tetramers, in mixtures.[442] The molecules may act as "tweezers", binding small oligomers if the central spacer has the correct length.

The dependence of fibrillization on fragment size was investigated for Aβ fragments containing the Aβ(16-20) sequence.[443] Electron microscopy suggested that the shortest fibril-forming sequence was Aβ(14-23), i.e. the decapeptide HQKLVFFAED. The KLVFF sequence was found not to form fibrils itself. However, more recent work shows that this peptide does form β-sheet fibrils under appropriate conditions in aqueous solution.[444] Meredith and coworkers later studied variants of KLVFF[284] and KLVFFAE[445] in which amide protons in alternate residues were replaced by *N*-methyl groups.[284,445] Ac-K(Me)LV(Me)FF-NH_2 was shown to form extended β-strands.[284] It is also more water soluble than KLVFF and can permeate phospholipid vesicles and cell membranes. This variant is also resistant to denaturation by addition of solvent, increase of temperature or pH. It is also a potent inhibitor of Aβ40 fibrillization, and

can break up preformed Aβ40 fibrils, being more effective than KLVFF in both regards[284] (as is heptapeptide NH_2-KLV(Me)F(Me)F(Me)A(Me)E-$CONH_2$[445]). These fragments are believed to form β-strands with distinct faces – one with unmodified groups capable of forming hydrogen bonds and the other containing non-polar methyl groups. This can disrupt the hydrogen bonded β-sheet structure of the Aβ peptide itself.

Other fragments have been designed to inhibit Aβ40 and Aβ42 fibrillization. Rational design principles based on the knowledge of the pentapeptide binding sequence led to a study on LPFFD,[446] now known as the β-sheet breaker peptide. This peptide incorporates proline, known to be a β-sheet blocker and was found to reduce amyloid deposition *in viv*o (rat model) and to disassemble pre-formed fibrils *in vitro*.[446] Prior investigations of Aβ(12-26) with proline substitutions had shown that Pro replacement of any residue in the Aβ(17-23) LVFFAED sequence leads to a loss of fibril formation[447] and this was confirmed via F19P substitution in Aβ42, although oligomeric species were still detected.[217] This observation also points to the role of Pro residues in forming the core of β-sheets. The capped version of the β-breaker peptide, CH_3CO-LPFFD-$CONH_2$, has been shown to improve spatial learning in a rat model.[448]

The retro-inverse peptide ffvlk (lower case indicates D-amino acids) binds Aβ40 fibrils with moderate affinity but this binding can be significantly enhanced by attaching multiple copies of this peptide to an eight-arm branched PEG. [449] Tandem dimers of ffvlk linked by a k(βA) (k denotes D-lysine) spacer or a difunctional PEG

chain also showed some enhancement of binding. All of these conjugates are effective in inhibiting fibrillization of the full Aβ40 peptide.[449]

TEM indicated that KLVFF itself forms fibrils in aqueous PBS solutions (pH 7.4),[284] contrary to the reports by Tjernberg *et al*.[443] There existed some controversy as to whether this fragment itself fibrillizes. This was resolved in a study which used cryogenic-TEM (cryo-TEM) among other techniques to confirm that KLVFF does form amyloid fibrils in aqueous solution, at sufficiently high concentration.[444] Cryo-TEM importantly avoids artifacts when drying to prepare samples for conventional negative stain TEM, this can be problematic in the case of weakly fibrillizing peptides. Fibril formation has been reported for the heptapeptide Aβ(16-22), CH_3CO-KLVFFAE-NH_2.[375] It has been suggested that "fibrils" of this peptide actually comprise nanotubes, based on electron microscopy, atomic force microscopy and small-angle scattering data, and a detailed model for the lamination of the β-sheets in the nanotube wall has been proposed.[450] The related capped peptide Aβ(16-22) E22L self-assembles into nanotubes in water/acetonitrile solution and the grooved surfaces of these were used to probe the binding of Congo red, which was found to bind parallel to the long axis.[451] Analysis of the 3D structure of Aβ42 from NMR (discussed in detail in section 4.1) indicates that residues Aβ(18-26) form a β-sheet as do residues 31-42 within the overall β-strand/turn/β-strand structure of residues 18-42 as shown in Fig.19 (residues 1-17 are disordered).[365] The sequence Aβ(17-23) which seems to be vital in amyloid self-assembly has also been shown to be important in forming the correct β-pleated sheet structure of the Aβ peptide.[439,447] As discussed in section 4.2, computer modelling predicts that Aβ(17-21) should be prone to β-sheet aggregation.[202c,452] Using algorithms based on the aggregation properties of the

