Journal of Peptide Science
J. Peptide Sci. **7**: 227–249 (2001)
DOI: 10.1002/psc.324

The Amyloid-β Peptide and its Role in Alzheimer's Disease

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Received 5 January 2001
Accepted 22 January 2001

Abstract: Amyloid formation plays a central role in the cause and progression of Alzheimer's disease. The major component of this amyloid is the amyloid-β (Aβ) peptide, which is currently the subject of intense study. This review discusses some recent studies in the area of Aβ synthesis, purification and structural analysis. Also discussed are proposed mechanisms for Aβ-induced neurotoxicity and some recent advances in the development of Aβ-related therapeutic strategies. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amyloid-β peptide; aggregation; Alzheimer's disease; amyloid; fibrilogenesis; metal ions; neurotoxicity; peptide synthesis; structural analysis; therapeutic strategies

INTRODUCTION

Alzheimer's disease (AD) is the most common form of senile dementia, being responsible for approximately 70% of all dementia cases in Australia [1]. Approximately 5% of people over the age of 65 currently suffer from AD, and the proportion increases to 20% for people over 75 [2]. It was estimated that, in 1998, 25 million people worldwide suffered from AD [3], and this figure is set to increase significantly with the ageing world population. Symptoms include breakdown of social skills and emotional unpredictability, coupled with severe and irreversible cognitive decline [3–6]. The average lifespan of sufferers from the time of diagnosis is between 7 and 10 years [6] and no cure is yet known. The disease was first described by Alzheimer in 1907 [7]. Pathology visible *post mortem* includes neurodegeneration, extracellular deposition of amyloid, both in neuritic plaques and diffuse deposits, amyloid deposition in the cerebral vasculature (amyloid angiopathy) and the formation of neurofibrillary tangles (NFT) and paired helical filaments. The AD brain also exhibits modified gross morphology, including enlargement of the ventricular system and atrophy of the frontal, temporal and parietal cortex together with the hippocampus and amygdala [8]. The major proteinaceous component of AD amyloid is the amyloid-β (Aβ), a 4-kDa peptide derived from proteolytic cleavage of the amyloid precursor protein (APP) [9,10]. In this paper we review recent literature on the role of the Aβ peptide in AD. The review does not aim to provide compressive coverage, but instead, to provide a summary emphasizing chemistry aspects of Aβ research. These include methods for the synthesis and purification of the difficult Aβ sequence, structural studies of Aβ solutions and solids, possible mechanisms of Aβ neurotoxicity and Aβ-based therapeutic strategies.

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BIOGRAPHIES

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undertook his doctoral studies at the Howard Florey Institute and School of Chemistry, University of Melbourne, under the joint supervision of Dr Colin Barrow and Associate Professor John Wade. The title of his thesis was 'The Chemistry of Alzheimer's Disease' and his studies focused on the chemial synthesis and conformational study of Aβ peptides. He is presently a Research Scientist at the Dunn Institute, Cambridge, United Kingdom.

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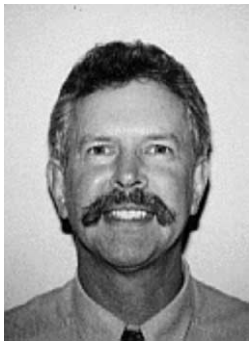

is an NHMRC Principal Research Fellow at the Howard Florey Institute, Melbourne, where he has long been actively involved in research on peptide and protein synthesis and chemistry. His primary focus is on synthesis methodology as applied to key reproductive hormones such as relaxin. Other areas of interest include peptide design and conformation. He serves on the editorial board of several peptide journals and has long been on the organizing committees of the Lorne Protein (Australia) and Australian Peptide Conferences series.

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The APP

The APP gene, located on chromosome 21, codes for multiple isoforms of a glycoprotein containing a single membrane spanning sequence, a long *N*-terminal extracellular region and a short *C*-terminal cytoplasmic tail (Figure 1). Alternative mRNA splicing results in the formation of APP_{770}, APP_{751}, APP_{714}, APP_{695}, APP_{563} and APP_{365}, where the number gives the total number of amino acids in each protein. The two latter isoforms do not contain an Aβ domain within their sequence [11]. APP expression occurs ubiquitously throughout the body and the primary APP isoform varies according to cell and tissue type [12]. Human neurons express high levels of APP_{695}, together with lesser amounts of APP_{751}, and are responsible for the majority of the APP produced in the brain [13]. Other brain cell types, such as meninges and glial cells, produce predominantly the longer APP isoforms, APP_{770}, APP_{751} and APP_{714}. Each of these, unlike APP_{695}, contain a Kunitz protease inhibitor domain [14] which is known to confer resistance to proteolysis by trypsin and chymotrypsin [15].

Proteolysis of the APP

Proteolytic cleavage of APP occurs in two distinct manners via either the α-secretase pathway or the β-secretase pathway. The former pathway involves neuronal secretion of $sAPP_{\alpha}$ by a putative α-secretase enzyme which precludes the formation of full-length Aβ, as cleavage occurs between residues 612 and 613 of APP_{695} corresponding to Aβ residues 16 and 17 (Figure 1) [13]. The 10-kDa *C*-terminal APP fragment generated by α-secretase cleavage can be further processed by a putative γ-secretase enzyme which cleaves APP_{695} between residues 637 and 638 or residues 639 and 640 yielding $A\beta_{17\text{-}40}$ or $A\beta_{17\text{-}42}$,

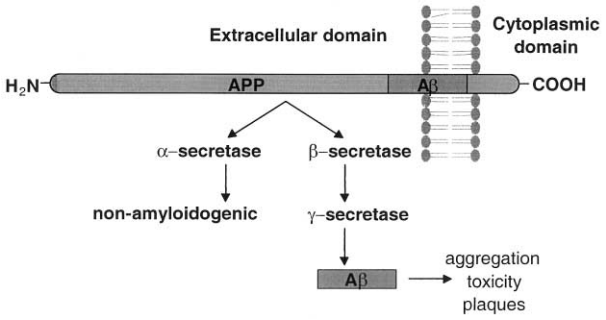

Figure 1 Proteolytic cleavage of APP.

also known as the p3 peptide. Traditionally the α-secretase pathway has been called the non-amyloidogenic pathway since full-length Aβ is not formed. However, p3 has since been shown to be a significant component of diffuse amyloid deposits [16–18] and, therefore, the description of this pathway as non-amyloidogenic is somewhat misleading. Full-length Aβ is generated by the β-secretase or amyloidogenic pathway (Figure 1), whereby APP_{695} is enzymatically cleaved by a β-secretase protease between residues 596 and 597. This yields a secreted APP species denoted $sAPP_{\beta}$ and a membrane bound peptide containing the intact Aβ domain (C-100) [13]. C-100 has been demonstrated to be highly amyloidogenic and neurotoxic to cultured neurons *in vitro* [19]. Further proteolysis of C-100 by γ-secretase generates full-length Aβ [13]. Rather than β-secretase cleavage of APP being the primary proteolytic event, followed by γ-secretase processing in an invariant manner, APP processed by γ-secretase without β-secretase has also been detected [20]. Interestingly the γ-secretase cleavage occurs within the transmembrane domain of APP. Only two other enzymes are currently known to cleave within or close to their transmembrane regions, S2P [21] and m-AAA [22]. It is believed that these proteases, as well as γ-secretase, act by forming a channel in the membrane to provide the room required to accommodate and cleave the α-helical transmembrane domain of the substrate [23]. Cleavage specificity has also been found to be dependent on the primary structure of the transmembrane domain [23]. APP metabolism by the β-secretase pathway was initially proposed to be an abnormal proteolytic processing event specific to, and with a causative role in, AD. However, proteolysis of APP via the β-secretase pathway has been found to be a normal process which occurs ubiquitously in both AD and non-demented individuals [24,25].

