Published in final edited form as:

Isr J Chem. 2017 July ; 57(7-8): 602–612. doi:10.1002/ijch.201600078.

Cerebrospinal Fluid Proteins as Regulators of Beta-amyloid Aggregation and Toxicity

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Abstract

Amyloid disorders, such as Alzheimer's, are almost invariably late-onset diseases. One defining diagnostic feature of Alzheimer's disease is the deposition of beta-amyloid as extracellular plaques, primarily in the hippocampus. This raises the question: are there natural protective agents that prevent beta-amyloid from depositing, and is it loss of this protection that leads to onset of disease? Proteins in cerebrospinal fluid (CSF) have been suggested to act as just such natural protective agents. Here, we describe some of the early evidence that led to this suggestion, and we discuss, in greater detail, two CSF proteins that have garnered the bulk of the attention.

Graphical Abstract

Keywords

Alzheimer's disease; amyloid beta-peptides; apolipoprotein E; glycoproteins; transthyretin

1. Introduction

Amyloid disorders are, with few exceptions, diseases of the aged. The diseases are caused by a variety of proteins, which differ in their native structure and function. Common to all amyloidoses is the production of alternatively folded or misfolded aggregates, all of which

have a fibrillar morphology and a cross- β sheet structure. Although the details differ, typically there is a progression from native monomer through soluble oligomers (a heterogeneous mix in size and conformation) to mature fibrils. The question arises: why does aging trigger amyloidogenesis? There are numerous possible explanations. Perhaps aging reflects an accumulation of bumps and bruises, mistakes and errors, and amyloidogenesis is the accumulated effect of a large number of small insults, none of which by themselves would be sufficient. Another possibility is that there is a loss of normal function. For example, the chaperone machinery that ensures proper folding could become inefficient, so a fraction of the newly synthesized protein is incorrectly folded. Or, the machinery for tagging and degrading improperly folded protein could run out of capacity. An intriguing idea is that there are sentinel proteins that are normally on the lookout for pre-amyloid oligomers, finding and sequestering them before damage is done, and that these proteins become less effective with age (due to decreased concentrations of sentinel protein, loss of sentinel protein function, or increased production of amyloidogenic species). It is this last idea that we will explore in this review article, as it pertains particularly to the Alzheimer-related peptide, β -amyloid.

2. β -Amyloid and Cerebrospinal Fluid Proteins

2.1 β -Amyloid and Alzheimer's Disease

There are several excellent review articles on β -amyloid and its relationship to Alzheimer's disease, to which the reader is referred for more details.^[1] Alzheimer's disease (AD) is characterized by three neuropathological features: extracellular fibrillar deposits of β -amyloid ($A\beta$) as senile plaques, intracellular neurofibrillary tangles (NFT) comprised of hyper-phosphorylated tau, and synaptic degeneration. Generally, it is believed that deposition of $A\beta$ precedes NFT formation. Typically, these features are found in the hippocampus and other regions of the brain involved with cognition. $A\beta$ is a 4 kDa proteolytic product of the membrane-spanning amyloid precursor protein, APP. Cleavage by β - and γ -secretase produces $A\beta$, as well as other fragments. Different cleavage patterns produce ragged N- and C- termini; of particular relevance are the most common isoforms, $A\beta_{1-40}$ and $A\beta_{1-42}$. The latter is considered to be more aggregation-prone and to have greater toxicity. APP can be alternatively cleaved by α -secretase, producing the soluble fragment sAPP α .^[1c] $A\beta$ is cleared from the brain by a number of pathways, including enzymatic degradation by neprilysin and insulin-degrading enzyme, receptor-mediated cellular clearance, and efflux through the blood-brain barrier. In healthy persons, $A\beta$ production and clearance are in balance. In AD, there is strong support for the hypothesis that imbalance in $A\beta$ production and clearance is the underlying cause of the disease.^[2]

The normal biological function of $A\beta$ is still a matter of debate, but there is some evidence that, at nanomolar concentrations, $A\beta$ serves a supporting role in neural development and neuron health. The peptide spontaneously self-associates in a concentration-dependent manner into soluble complexes; these go by a bewilderingly diverse set of names (oligomers, ADDLs, protofibrils, prefibrillar assemblies, and others). We will use the term "soluble oligomers" to describe an ensemble of complexes that are heterogeneous in size (dimer to multimers of 30, 50, or 100s of monomers), shape (globular or elongated), and secondary

structure (disordered, β -sheet).^[3] Oligomers are dynamic species that are affected by changes in concentration, solution conditions, or handling. Much work has been done to characterize oligomer solutions in vitro, which poses substantial analytical challenges, because of their heterogeneity and instability.^[2, 3] Some soluble oligomers eventually mature into insoluble fibrils with typical features of cross- β sheet structure, a twisted appearance, a diameter of ~ 10 nm diameter, a length of up to 1 micron or more, and positive staining by amyloid dyes such as Congo red or thioflavin T (ThT). Some oligomers are thought to be “off-pathway” in that they never directly mature into fibrils. Conversely, fibrils might act as sources of soluble oligomers, or as catalysts for conversion of monomers to oligomers. A β is also found intracellularly, and generated either by intracellular processing of APP or re-uptake of A β from the environment.^[1c] Most of the detailed characterization of A β oligomers has been done in vitro, as in vivo characterization is even more challenging. That said, oligomers have been detected in vivo in cerebrospinal fluid (CSF), intracellularly, and attached to cellular membranes.^[1b]