constituent amino acids, Kallberg *et al.*[453] suggest that Aβ(16-23) is a so-called discordant sequence of amino acids, in the sense that this sequence is predicted to adopt a β-strand conformation, whereas the full protein structure in the protein data base (ref 1ba6) indicates an α-helix for this region of Aβ40. The protein database structure 1ba6[387] is for Aβ40 with oxidised methionine (residue 35) in aqueous SDS solution, a solvent which is known to favour α-helices, observed between residues 16 and 24 (section 4.1). NMR data on Aβ40 in aqueous solution also indicate an α-helix for residues 15-24 in a water/TFE solution[454] and for residues 1-36 in SDS solution[455] (the data from Sticht *et al.*[454] gives pdb structure 1AML). As mentioned in Section 4.1, NMR in aqueous solution[365] indicates a β-sheet in this region of Aβ42. Different methods to predict secondary structure indeed lead to different predictions for the conformation of KLVFF. The method of Garnier predicts α-helices for KLVFF, whereas the Chou-Fasman method predicts residues KLV are in β-strand and FF in α-helix structures.[202c] Aβ(9-11), i.e. NH_2-GYE-OH, forms amyloid-like fibrils in aqueous solution.[456] MTT assays indicate that the peptide is toxic to neurons.

N-terminal variants of Aβ may also play an important role in AD pathophysiology.[192,457] Hilbich *et al*. used CD and EM to investigate the fibril-forming properties of Aβ43 and N-terminal truncated variants along with variants of Aβ(10-23) with substitution of hydrophobic residues.[361] Peptides in which phenylalanine residues are substituted for less hydrophobic residues show an enhanced solubility in salt solution compared to the native sequence. These authors also investigated variants in which pairs of residues (D23K28, V24G29, G25A30) in the Aβ(10-43) peptide were replaced by pairs of cysteines, in order to examine the influence of disulfide bridging,

i.e. to fix an artificial turn structure. As discussed in section 4.1, the native peptide is believed to adopt a turn structure in the Aβ(23-30) domain.[361]

The fibrillization of Aβ variants with N-terminal glutamines replaced with pyroglutamyl residues has been investigated. Peptides with this form of post-transcriptional modification are observed *in vivo,* in the brains of AD and Down's syndrome patients [457-458] due to presenilin 1 mutations.[55a] In particular, the aggregation of pGlu-Aβ(3-42)[458] and pGlu-Aβ(11-42) has been examined.[459] These peptides exhibit accelerated aggregation compared to the unmodified Aβ40 and Aβ42 and their possible role in seeding aggregation *in vivo* was noted.[459]

The important role of the C-terminal hydrophobic domain, in particular Aβ(34-42) in driving fibril formation has been examined.[34,372,460] The length of the C terminus critically influences the rate of amyloid formation but only has a minor effect on the solubility.[34] Peptides containing the C terminal sequence Aβ(36-42/43) can seed fibrillization by peptides lacking the C terminal residues (Aβ40-42).[34]

4.6 Micelles

Aβ has surfactant-like properties, for example it is able to reduce surface tension in a concentration-dependent manner and both Aβ40 and Aβ42 and shorter variants (C-terminal truncations) exhibit an apparent critical micelle concentration (*cmc*).[461] The "micelles" may in fact correspond to proto-fibrillar species believed to be involved in the nucleation of fibril formation (section 4.3.1). The formation of SDS-stable aggregates was also confirmed by SDS-PAGE. For a series of C-truncated peptides, the *cmc* was found to be 25 μM in aqueous solution, and measurements on the

partitioning of a fluorescent dye DPH (1,6-diphenyl 1,3,5-hextriene) suggested that residues 29-42 form the interior hydrophobic domain.[461] The *cmc* of Aβ40 has also been deduced from the concentration dependence of fibril growth kinetics and was found to be $c^* = 0.1$ mM in acidic aqueous solution.[400] Surface-pressure area experiments and pyrene fluorescence measurements indicate $c^* = 17.5\text{-}17.6$ μM in aqueous Tris buffer solution.[462] Wetzel and coworkers report a value $c^* = 0.7 – 1$ μM in aqueous PBS solution, measured by SPR and also deduced from fibril dissociation equilibrium constants, obtained from kinetic measurements of ThT fluorescence.[420] Linse *et al*. obtained $c^* = 0.2$ μM for Aβ(M1-42) in the buffer system they studied, and noted that this is lower than the value observed by Wetzel and coworkers for Aβ40 due to differences in ionic strength of buffer as well as the peptide length.[408] The aggregation number of the micelles (N = 25) was estimated using fluorescence quenching techniques. The *cmc* of Aβ(11-25) determined from FRET experiments is 3 μM at pH 5 and 70 μM at pH 7.4.[463]