A number of enzymes with secretase-like activity have been described. α-secretase is thought to reside at the plasma membrane [26] in microdomains called caveolae [27] or in the trans-Golgi network [28]. Unlike β- and γ-secretase, products of the cleavage of APP by α-secretase are relatively homogeneous. Sisodia reported that the cleavage activity of α-secretase was mediated largely by the α-helical conformation around the cleavage site, and an optimal distance of 12 residues from the plasma membrane, rather than by the primary sequence of the peptide [29]. The proposal that α-secretase is a calcium-dependent protease [30] appears to have been discounted due to the α-secretase-like activity observed for metalloproteinases such as the disintegrin metalloproteinase ADAM 10 [31] and the zinc metalloproteinase angiotensin converting enzyme secretase [32,33].

Proteolysis of APP by β- and γ-secretase was initially thought to occur in late endosomal or lysosomal compartments [24,34,35]. However, other reports have demonstrated activity of both secretases in the endoplasmic reticulum and the Golgi [36–39]. Although the β-secretase cleavage is probably specifric there are a number of *N*-terminally modified and truncated forms found in brain. *N*-terminal truncation is probably a result of non-specific proteolysis subsequent to release of full-length Aβ. Aβ truncated at position 3 ($A\beta_3$pE) and position 11 ($A\beta_{11}$pE) is particularly abundant in the brain [40–44]. The *N*-terminal glutamic acid residues of these peptides are post-translationally modified to form pyroglutamyl species, making these peptides less susceptible to further proteolysis [44]. This resistance to proteolysis probably explains the high abundance of $A\beta_3$pE and $A\beta_{11}$pE relative to other *N*-terminally truncated Aβ forms.

A number of enzymes with β-secretase-like activity have been described [13], including cathepsin G which led to the suggestion that β-secretase may be a chymotrypsin-like serine proteinase [45]. The novel membrane-bound aspartyl proteinase Asp 2 has been reported to possess β-secretase activity [46,47]. Vassar *et al.* [48] recently described a β-site APP cleaving enzyme (BACE) which possesses all the known characteristics of β-secretase. BACE is predicted to be a type-1 transmembrane protein, and is expressed most highly in the Golgi and the endosomes. Over-expression of BACE caused increased β-secretase activity, with APP cleavage occurring specifically at the β-secretase site. Furthermore, inhibition of BACE expression with antisense oligonucleotides inhibited β-secretase activity *in vitro*. These data strongly support BACE as a candidate β-secretase enzyme.

There is also significant heterogeneity inherent in the γ-secretase cleavage site, with the *C*-terminus of Aβ varying between amino acids 39 and 43. The $A\beta_{1\text{-}40}$ form constitutes approximately 90% of secreted Aβ and the $A\beta_{1\text{-}42}$ form making up the majority of the remaining 10% in non-AD individuals (Figure 2) [13,20]. This heterogeneous cleavage can be explained by γ-secretase actually being multiple enzymes, or by Aβ generation occuring in different cellular sites where conditions affect enzymic specificity [13]. $A\beta_{1\text{-}42}$ production has been demonstrated in neuronal endoplasmic reticulum and

Asp_1-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-**Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala_{42}**

Figure 2 Primary structure of A$\beta_{1\text{-}42}$ in three letter code. Hydrophobic membrane-derived portion shown in bold.

nuclear envelope [37,38], whereas A$\beta_{1\text{-}40}$ production has been observed in the trans-Golgi membrane [38]. However, different sensitivity to cleavage at positions 40 and 42 has been shown by studies with protease inhibitors, supporting the multiple enzyme hypothesis [49–51]. A combination of the two hypotheses whereby different enzymes reside in separate cellular compartments is also possible [13]. The lysosomal aspartyl protease, cathepsin D, possesses similar proteolytic specificity and cellular localization to γ-secretase and has been proposed as a possible γ-secretase candidate [52]. Studies indicate that the product of a gene associated with early onset AD, presenilin-1 (PS-1), is required to enable normal γ-secretase activity [53]. Furthermore it has been proposed that PS-1 is itself a γ-secretase [54–57].

The Aβ Hypothesis of AD

The hypothesis that Aβ amyloid formation and deposition is a causative factor in AD [58] (see Small and McLean [59] for a recent review) is supported by the observation that mutations which increase the aggregation propensity of Aβ, such as the dutch cerebral haemorrhage [60,61] and presenilin mutations [62,63], cause symptomatically severe early onset forms of AD. Individuals with Down's syndrome (trisomy 21) develop AD in the fourth or fifth decade of their lives and express APP at a level approximately 1.5 times that of normal individuals, presumably owing to a gene dosage effect [12,20]. The Swedish double mutation of APP mediates a significant increase in Aβ secretion compared with cells expressing wild type APP, and individuals with this mutation develop severe, early-onset AD [25,64]. In an attempt to obtain an animal model of AD, transgenic mice which over-express either mutant or wild-type human APP have been produced and these animals have been found to develop AD-like pathology [65–67]. Furthermore, *in vitro* studies of A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ have indicated that aged solutions of these species are toxic to cultured neurons [68], and the aggregation propensity of Aβ has been correlated with neurotoxicity [69–71].

SYNTHESIS AND PURIFICATION OF Aβ PEPTIDES (SPSS)

SPPS has been demonstrated to provide a facile means for the preparation of a wide range of peptide sequences. However, despite the substantial optimization and development the method has undergone since its introduction, a subset of sequences still prove problematic to prepare. These so-called 'difficult' sequences, of which Aβ is a prime example, are poorly solvated while bound to the solid phase, which results in reduced N^{α}-amino acid acylation and deprotection yields [72–76]. Possible explanations for the poor reagent accessibility exhibited by 'difficult' sequences have been proposed to include intramolecular peptide self-association, incomplete solvation of the peptide-resin matrix and intermolecular association (see Figure 3), all of which would result in *N*-terminal masking of the peptide chain [76,77]. Studies have indicated that intermolecular and intramolecular self-association and aggregation of resin-bound peptide chains occurs during the assembly of these peptides [75]. The peptide chains aggregate and are thought to form β-sheet structures in an analogous manner to peptide aggregation in solution [72,75], which results in a significant increase in *N*-terminal steric hindrance [76]. Chain aggregation is sequence specific, with difficult acylation or deprotection reactions during assembly being caused by the primary sequence rather than the residues in question [75]. Intermolecular aggregation also introduces additional crosslinks within the peptide-resin matrix, causing resin collapse, and this in turn causes a significant decrease in reagent permeability of the solid support [76].

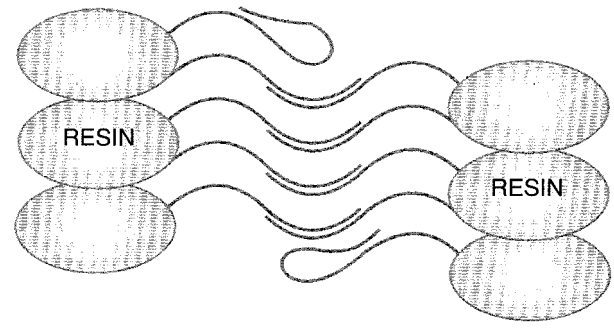

Figure 3 Schematic representation of peptide aggregation on a solid support during solid phase peptide synthesis. Peptide chains can self-associate either intramolecularly (top and bottom) or intramolecularly (centre strands) resulting in incomplete solvation of the peptide-resin matrix.

