Apolipoprotein E: from lipid transport to neurobiology

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Abstract

Apolipoprotein (apo) E has a storied history as a lipid transport protein. The integral association between cholesterol homeostasis and lipoprotein clearance from circulation are intimately related to apoE's function as a ligand for cell surface receptors of the low density lipoprotein receptor family. The receptor binding properties of apoE are strongly influenced by isoform specific amino acid differences as well as the lipidation state of the protein. As understanding of apoE as a structural component of circulating plasma lipoproteins has evolved, exciting developments in neurobiology have revitalized interest in apoE. The strong and enduring correlation between the apoE4 isoform and age of onset and increased risk of Alzheimer's disease has catapulted apoE to the forefront of neurobiology. Using genetic tools generated for study of apoE lipoprotein metabolism, transgenic "knock-in" and gene-disrupted mice are now favored models for study of its role in a variety of neurodegenerative diseases. Key structural knowledge of apoE and isoform specific differences is driving research activity designed to elucidate how a single amino acid change can manifest such profoundly significant pathological consequences. This review describes apoE through a lens of structure-based knowledge that leads to hypotheses that attempt to explain the functions of apoE and isoform specific effects relating to disease mechanism.

Keywords

1. Introduction

Apolipoprotein E (apoE) is a potent modulator of plasma lipoprotein and cholesterol levels whose mode of action is mediated by interaction with members of the low-density lipoprotein (LDL) receptor family. Transgenic mice over-expressing apoE have decreased plasma cholesterol and are resistant to diet-induced atherosclerosis [1]. On the other hand, apoE-null mice manifest elevated plasma cholesterol and increased susceptibility to diet-induced atherosclerosis [2,3]. In humans, the absence of apoE, or the presence of defective

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apoE, leads to type III hyperlipoproteinemia, characterized by premature atherosclerosis and accumulation of plasma cholesterol [4]. ApoE is synthesized with an 18 amino acid N-terminal signal peptide that undergoes intracellular processing before secretion of a mature 35 kDa glycoprotein containing 299 amino acids [5-7]. The protein is encoded by a 3.6 kbp, four-exon gene located on chromosome 19 [8,9]. Plasma apoE originates predominantly from liver and, to a small but functionally significant extent, macrophages [10-12]. At the same time, apoE is expressed in a wide variety of other tissues including brain, spleen, lung, adrenal gland, ovary and kidney [13]. This broad tissue expression suggests apoE participates in additional biological processes, both related to and disparate from, lipid metabolism and cardiovascular disease. Indeed, support for this concept has emerged from studies linking apoE to innate immunity, normal brain function and a host of neurodegenerative disorders [14].

2. ApoE Structural Organization

In general, members of the class of exchangeable apolipoproteins are related by their high amphipathic α-helix content, which is critical to their function in lipoprotein particle stabilization. The opposing hydrophobic and hydrophilic faces of these helices allow exchangeable apolipoproteins to exist in alternate lipid-free and lipid-associated states and explains their activity as "detergents" capable of solubilizing lipophilic molecules [15]. Primary sequence [16,17] and spectroscopic analyses [18], [19] reveal that apoE possesses a high content of α-helix secondary structure (~62% in aqueous solution). Structure-function studies investigating its role in disease and cholesterol homeostasis revealed apoE is a two-domain protein [20]. Studies monitoring secondary structure content as a function of chaotrope concentration yielded a biphasic curve with transition midpoints at 0.7 and 2.5 M guanidine HCl. Limited proteolysis of full-length apoE generated 22 kDa and 10 kDa fragments that correspond to distinct N- and C-terminal domains, respectively [20]. This finding is consistent with the concept that the domains are separated by a flexible, protease sensitive loop. The C-terminal domain facilitates lipid binding and, in isolation, has lower stability and greater conformational flexibility than the N-terminal fragment. Analytical ultracentrifugation studies and C-terminal truncation analysis [21,22] revealed that, as with other exchangeable apolipoproteins, full-length apoE forms multimeric complexes in aqueous solution [23]. Full-length apoE displays a propensity to form tetramers and this has been attributed to the C-terminus since the isolated N-terminal domain remains monomeric at concentrations up to 15 mg/mL [18]. Consistent with these observations, substitution of a limited number of bulky residues in the C-terminal domain by smaller polar residues results in a protein that is resistant to cross-linking, suggesting these residues play a role in apoE self association [24,25].