It is a generally accepted paradigm that these soluble oligomers are toxic to neurons. Although this has been fairly well established in cell culture, it is difficult to prove the role of oligomers in human disease. Further complicating this hypothesis is that, since the oligomers are heterogeneous in size, structure and morphology, they may also be heterogeneous in toxicity.^[2, 3] Furthermore, in vivo A β might be chemically modified or bound to lipids or other proteins. A β isolated from tissue is typically two or more orders of magnitude more toxic than in vitro A β preparations.^[2]

Many different mechanisms of A β toxicity have been proposed: these include disruption of calcium flux, loss of glutamine homeostasis, or generation of reactive oxygen species. A β binding to more than a dozen cell-surface receptors has been reported; the large diversity of reported receptors may simply be a function of the stickiness of the oligomers, as well as their heterogeneity. A β oligomers are known to interact with cellular membranes, and it has been postulated that interactions with lipids, rather than with a specific receptor, cause neuronal dysfunction. Even fuzzier is our understanding of how the A β binding event translates into a downstream toxic response, but it is reasonably well established that this response includes tau hyperphosphorylation. An inflammatory response, possibly to deposited A β plaques, and secondary to the primary disease mechanism, is observed as well.

Currently, only symptomatic treatments for AD are available, such as acetylcholinesterase inhibitors and NMDA receptor agonists.^[4] Several curative approaches are under investigation, including: inhibitors of enzymes that cleave APP, immunization to trigger an innate immune response against A β , dissolution of plaques to facilitate natural degradative processes, and compounds that bind A β and prevent its aggregation.^[5] As an example of the last approach, antibodies to A β as therapeutics have been tested in clinical trials, with minimal success.^[6] One issue that arises is the nature of the antigen. In early studies, oligomers were not selectively targeted by the antibody, but newer antibodies are designed to specifically bind to oligomers. Several clinical trials are underway, but past disappointments must temper any optimism that an effective anti-A β antibody treatment will be available in the near future.

2.2 CSF as an Anti-amyloid Sequestering Fluid

The choroid plexus is an organ at the blood-CSF barrier that secretes CSF, provides nutrients to the brain, and removes waste products from the brain extracellular space.^[7] CSF is a complex fluid containing salts, vitamins, nutrients, growth factors, and proteins.^[8] Proteins in the CSF are produced primarily by the choroid plexus (TTR, BDNF and IGF-2 for example), but are also obtained by active (e.g., leptin) or passive (e.g., albumin) transport from the blood. The concentration of proteins in the CSF is also affected by metabolism, synthesis, and transport in neurons, microglia, and astrocytes. The total protein content of CSF is lower than that of the blood ($\sim 0.25 \text{ mg mL}^{-1}$ in CSF versus $\sim 70 \text{ mg mL}^{-1}$ in plasma)^[8] and the relative protein composition of CSF is significantly different. Table 1 contains a list of many of the common proteins found in CSF, along with their typical concentration and major function(s). Not shown in the table, A β has been reported to exist in the human brain at concentrations ranging from high picomolar to low nanomolar, with both total A β and A β_{1-42} being significantly increased in AD brain tissue.^[9] Several studies have shown that total CSF protein concentration decreases in AD.^[10]

The first hints that cerebrospinal fluid might contain compounds that inhibit A β fibrillogenesis came in the early 1990s. In this work, synthetic A β was mixed with CSF from healthy volunteers; it was observed that CSF inhibited fibril formation, as assessed by both thioflavin (ThT) fluorescence and transmission electron microscopy (TEM).^[18] The researchers hypothesized that the inhibitory compound was a protein, because ultrafiltered CSF was no longer inhibitory.

Rapidly following the discovery that CSF inhibits A β aggregation, two groups reported their efforts to identify the specific CSF protein(s) responsible for this activity. By immobilizing A β onto a membrane and then probing with CSF, Strittmatter *et al.* observed that several proteins, including Apolipoprotein E (ApoE) (identified by Western blot), bound with high avidity.^[19] ApoE was of particular interest, because of the then newly discovered connection between inheritance of the ApoE4 allele and higher frequency of sporadic AD.^[20] In a follow-up study, synthetic A β was shown to bind to delipidated ApoE3 and ApoE4, but with distinct differences in the kinetics and pH sensitivity of binding.^[21] Residues 244–272 of ApoE, and residues 12–28 of A β , were identified as critical for binding interaction. No investigation of the effect of ApoE on A β aggregation was undertaken at that time.

Using synthetic A β as a probe, Schwarzman and coworkers identified the A β binding partner to be transthyretin (TTR).^[22] In contrast to the ApoE study, this investigation was unbiased in that the binding event between A β and CSF occurred in solution, rather than on a membrane, and the protein was identified via sequencing. In addition, the group demonstrated, using ThT and TEM, that TTR inhibited A β amyloid formation at a remarkable 300:1 A β /TTR molar ratio. This result was later confirmed in a *C. elegans* model.^[23] These researchers proposed a “sequestration hypothesis”: that proteins in CSF, particularly TTR, normally sequester A β and prevent its conversion to amyloid, and that AD is a consequence of the failure of sequestration.^[24] This conclusion was questioned by researchers from other laboratories, who suspected that TTR preparations were contaminated or who had observed that TTR enhanced A β aggregation.