Synthetic Methods for the Preparation of the Aβ Sequence

Aβ peptides have been successfully prepared by both Boc-SPPS and Fmoc-SPPS for use in a range of structural and biological studies. Automated Boc-SPPS was used for the preparation of $A\beta_{1-42}$, $A\beta_{1-39}$, $A\beta_{1-28}$ and $A\beta_{29-42}$ in the studies by Barrow *et al.* [77–79]. More recently, Liu *et al.* [80] and He and Barrow [44] have reported the successful use of *in situ* neutralization Boc-SPPS methodology [81] for the preparation of $A\beta_{1-28}$, $A\beta_{1-40}$ and analogues thereof. Improved yield and purity of these and other 'difficult' peptides has been reported to be obtained when they were prepared using Boc chemistry rather than Fmoc-SPPS [82]. This has been attributed to the residual charge on the peptide *N*-terminal following Boc deprotection with TFA, which would inhibit peptide chain interaction owing to charge repulsion, thereby improving peptide-resin solvation [83].

The synthesis of $A\beta_{1-28}$, $A\beta_{1-30}$, $A\beta_{1-33}$, $A\beta_{1-36}$, $A\beta_{1-39}$, $A\beta_{1-42}$, $A\beta_{1-47}$ and $A\beta_{1-52}$ by Fmoc-SPPS was undertaken by Burdick *et al.* [84] as part of a systematic analysis of the role of the hydrophobic *C*-terminal region of Aβ. In this study, the assembly and purification of all of the peptides up to $A\beta_{1-42}$ was found to proceed relatively smoothly, with adequate peptide yield and purity being obtained throughout. However, $A\beta_{1-42}$ proved problematic to purify and the adequate characterizations of the $A\beta_{1-47}$ and $A\beta_{1-52}$ samples prepared were not achieved. More recently, the stepwise Fmoc-SPPS of $A\beta_{1-39}$ and $A\beta_{1-40}$ have been performed and the identities of the substantial amounts of side products formed during these syntheses investigated [85,86]. Further optimization of the Fmoc-SPPS of 'difficult' sequences by the use of HATU activation for regions of the target sequence known to be prone to aggregation has also been reported to enable preparation of Aβ and other aggregating sequences in high yield and better than 90% initial purity [87].

The synthesis of $A\beta_{1-42}$ and $A\beta_{1-43}$ has also been achieved by the segment condensation of fully-protected peptide fragments previously prepared by Boc-SPPS in organic solution [88]. $A\beta_{1(3)-9}$, $A\beta_{10-19}$, $A\beta_{20-25}$, $A\beta_{26-29}$, $A\beta_{30-33}$ and $A\beta_{34-42(43)}$ were ligated in a stepwise manner using a mixture of chloroform and phenol to solubilize these sparingly soluble fragments. Fragment activation was mediated by a water soluble carbodiimide [89], together with 3,4-dihydroxo-3-hydroxo-4-oxo-1,2,3-benzotriazine (HOOBt) and minimal epimer formation was observed following the ligation steps [88].

The preparation of $A\beta_{\mathbf{1}-43}$ and $A\beta_{25-35}$ have also been successfully performed using Fmoc-SPPS together with Hmb backbone amide protection [90–93]. $A\beta_{1-43}$ was initially prepared using Hmb protection of Gly^{38}, Gly^{33}, Gly^{29}, Gly^{25} and Phe^{20} [90]. Hmb groups were incorporated using *N*,*O*-bisFmoc-Hmb amino acid Pfp esters, and their acylation was performed using *N*-carboxy anhydrides in DCM. The positioning of the Hmb amino acids was largely dictated by the previously noted ability of these protecting groups to abolish peptide aggregation for five or six residues after their incorporation during 'difficult' sequence assembly [74,94]. Crude $A\beta_{1-43}$ was obtained in satisfactory purity and phenolic acetylation of the Hmb groups facilitated the RP-high performance liquid chromatography (HPLC) purification of the penta(acetyl-Hmb) protected peptide. Following purification, de-*O*-acetylation and Hmb cleavage were performed to afford purified native $A\beta_{1-43}$ [90]. Upon investigation of $A\beta_{34-42}$ aggregation it was found that Hmb protection of Gly^{37} inhibited fibril formation to a much greater extent than protection of Gly^{38} [93]. This was attributed to more effective disruption of β-turn formation around the Gly^{37}-Gly^{38} bond by Hmb-Gly^{37} and enabled the re-synthesis of $A\beta_{1-43}$ in significantly improved yield and purity relative to the earlier assembly via an otherwise similar protocol [91,93].

We have recently synthesized $A\beta_{1-40}$ with acetyl-Hmb backbone protection [95], using a modification of the methods used for the synthesis of penta(acetyl-Hmb)$A\beta_{1-43}$ [93], but using *N*-Fmoc-Hmb glycine in the free acid form, rather than the commonly used, but expensive and difficult to prepare *N*,*O*-bis-Fmoc-Hmb glycine pentafluorophenol esters. 'Semi-on-line' MALDI-TOF-MS [96] was used to monitor acylations onto the sterically hindered terminal Hmb substituted residues. These difficult acylations cannot be monitored by normal colourimetric methods because of the absence of a primary amine. The ability to monitor the progress of these acylations enabled us to use standard coupling conditions throughout the synthesis, significantly simplifying the incorporation of Hmb backbone protection.

The conformational and amyloid forming behaviour of penta(acetyl-Hmb)$A\beta_{1-40}$ was studied by circular dichroism and electron microscopy [95,97]. In the α-helix-inducing solvent trifluoroethanol, penta(acetyl-Hmb)$A\beta_{1-40}$ adopted approximately

25% α-helical structure and 70% random coil, compared with 81% α-helix for Aβ_{1-40}. The Hmb groups probably inhibit the formation of α-helical structure in regions close to these groups, leading to a reduced α-helical propensity. In phosphate buffer, penta(acetyl-Hmb)Aβ_{1-40} adopts some β-sheet structure, but retains significant random coil, even after 24 h. Penta(acetyl-Hmb)Aβ_{1-40} does not aggregate over time and only monomers were observed using Tris/Tricine SDS-PAGE, whereas Aβ_{1-40} forms time-dependant aggregates that form multiple bands on SDS-PAGE. Electron microscopy indicated that penta(acetyl-Hmb)Aβ_{1-40} did not form fibrils, but did form a more disordered branched filamentous structure. The Hmb groups probably inhibit amyloid formation by inhibiting β-sheet formation in regions close to the Hmb containing residues. Penta(acetyl-Hmb)Aβ_{1-40} offers a good tool for investigating the relationship between primary sequence, amlyoid formation and Aβ neurotoxicity.

STRUCTURAL ANALYSIS OF Aβ

Aβ Fibrilogenesis Studies

In AD brain, Aβ has been found to exist predominantly as amyloid fibrils [25]. The morphology of fibrils formed *in vivo* and those formed from synthetic Aβ was found by electron microscopy (EM) to be unbranched and straight or slightly curved [98,99]. Amyloid fibrils generally range between 6–10 nm in diameter [100], with the majority being approximately 8 nm in diameter [99], and are of indeterminate length [101,102]. Analysis of AD amyloid fibrils by X-ray diffraction indicated that in these structures Aβ was arranged as antiparallel β-pleated sheets, four of which were stacked together in each fibril [103]. Directly analogous structures were also observed to be formed by synthetic Aβ [98]. However, a recent study of the structure of fibrils formed by Aβ_{10-35} using solid state NMR found that the long axis of the propagating fibre was defined by strands of Aβ oriented in a parallel manner rather than antiparallel, and exactly in register [104]. In contrast, Tjernberg *et al.* [105] found that Aβ_{14-23} constituted the minimum structural requirements for fibrilogenesis with well-defined Aβ morphology. Molecular modelling indicated that the strands aligned in an antiparallel manner, thereby optimizing hydrogen bonding and salt bridges [105]. A recent fibril structure determined by synchrotron X-ray analysis also indicated an antiparallel configuration, with each Aβ molecule adopting a strand-turn-strand motif [106], the stability of which was further supported in a molecular modelling study [107]. Therefore, the orientation of Aβ in amyloid fibrils is currently a matter of some contention.