Characterization of apoE from human subjects has revealed intriguing heterogeneity. Seminal isoelectric focusing experiments [26,27] indicated the presence of charge variants while experiments with neuraminidase provided evidence that apoE is glycosylated [28]. Glycosyl moieties present include galactose, glucosamine, galactosamine, sialic acid, N-acetylglucosamine, and N-acetylgalactosamine [29]. Amino acid analysis identified Thr194 as the sole glycosylated residue and glycosylation was shown not to be necessary for apoE expression [30]. While some studies have linked alterations in apoE glycosylation state to disease phenotypes, the exact role of specific sugar moieties remains unknown. Hypotheses have also been advanced that propose glycosylation protects the loop region in which it resides from proteolytic cleavage [13].

Understanding of the relationship between apoE structure and function was greatly enhanced by determination of the three-dimensional structure of the N-terminal domain by X-ray crystallography [31] and, more recently, by nuclear magnetic resonance (NMR)

spectroscopy [32] (Figure 1). These structures reveal four amphipathic helices that align in an up-and-down manner, forming an elongated globular helix bundle. The boundaries of the four major helices: helix 1 (residues 24-42), helix 2 (residues 54-81), helix 3 (residues 87-122) and helix 4 (residues 130-164) are augmented by helix 1′ (residues 44-53) and, in the NMR structure, helices N and C (residues 12-22 and 173-181, respectively). Although a segment of the C-terminal domain (residues 223-272) has been crystallized [33], a high-resolution structure has yet to be reported. Computer-based sequence algorithms predict residues 210-266 and 268-289 form α-helices [15,34], with the latter believed to be involved in protein-protein interactions and/or self-association. Consistent with this prediction, recent biophysical studies of the C-terminal domain suggest that these helices are involved in close helix-helix contact, likely driving self-association of lipid-free apoE [35,36].

2.1 Isoforms

The first experiments to suggest apoE has amino acid sequence heterogeneity came from studies designed to isolate and characterize proteins found on very low-density lipoprotein (VLDL) particles by ion-exchange chromatography [37] and 2-D gel electrophoresis [38]. Speculation that amino acid sequence differences among apoE variants correlate with hyperlipoproteinemia was confirmed upon identification of the three major alleles [39,40], ε2, ε3, and ε4. The three isoforms, apoE2, apoE3 and apoE4, differ only at positions 112 and/or 158 (Figure 2). ApoE3 contains a cysteine at position 112 and an arginine at 158 while apoE2 contains cysteine at positions 112 and 158 and apoE4 contains arginine at these sites.

Further investigation has linked apoE2 with Type III hyperlipoproteinemia and premature atherosclerosis. Interestingly, the LDL receptor binding activity of apoE2 is only ~1% that of the parent isoform, apoE3 [41]. Despite being a rare genetic disorder, the phenotypic incidence of Type III hyperlipoproteinemia among apoE2 homozygotes (less than 5%) is even lower than predicted by genetics alone suggesting additional environmental factors are required to trigger the phenotype [42]. Studies designed to reveal the mechanism behind the reduced LDL receptor binding activity of apoE2 focused on Cys158. Following treatment of apoE2 N-terminal domain with cysteamine to convert the cysteines at positions 112 and 158 to positively charged, lysine analogs, LDL receptor binding activity increased to normal levels [43]. A mechanistic explanation for differences between apoE2 and E3 with respect to LDL receptor binding came from X-ray crystallographic comparison of the isoforms [44]. These structures revealed a salt bridge between Arg158 and Asp154 in apoE3 that is absent in apoE2. Moreover, in apoE2, an alternative salt bridge forms between Arg150 and Asp154, effectively eliminating the availability of Arg150 for interaction with the LDL receptor. In keeping with this, an alanine to arginine substitution at position 150 in apoE3 decreased receptor binding to 24% of normal [45]. Although much higher than seen with apoE2, these data suggest salt bridge interactions strongly influence receptor binding, presumably by changing the orientation or patterning of basic residues in the receptor recognition sequence (residues 136-152; see Figure 1). These authors further hypothesized that dietary factors (e.g. a high-fat diet) influencing lipoprotein size and lipid composition can, in turn, affect apoE2 Arg150 side chain conformation and, consequently, the presence or absence of the Arg150-Asp154 salt bridge [44]. Modulation of the Arg150-Asp154 salt bridge under various physiological conditions would be expected to affect LDL receptor binding activity, thus providing a possible explanation for the influence of secondary environmental factors on the occurrence of Type III hyperlipoproteinemia in apoE2 homozygotes.