In the same time frame, a few reports surfaced in which both TTR and ApoE were compared with respect to A β interaction. In one study, ApoE codeposited with A β , and ApoE induced intracellular accumulation of A β deposits in brain vascular smooth muscle cells, with ApoE3 triggering more accumulation than ApoE4. Addition of TTR blocked ApoE-induced A β accumulation. The authors suggested that this was due to competitive binding to A β between ApoE and TTR, with ApoE acting as a transporter of A β across cellular membranes.^[25] Serot *et al.* reported that TTR inhibited fibrillogenesis, while ApoE promoted fibrillogenesis, and that TTR, but not ApoE, was reduced in concentration in the CSF of patients with AD relative to age-matched controls.^[26]

Although these investigators suggested that there were specific interactions between CSF proteins and A β , interest in the area declined following these early publications for about a decade. Given the “stickiness” of A β , it was not unreasonable to consider the observations of binding between A β and TTR or ApoE as “nonspecific” and biologically irrelevant. More recently, however, there has been a growing appreciation for the role of so-called low-affinity promiscuous interactions in biological networks, particularly involving proteins, like A β , that lack a single, defined, stable, native structure.^[27] In the remainder of this review, we take a closer look at more recent detailed studies of interactions of TTR and ApoE with A β , with a brief foray into other CSF proteins that may also interact with A β .

2.3 Transthyretin as a CSF Inhibitor of A β

Transthyretin is a 55 kDa homotetrameric protein, synthesized in the liver and secreted into the blood (165–385 mgL⁻¹), and synthesized in the choroid plexus and secreted into CSF (12–27 mgL⁻¹). Each monomer contains two four-stranded β -sheets, an inner sheet comprised of strands D, A, G, and H, and an outer sheet of strands C, B, E and F, with a single α -helix between strands E and F. Monomers assemble into a dimer stabilized by extensive hydrogen bonding, while dimer-dimer association is driven primarily by hydrophobic interactions.^[28] TTR is a carrier for the thyroid hormone thyroxine, which sits in a central hydrophobic cavity formed when the tetramer assembles. TTR also forms a complex with retinol-binding protein, which transports retinol extracellularly.

Interest in TTR's role in AD languished for about a decade, until two studies involving transgenic mice were published. It was known that transgenic mice, carrying the Swedish mutation in APP (APP_{Sw}), produced high levels of A β in the brain, but did not develop amyloid plaques until 12 months, and showed no significant neuronal loss or neurofibrillary tangles, even at 16 months. This model raised doubts about the dominant “amyloid cascade” hypothesis, that A β amyloid deposition causes AD. To explore this quandary, gene expression profiles were obtained which showed a manyfold increase in TTR mRNA and protein in the hippocampus,^[29] a result supported by later studies.^[30] Furthermore, infusion of anti-TTR antibodies led to greater neuronal loss and tau hyperphosphorylation.^[31] This led to the hypothesis that increased TTR expression was a protective response to high levels of A β , thus explaining the lack of pathology in APP_{Sw} mice. Additional studies demonstrated that synthetic A β was toxic to hippocampal slices, and addition of TTR prevented A β -induced toxicity.^[31]

Other researchers have confirmed that TTR protects neurons against A β toxicity in vitro.^[30b, 32] Animal studies have provided additional evidence that TTR has a neuroprotective role against A β . Buxbaum and coworkers observed improved cognition when APP_{Sw} mice were crossed with mice expressing human TTR.^[33] AD mice raised in an enriched environment had greater TTR levels and performed better on cognitive assays.^[34] A β deposition in brain tissue was significantly higher in APP_{Sw} mice when TTR was partially knocked out.^[35] Using a novel method, A β oligomers were repeatedly and directly injected into mouse hippocampus, where they caused neuronal loss and tau hyperphosphorylation; TTR was able to attenuate these toxic effects.^[36] But not all studies support a connection between TTR and attenuation of A β . In one study, there was little to no difference in plaque burden or cognitive defects whether or not TTR was knocked out;^[37] the authors speculated that this contradictory result was obtained because they used an especially aggressive AD mouse model.

In contrast to the increased TTR gene expression observed by Stein *et al.*, TTR gene expression dropped precipitously in aged (24 month) transgenic mice, compared with wild-type (wt).^[38] A threefold lower expression of TTR was observed in a triple transgene mouse model (APP_{Sw}/PS1/tauP301L) at 16 months, compared with wt mice.^[39] Since these studies were completed with very old mice or with a very aggressive disease model, the drop in TTR may be attributable to widespread loss of functional choroid plexus, and thus, is a consequence, rather than a cause, of disease. This conclusion would be consistent with the observations of a substantial decrease in TTR in CSF of patients with advanced AD.^[40] On the other hand, no significant difference in CSF TTR levels between AD and control groups was found by Schultz *et al.*,^[41] so whether TTR is an effective biomarker for AD is still a matter of debate.