Kinetic studies have indicated that fibril formation is a two step process, comprising an initial nucleation step which is rate limiting, followed by a rapid fibril elongation stage [99,108]. Aβ has been found to have surfactant qualities in surface tension studies [109], leading Lomakin and co-workers to propose that above a critical Aβ concentration of 0.1 mM the nucleation event is a result of Aβ micelle formation [99]. The micelles then self-associate and collapse to form a dense nucleus or, alternatively, can associate on a pre-formed heterogeneous seed [99]. Fibril elongation has been observed to initially occur via the formation of small protofibrillar intermediate species [100,107,110–112], which are toxic to cultured neurons *in vitro* [112,113]. There is evidence that protofibril assembly is dependent on interactions with biological surfaces [114]. Interaction of Aβ with a hydrophobic graphite surface *in vitro* has been found to induce self-association of Aβ in a β-sheet configuration. Interaction of Aβ with a hydrophilic mica surface-induced micelle-like structure formation. Therefore, protofibril assembly may be induced by a local hydrophobic environment after initial micelle formation [114]. Protofibrils have a typical diameter of approximately 3 nm, and range between 20 and 70 nm in length [115]. The strikingly similar morphology of fibrils and protofibrils from a number of amyloidoses, despite significantly different protein constituents, has led to the proposal that the basic structure of amyloid fibrils may be common to all forms of amyloid [3]. Synchrotron X-ray diffraction studies of both transthyretin fibrils associated with familial amyloidotic polyneuropathy [116,117] and Aβ indicated that these protofibrils have a twisted structure, with 24 β-stranded peptide units comprising each complete helical turn [106,116,117]. However, molecular modelling of protofibrils using the structure determined by Sunde *et al.* as the starting point has been unable to confirm the stability of the twisted structure. Aβ strands were found to prefer to adopt a linear ribbon-like structure [107]. Protofibrils are of relatively diverse morphology, with linear, curved and branched structures being formed [110]. The conversion of protofibrils to mature fibrils is thought to be driven by the entropic benefit gained through reduction of solvated area and the formation of a hydrophobic core [107]. Four

protofibrils were initially thought to associate to form a mature fibril [98] but this number now appears to be variable with each mature fibril usually containing between three and six protofibrils [107]. In his extensive review of the subject, Teplow [3] proposed that fibril assembly can be described as three steps, although these are unlikely to occur as discrete processes *in vivo*. First, Aβ aggregates in solution to form extended β-sheet helices of 1.0–1.5 nm diameter, termed sub-protofibrils. Secondly, multiple sub-protofibrils pack face to face to form helical protofilaments with diameters of 2.5–3.5 nm. Finally, the protofibrils associate laterally to form amyloid fibrils of 7.5–8.0 nm in diameter.

Fibril formation and deposition has long been associated with pathogenic states where abnormal physiological conditions, or possibly irregular protein control or expression, result in conditions where protein polymerization becomes favourable. It is possible that protein aggregation and deposition could be used as a protective mechanism by the cell in order to dispose of over-expressed proteins which may otherwise result in cellular damage [118]. However, the highly intractable nature of amyloid fibrils and the apparent association of their formation with toxicity in amyloidogenic disorders such as AD make this theory appear unlikely [119]. Evidence is also accumulating that amyloid fibril formation, rather than being specific to a small subset of strongly amyloidogenic proteins, may be an intrinsic property of a wide spectrum of globular proteins [119,120]. Protein folding is known to be highly regulated in the cellular environment by mechanisms such as pH and the presence of molecular chaperones. Therefore, the breakdown of these regulation mechanisms may, in a wide range of cases, result in protein aggregation even where this is not known to be associated with a disease state [119].

Aβ Secondary Structural Analysis by NMR Spectroscopy

Analysis of the secondary structure of $A\beta_{1-28}$ using NMR spectroscopy was performed by Barrow and Zagorski [78] using a peptide concentration of 3.5 mM in aqueous 60% TFE (v/v). The nuclear Overhauser enhancement spectroscopy analysis performed indicated that the residues from Tyr^{10} or Glu^{11} to Ser^{26} formed an α-helix. A shorter and less stable α-helix was also detected between Ala^{2} and His^{6} [78,121]. These workers also predicted that the region between the two helices, Asp^{7} to Tyr^{10}, constitutes a reverse turn [78]. Increasing the temperature of the sample to 35°C was found to induce the short *N*-terminal helix to become unstructured, while His^{13} to Phe^{20} remained α-helical [121]. Studies investigating the pH dependence of this structure found that at pH 1.0 $A\beta_{1-28}$ adopted a completely α-helical structure. When the pH of the peptide solution was increased to between pH 1 and 3, the two helix structure observed initially formed, and at above pH 3, the short *N*-terminal helix was found to become unstructured, as observed in the temperature dependence studies [121]. The formation of this structure was recently confirmed during an NMR study of the pK_a values of Asp, Glu, His and Tyr in $A\beta_{1-28}$ [122]. It was found that Glu^{22} and Asp^{23} deprotonate at above pH 5.3 and His^{13} and His^{14} protonate below pH 5.7. In this critical pH range, unfavourable interactions between the charged side chains and the helix macrodipole (negative at *C*-terminal and positive at the *N*-terminal end of the helix) [121,123] induces an α-helix to β-sheet conformational shift. This pH dependent change from α-helix to β-sheet structure may be the initiator for Aβ aggregation in AD [122].

The formation of micelles by SDS in aqueous buffers is commonly used to simulate a membrane-like environment for use in peptide and protein secondary structural studies [124,125]. Interaction of Aβ with SDS micelles has been observed by circular dichroism (CD) to induce α-helix formation [44]. Coles *et al.* [126] recently performed NMR analysis of $A\beta_{1-40}$ in 100 mM SDS, as Aβ is known to remain soluble in SDS for extended periods at various pH values. This approach enabled them to analyse the effect of solution pH on peptide structure by NMR, and avoided the use of the helix inducing solvent TFE that was required in previous studies to ensure adequate peptide solubility [78,127]. They found that residues 15–36 of the Aβ sequence are largely helical. However, residues 25–27 form a kink, which was proposed to be a hinge region between two well-defined helices. They, therefore, described the solution structure of Aβ as consisting of an unstructured *N*-terminal region (residues 1–15), helix 1 (residues 15–24), a kink (residues 25–27), helix 2 (residues 28–36), and an unstructured *C*-terminus (residues 37–40). The helical region of His^{13}-Phe^{20} observed by Zagorski and Barrow [121] corresponds reasonably well to the position of helix 1 found in this study [126]. This structure is also consistent with the NMR structure of $A\beta_{25-35}$ determined by Kohuo *et al.* [128], and is in general agreement with the $A\beta_{1-40}$ NMR structure obtained

by Sticht *et al.* [127], who found two helical regions between residues 15 and 23 and 31 and 35, with the remainder of the peptide being unstructured. However, this study was performed in 40% TFE at pH 2.8, so some differences between the structures obtained are to be expected [126].

Secondary Structural Analysis of Aβ Using CD Spectroscopy

CD spectroscopy can provide qualitative and semi-quantitative information about the secondary structure of proteins and peptides in solution. The technique operates by the differential absorption of the left and right handed components of circularly polarized light by chiral molecules in solution [129]. An adequate representation of the CD spectra of globular proteins is possible by considering them as a combination of CD spectra for pure α-helix, β-sheet and random coil determined using poly-L-Lys [130]. By considering all spectra as a combination of these curves, it is possible to estimate the amount of these secondary structures in a given protein using curve fitting algorithms [131]. Furthermore, α-helical content can be determined by comparing the intensity of the helical minima at 208 and 222 nm to the intensity determined for pure α-helical poly-L-Lys [130,132]. This approach provides results that correlate reasonably well to X-ray crystallographic studies of globular proteins [130]. However, the estimation of the helical content of peptides by CD is somewhat less quantitative due to variations in signal, induced by side-chain absorbance, as the reference spectra used for calculating structural content were determined using homopolymers [133]. Despite this, CD spectroscopy is an extremely useful method for determining secondary structural propensity in peptides.