While apoE3 is considered the "wild-type" isoform in humans because of its high allelic frequency and lack of strong association with a human disease phenotype, apoE4 appears to be the ancestral form since the Arg at sequence positions 112 and 158 are strongly

conserved across almost all animal species possessing apoE [42]. In ε4 homozygous human carriers, the plasma concentration of apoE is lower than that for individuals possessing two ε3 alleles. Furthermore, such individuals manifest elevated plasma cholesterol and LDL as well as increased cardiovascular disease risk [46,47]. Additionally, and more strikingly, inheritance of apoE4 is correlated with cerebral amyloid angiopathy, tauopathies, dementia with Lewy bodies, Parkinson's disease, multiple sclerosis and a higher incidence, and significantly earlier onset, of Alzheimer's disease (AD) [48]. While the molecular basis for the correlation between apoE4 and disease is not established, a unique feature of apoE4, termed "domain interaction", may play a role. Studies have shown that arginine at position 112 causes amino acid side chain reorientation within the protein that promotes N- and C-terminal interaction via a unique salt bridge [14,49,50]. Domain interaction in apoE4 induces a more compact structure while the Arg at position 112 increases its molten globule like properties of this isoform [21,51-53]. Reduced apoE4 levels in plasma, compared to apoE3, results from enhanced clearance of apoE4-containing particles [54]. Additionally, it has been noted that apoE4 displays a preference for larger lipoprotein particles, such as VLDL and chylomicron remnants, and this preference has been noted as the causative factor for the association of apoE4 with increased plasma LDL [54,55]. Introducing human apoE4 'domain interaction' into mouse apoE (by substituting Thr for Arg at position 61) results in a phenotype resembling human apoE4 subjects, including reduced abundance and binding preference for VLDL [56].

2.2 ApoE lipid particles

For the most part, biologically active apoE is associated with lipid. Indeed, lipid association is a prerequisite for apoE binding to the LDL receptor. Reconstituted HDL particles formed by combining apoE with purified phospholipids of varying fatty acid chain length and polar head group composition represent an extensively utilized model for facile reproduction of the bioactive conformation of the protein. This model system allows for the controlled manipulation and study of apoE conformational changes required for LDL receptor binding, making these particles a reliable model of apoE function *in vivo*. Phospholipids, including dimyristoylphosphatidylcholine (DMPC), palmitoyloleoylphosphatidylcholine or dipalmitoylphosphatidylcholine (DPPC) have been used to create nanometer scale particles wherein the apolipoprotein circumscribes the periphery of a disk-shaped phospholipid bilayer [57-59]. The exact conformation adopted by apoE in these particles remains controversial, however. Whereas some data indicate apoE aligns in such a way that the hydrophobic face of their constituent amphipathic a-helices interact with acyl chains at the edge of the phospholipid bilayer [19,60-62] other models have been proposed. Indeed, X-ray [63], electron paramagnetic resonance [64] and recently, electron microscopic [65] data are consistent with an ellipsoidal shape for apoE-phospholipid complexes. In this model apoE a-helices align in such a way that their hydrophobic faces interact with one another while the polar faces contact phospholipid.

Regardless of the ultimate conformation adopted by apoE in lipid complexes, it is generally agreed that the protein undergoes a lipid binding-induced conformational change. On the basis of biophysical and spectroscopic studies, two models, the "open conformation" [66] and the "extended belt", suggest possible ways in which the N-terminal helix bundle may alter its structure upon lipid association (Figure 3). In both of these models, the conformational change in apoE appears to be initiated by helix bundle opening via a "hinge" region between helices 2 and 3, permitting exposure of hydrophobic residues normally sequestered in the bundle interior. Fluorescence resonance energy transfer (FRET) studies revealed that interaction with DMPC results in increased separation between helices 1 and 3, consistent with the "hinge" hypothesis [67]. This conformational change also allows surface-exposed hydrophilic residues to retain contact with the aqueous environment and