Above the cmc, Lomakin *et al*. found that the initial rate of elongation and the final size of fibrils were independent of Aβ concentration due to the monomer-micelle equilibrium.[400] The surfactant *n*-dodecylhexaoxyethylene glycol monoether ($C_{12}E_6$) slowed nucleation and elongation of Aβ40 fibrils in a concentration-dependent manner.[400] The hydrodynamic radius of the micelles was approximately 7 nm. Detailed SANS experiments later provided a model for the shape and dimensions of "spherocylindrical" micelles.[464] The authors identified these species as aggregates comprising 30-50 monomers, therefore they seem to correspond to proto-fibrils rather than oligomers.

SDS micelles hinder the formation of β-sheet fibrils by Aβ40 and Aβ42, and instead stabilize helical conformations.[373] However, at lower concentration (below the *cmc*), SDS promotes the formation of oligomers by Aβ42 (not Aβ40).[465] The interaction of Aβ40 with SDS has been investigated by small-angle X-ray and neutron scattering (SAXS and SANS).[466] It was shown by time-resolved SAXS that Aβ40 rapidly forms a complex with pre-existing SDS micelles due to interaction between the sulfate groups and the hydrophilic headgroup units of the surfactant.[466] Below the critical micelle concentration of SDS, globular core-shell aggregates were also formed but lagging behind the CD-observed transition in secondary structure of Aβ40 from random coil to (predominantly) α-helical. These measurements also showed that aggregation of Aβ40 in the presence of HCl can be monitored by SAXS. Aggregates of Aβ42 with SDS (sub-micellar concentrations) have been observed via AFM, and the effect of SDS on secondary structure was probed by CD and FTIR.[467] The cationic surfactant hexadecyl-*N*-methylpiperidinium bromide is able to inhibit Aβ aggregation well below its *cmc*, pointing to a mechanism not involving micellar solubilisation.[468] A similar phenomenon is reported for a tetrameric quaternary ammonium cationic surfactant which can also disassemble existing Aβ40 fibrils.[469] Instead, it was suggested that a specific binding surface on Aβ40 (absent for other amyloid forming proteins) is able to bind such amphiphilic molecules.[468] A cationic surfactant containing an azobenzene moiety influences Aβ40 fibrillization differently depending on its conformation, which can be photo-switched.[470] The interaction of Aβ(12-28) with SDS has been investigated by CD and NMR, an α-helical conformation for residues 16-24 was noted (see also section 4.1).[374]

The interaction of Aβ40 with the bio-derived surfactant surfactin is also dependent on the aggregation state of the surfactant.[471] Below the *cmc*, surfactin causes Aβ40 to unfold and to fibrillize. Well above the *cmc*, β-sheet fibril formation is inhibited.

4.7 Interactions with Lipid Membranes

The importance of lipid interactions with Aβ is highlighted by the fact that ApoE, (especially the ε4 allele) a key genetic risk factor for AD (section 2.1), is involved in lipid metabolism.[472] Lipid membranes have a number of important roles in modulating amyloid fibrillization. These include: (partially) unfolding the peptide, increasing the local concentration of peptide bound to the membrane, orienting the bound protein in an aggregation-prone manner and variation of penetration depth into the membrane affecting the nucleation propensity.[473] Lipid rafts are implicated in Aβ dimer and oligomer formation.[474] and may provide platforms for selective deposition of different Aβ aggregates (this also depends on the ordering of the lipids within the membranes which may be different in the rafts[475]).[476] Ganglioside-rich lipid rafts induce Aβ oligomerization, for which cholesterol appears not to be essential.[474b] The involvement of different types of membrane structure (vesicles formed by different phospholipids and gangliosides) in Aβ fibril growth has been discussed in reviews of membrane-amyloid interactions.[224g,473,477] The role of membranes in the formation of annular structures that may comprise arrays of oligomers has been revealed by AFM.[224g,226] and the role of oligomers in creating pores/ion channels has been revealed by membrane conductance measurements (see also section 2.7).[215a]

Aβ is generated via regulated intramembrane proteolysis (RIP),[6e] which involves the shedding of the ectodomain of APP through membrane-anchored secretases (section 2.2.3). The membrane-bound stubs can then be cleaved within their transmembrane domains to release small peptides (Aβ in the case of APP) into the extracellular space and intracellular domains into the cytoplasm.[6e]