Detailed secondary structural analysis by CD spectroscopy indicated that Aβ_{1-42} formed approximately 90% β-sheet in aqueous buffer at pH 7.3. In contrast, Aβ_{1-39} adopted a mixed random coil/β-sheet structure in a ratio of approximately 1:1 [134]. The *C*-terminal Aβ_{29-42} peptide fragment was found to adopt approximately 100% β–sheet structure under these conditions. The addition of 25% (v/v) TFE, which promotes intra-chain hydrogen bonding, thereby stabilizing α-helical structure [134,135]; induced α-helical structure for Aβ_{1-42}, Aβ_{1-39} and Aβ_{1-28}, but Aβ_{29-42} remained in a β-sheet conformation. Aβ_{1-40} has recently been shown to form a mixed random coil/β-sheet structure in aqueous buffer at pH 7.2, and to convert to α-helix as TFE content of the buffer is increased in a similar manner to Aβ_{1-39} [44]. β-Sheet formation by Aβ was observed to be concentration dependent [134,136]. These data indicated that the hydrophilic *N*-terminal of Aβ can form α-helix, random coil and β-sheet structures, depending on solution conditions, and that Aβ_{1-42} formed more stable β-sheet structure than Aβ_{1-39} or Aβ_{1-40}, indicating that the *C*-terminal amino acids encourage β-sheet formation [44,134,136]. The observation that Aβ_{29-42} remained in a β-sheet conformation, regardless of TFE content, pH or temperature suggested that this segment of the peptide is largely responsible for the propensity to form β-sheet exhibited by Aβ [134,136]. However, rodent Aβ, which differs from human Aβ only by three amino acid substitutions, namely residues 5 (Arg → Gly), 10 (Tyr → Phe) and 13 (His → Arg), has been suggested by CD spectroscopy to have a lower propensity to form β-sheet than the human analogue in aqueous and membrane mimicking solvents [137]. This indicates that the hydrophobic *C*-terminal of Aβ is not solely responsible for inducing β-sheet structure as the *N*-terminal region of Aβ also plays a critical role.

Peptide aggregation and precipitation was observed to occur following β-sheet formation by Aβ_{1-39} and Aβ_{1-42} in a time dependent manner indicating that formation of β-sheet structure is directly related to peptide aggregation [134,136]. β-Sheet formation and peptide aggregation exhibited pH dependence and were most rapid at pH 5.5, as evidenced by changes in the CD spectra corresponding to complete β-sheet formation. This was followed by a shift of the 217 nm β-sheet minima to higher wavelength and reduction in signal intensity until the spectra were indistinguishable from the baseline [134]. The observation that β-sheet formation by Aβ was promoted at low pH is, particularly, of interest as the pH in AD brain has been found to be slightly lower than in normal brain [138], and this acidolysis may result in enhanced Aβ deposition [136]. These studies indicated that Aβ was not intrinsically insoluble, but that its insolubility was a result of β-sheet formation in response to solution conditions [134,136].

Aβ_{3-40} and Aβ_{11-40}, both of which bear an *N*-terminal pyroglutamyl residue are major components of plaque-core amyloid deposits [40,42,43,139]. A recent CD spectroscopy and aggregation study of these species together with Glp^{3}Aβ_{3-28} and Glp^{11}Aβ_{11-28} indicated that they have enhanced propensity to form β-sheet relative to full-length Aβ_{1-40} and Aβ_{1-28}, respectively [44]. Whereas

$A\beta_{1-40}$ adopts β-sheet structure optimally around pH 5, with increased random coil content at higher and lower pH, the pyroGlu analogues form a greater amount of β-sheet at all pH values with optimal β-sheet formation by pyroGlu$^{3}A\beta_{3-40}$ and pyroGlu$^{11}A\beta_{11-40}$ occuring at pH 5 and pH 4, respectively [44]. The *N*-terminally truncated analogues also require greater TFE concentrations to induce conversion of β-sheet structure to α-helix relative to $A\beta_{1-40}$, indicating β-sheet stability is also enhanced in these species. β-sheet formation and stability was also observed to be increased for pyroGlu$^{3}A\beta_{3-28}$ and pyroGlu$^{11}A\beta_{11-28}$, since $A\beta_{1-28}$ forms random coil structure in aqueous solution at all pH values tested, whereas the *N*-terminally truncated peptides exhibit some β-sheet formation. Aggregation assays confirmed that the increased propensity of β-sheet formation of these peptides was correlated with increased aggregation propensity [44]. Their enhanced β-sheet propensity and aggregation propensity is thought to be owing to the loss of the charged *N*-terminal residues, which may decrease inter-strand repulsion when intermolecular β-sheet formation occurs [44]. Alternatively, this effect may result from destabilization of α-helical structure caused by the loss of carboxylic acid functionality proximal to the *N*-terminus, thereby reducing favourable charge–dipole interactions [140]. It is possible that these strongly aggregating species may be responsible for seeding amyloid deposition in AD [44].

Aβ containing isoaspartic acid residues in position 1 and 7 is another *N*-terminally modified analogue of Aβ, which has been found in significant amounts in AD brain [141–144]. Fifty-four percent of the Aβ in samples derived from AD parenchymal extracts were found to contain isoAsp residues [142]. CD and FTIR studies indicated that isoAsp$_{1,7}A\beta_{1-42}$ had a significantly greater propensity for β–sheet formation than native $A\beta_{1-42}$ in aqueous solution at pH 7 to 12 and in membrane mimicking solvents such as octyl-β-L-glucoside and aqueous acetonitrile (ACN) [145]. The presence of isoAsp residues has been demonstrated by molecular modelling to destabilize Aβ α-helices [146], thereby facilitating the formation of β-sheet structure [145,146]. The substitution of L-amino acids from residues 17–22 of Aβ with their D-isoforms has also been demonstrated by CD spectroscopy to significantly decrease β-helix formation and stabilize β-sheet [147]. Therefore, the increased propensity for β-sheet formation exhibited by isoAsp Aβ may lead to the seeding of fibril formation in AD [3,142,145,146]. Furthermore, enzymatic degradation of these species would also be expected to occur at a reduced rate owing to the stereospecificy of the proteases involved. It has therefore been proposed that these species may also cause reduced protease susceptibility [142], which is a characteristic of AD amyloid deposits. This has been supported in model studies indicating that serum stability of isomerized *N*-terminal 10-mers derived from Aβ was significantly greater than the native peptide [146].

Aggregation Propensities of Aβ Isoforms

The propensity of $A\beta_{1-42}$ to form β-sheet has been found to be significantly greater than $A\beta_{1-39}$ and this increase was correlated with increased propensity for peptide aggregation [78]. This qualitative observation was supported by Burdick *et al.* [148], who found that Aβ peptides of 42 residues and longer exhibited significantly lower solubility than shorter analogues and that aggregation of these species was dependent upon time, peptide concentration and solution pH. Gel electrophoretic analysis performed by Burdick *et al.* indicated that $A\beta_{1-42}$ migrates as a range of high molecular weight aggregate species, unlike $A\beta_{1-28}$ and $A\beta_{1-39}$, which were observed to migrate as monomers. A recent study of Aβ aggregation, using dynamic light scattering, found that, under identical solution conditions, $A\beta_{1-42}$ aggregated from solution approximately 70 times faster than $A\beta_{1-40}$ [149]. Jarrett *et al.* performed turbidity based aggregation assays on $A\beta_{1-39}$, $A\beta_{1-40}$ and $A\beta_{1-42}$, as well as $A\beta_{26-39}$, $A\beta_{26-40}$, $A\beta_{26-42}$ and $A\beta_{26-43}$ [150]. In this study, Aβ aggregation was found to proceed by a nucleation dependent mechanism. Seeding of aggregation by the addition of a trace amount of amyloid fibrils to filtered solutions of Aβ resulted in rapid peptide aggregation and precipitation, whereas, when seeding was not performed, there was a significant lag time before detectable aggregate formation was observed [150]. Therefore, it was proposed that Aβ aggregation occurs in two stages – a slow nucleation stage, followed by a rapid aggregation stage and strong dependence of the rate of aggregation on the length of the *C*-terminal region of the Aβ species present was also reported [150]. A critical role of $A\beta_{1-42}$ in seeding soluble Aβ aggregation and fibril formation *in vitro* was recently proposed based on fibrilogenesis studies [151].