effectively substitutes helix-lipid contacts for the helix-helix interactions that stabilize the helix bundle state. The "extended belt" and "open conformation" models differ in the ultimate conformation adopted in a given reconstituted HDL particle. In the "extended belt" conformation, the hinge between helices 2 and 3 initiates further unfurling to create a fully extended helical protein that wraps around the perimeter of the disk wherein a second molecule aligns in the opposite direction to form a double belt. In contrast, the "open conformation" model preferentially retains contact between the helix 1 and 2 and helix 3 and 4 pairs wherein half-opened molecules wrap around the disk perimeter end to end [66]. Support for these models have come from a combination of FRET-based measurements [68], Fourier-transformed infrared spectroscopy [60], and tryptophan fluorescence depth quenching studies using the parallax method [62]. A similar extended conformation was reported for the C-terminal domain of apoE bound to discoidal DMPC particles [69]. An alternative, hybrid model that combines features of the "open" and "extended belt" conformations was described from FRET analysis of the NT domain of apoE3. In these experiments, intermolecular FRET was observed between helix 3 of one molecule and helix 4 of a second apoE N-terminus in which two partially extended apoE molecules interlock to encircle the disk [67]. It should be noted that an alternative "picket-fence" model, wherein anti-parallel ~17-residue helices surround the bilayer disk and orient parallel to the lipid acyl chains, has also been advanced [19]. This model, however, is difficult to reconcile with the known helical boundaries of the lipid-free crystal structure and multiple studies that support the aforementioned conformation in which the long axis of apoE a-helices align perpendicular to the phospholipid fatty acyl chains.

A comprehensive study comparing particle size, apoE protein copy number and conformational parameters as they relate to changes in lipid composition and apoE isoform, has been reported [70]. These authors found that, on average, disks contained three apoE N-termini or four full-length proteins with between 200 - 250 lipid molecules per particle. While no significant differences in particle architecture were noted between apoE isoforms, differences in lipid order parameter and protein to lipid ratio were seen when comparing apoE to apoA-I [70,71]. These observations led to the conclusion that apoE can adopt one of two predominant conformations on discoidal reconstituted HDL particles, either the canonical "belt" conformation around the disk perimeter or an alternative conformation wherein apoE helices embed horizontally within the interfacial region of the bilayer and perturb phospholipid head group organization [70].

A model of lipid-bound apoE that is distinct from previous models has emerged from studies of full-length apoE4. Fluorescence analysis using spatially sensitive probes revealed that, in DMPC reconstituted HDL, apoE4 adopts an extended conformation that loops back on itself around the periphery of the discoidal particle [72]. A similar "helical hairpin" conformation was suggested for apoE4 bound to DPPC using X-ray crystallography [63,64]. These lipidated particles, resolved to 10 Å, display an ellipsoidal shape and contain two interlocking apoE4 molecules. In the absence of a clear delineation of secondary structure at 10 Å resolution, a predominantly helical full-length apoE4 molecule was modeled such that each extended molecule doubled back on itself and folded into a curved, horseshoe-like conformation with a 310º axis of rotation. In this model, two horseshoe shaped proteins pack into an incomplete "toroid" containing DPPC intercalated within the opposing and slightly rotated apoE molecules to form an ellipsoidal space-filling model. Support for this model was obtaining by strategic placement of electron paramagnetic resonance sensitive probes in apoE4 followed by determination of the effect of probe sequence position on side chain dynamics [73].

Structural and biophysical data on full-length apoE have led to the concept that the C-terminal domain mediates initial contact with spherical lipoprotein surfaces, effectively

anchoring the N-terminal helix bundle at the particle surface [74-76]. Once localized at the lipoprotein surface, the N-terminus may either retain a receptor-inactive four-helix bundle or alter its conformation by binding to the lipid surface to adopt a receptor competent state. Thus, it may be considered that the balance between alternate conformational states of lipid associated full-length apoE will determine the extent to which apoE can mediate lipoprotein particle clearance from the plasma via interaction with LDL receptor family members. By extension, it is conceivable that isoform-specific differences could affect this balance, providing a molecular rationale for pathological consequences associated with different apoE genotypes. For example, an increased proportion of apoE4 molecules in a receptor inactive state could result in increased VLDL remnant conversion to pro-atherogenic LDL.

2.3. ApoE receptor interactions

A fundamental role of apoE is its function as a ligand for cell-surface receptors. ApoE3 is known to be a high affinity ligand for the LDL receptor, the LDL receptor related protein 1 (LRP1), apoE receptor 2 (apoER2) and the VLDL receptor. The LDL receptor is the prototype of a family of integral membrane proteins that act via ligand-activated, clathrin coated pit-mediated endocytosis to internalize plasma lipoproteins. This 839 amino acid protein is composed of five distinct regions i) an amino terminal ligand binding domain containing seven cysteine-rich LDL-A repeats, each roughly 40 amino acids in length; ii) an epidermal growth factor (EGF) precursor homology domain containing three EGF-like, cysteine-rich repeats and a β-propeller domain that mediates ligand release in the endosome via a pH-dependent conformational change preceding receptor recycling [77], iii) an O-linked sugar domain, iv) a single-pass transmembrane domain, v) and a cytoplasmic tail domain that contains an NPxY motif that facilitates receptor clustering in clathrin coated pits [78].