Aβ, which is a cationic peptide at neutral pH, (residue-specific pKa values are available[374,478]) interacts with anionic lipid membranes through electrostatic interactions, depending on pH.[224g,477a] On the other hand, it has been reported that Aβ can interact with cationic or zwitterionic lipids as readily as anionic lipids.[479] This suggests that association of Aβ with lipid membranes is driven to a substantial extent by hydrophobic interactions with hydrophobic regions in the peptide. Preferential incorporation of Aβ into anionic lipid membranes is however noted.[480] The interaction of Aβ with negatively charged lipids is driven by electrostatic interactions whereas insertion into the membrane is driven by the hydrophobic tail of Aβ. It has been demonstrated that Aβ40 spontaneously inserts into anionic DPPG (1,2-dipalmitoyl phosphatidylglycerol) membranes but not zwitterionic DPPC (1,2-dipalmitoyl phosphatidylcholine) membranes.[481] Furthermore, the DPPG membrane induces β-sheet "crystallization" of Aβ,[481] although this interaction was eliminated above pH 7.4 where Aβ becomes anionic. Enhanced binding of Aβ40 (tryptophan labeled Y10W for fluorescence experiments) oligomers to vesicles has been observed when DPPG is incorporated as compared to pure DPPC vesicles.[482] Terzi *et al*. studied Aβ(25-35) [and Aβ(25-35Nle)] on anionic lipid membranes and noted an increase in β-sheet formation (in solution both random coil and β-sheet structures coexist for this peptide)

as probed by CD spectroscopy, in the presence of the negatively charged vesicles.[483] These authors were also able to measure binding enthalpies.[483] The C terminus capped version of this peptide however adopts a random coil structure on binding to the lipid vesicles, pointing to the role of electrostatics in the binding process, also confirmed by salt screening experiments.[483] This group found that Aβ40 can insert into anionic monolayers at sufficiently low packing density.[480] These authors also noted transitions in secondary structure random coil – β-sheet – α-helix depending on the lipid-to-peptide ratio.[480] A shift to an initial α-helical conformation has been noted upon binding of Aβ peptides to membranes,[374] however the enrichment of peptide concentration close to the membrane may subsequently favour β-sheet formation.[224g,477a] Thus, membranes can inhibit fibrillization at high lipid-to-peptide ratio, but accelerate it at low relative lipid concentration.[224g] Biological lipids (in liposomes) can resolubilize Aβ42 fibrils, and convert them into more toxic oligomeric forms, and the potential biological relevance of this was noted.[484]

Aβ can form cation-selective channels when incorporated in lipid bilayers as revealed by conductance measurements.[225a-c,485] Aβ peptides disrupt membranes comprised of negatively charged phospholipids, in a pH-dependent manner (which is important in the context of different pH levels in endosomes vs. the extracellular matrix, for example).[486] AFM shows the formation of channel structures by Aβ42 reconstituted in planar lipid bilayers.[226a] On the other hand, Kayed *et al*. report that Aβ oligomers increase permeability (quantified via conductance measurements) without any evidence for pore formation or ion selectivity.[215a] Serpell and coworkers have observed that the Aβ aggregation state influences its ability to permeate vesicles (probed via calcein fluorescence measurements), and that oligomers have the highest

membrane disruption activity.[487] The less fibrillogenic Aβ40 has a reduced tendency to permeabilize membranes than Aβ42. The role of GM1 ganglioside receptors was also highlighted.[487]

Nussinov and coworkers have performed MD simulations of Aβ in lipid bilayers, focussing on Aβ(17-42) protofibrils, and Aβ pore structures were examined.[366,488] The formation of subunit structures within the channels was observed. The selectivity of the channels for Ca^{2+} observed experimentally was also confirmed from the models.[488] Consistent with AFM images of Aβ40 in a DOPC (dioleoyl phosphatidylcholine) bilayer,[226b] break-up of the channels into subunits was observed (Fig.26). [488] Strodel *et al*. have also performed MD simulations on Aβ pore structures, for Aβ42, modeling oligomers.[489] They found that membrane-spanning β-sheets adopt ordered configurations for dimers to hexamers, however separation into subunits was only observed for octameric oligomers which separated into distinct subunits (*cf*. Fig.24).[489] A molecular dynamics simulation of the interaction between Aβ42 and zwitterionic and anionic lipids indicated that peptide-peptide interactions are favoured in the vicinity of the membrane, driving oligomerization in the case of the anionic lipid membranes.[490] The dependence on pH was also modelled.