A study of the aggregation behaviour of $Glp_{3}A\beta_{3-40}$ and $Glp_{11}A\beta_{11-40}$, together with $Glp_{3}A\beta_{3-28}$ and $Glp_{11}A\beta_{11-28}$ was recently

performed [44] using the fluorescamine aggregation assay [71], which quantitates the amount of peptide remaining in solution after aggregation and centrifugation. The results of this study indicated that pyroglutamyl *N*-terminal Aβ analogues aggregate at a significantly higher rate than the corresponding full-length peptides [44]. Aggregation assays were performed at pH 5 and pH 7.2 for each of the peptides and their controls. All the peptides tested were found to aggregate more rapidly at pH 5 than at pH 7.2 and aggregation was also faster at higher peptide concentration. $Glp_3A\beta_{3-28}$ and $Glp_{11}A\beta_{11-28}$ were each found to sediment to a similar degree, and significantly more rapidly than $A\beta_{1-28}$. $Glp_3A_{\beta 3-40}$ and $Glp_{11}A\beta_{11-40}$ were observed to aggregate maximally and to a similar extent within 20 min at both pH 5 and pH 7.2, whereas detectable $A\beta_{1-40}$ aggregation was not observed until 40 min. Similar data was obtained at peptide concentrations of 50 and 100 μM. These data indicated that *N*-terminally truncated analogues of $A\beta_{1\text{-}40}$ and $A\beta_{1-28}$ bearing terminal pyroglutamyl residues exhibit significantly increased aggregation propensities compared to the full-length forms [44].

Induction of Aβ Aggregation by Metal Ions

The hypothesis that Aβ neurotoxicity is mediated, at least in part, by interactions with trace elements has attracted significant attention in AD research. Aluminium, iron, zinc and copper have all been reported to interact with Aβ and induce aggregation, thereby posing a possible link between abnormal levels of trace metals in the brain and the progression of AD [152,153]. Aluminium was first implicated in the progression of AD when it was observed that intracerebral injection of this element into rabbits induced the formation of neurofibrillary tangles [152,154,155]. Aluminium is known to localize at high concentrations in AD amyloid deposits [156], but studies aimed at determining if it is elevated in AD brains have not achieved consensus [152]. Mantyh *et al.* [157] have reported that it can induce the aggregation of Aβ, but the metal concentration used in this study has since been claimed to be significantly above physiological levels, and when Aβ was exposed to low micromolar levels of aluminium, no Aβ aggregation was induced [158]. Overall, it appears that although this element may be a contributing factor to the neurodegeneration in AD, it does not appear to have a causative role [152].

Iron, in both ferric and ferrous forms, has also been found to induce Aβ aggregation from solution *in vitro* at a concentration of 1 mM when peptide concentration was 10^{-10} M, which is approximately the level found *in vivo* [157]. Elevated levels of Fe have been found in NFT containing neuronal cells by microprobe mass spectral analysis [156], suggesting that disturbed iron metabolism occurs in AD sufferers [152,159].

A number of studies have reported enhanced rates of Aβ amyloid formation *in vitro* upon addition of physiological concentrations of zinc(II) [157,159–163]. Aβ and sAPP aggregation from cerebrospinal fluid in response to Zn^{2+} has also been reported [164]. Aggregation of $A\beta_{1-40}$ mediated by Zn^{2+} has been found to be almost instantaneous [161,165], in contrast to the slow aggregation observed in its absence [150]. The strong modulation of Aβ aggregation by this element is thought to be owing to a discrete zinc binding domain within the Aβ structure [158]. Bush *et al.* reported that Zn binding by Aβ is specific and saturable with a high and low affinity mode. The stoichiometry for high affinity Zn^{2+} binding by Aβ is 1:1 (Zn:Aβ), and the K_D for this interaction is 107 nM. Low affinity binding of Zn to Aβ occurs in a 2:1 manner, with a K_D of 5.2 μM [158,166]. Interestingly, rodent Aβ, that has just three amino acid substitutions, binds Zn^{2+} significantly less strongly than the human peptide, the K_D for this interaction being 3.8 μM when in 1:1 stoichiometry [158]. Rodent Aβ does not aggregate when exposed to concentrations of this metal found in humans *in vivo*. This has been proposed as a reason why rodents form minimal amounts of amyloid deposits [167,168] since the solubilities of the rat and human Aβ analogues were indistinguishable in the absence of Zn^{2+} [161]. The binding of Zn^{2+} by Aβ has been observed to depend on the peptide adopting an α-helical structure followed by Aβ dimerization which facilitates further aggregation [163]. Although the specific zinc binding site of Aβ is not entirely resolved, a recent study indicated that His_{13} is a critical residue for binding, and that the His_{13}Arg substitution in rodent Aβ is responsible for the modified Zn^{2+}-induced aggregation behaviour of rodent Aβ [169]. This is supported by the report that non-specific side chain modification of His residues in Aβ with diethyl pyrocarbonate abolishes Zn^{2+} binding [153]. This *in vitro* evidence coupled with the observations of abnormal zinc metabolism and regulation associated with AD (for review, see [160]) appears to indicate a significant role of Zn^{2+} mediated Aβ aggregation in AD.

The binding of copper to Aβ has also been found to be associated with rapid formation of amyloid. The binding of a single Cu^{2+} atom to an Aβ dimer has been found to be sufficient to cause peptide aggregation [170], although between two and three Cu^{2+} ions are usually bound to each Aβ unit in amyloidβ [153,170]. At pH 7.4, this binding is largely attenuated, but at pH 6.6, corresponding to the slight acidolysis associated with AD brain, Aβ is thought to adopt a conformation which allows the binding of Cu^{2+}, presumably a soluble dimer [153]. $A\beta_{1-42}$ binds copper in two discrete manners, a low affinity binding and a very high affinity binding, with a K_D of 2×10^{-17} M, associated with the rapid formation of Aβ amyloid [170]. $A\beta_{1-40}$ binds Cu^{2+} in a similar manner but with significantly lower affinity, the K_D for the high affinity interaction being 2×10^{-10} M [170]. Copper binding was found to partially compete with zinc for Aβ binding but, unlike Zn^{2+}, the modification of His_{13} reduced but did not abolish Cu^{2+} binding [153]. Therefore, the Cu^{2+} and Zn^{2+} binding sites appear to be distinct, and it is possible that Aβ could bind both metal ions at the one time [153]. A significant enrichment of Zn^{2+}, Cu^{2+} and Fe^{3+} has been observed in amyloid plaques [159]. This observation, coupled with the high affinity of Aβ binding to these species, led to the proposal that metal ion-induced aggregation of Aβ is a protective mechanism that provides a sink for abnormally high levels of trace metals [153]. However, the binding of Cu^{2+} to Aβ has been observed to induce the formation of SDS insoluble amyloid, indicating that the binding has, in some way, damaged Aβ, possibly by an oxidative mechanism [170]. Interestingly, copper also seems to protect against AD amyloidosis, as it has been demonstrated to stimulate the β-secretase pathway for APP proteolysis which precludes Aβ formation [171].