Studies to define *in vivo* ligands of the LDL receptor revealed that transgenic mice overexpressing this receptor manifest a >90% reduction in plasma apoE and apoB-100 levels, while apoA-I levels were unchanged [79]. Additional evidence emerged from studies of LDL receptor null mice wherein plasma LDL and cholesterol levels were dramatically elevated due to impaired receptor-mediated clearance of apoE and apoB containing lipoproteins [80]. By binding to the LDL receptor, apoE and apoB containing lipoproteins are cleared from plasma, thereby regulating plasma cholesterol levels [81,82]. At neutral pH, receptor-ligand complexes are internalized into vesicles that become endosomal compartments. Subsequent pH lowering releases the lipoprotein ligand, facilitating receptor recycling and lysosomal degradation of LDL [83,84].

As Brown and Goldstein were elucidating the LDL receptor endocytic pathway, Mahley and coworkers identified a limited, but highly conserved, sequence similarity between apoB and apoE [85,86]. Shortly thereafter, Mahley *et al.* demonstrated the importance of this stretch of charged residues by showing that treatment of apoE with cyclohexanedione (an arginine-specific modifier) abolished all receptor activity [87]. From these studies, the conserved LDL receptor recognition sequence was identified. Analysis using cyanogen bromide to digest apoE at methionine residues revealed a peptide (residues 126-218) that, when complexed with DMPC, bound the LDL receptor with the same affinity as LDL [88]. This region was further delineated by abolition of receptor binding by an antibody that recognized residues 139-169 [89]. Further refinement emerged from mutational analysis. Substitution of positively charged residues at positions 142, 145, 146, and 158 for neutral amino acids markedly reduced apoE binding to the LDL receptor [13].

The region of apoE responsible for receptor recognition was further probed by generating truncation mutants and measuring receptor binding activity [90]. While apoE(1-170) and apoE(1-174) fragments retain 1% and 19% LDL receptor binding activity, respectively,

apoE(1-183) possessed 85% of binding compared to full length protein. Importantly, this was the first detailed study implicating residues outside of the putative LDL receptor recognition sequence (residues 136-152) in receptor binding. Subsequent mutagenesis analysis noted the contribution of Arg172 to receptor binding activity [91]. This study confirmed the general importance of residues 170-183 by confirming that their removal reduced binding activity to 15% of full-length apoE3 levels, but strikingly, a 98% drop in binding activity was seen with a single Arg172Ala substitution mutation. Notably, an Arg172Lys substitution showed only 6% of normal activity, suggesting that arginine is required at this position to preserve the conformation necessary for receptor binding. Given that Arg172 is well outside the classical LDL receptor recognition motif and, in lipid free apoE, resides in an unstructured region beyond the boundary of helix 4, it is conceivable this segment of the protein may explain the requirement that apoE associate with lipid to be conferred with receptor recognition capability. Heteronuclear multidimensional NMR spectroscopy of an apoE-derived peptide corresponding to residues 126 – 183 [92,93] revealed that, in the presence of trifluoroethanol or when bound to dodecylphosphocholine micelles, helix 4 extends beyond residue 165 to encompass Arg172. This hypothesis was further examined in the isolated N-terminus of apoE using site-specific electron paramagnetic resonance spectroscopy [94]. Gupta *et al.* showed that lipid association induced fixed secondary structure in a region of the molecule known to exist as random coil in the lipid-free state. Thus, extension of helix 4 beyond the boundary defining its lipid-free conformation may represent a key conformational change necessary for manifestation of the LDL receptor recognition properties of apoE.

In studies designed to address whether apoE binding to the LDL receptor is multivalent, the ratio of active to inactive apoE was varied on DMPC particles containing an average of four apoE molecules per particle [58]. LDL receptor binding activity was affected such that, when the number of active apoE per particle approached one, binding affinity approximated that of LDL. Other studies have shown that optimal receptor binding is achieved with spherical lipid microemulsion particles that contain at least four apoE molecules per particle [95]. This model implies that the presence of multiple apoE on discoidal and spherical particles enhances LDL receptor binding efficiency compared to particles containing a single apoB-100. In another approach, Fisher *et al.* employed single chain multimers of the N-terminus to show that, when bound to lipid, more than one apoE is required for high affinity binding to the LDL receptor [96]. Thus, in addition to inducing a conformational change in the structure of apoE, lipid association enhances the affinity of apoE for the LDL receptor in part by creating a multivalent ligand.