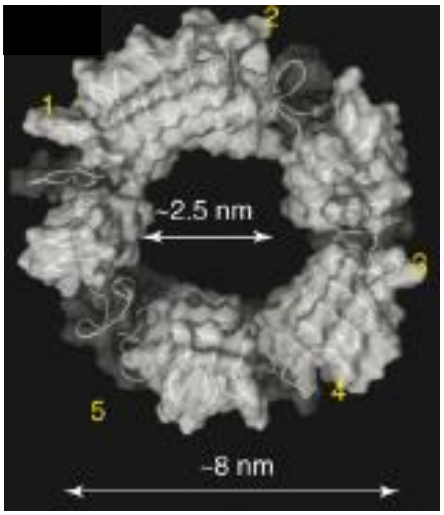

Fig.26. MD simulation of a 24-mer channel formed by Aβ(17-42) in a DOPC lipid bilayer.[366,488] The yellow numbers label sub-units. Reprinted from Jang, H. B. *et al*. *Trends in Biochemical Science* **2008**, *33*, 91, Copyright 2008, with permission from Elsevier.

The binding of Aβ and gangliosides [ganglioside = glycosphingolipid] has been the subject of several studies, although with conflicting conclusions.[477a,491] Ganglioside membranes have been reported to accelerate Aβ aggregation[300,474c] or to inhibit it and stabilize α-helical structures[492] or to induce β-sheet structure.[493] Another report claims that gangliosides induce Aβ to adopt a mixed α/β conformation at neutral pH.[486] Actually, a transition from random coil to α-helix might be favoured at low peptide-ganglioside lipid ratios, whereas higher ratios promote the adoption of a β-sheet conformation.[224g,493b] It has been proposed that ganglioside clusters may form sites that seed Aβ fibril formation.[224g,477a] The interaction of Aβ40 and Aβ42 with phosphatidylinositol (PI) with different inositol headgroups was examined.[276] The

formation of β-sheet structures was found to be pH-dependent in the presence of PI vesicles – both Aβ40 and Aβ42 aggregate at pH 6 (close to the pI = 5.5)[491,494] but only Aβ42 aggregates at pH 7. The work with different PI vesicles formed part of the basis for the development of an inositol-based inhibitor of Aβ aggregation,[276] as discussed further in Section 3.2.1. formation of Aβ40 fibrils is accelerated in the presence of ganglioside-containing POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) vesicles.[300,495] The binding of the peptide to the membrane was analysed quantitatively. The binding was found to be ganglioside-specific.[300,495] AFM and TEM imaging show aggregation and fibril formation of Aβ40 and Aβ42 on total brain extract lipids.[496]

Bokvist *et al*. used MAS-NMR (MAS: magic angle spinning) and CD to probe the interaction of Aβ40 in different lipid membranes.[497] Charged membranes can act as templates for aggregation of surface-associated Aβ40 in the case that the peptide is released in soluble form. However, membrane inserted Aβ40 is anchored by the K28 residue by electrostatic interactions with negatively charged lipids.[497-498] By studying a series of full length peptides and fragments, Chauhan *et al*. were able to highlight the role of aliphatic residues at the C terminus of Aβ interacting with the fatty acid chains as well as the electrostatic interaction involving K28.[498] Several groups have noted that the interaction of Aβ with membranes depends on the ordering of the lipid membranes.[479b,499] Murphy and coworkers used fluorescence anisotropy of an inserted probe to examine interaction of Aβ40 with lipid membranes.[479b,491] They observe that Aβ aggregates only affect lipid membrane fluidity above, and not below, the lipid chain melting temperature.[479b] Aggregated Aβ decreases membrane fluidity, but Aβ

monomer does not. Aggregation rate and surface hydrophobicity were greater for Aβ40 prepared at pH 6 compared to pH 7.[491] However, Wood *et al*. observe that Aβ40 does not form amyloid fibrils at pH 5.8 (approximate pH of endosomes and conditions for proteolytic cleavage), but rather forms larger aggregates which lead to turbidity of the solution.[422] Inhibition of Aβ fibril formation was observed to depend on the state of the lipid membrane in studies of the interaction of Aβ(M1-40) and Aβ(M1-42) (recombinantly expressed in *E. Coli*, Section 2.2.2) with liposomes.[499] The largest retardation is observed when DPPC bilayers are in the solid gel phase. The mobility of Aβ42 prefibrillar and fibrillar oligomers on the membrane of living cells or lipid membranes has been investigated via single particle tracking techniques (using quantum dots as labels).[500] The dynamic behaviour is distinct depending on the aggregation state (and conformation) of the peptide, although motion for oligomers is largely confined. Diffusion coefficients were obtained and differ significantly for fibrillar and prefibillar oligomers recognized by the A11 antibody.[500]