MECHANISMS OF Aβ NEUROTOXICITY

Enhanced membrane permeability leading to cellular dysfunction is one proposed mode of Aβ toxicity. Over-expression of Aβ in cell culture studies has been observed to cause membrane ruffling [172,173], and the formation of cation selective channels in cellular membranes, together with the activation of voltage dependent Ca^{2+} channels in response to Aβ has also been reported [112,113,174–177]. Formation of these channels has been shown to result in ion homeostasis malfunctions and disregulation of cellular signal transduction, which may lead to cell death [113,174,175,178]. Interestingly, these channels can be selectively blocked with Zn^{2+}, which indicates a protective role for this ion [179]. The membrane disruption hypothesis is supported by studies which have indicated Aβ interacts strongly with phospholipid membranes, and can cause disruption of acidic phospholipid containing membranes by specifically binding to the membrane components [180–182]. A recent study of $A\beta_{1-40}$ interactions with ganglioside membrane preparations by CD and FTIR found that Aβ orientates itself in an anti-parallel β-sheet across the surface of the membrane, the formation of which was dependent on the ganglioside concentration of the membrane. The formation of this β-sheet parallel to the plane of the membrane was found to perturb membrane structure and cause dehydration of lipid interfacial groups and displacement of the acyl chains [183]. Electrostatic interactions are a primary driving force behind these peptide–phospholipid interactions [184], although hydrophobic interactions may also be significant [122]. An alternative explanation for the membrane activity related neurotoxicity of Aβ was proposed by Avdulov *et al.* [185], who found that aggregates of $A\beta_{1-40}$ bound specifically and strongly to cholesterol in preference to phosphatidylcholine and fatty acid preparations. Non-aggregated $A\beta_{1-40}$ was found to not bind cholesterol in this preferential manner. They propose that this may result in the stripping of cholesterol from neuronal membranes in AD, resulting in impaired membrane function and subsequent cellular dysfunction.

In two recent publications, Aβ neurotoxicity has been reported to be due to the induction of apoptosis by caspase enzymes which are members of the family of Cys proteases [186,187]. Nakagawa *et al.* found that caspase 12, a member of the interleukin-1β converting enzyme subfamily of caspases, was activated in response to endoplasmic reticulum stress, including Ca^{2+} dysregulation [186]. Furthermore, neurons from caspase-12 deficient mice were found to be insensitive to treatment with Aβ, but underwent apoptosis in response to other cell death stimuli [186]. Caspase-2 has also been reported to modulate Aβ-induced neuronal death. Down-regulation of caspase 2 was found to prevent $A\beta_{1-42}$-induced cell death, unlike downregulation of caspase-1 and caspase-3, and neurons from caspase-2 null mice were resistant to $A\beta_{1-42}$ toxicity [187].

Microglia are consistently localized in and around AD amyloid deposits, and their interaction with Aβ is thought to induce cytokine release and activation of the complement pathway, both of which result in inflammation [188,189]. Aβ and amylin fibrils have both been demonstrated to induce microglial production of the inflammatory cytokine interleukin-1β, together with the pro-inflammatory cytokines TNFα and interleukin-6 [189]. Production of the chemokines interleukin-8, macrophage inflammatory protein-1α and macrophage inflammatory protein-1β was also induced. These data indicate that amyloid fibrils can induce the inflammatory response, which may cause significant neurodegeneration in AD brain [189] (see [188] for a recent review). Activation of Ca^{2+} channels by Aβ and the associated neuronal Ca^{2+} dysregulation is also thought to be modulated by the release of interleukin-1β [177]. Microglia are known to phagocytose amyloid fibrils, and to disaggregate a proportion of this material in an attempt to clear the proteinaceous deposits [190]. However, a significant amount of the fibrilar Aβ taken up by the microglia is simply released as soluble Aβ, and thus, the amount of protection against amyloid accumulation offered by these cells is somewhat limited [190].

The hypothesis that Aβ derived free radical species cause protein and lipid oxidation which leads to a loss of ion homeostasis and cell death [191] is supported by a number of studies. Free radicals formed by cells in response to Aβ have been found to have significant toxicity *in vitro*, inducing loss of enzymatic activity, lipid peroxidation, inhibition of cellular signalling, and elevation of intracellular calcium leading to apoptosis [191–193]. Blanc *et al.* [193] found that Aβ-induced apoptosis of vascular endothelial cells was successfully blocked by the use of antioxidants, a chelator of intracellular calcium and a calcium channel blocker. These data strongly supported the role of Aβ-induced free radical damage in causing dysregulation of Ca^{2+} influx and cell death [187–191]. It is thought that the interaction of Aβ with vascular endothelial cells results in excess production of superoxide radicals, which then cause destruction of these cells [192]. Free radical-induced oxidative damage has also been implicated in damage to glutamine synthetase in AD, which may result in excitotoxic cell death [193,194], and in mitochondrial dysfunction and uncoupling, a process by which oxygen radicals are known to be produced owing to incomplete reduction of oxygen [195,196].

The mechanism(s) by which free radicals are generated by Aβ is currently under intense scrutiny [197–203]. Neurons treated with Aβ have been found to release hydrogen peroxide concurrently with cell death [202], as well as superoxide radicals, glutamate [199] and nitric oxide radicals [203]. The interaction of fibrilar Aβ with the enzyme NADPH oxidase in microglia, monocytes and neutrophils also induces production of superoxide radicals. Superoxide production by this enzyme is associated with inflammatory reactions, which is one of the characteristics of AD, and is also a current therapeutic target [204]. Class A scavenger receptors on microglia have also been reported to bind to Aβ coated surfaces, resulting in secretion of reactive oxygen species (ROS) such as hydrogen peroxide. Microglia localize in amyloid plaques in AD, and ROS production may explain the neurodegeneration around these structures [205]. Furthermore, Aβ is known to bind to catalase and prevent the breakdown of H_2O_2 by this enzyme, which would further enhance the probability of oxidative damage in AD [206]. There is also evidence that Aβ interacts with the receptor for advanced glycation endproducts (RAGE) in neurons, microglia and vascular endothelial cells, which is thought promote the production of ROS through a range of oxidant pathways in the target cell [207]. Cells producing RAGE have been found to exhibit increased lipid peroxidation in response to Aβ when compared to cells without this receptor [207,208]. RAGE is crucial for the removal of glycated proteins formed through oxidative damage, and the interaction of amphoterin with RAGE is known to have beneficial effects on neurite outgrowth during development. Why a change in receptor activity is observed with increasing cell age and development remains unclear [200,207].

An alternative possibility for Aβ-induced production of free radicals and ROS is the interaction between Aβ and redox-active species, such as iron and copper. Smith and co-workers have reported that iron, which accumulates in AD amyloid deposits owing to dysregulation of iron metabolism, is redox active [209]. The oxidation of Fe(II) to Fe(III) with hydrogen peroxide by the Fenton reaction is known to result in the formation hydroxyl radicals, and this process is known to be catalytic owing to the presence *in vivo* of agents capable of reducing Fe(III) [209]. This study demonstrated that the coordinated iron found in amyloid deposits was capable of undergoing oxidation in response to H_2O_2, thereby implying that free radical generation by Fenton type chemistry was feasible in AD. In

support of this model, Huang *et al.* [210] recently reported that $A\beta_{1-42}$ and, to a lesser extent, $A\beta_{1-40}$, are capable of reducing Fe(III) and Cu(II) to Fe(II) and Cu(I), respectively. Analogues with little or no neurotoxicity, such as rat $A\beta_{1-40}$, reversed human $A\beta_{1-40}$ and $A\beta_{1-28}$, were unable to reduce either Fe(III) or Cu(II). Metal-induced aggregation of Aβ was found to be independent of the extent of reduction, as aggregation is significantly enhanced at pH 6.8 compared with pH 7.4, but only a small increase in the amount of reduced metals produced was observed at pH 6.8 compared with pH 7.4. Chelation attenuated the reduction, indicating that the metals need to be coordinated to Aβ in order for the reduction to occur. These authors propose that the reduction of Fe(III) and Cu(II) could result in the formation of covalently bridged dimeric species via a two-step mechanism:

$$(A\beta)_2 + M^{(n+1)+} \rightarrow A\beta{:}A\beta^{+} + M^{n+} \quad (1)$$

$$A\beta{:}A\beta^{+} + M^{(n+1)+} \rightarrow A\beta - A\beta + M^{n+} \quad (2)$$