Our understanding the structural determinants of ligand binding to the LDL receptor was significantly advanced by the X-ray crystallography studies of Rudenko *et al.,* who determined the structure of an extracellular portion of the LDL receptor at pH 5.3 [97]. In addition to confirming all known structural features of the receptor, this crystal structure gave rise to a comprehensive model to explain the mechanism of ligand release in the endosomal compartment. In this structure, LDL-A repeats 4 (residues 127-163) and 5 (residues 176-210) make contact with β propeller residues 377-642 of the EGF precursor domain. Thus, at endosomal pH, the molecule folds onto itself in a manner anticipated to result in ligand discharge. The authors suggest the strength of the interfacial interaction can be modulated as a function of pH-dependent aspartate, glutamate and histidine side chain protonation within the contact region [78,97]. Using site-directed mutagenesis, Yamamoto et al. [98] provided evidence for a pH dependent "histidine switch" mechanism wherein ligand discharge occurs via conformational reorganization of the receptor [99]. However, other research suggests that the intramolecular contact does not drive release through a competitive mechanism and the key His residues (His190, His562, and His586) function as part of an allosteric mechanism that drives lipoprotein release [100]. Extending this work,

Zhao and Michaely point out that, in addition to low pH, endosomes possess low concentrations of free calcium [101]. Using fibroblasts that express either a normal LDL receptor or a variant that is incapable of acid-dependent ligand release, these authors showed that endosomal concentrations of free calcium are sufficient to drive lipoprotein release. Thus, it is plausible that calcium-dependent and acid-dependent mechanisms cooperate to facilitate lipoprotein release from the LDL receptor.

3. Neurobiology of apoE

The importance of apoE in maintenance of cellular cholesterol homeostasis is not limited to the peripheral circulation. Indeed, its role in neurological phenomena, including neuronal plasticity, neurite outgrowth and synaptogenesis is a rapidly advancing field. While the bulk of plasma apoE originates from liver and macrophages, apoE found in the central nervous system (CNS) is produced locally. Exchange between liver and brain-derived apoE does not take place owing to the blood-brain barrier (BBB). Consistent with this, no liver derived apoE was recovered in cerebrospinal fluid (CSF) of liver transplant recipients [102]. While apoE is the predominant apolipoprotein found in the CNS, other apolipoproteins, such as apoJ, apoD, apoA-I and apoA-IV are also present. In adult brain tissue, apoE is primarily synthesized by astrocytes [103,104], although microglia and neurons also synthesize the protein under select physiological and pathological conditions [105-107]. Under basal conditions, glial cells produce two to three times more cholesterol than neurons and also manifest elevated apoE expression. It has been demonstrated that apoE associates with lipoproteins in the brain, though astrocyte-secreted apoE-containing lipoprotein particles differ from peripheral apoE-containing lipoproteins in that they are discoidal in shape and comprised mostly of phospholipid and unesterified cholesterol [108,109]. It is presumed that some astrocyte-secreted apoE-containing lipoproteins acquire a cholesteryl ester core on their way to the CSF since both discoidal and spherical lipoproteins have been isolated from this site [108,110].

Accumulating evidence indicates a role for apoE in aging [111]. In this context, apoE-null mice represent a useful model for understanding its effects on natural aging. In certain studies no signs of synaptic degeneration were noted in apoE-null mice [112], with normal brain histology, an absence of neurodegenerative markers [113], normal cholinergic activity and neuronal function [114-117]. However, other studies have found that apoE-null mice develop mild to severe spatial learning and memory deficits [118,119]. Memory impairment was accompanied by cholinergic deficits, highlighting the importance of apoE in cognition and memory [120,121]. Studies demonstrating that apoE deficient mice are more susceptible to neurodegeneration than their wild-type counterparts implicate this protein in age-related neuropathology [122,123].