The insertion of the transmembrane fragment Aβ(25-35) into phospholipid bilayers has been examined by neutron diffraction using deuterium labeling techniques.[501] The location of the peptide C terminus was studied in two different lipid compositions and was found to be dependent on the surface charge of the membrane (zwitterionic or anionic). The adsorption of Aβ40 on phospholipid monolayers has been probed using grazing incidence x-ray diffraction and IRRAS.[502] On negatively charged monolayers, the peptide adsorbs at the air-water interface and inserts into the monolayer, although it is squeezed out at high surface pressure. Aβ40 can also penetrate into disordered anionic monolayers in buffer due to salt screening of electrostatic interactions.[502] The influence of Aβ(25-35) and Aβ(22-40) on the picosecond dynamics of lipid

membranes has been examined by quasi-elastic neutron scattering.[503] The main influence was on long-range translational diffusion, although localized diffusion was also considered. By selection of the Aβ(11-22) fragment, the role of charge, and the influence of pH, in governing the interaction of Aβ with lipids was examined.[504] Only at endosomal pH (approximately 6) does the peptide insert into negatively charged membranes, with a conformation change (increase in α-helix content in the presence of lipids) detected by CD. Specifically, the protonation state of H13 and H14 was found to be important (as in the case of interactions with metal ions, section 4.8).[504]

Aggregation of Aβ42 proceeds with distinct aggregate morphology (and kinetics) when adsorbed on hydrophilic mica or hydrophobic graphite.[505] and this was related to fibrillization at interfaces such as membranes.

The role of cholesterol in AD has been examined due to the mutal interaction of Aβ and cholesterol (the metabolism of which is modulated by ApoE).[472a,474c,477a,506] Cholesterol mediates Aβ aggregation and reciprocally Aβ influences cholesterol dynamics in neurons, leading to tauopathy.[472a] Whether cholesterol inhibits or promotes Aβ fibrillization may depend on its content within the membrane,[224g,507] and the presence of metal ions.[508] Serum and CSF levels of cholesterol may provide a biomarker for AD (others are discussed in Section 2.4) although the relationship between HDL (high density lipoprotein) level and AD is not clearly established at present.[472a] A correlation between cholesterol (uncharged) content in the model membranes and Aβ42 deposition was noted, due to the effect of cholesterol on membrane rigidity (addition of Aβ40 increased vesicle rigidity).[496a] The structure of lipid membranes extracted from AD patient brain tissue has been compared to control,

using SAXS to obtain electron density profiles and differences were ascribed to the increase in cholesterol level in the AD brain.[509] The changes in membrane rigidity in turn influence Ca^{2+} ion transport across neuronal cell membranes.[485] An inverse correlation between membrane cholesterol level and Aβ-cell surface binding and cytotoxicity was observed.[496a] The role of cholesterol in amyloid aggregation and tau phosphorylation has been reviewed. [472,510] Statins may have a role in alleviating AD pathologies associated with cholesterol since they can influence cholesterol metabolism in the human brain. [472a,506,511] Several cholesterol derivatives have been found to enhance Aβ42 fibrillization, and aspirin can inhibit this cholesterol-mediated fibrillization.[512] The formation of the GM1 ganglioside-bound Aβ (GM1/Aβ) complex (found in the brains of AD patients and proposed to seed Aβ aggregation) is cholesterol-dependent.[513] Cholesterol byproducts from antibody-induced ozonolysis during inflammation have been detected in human brains.[514] These compounds which contain aldehydes dramatically accelerate Aβ aggregation *in vitro*. The authors note that these observations relate to some common features of AD and atherosclerosis, in particular in terms of inflammation.[514] The mechanism of interaction of these cholesterol metabolites with Aβ40 was subsequently analysed in detail.[515]

4.8 Effect of Metal Ions

Aggregation of Aβ may be promoted by metal ions.[16d,516] High concentrations of metal ions (Cu^{2+}, Fe^{3+}, Zn^{2+}, Al^{3+}…) are found to be co-localized at abnormally high concentration with senile plaques in AD brains.[517] Aβ rapidly aggregates in the presence of physiological concentrations of Zn^{2+} at pH 7.4.[106b,518] Enhanced Cu^{2+}-induced aggregation is noted when the pH is lowered to 6.8.[518b] Mildly acidic

conditions are often associated with inflammation. APP binds copper in the N terminal domain within the APP(135-175) sequence.[16d] The copper binding domain contains a His-X-His motif.[16d] Metal ions are involved in processes associated with inflammation in AD patients (*vide infra*). Treatment with metal ion chelators can reduce the deposition of Aβ in brains, as discussed further in section 3.2.1.[242a,242d,259] The focus of most studies has been Cu^{2+}, Zn^{2+} Al^{3+} and Fe^{3+}, which enhance fibril formation.