$A\beta_{1-42}$ and $A\beta_{1-40}$ were found to not only reduce metal ions, but also to trap molecular oxygen, which can react with the reduced metal centre to produce hydrogen peroxide. H_2O_2 production was increased at higher O_2 tension, and abolished in the presence of chelators. As with the reduction, H_2O_2 production was more greatly facilitated by $A\beta_{1-42}$ than by $A\beta_{1-40}$. An assay designed to detect the production of hydroxyl radicals, as predicted by the Fenton reaction, also appeared to support their production, indicating that a catalytic cycle producing ROS is possible in this system [210,211]. Although this hypothesis provides an elegant explanation of Aβ neurotoxicity and its apparent link to oxidative stress, it remains somewhat contentious as free radicals and lipid peroxidation were recently reported to not mediate neuronal death in response to Aβ [212].

Aβ BASED THERAPEUTIC STRATEGIES

Because increased oxidative stress may play a significant role in the neurodegeneration associated with AD antioxidants have been proposed as potential therapeutics. L-Deprenyl is an inhibitor of monoamine oxidase B and has antioxidant properties attributed to its ability to scavenge free radicals [213–215]. Vitamin E is also a potential antioxidant for use in AD therapy, as it scavenges free radicals and prevents lipid peroxidation [213,216,217]. Both of these compounds have been shown to delay the onset of disabilities caused by early Parkinson's disease [213]. Vitamin E has proved effective in the prevention of apoptosis in cells expressing the presenilin-1 mutation which leads to increased oxidative stress and early onset AD [218]. The peptide hormone melatonin has been found to possess antioxidant properties and prevents lipid peroxidation, rises in intracellular Ca^{2+} and cell death in response to $A\beta_{25-35}$ [219], which has been attributed to free radicals formed in response to Aβ [194,219]. Further investigation of the antioxidant activity of melatonin and its possible application to AD therapy may therefore be warranted [219].

Induction of inflammatory responses is known to play a role in the progression of AD, and is proposed as a mechanism by which Aβ could modulate its toxic effects. Aβ has been shown to stimulate macrophages and microglia, induce the release of cytokines, and activate the classical complement cascade [213,220]. Consequently, anti-inflammatory agents may be beneficial in slowing the rate of neurodegeneration in AD. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), was shown to significantly reduce cognitive decline, but also induced severe gastrointestinal side-effects in some patients [213]. An inverse correlation between the onset of AD and use of anti-inflammatory drugs including aspirin, steroids and NSAIDs taken for treatment of arthritis has also been reported [213]. Clinical trials of other anti-inflammatory agents, including the steroid prednisone, have also been performed [216]. Other recently developed NSAIDs, such as celecoxib, a cycloxygenase II inhibitor, appear be relatively side-effect free, and may lead to significant improvements in the applicability of anti-inflammatory therapy in AD [221].

As the formation and deposition of amyloid is a pathological hallmark of AD, and Aβ is neurotoxic in the aggregated state [69,70], the inhibition of amyloid formation, or its subsequent disaggregation, could potentially lead to effective therapeutic strategies for AD. The secretory protein gelsolin has been reported to bind to Aβ and prevent fibril formation, as well as disaggregating pre-formed Aβ fibrils [222]. Both 11-mer (RDLPFFPVPID) and 5-mer (LPFFD) peptides derived from residues 15 to 25 and 17 to 20 of Aβ, respectively, have been reported to bind Aβ with high affinity and inhibit $A\beta_{1-42}$ fibrilogenesis *in vitro* [223–225]. The 5-mer also disaggregated pre-formed Aβ fibrils, and inhibited $A\beta_{1-42}$ neurotoxicity [224,225]. Other agents known to inhibit Aβ fibrilogenesis include congo red

dye [226], 3-*p*-toluoyl-2-[4′-(3-diethylaminopropozy)-phenyl]-benzofuran [227], the tetracyclic compounds daunomycin and rolitetracycline [228], and the substituted carbazole carvediol [228], together with many others (for review see [228]).

Schenk *et al.* recently reported that immunization with Aβ attenuated AD pathology in transgenic PDAPP mice [229,230]. The PDAPP mouse overexpresses human APP bearing the pathogenic Val^{717} to Phe mutation and exhibits amyloid deposition, plaque formation and other characteristics of AD pathology from an early age [66,67]. Young animals immunized with human Aβ were found to develop almost no AD type pathology and high titres of Aβ antibodies were observed. Furthermore, immunization of older PDAPP mice already expressing AD pathology was found to significantly retard further pathological development [229]. The mechanism by which plaque deposition was inhibited in these studies remains unclear, and the effect of immunizing humans with Aβ could be significantly different to the mouse model which, at best, represents only partial AD pathology, as these animals do not form NFTs [230]. Therefore, further research into this discovery is required to determine if the effect is robust and therapeutically useful in the treatment of AD.

Reduction of Aβ deposition by controlling APP proteolytic cleavage is potentially one of the most effective therapeutic strategies for AD. If specific inhibitors to the secretase enzymes could be developed, it should be possible to prevent production of Aβ, which may lead to the ability to prevent the progression of AD altogether. Unfortunately, this approach has, to date, been hampered by the inability to clearly define the secretase enzymes responsible for APP metabolism. However, the recent report by Vasser *et al.* that the membrane-bound aspartic protease BACE possesses highly specific β-secretase activity may lead to the development of a specific inhibitor for this enzyme, thereby enabling therapeutic control of the β-secretase pathway and Aβ generation in AD [48]. Indeed, the development of a tripeptide aldehyde inhibitor of β-secretase has recently been reported [231]. Further research in this area may more clearly elucidate the role of Aβ in AD, and could potentially lead to a highly effective neuroprotective strategy [232].

CONCLUSIONS

The Aβ peptide is clearly central to the neuropathology of AD, and provides a valid therapeutic target for AD. Most of the problems associated with its synthesis and purification have been overcome, although most methods of production are relatively expensive, and still provide a low recovery after purification. Economically viable high yielding synthesis and purification methods will be particularly important if immunization with Aβ is proven to be therapeutically useful. Although some forms of aggregated Aβ are clearly neurotoxic, it is still unclear which aggregated species is the toxic species in AD. Amyloid plaque itself is probably not the major species responsible for neurodegeneration, but rather a soluble intermediate aggregate on the pathway to amyloid formation is probably the major neurotoxic species. Identification of the toxic aggregated species is particularly important for designing therapeutic strategies that inhibit amyloid formation, especially when these strategies do not decrease soluble Aβ levels in brain. The roles played by endogenous Aβ isoforms and metal ions in Aβ-induced neurotoxicity remains unclear, although both are currently hot areas of Aβ research. Recently, the metal-chelator clioquinol has been shown to clear amyloid plaque in transgenic mice (private communication) supporting a role for metals in amyloid formation *in vivo*. Metal chelation, inhibition of Aβ formation, aggregation, or associated neurotoxicity, and immunization with Aβ, are all promising strategies for the treatment of AD. The next few years will be an exciting time for AD research, with several new amyloid therapies currently in clinical trials. With the intensity of current research into Aβ and AD, there is a realistic hope that a new generation of drugs will make AD a treatable disease.

Acknowledgements

The reported work that was carried out in the authors' laboratories was generously supported by an ARC Large Grant (ABC and CJB) and an NHMRC Institute Block grant (Grant Reg key # 983001) (ABC and JDW).

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