The predominance of apoE containing lipoproteins in brain suggests it functions in cholesterol transport and clearance. In brain, cholesterol is highly abundant and is required for synapse development [124], dendrite formation [125], long term potentiation [126] and axonal guidance [127,128]. Cholesterol delivered to neurons on apoE-containing lipoprotein particles increases synapse formation [124] by promoting biogenesis of synaptic vesicles and up-regulating the machinery necessary for their release [129,130]. Cholesterol depletion, or a lack of cholesterol delivery, causes synaptic and dendritic spine degeneration and results in failed neurotransmission and decreased synaptic plasticity [131]. Following neuronal cell damage, cell death, traumatic brain injury or terminal differentiation, large amounts of cholesterol are lost due to membrane and myelin degeneration [132]. In response to these events, apoE is up-regulated in astrocytes and macrophages where it is presumably involved in clearance and redistribution of cholesterol and lipid debris [133,134]. This suggests a role for apoE as a scavenger of lipophilic molecules during nerve regeneration

[135,136]. The fact that LDL receptors are up-regulated in regenerating peripheral nerves suggests enhanced lipoprotein uptake occurs during nerve growth and regeneration [137]. Moreover, it has been shown that cholesterol-containing apoE-lipoproteins secreted by astrocytes are required for synapse formation *in vitro* via a mechanism that is dependent upon functional apoE receptors [124,138].

Emerging from statistical correlations examining the relationships between apoE isoforms and neurodegenerative disease progression, outcome and average age of onset, studies have focused on the differential effects of apoE3 and apoE4 on synaptic plasticity and synaptogenesis. Although both isoforms have the ability to reverse presynaptic deficits and cognitive impairment seen in apoE-null mice [139], evidence suggests apoE4 is less efficient in promoting neurological repair and maintaining proper brain function. Buttini and colleagues showed that expression of apoE3, but not apoE4, protects against neuronal damage and age-related neurodegeneration seen in apoE-null mice [140]. In contrast to apoE3-expressing mice, apoE4 mice display synaptic deficits and lower excitatory synaptic transmission, even in the absence of neuropathology [141]. In addition, apoE4 expressing mice have impaired long term potentiation, decreased numbers of synapses per neuron and reduced dendritic spine formation compared to their apoE3-expressing counterparts [142]. Significant correlative associations have emerged that suggest apoE plays a critical role in response to traumatic head injury, presumably by transporting cholesterol and lipid metabolites from the site of injury, facilitating repair. Studies examining isoform-specific effects in the context of brain injury indicate that, compared to an apoE3 cohort, apoE4 carriers display decreased recovery efficiency [143-145]. Additionally, apoE4-containing mice do not recover as efficiently from traumatic brain injury [146] and are more susceptible to cerebral ischemia [147]. Unlike under normal conditions, brain injury induces significant neuronal production of apoE, augmenting the basal production by astrocytes and microglia [148-150]. This has led to inquiry regarding the relative contribution of astrocyte versus neuron-derived apoE in the repair process. While *in vitro* evidence suggests apoE4 has a detrimental effect on cultured astrocytes and neurons [151-153] recent *in vivo* excitotoxic injury experiments have shown that expression of apoE4 by neurons preferentially causes greater cellular toxicity than does apoE4 generated by astrocytes or apoE3 generated by either neurons or astrocytes [154]. This finding supports the significant supportive role of astrocytes following injury but further suggests that apoE4 causes increased cellular burden due to its neuron specific upregulation following brain trauma.

Despite these notable injury-related pathophysiological effects of apoE4, most studies demonstrate that both apoE3- and apoE4-expressing mice perform better on cognitive tests than apoE-null mice [155]. In the case of dendritic spine morphology, the phenotype seen in apoE4-expressing mice was similar to that of apoE-null mice, suggesting an apoE4 isoform functional deficit in dendritic spine maintenance [142]. This effect, however, is age-related since an apoE4-dependent reduction in dendritic spine formation was observed only in one-to two-year old mice. This observation suggests that apoE isoform-specific effects might relate to increased risk of dementia in aged humans expressing apoE4. In older patients, with and without AD, apoE4 gene dose is inversely correlated with dendritic spine density [142]. Although a considerable number of studies implicate apoE as a significant factor in nervous system maintenance, more work is needed to address mechanistic aspects of this role. Indeed, apoE-null mice do not show gross defects in normal nerve repair and development and only manifest a neurodegenerative phenotype upon aging or injury [114,156]. Other studies show that apoE-null mice display massive infiltration of injected dyes in brain parenchyma, indicative of BBB leakage [157-159]. This defect is selective to brain micro vessels and is exacerbated in older mice, suggesting an independent effect of aging (e.g. oxidative stress). A similar breach in BBB integrity is noted in post mortem brains of subjects with AD and cerebral amyloid angiopathy [160].