Transition metal ions may interfere with transport across ion channels, eg. Al^{3+} ions[225a] and Zn^{2+} ions[225d,226a,229,266b] have been shown to block these channels. In the latter case, this can be reversed using a Zn^{2+} chelator.[225d] Arispe *et al.* also point to the formation of channels in bilayer membranes as a possible tool to screen for possible therapeutic compounds.[225a] Zn^{2+} and Cu^{2+} ions induce Aβ42 and Aβ40 insertion into vesicles in a suitable pH range with an accompanying formation of α-helical structures.[508]

Surface plasmon resonance (SPR) biosensing has been used to investigate the aggregation of Aβ40 in the presence of Cu^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+}.[519] All ions promoted Aβ aggregation, but with different rate constants, that for Cu^{2+} being highest, although the aggregates were unstable. The chelator EDTA (ethylenediamine tetraacetic acid) can dissociate metal-ion induced Aβ aggregates.[519] Rottkamp *et al.* showed that if Aβ is pretreated with the iron chelator deferoxamine, neuronal toxicity is significantly reduced while conversely, incubation of Aβ with excess free iron restores toxicity to original levels.[520]

The aggregation of human Aβ is stimulated by the presence of zinc ions at sufficiently high concentration due to binding mediated by histidine.[106b] The importance of the H13 residue in Aβ in binding Cu^{2+} and Zn^{2+} in a pH-dependent manner was highlighted (the other N terminal histidines H6 and H14 also play a role).[259b,518b,521] The Cu^{2+} binding site can also involve D1 or E11.[522] A solid state NMR study has recently elucidated the Cu^{2+} binding site of Aβ40.[523] It was reported that under physiological conditions, aluminium, iron, and zinc strongly promote Aβ aggregation (rate enhancement of 100-1,000-fold) whereas the other metal ions (including calcium, copper and sodium) studied do not.[524] The aggregation of Aβ induced by iron or aluminium ions is distinguished from that of Zn^{2+} by its rate, extent and pH- and temperature-dependence as probed via sedimentation experiments using ^{125}I-labelled Aβ(1-40).[524] It has been proposed that rapid formation of a pre-oligomeric peptide/metal/peptide complex follows binding of Cu^{2+} to Aβ, leading to inhibition of oligomer formation,[525] as observed at low Cu^{2+} concentration.[526]Ascorbate-dependent hydroxyl radical generation, is inhibited by Aβ(1-16) or Aβ42 for Cu^{2+} or Fe^{3+}.[521e]

AFM imaging directly illustrates the influence of copper and zinc ions on the aggregation of Aβ42, preventing fibrillization even in trace amounts.[527] Using immobilized Aβ seeds (Section 4.3.1), Cu^{2+} and Zn^{2+} at neutral pH are found to accelerate the deposition of Aβ40 and Aβ42 but produce amorphous aggregates whereas Fe^{3+} induces the formation of fibrils.[418b] The effects of mixtures of Cu^{2+}, Zn^{2+} and Fe^{3+} ions on Aβ42 aggregation has also been examined via AFM using the same technique. [528]

The role of aluminium in the etiology of AD is controversial.[516d,529] It was originally found to be associated with plaques in AD patients, in the form of aluminosilicates[530] specifically associated with neurofibrillary tangles.[529b,531] Aluminium has significantly higher cytotoxicity in complexes with Aβ42 than Cu^{2+}, Zn^{2+} and Fe^{3+} and the aggregation properties of Aβ in the presence of Al^{3+} are also substantially different.[532]

The effect of metal ions on Aβ aggregation has been correlated to oxidative stress.[517b,520] Both iron and copper have high affinity for Aβ and are reduced by it, with the subsequent production of hydrogen peroxide and oxidised Aβ.[533] Iron has been implicated as a key species in oxidative stress, due to its involvement in the creation of free radicals (in particular hydroxyl radicals[517b]) from H_2O_2 via the Fenton reaction.[534] It has been suggested that iron enhances the toxicity of Aβ by delaying the deposition of the peptide into well-defined fibrils.[535] The cytotoxic effects of Aβ can be attenuated by antioxidants and free radical scavengers such as vitamin E.