Overall, the literature provides sufficient support for the hypothesis that TTR plays a neuroprotective role against A β , motivating detailed inquiry into the biochemical and biophysical nature of the TTR–A β interaction. Several studies have aimed to confirm that TTR binds selectively and specifically to A β , and to establish the binding affinity. Using radio-iodinated TTR in a competitive binding assay, with A β adsorbed onto plates, Costa *et al.* reported an apparent K_D of 28 ± 5 nM.^[32b] A solution-based assay, in which quenching of TTR's tryptophan fluorescence was used to detect binding, resulted in an estimate of a much weaker binding interaction, with $K_D \sim 430$ μ M.^[42] Using isothermal titration calorimetry, another group estimated $K_D \sim 24$ μ M.^[43] Although not a direct estimate of K_D , the same researchers estimated an IC_{50} of ~ 80 – 140 nM TTR from a dot-blot assay, with A β immobilized. Taken together, these data illustrate the difficulty of ascertaining a reliable estimate of K_D ; in particular, there is a large disparity between estimates obtained from solution-based versus surface-based estimates.

One complicating factor is the heterogeneity in conformation and oligomerization that arises in A β preparations. Costa *et al.* prepared monomeric, oligomeric, and fibrillar A β solutions and concluded that TTR binding was independent of A β aggregation status.^[32b] Du and Murphy used an ELISA protocol, with TTR adsorbed, and concluded, in contradiction to Costa *et al.*, that greater binding is exhibited with A β oligomers than monomers, or to a lesser extent, fibrils, although no attempt was made to extract a quantitative measure of

affinity.^[44] Li *et al.* also concluded that binding of TTR to A β monomers was much less than to oligomers.^[43] Several groups have reported that TTR inhibits A β toxicity at substoichiometric ratios with TTR concentrations down to ~100 nM.^[32c, 45] Given these results, the preponderance of the evidence indicates that TTR binds with high affinity to A β oligomers, and less or not at all to A β monomers.

There is strong experimental evidence that TTR inhibits A β fibrillogenesis. By TEM, TTR decreased the number and length of A β fibrils,^[32b] with a monomeric mutant being particularly effective.^[44] Similar conclusions were drawn from light-scattering and nanoparticle-tracking analysis,^[32c, 42] AFM imaging, and ThT fluorescence.^[43] Data were fit to a kinetic model, and shown to be consistent with the hypothesis that TTR inhibits A β aggregation by suppressing the growth of aggregates, rather than by preventing initial A β self-association.^[42]

An additional complexity arises because TTR tetramers can dissociate into monomers. Although the tetramer is the native state, about 0.5 % is expected to be dissociated at the concentrations typical in blood, and about 5% in CSF.^[46] Using an engineered TTR mutant that is stable as a monomer (mTTR), two groups observed greater binding of A β to mTTR than to TTR tetramers.^[43, 44] Several single-point mutations in TTR change the stability of the tetramer without disrupting the protein's structure, as does oxidation at the lone free cysteine. In general, binding of TTR to A β , and inhibition of A β aggregation, increases with a decrease in TTR tetramer stability,^[43, 46, 47] although one group reported the opposite trend.^[32b]

Binding of A β to TTR involves residues in strand G, in the "inner" β -sheet of TTR (Figure 1). L110 in strand G was identified as a critical residue;^[47] other residues in strands G and H also contribute to A β binding.^[48] Mutation of L110 to alanine abrogated A β binding; the L110A TTR mutant was a poor inhibitor of A β aggregation, and, critically, was no longer able to inhibit A β toxicity.^[32c] These experiments showed that the binding interaction between A β and TTR was directly necessary for TTR's protective action. Using NMR, Li *et al.* pinpointed TTR residues involved in binding with A β ,^[43] specifically, strand G and H residues I107, A108, A109, L110, A120, V121, and V122, as well as K15 and L17, both residues near the thyroxine-binding site. Surprisingly, these researchers did not observe any chemical shifts with mTTR and A β , a result that is difficult to reconcile with other binding data. One explanation proffered was that TTR tetramers bind A β monomers, but mTTR binds A β oligomers, which did not develop in the solution during the time course of the experiment.^[43] There is some evidence for a weak secondary interaction between A β and TTR's EF helix,^[32c, 44, 47] although this conclusion has been challenged by another group.^[43] On A β , the hydrophobic central domain, specifically residues 18–21, are involved with binding to TTR.^[43, 44]

The specific mechanism by which TTR binds to and inhibits A β aggregation and toxicity remains an open question. Our group has hypothesized that binding of A β oligomers to TTR's EF helix triggers partial destabilization of TTR tetramers, opening up the interior hydrophobic cavity for further A β binding, and that the small fraction of TTR that is naturally monomeric may play an outsize role in sequestering A β oligomers.^[32c, 44, 46]

Others have proposed that it is TTR tetramers binding to A β monomers and A β oligomers that is the major mechanism of fibrillogenesis inhibition.^[43]

Finally, the question as to why TTR binding is neuroprotective needs to be answered. Given the consensus that A β oligomers are more toxic than fibrils, the fact that TTR arrests A β aggregation and inhibits fibril growth might potentially be harmful rather than protective! However, it is plausible that, by binding A β oligomers, TTR competes with binding of A β to cell surfaces. Alternatively, sequestration of A β oligomers may render them more sensitive to degradation by proteolysis. To our knowledge, there is not yet data to provide a definitive explanation of the mechanism by which TTR prevents A β toxicity.

2.4 Apolipoprotein E and its Interaction with A β

ApoE is a 34 kDa glycoprotein that is synthesized in the liver and in astrocytes, microglia, vascular smooth muscle cells, and choroid plexus. ApoE is secreted into both the blood (40–70 mg L⁻¹) and CSF (1.9–7.5 mg L⁻¹).^[49] In the central nervous system, ApoE facilitates the transport and delivery of cholesterol and other lipids to neurons via ApoE receptors, which are members of the low-density lipoprotein receptor (LDLR) family. The N-terminus of ApoE forms a four-helix bundle, with residues 134–150 and Arg172 comprising the receptor-binding region.^[49, 50] The lipid-binding region is localized to the C-terminal residues 244–272.^[49, 50] There are three common ApoE alleles that differ by a single amino acid. In ApoE2, Arg158 is substituted with Cys158.^[51] This substitution disrupts the salt bridge between Asp154 and Arg158; instead, a salt bridge is formed between Asp154 and Arg150, greatly decreasing the capability of the LDLR-binding site on ApoE2.^[49, 50] ApoE4 contains a Cys112 to Arg112 substitution that alters the orientation of Arg61 to a more exposed position, thereby allowing the N terminus (Arg61) to interact with the C terminus (Glu255).^[50, 51] The ApoE isoforms also differ in the stability of their N-terminal domains, with ApoE2 being the most resistant to thermal and chemical denaturation and ApoE4 being the least resistant.^[52]

ApoE is a particularly intriguing CSF protein, because whether it inhibits or aids in AD pathology is dependent upon which ApoE allele is present. ApoE3 is the most common allele, but people carrying the ApoE2 allele have a decreased risk of AD.^[50, 51] In contrast, ApoE4 is considered the strongest genetic risk factor for the sporadic form of the disease, as well as a risk factor for early-onset AD.^[51a] ApoE is codeposited with A β in senile plaques in AD brain tissue.^[51a] Although there have been some conflicting reports, ApoE levels tend to be lower in patients with AD, and decreased CSF ApoE levels are correlated with increased A β deposition in the brain.^[49] CSF ApoE levels are also decreased in carriers of the ApoE4 allele^[49] possibly explaining the increased risk of AD associated with this allele. Overall, studies indicate ApoE affects AD pathology, but these effects differ drastically depending on allele type.

Several animal and cell studies have compared the biological responses to A β as a function of the specific ApoE allele. Compared with ApoE3, ApoE4 increases A β production and deposition, and decreases A β clearance and degradation in mice.^[50, 51, 53] Additionally, AD mice expressing ApoE4 have greater A β deposition and more compact plaques than mice expressing ApoE2 or ApoE3.^[49, 54] ApoE4 is also reported to cause membrane disruption

and apoptosis in A β -treated neuronal cells, whereas ApoE3 has a protective effect.^[51a, 55] Neurons cocultured with ApoE4-expressing glia cells experience increased toxicity when treated with oligomers than neurons cocultured with either ApoE2 or ApoE3-expressing glia cells.^[56] In contrast, compared with ApoE3, ApoE2 decreases membrane disruption and A β deposition/burden.^[50, 51, 54a, 55, 57] The source of the ApoE is an important factor, with the presence of ApoE in Tg2576 mice increasing A β deposition,^[49] while replacing the mouse ApoE gene with the human gene decreases A β deposition.^[49, 54c]

ApoE's ability to attenuate A β toxicity appears to vary with the A β aggregation state. ApoE2 and ApoE3 both reduce the toxicity of nonfibrillar A β in cortical neurons, but have no effect on fibril toxicity. In contrast, ApoE4 does not reduce toxicity for either nonfibrillar or fibrillar A β species.^[58] One study challenged the finding that ApoE attenuates A β toxicity, reporting no significant change in toxicity for PC12 cells cotreated with A β and ApoE, compared with cells treated with A β alone.^[59]

The accumulated evidence, that ApoE can either inhibit or exacerbate A β pathology depending on ApoE allele type, and that ApoE reduction of A β toxicity depends on the type of A β aggregate, has spurred more detailed inquiries into ApoE allele-A β interactions. In addition, the reader is directed to several recent reviews^[49, 51a, 60] focusing solely on ApoE in AD that explore many other facets of ApoE.

Regardless of allele type, ApoE is reported to bind one molecule of A β per ApoE.^[61] A K_D of 20 nM has been observed for A β binding to both ApoE3 and ApoE4.^[62] This binding can occur at either the N or C-terminus of ApoE and involves residues 12–28 of A β .^[19, 61, 63] Even though each ApoE allele binds to A β , the kinetics of binding were reported to be faster with ApoE4 than with ApoE3.^[19] Other studies report that, among the alleles, ApoE4 binds with the lowest affinity to A β ,^[63, 64] resulting in decreased A β clearance.^[51a, 53a]

Contradictory results on ApoE–A β binding from various groups have been attributed to the source of ApoE and its purity.^[49, 65] In particular, ApoE structure is strongly affected by the binding of lipids. While lipid-free ApoE folds into a compact protein with an N-terminal 4-helix bundle and a C-terminal helix, lipidated ApoE adopts an extended conformation wrapped around a lipid core.^[66] Recent experimental evidence suggests that the ApoE-lipid complex binds to both A β oligomers and fibrils.^[66] Given the marked effect of lipids on protein conformation, as well as the potential for direct interaction of lipids with A β , it is not surprising that the lipid status strongly affects the ApoE–A β interaction.