The zinc binding motif, strongly conserved among members of the APP family has been identified.[536] APP can catalyze the reduction of H_2O_2 and ensuing oxidation of Cu^{+} to Cu^{2+} in a peroxidative reaction *in vitro*, leading via a Fenton-type reaction to free radical formation.[536c]

4.9 Conjugates of Aβ with Polymers and Lipid Chains

Conjugation of peptides to synthetic polymers such as PEG may lead to improved solubility, enhanced stability against dilution, reduced toxicity and

immunogenicity.[537] The solution self-assembly of peptide-containing copolymers has been reviewed.[538]

In a pioneering series of papers, Meredith and coworkers have confirmed the formation of fibrils in aqueous solutions of PEG-peptide diblocks where the peptide block was based on the central hydrophobic domain Aβ(10-35) of the β-amyloid peptide and the PEG block had a molar mass of 3000 g mol^{-1}.[539] They found from SANS and TEM that the PEG forms a coating around the fibril, thus acting as a "steric stabilization" layer. The self-assembly in aqueous solution of PEG-peptides, with peptide sequences based on KLVFF Aβ(16-20) motif has been investigated, with PEG molar mass in the range 1000 – 3000 g mol^{-1}. Conjugates FFKLVFF-PEG[540] and YYKLVFF-PEG[541] form core-shell fibrils and aggregation into nematic and hexagonal columnar liquid crystal phases is observed at high concentration. Conjugate βAβAKLVFF-PEG was used in studies of enymatic cleavage (using α-chymotrypsin to cleave between the two phenylalanine residues).[542] The conjugate forms spherical micelles which are degraded by the enzyme, releasing peptide βAβAKLVF (which does not aggregate into amyloid).

The formation of amyloid-like structures at the air/water interface was reported for peptide amphiphiles (PAs) with a peptide sequence based on Aβ(31-35), i.e. IIGLM, attached to a C_{18} chain.[543] Epifluorescence microscopy showed the formation of threadlike and needle-like aggregates. Yilin Wang's group have shown that PA C_{12}-Aβ(11-17) forms fibrils.[544] The double tail analogue PAs $2C_{12}$-Lys-Aβ(12-17) and C_{12}-Aβ(11-17)-C_{12} self-assemble into fibrils or twisted ribbons/tapes respectively, the latter dependent on pH.[545]

5. SUMMARY AND OUTLOOK

This review has focussed on the biological and biophysical properties of the Amyloid β (Aβ) peptide and its role in Alzheimer's disease. The biological, biochemical and neurochemical characteristics of AD, involving Aβ and APP have been discussed. Therapeutic treatments including those on the market, or those that have been or continue to be the subject of clinical trials have been described. Finally, aspects of the biophysical chemistry of Aβ and Aβ fragment peptides have been outlined, including various aspects of structure at the molecular and supra-molecular level, and aggregation mechanisms and kinetics.

It is now more than a century since Alzheimer identified the disease now named after him, and more than a quarter century since aggregation of amyloid beta peptide was first associated with the condition. As a major global healthcare challenge, there has been intense research activity in this period. This has led to deep insights into the causative agents, the current consensus being that Aβ is the primary suspect. However, the progression of AD is not straightforward, and other factors are almost certainly involved, including other proteins such as tau, but also influences such as oxidative stress. Further large-scale genetic and proteomic screening studies will almost certainly reveal other risk factors.

A number of compounds are available to amelioriate the early stage symptoms of AD, however there is still no effective treatment that can halt or reverse progression into the debilitating late stage of the disease. Several approaches have led to compounds

that have reached phase III clinical trials, some of which failed. However, given the complexity associated with treatment of brain disease and the fact that it involves a subtle target, i.e. a protein misfolding process, some setbacks along the road are probably inevitable. The problem of finding a selective therapeutic agent is challenging given the diversity of biochemical pathways involved in brain signalling and other neuronal growth and differentiation processes. Nonetheless, there is reason for optimism as clinical trials of a number of therapeutic agents continue. There has been some refocusing by big pharma in the brain (and central nervous system) disease field, but some significant players are still very active in R&D related to AD therapies. Furthermore, governments internationally are increasingly recognising the magnitude of the problem of diseases of aging, especially AD, and there is currently substantial investment in further research through various funding agencies and networks. Probably closer to realisation are effective diagnostic systems based on blood or plasma analysis, or brain scanning methods.

In terms of biophysical measurements, it has to be noted that Aβ is a difficult peptide to work with, in the sense that its aggregation properties are highly sensitive to sequence, purity and preparation conditions. The latter include initial dispersal solvent, nature and concentration of the aqueous or buffer solutions, but also the effect of shear during mixing and potentially the nature of the surface of the vessel. Small differences in preparation conditions can lead to distinct polymorphs, which can propagate, as discussed in section 4.4.

Despite these caveats, there is now a great wealth of data on the fibrillization properties of Aβ, variants and fragments under defined conditions. These

measurements provide a strong framework to underpin the ongoing biological research activity.

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