Based on molecular dynamics simulation, one group proposed that ApoE4, but not ApoE2 or ApoE3, unfolds upon binding to A β .^[67] This unfolding is not observed for ApoE2 or ApoE3, correlating with their higher clearance of A β .^[51a, 53a, 67] Clearly, experimental studies are required to test this hypothesis and to more fully understand the nature and consequence of this protein's interaction with A β .

The binding of any of the three ApoE alleles to A β results in a decrease in fibril formation and an increase in smaller aggregate species, as shown via ThT and TEM.^[61, 68] Additionally, each allele is able to inhibit A β aggregation, even when aggregation has been stimulated with fibril seeds.^[69] Most studies support the idea that ApoE4 inhibits fibril

formation, with one study determining that ApoE4 inhibits fibril formation at a lower ApoE/A β ratio than ApoE3 (1:75 compared with 1:25).^[68a] Contrary to the predominant view, one group of investigators reported, based on ThT analysis, that ApoE4 increases, rather than inhibits, aggregation of both A β ₁₋₄₀ and A β ₁₋₄₂.^[59] Others report that ApoE4 increases the amount of A β oligomeric species, compared with ApoE2 and ApoE3.^[68a, 70] Overall, the findings support the theory that ApoE4 forms a stable complex with A β faster than the other alleles, thereby trapping A β in the oligomeric stage, resulting in fewer fibrils, but increased toxicity.

While it is clear that ApoE binds to A β , several questions remain. If all three alleles inhibit fibril formation and increase smaller aggregates, why does only ApoE4 increase toxicity? Is this due to ApoE4 inhibiting fibril formation at the cost of increasing oligomer concentration? Or is this a result of ApoE4's decreased clearance of A β ? And why does ApoE4 have decreased A β clearance? Questions about mechanism aside, ApoE2 and ApoE3 do exhibit beneficial effects and could serve in the design of new AD therapeutics.

2.5 Other CSF proteins

While we have focused our discussion on two CSF proteins, TTR and ApoE, other CSF proteins are worth briefly mentioning, because of evidence of their connection with AD.

S100 calcium-binding protein β (S-100 β) levels are elevated in AD brain tissue, predominantly localized to astrocytes.^[71] Since S-100 β levels are also elevated in Down's syndrome,^[71a] a condition which greatly increases the risk of AD, and because S-100 β increases intracellular calcium,^[72] the increase in S-100 β was first postulated as contributing to AD pathology. However, S-100 β also protects hippocampal neurons from damage^[73] and is credited with elevating neurotrophic activity in AD brain tissue.^[71b] These findings suggest that S-100 β may be a protective agent against AD pathology rather than a contributing factor.

Soluble Intercellular Adhesion Molecule 1 (sICAM-1) is another protein that is elevated in the serum of patients with AD.^[74] sICAM-1 levels are positively correlated with severity of AD^[74] and the protein has been shown to accumulate in senile plaques.^[75] One study determined that the presence of sICAM-1 increased the level of neprilysin, an A β -degrading enzyme, in both microglia cells and mice.^[76] Additionally, knockout of sICAM-1 reduced both neprilysin expression and A β degradation in microglia cells.^[76] These discoveries suggest that sICAM-1 may be synthesized as an attempt to protect against AD. Whether sICAM-1 functions by directly interacting with A β is unknown.

In AD Tg2576 mice, neuron-specific enolase (NSE) is overexpressed predominantly in microglial cells near senile plaques.^[77] Furthermore, treatment with A β increases the amount of active NSE in microglial cell culture.^[77] This upregulation appears to be neuroprotective, because pretreatment with an NSE peptide greatly decreasing A β toxicity in PC12 cells.^[77] However, the mechanism by which NSE reduces A β toxicity is not yet fully understood. In fact, the level of NSE in patients with AD is itself a point of contention with studies reporting a reduction,^[78] increase,^[79] or no change^[80] in NSE expression.

The β trace protein, also known as lipocalin-type prostaglandin D synthase (PGDS), has been investigated for its ability to bind to A β and inhibit A β aggregation. Kanekiyo *et al.* discovered that purified human β trace binds to immobilized A β monomer and fibrils with K_D values ranging between 40–50 nM.^[81] Their data shows that the binding interaction involves the central section and/or C-terminus of A β . Evidence also suggests this binding occurs *in vivo*, with β trace localized in amyloid plaques in both Tg2576 mice and human AD frontal cortex.^[81, 82] ThT and AFM studies revealed that binding of β trace greatly reduces A β fibril formation at a ratio of 1:10 with A β and that fibril formation inhibition by CSF is significantly reduced upon the removal of β trace from CSF.^[81] Human L-PGDS-transgenic mice brains were observed to possess 77% fewer A β deposits than wt mice after injection with biotin-labeled human A β ,^[81] demonstrating β trace prevents A β deposits *in vivo*. These results indicate that the β trace protein may act as a natural, preventive agent against A β aggregation and deposition. However, studies on this protein are limited, and little is known about the specific mechanism of interaction.

α 1-antitrypsin (A1AT), also called α 1-proteinase inhibitor, originally showed promise as a potential inhibitor of A β aggregation. As a serpin, A1AT possesses a structure ideal for binding to β -sheet proteins such as A β aggregates,^[83] and A1AT is present in senile plaques in AD brain tissue.^[84] However, at a concentration similar to that found in CSF, A1AT was unable to inhibit fibril formation.^[85] It should be noted though, that this experiment used an A β fragment, rather than full-length A β , so A1AT could bind to residues not included in this A β fragment. Despite the failure to inhibit fibril formation, A1AT protected human red blood cells from A β fibril-induced lysis.^[85] Additionally, in primary microglial cells, A1AT increased cell viability and reduced the release of both TNF- α and IL-6 stimulated by A β oligomer treatment.^[86] This protective effect was not attributable to inhibition of aggregation, as western blot analysis showed no change in A β oligomer formation upon incubation with A1AT. These results suggest that A1AT attenuates A β toxicity indirectly, possibly through its anti-inflammatory function. However, inhibition of oligomer formation was assessed at a 1:13 ratio A1AT/A β , whereas the neuroprotective effects by A1AT were obtained at a 9:1 ratio.^[86] This large discrepancy precludes any firm conclusions connecting inhibition of aggregation to inhibition of toxicity. Still, the evidence indicates that A1AT is a much weaker inhibitor of toxicity than other CSF proteins.

Haptoglobin (Hp) and α 2-macroglobulin (A2M) show opposing trends in patients with AD: Hp concentration is decreased, while A2M is increased in AD, compared with healthy controls.^[87] Several studies have investigated both Hp and A2M as potential inhibitors of A β aggregation and toxicity due to their natural function as chaperones. Both proteins bind more strongly to prefibrillar A β than to A β monomer or fibrils, resulting in the formation of stable complexes.^[88] Studies on the binding affinity of A2M to A β revealed K_D values of 350 nM and 0.38 nM for biotinylated^[88b] and iodinated^[89] A β , respectively. Binding is believed to occur at the C-terminus of A2M.^[88b] Binding of either A2M or Hp to A β results in a significant decrease in fibril formation, as determined via ThT analysis, CD, and TEM imaging, and a parallel increase in soluble A β species.^[87, 88, 90] Hp and A2M reduce A β toxicity in SH-SY5Y cells.^[45, 91] Interestingly, Hp and A2M are heavily oxidized in AD brain tissue versus non-AD brain tissue, resulting in a loss of fibril inhibition in the case of

Hp.^[87] This may indicate that both proteins inhibit the progression of AD pathology, but the oxidation of the proteins as AD progresses decreases their effectiveness.

One interesting facet of A2M is the ability to form complexes with various proteinases that can degrade proteins, even when caged within A2M. When A2M forms a complex with trypsin, α -chymotrypsin, or bromelain, it is able to degrade radiolabeled A β , suggesting another mechanism by which A2M may reduce A β toxicity.^[92] Additionally, once A2M has trapped a protease and undergone a conformational change (becoming activated A2M, A2M*) it decreases soluble A β levels in both Tg2576 mice and various cell lines through clearance of A β via a LRP-mediated pathway.^[88c, 93] This pathway is believed to be the primary route by which A2M* clears A β , with A2M* reducing A β -induced toxicity in LAN5 cells (a normally LRP-negative cell line) only if the cells are transfected to express high levels of LRP.^[94] Combined, these studies show that both Hp and A2M have promise as protective agents against AD pathology.

3. Outlook

Because of its central role in AD, the small, but potent, A β peptide has garnered a remarkable degree of attention. This natively disordered peptide self-associates into a heterogeneous mix of multimers of diverse size, conformation, and morphology. Soluble A β oligomers have attracted particular attention, and there is now a general (but not universal) consensus that the oligomers are toxic to neurons. The oligomers, or a subset, appear to be surface-active. Interactions with diverse receptors, proteins, and lipids have been reported.

Given that A β deposits are localized primarily to the brain, and the relatively heterogeneous nature of A β oligomers, it is not surprising that proteins in the CSF are suspected of binding to A β . From the synopsis given in this review, there is evidence that at least 10 of the proteins listed in Table 1 do, in fact, bind to A β . This raises the possibility that such interactions are not specific in the classic sense; rather, these proteins may simply be available as partners. (It is interesting that albumin, IgG, and transferrin, 3 abundant components of CSF, have generally not been identified as A β binders.) Binding does not necessarily translate into alteration of aggregation. Still, it was the fibril-inhibitory activity of CSF, and not binding *per se*, that first brought attention to at least two of the proteins, TTR and ApoE, which were discussed in some depth in this review.

There is solid evidence that TTR binds A β oligomers preferentially, that it traps A β oligomers and inhibits fibrillogenesis, that it inhibits A β toxicity at ratios well below stoichiometric, and that binding is directly linked to TTR-mediated inhibition of toxicity. There are now several transgenic animal studies that provide further support to the hypothesis that TTR is neuroprotective in vitro against A β deposition. Emerging data points to strand G and the thyroxine-binding pocket as the major site of interaction between TTR and A β . Notably, TTR's strand G is, by itself, highly amyloidogenic, and one wonders if that property contributes to the TTR–A β association. Some progress has been made in teasing out the specific mechanism and pathways by which TTR binds A β and inhibits its aggregation and toxicity, but there is much work still to be done. The experimental data are mixed as to whether TTR in human CSF is increased or decreased in patients with AD. It

may eventually emerge that early in the disease state, TTR synthesis is ramped up in an attempt to deal with the increased A β load, but that the generalized cellular death occurring as the disease progresses leads to an overall loss of protein synthesis in the choroid plexus and a drop in TTR levels.

The biological significance of ApoE–A β interaction arises from the strong (but not perfect) correlation between ApoE allele type and the probability of developing AD. The biophysical situation with ApoE is much more complex than with TTR, because ApoE is a less stable protein, and lipid binding greatly alters the protein's conformation, and likely, its association with A β . Most experimental data points to the conclusion that ApoE inhibits A β aggregation, although the specific alleles may be subtly distinctive in the strength or kinetics of inhibition. These results are puzzling; the biophysical data do not provide an obvious explanation for why the ApoE alleles should differ so much in their correlation with AD. Perhaps the explanation lies in other properties of ApoE (for example, their lipid transport properties) rather than in the direct interaction with A β .

Several other CSF proteins have been identified that may interact with A β in a biologically relevant way. The β trace protein is particularly worthy of further investigation, in part because of its relatively high abundance in CSF. Much of the early biochemical and biophysical work with A β considered the peptide's properties in isolation. It is becoming apparent that A β must be considered in the context of its milieu, in CSF and brain tissue, as one player in a complex network of interacting species.

Acknowledgments

The authors gratefully acknowledge financial support from the National Institutes of Health grant R01AG033493.

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Figure 1.

(a) Structure of transthyretin (TTR) tetramer, showing putative sites of binding of A β . Two sites are highlighted: high-affinity strand G (red), with critical residue Leu110 drawn in detail, and low-affinity EF helix (blue), with critical residue Leu82 drawn in detail. (b) TTR tetramer showing putative binding sites along with TTR's natural ligands, thyroxine (in the hydrophobic cavity in the center) and two molecules of retinol-binding protein (green). (c) A monomer of TTR, with strand G and adjacent strand H highlighted in red.

Table 1

Concentration and function of common CSF proteins.

Protein	Alternative Name(s)	Concentration (mg L ⁻¹)	Function	Refs.
Albumin		120–300	Regulation of the colloidal osmotic pressure of blood	[11]
IgG		12–40	Anti-infection, cell-mediated cytotoxicity, intracellular pro-teolysis	[11b–d]
β trace protein	PGDS	16.6–26.0	Conversion of prostaglandin H2 to prostaglandin D2	[11b, c]
Transthyretin	Prealbumin	12–27	Transport of thyroxine and retinol-binding protein	[11]
Transferrin		7–22	Regulation of free iron	[11a, b, d]
α1-glycoprotein		14	Copper transport	[12]
α1-antitrypsin	α1proteinase inhibitor	6–15	Protease inhibition, anti-inflammatory	[11b, d, e]
Cystatin C	γ trace	3.1–7.3	Cysteine proteinase inhibitor	[11a–c, e]
ApoE		1.9–7.5	Lipid metabolism	[11b, e, 13]
Apo A-1		1.5–6.0	Promotion of fat transport to the liver for excretion	[11b, d]
α1-acid -glycoprotein	Orosomucoid	1.5–4.5	Lipophilic compound carrier	[11b, d, e]
ApoH	β2-glycoprotein	1–3	Metabolism of lipoproteins and coagulation	[11b, 12]
α2-macroglobulin		1.3–3.0	Broad spectrum protease inhibitor	[11b, e, 14]
α1-lipoprotein	α1-high-density lipoprotein	1.45–2	Transports lipids and hormones	[12, 15]
β2-microglobulin		0.6–2.2	Component of the MHC class I molecule, involved in immune system function	[11a, b, 16]
Haptoglobin		0.5–2.1	Hemoglobin-binding protein	[11b, d]
ApoB		0.5–2	Primary organizing protein of LDL	[11d]
IgA		0.2–2.1	Antibody involved in immune function in mucous membrane	[11b–d]
IgM		0.15–1.2	Antibody responsible for B-cell activation	[11b–d]
β-lipoprotein		0.59	Cholesterol transport in the blood	[11b]
IGF-II		0.05	Growth promoting hormone	[11a]
NSE	γ-enolase, enolase 2	0.010–0.017	Neurotrophic and neuroprotective	[11b, 17]
IGF-I		0.002	Growth promoting hormone	[11a]
S-100B		0.0015	Regulation of proliferation, differentiation, and migration	[11b, c]
s-ICAM	CD54	0.0015	Stabilization of cell-cell interactions, aid in leukocyte endothelial transmigration	[11c]
Tau protein		0.0002	Stabilization of microtubules	[11c]