3.1 ApoE and Alzheimer's Disease

There is mounting evidence that apoE plays a central, if not direct, role in the pathogenesis of AD. The clinical symptoms of AD include progressive loss of cognition, dementia and the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles [161,162]. ApoE4 has been shown to be a significant risk factor for development of the disease, including early and late onset non-familial [48,163] and sporadic forms [164-166]. Among individuals that manifest late onset AD, the ε4 allele is present at a two- to three-fold higher rate compared to the general population and some studies indicate up to 65% of clinically diagnosed cases carry at least one ε4 allele [167]. Genetic and epidemiological evidence linking the allelic dose of apoE4 to premature onset and increased severity of AD continues to drive research aimed at elucidating the molecular mechanisms whereby this protein leads to neurological and neurodegenerative disease [168]. While genetic associations between apoE and AD are striking, the relationship between apoE protein expression and amyloid burden is not as straightforward. Absence of apoE in an amyloid mouse model background dramatically reduces amyloid burden without affecting amyloid-β (Aβ) peptide production [169]. This has led to the concept that apoE may contribute to conversion of Aβ to its more toxic oligomeric or fibrillar forms. However, in LDL receptor null mice, apoE levels are increased by ~50% yet this had no effect on amyloid deposition [170]. Interestingly, apoE4 has been shown to be preferentially degraded by astrocytes, which may explain why mice display genotype-dependent effects on total apoE levels in brain and CSF (with protein levels following apoE2/2 > apoE3/3 > apoE4/4) [171] and suggests a complex mechanistic link between apoE protein levels, activity and subcellular localization and Aβ production and clearance that likely involves the coordination of multiple brain cell types.

The cholesterol transport function of apoE involves interaction with a cell surface, ATP Binding Cassette Transporter A1 (ABCA1) that promotes efflux of cellular cholesterol to acceptor proteins (e.g. apoE). In neuronal and astrocyte culture models, apoE4 is blunted in its ability to promote cholesterol efflux, presumably through a mechanism that depends on ABCA1-mediated transport [172,173]. In ABCA1 deficient mice, apoE expression levels decrease by ~70-80% and this is associated with decreased cholesterol efflux, poor apoE lipidation and increased amyloid burden [174-177]. On the other hand, overexpression of ABCA1 increases apoE lipidation in the CNS and decreases amyloid plaque formation [178]. These findings point to Liver-X-Receptors (LXRs), transcription factors that modulate expression of apoE and ABCA1 [179], as key regulators of brain lipid homeostasis. Indeed, deficiency of LXR-α or β increases AD pathology [180] while treatment of AD model mice with synthetic LXR agonists reduces amyloid burden and improves cognitive function [181-183]. These findings raise the intriguing possibility that LXR activation by synthetic agonists represents a potential therapeutic strategy for treatment of AD [184].

Potential reasons for the increased neuropathology of apoE4 compared to other isoforms have been investigated [111]. Analysis of the isolated N-terminal domain of the three human apoE isoforms showed that apoE4 is least resistant to chemical and heat denaturation, while apoE2 is most resistant at neutral and low pH [51-53]. Lipid binding affinity is reportedly higher for apoE4, although maximal lipid binding capacity appears equivalent among the isoforms [21,76]. While apoE4 displays a preference for VLDL sized lipoproteins *in vitro* [54,55], how this may affect brain physiology is unclear since only HDL-sized lipoproteins have been found in brain. Nevertheless, this preference has been attributed to domain interaction in apoE4, a structural feature that has also been proposed as a causative factor for cellular effects leading to AD pathology. Although the mechanism whereby domain interaction may lead to propagation of neurological defects remains elusive [151,185,186], Huang and colleagues documented domain interaction in living neuronal cells expressing

apoE4 [187]. A novel approach to understanding the pathological significance of this phenomenon is to identify potential therapeutic small molecule inhibitors or "structure correctors" of domain interaction [188].

ApoE4 also appears to be more susceptible to proteolysis than apoE3 [189,190] leading to speculation that apoE4 digestion products contribute to amyloid plaque formation and AD pathology, especially since these fragments have been detected in plaque from AD positive brains [191]. Furthermore, proteolysis of apoE4, and subsequent fragment accumulation in the cytosol of neurons alters cytoskeletal organization and disrupts mitochondrial energy balance. Whether this is necessary and sufficient for the progression of AD pathology remains unclear [190,192]. Other evidence suggests the isolated N-terminal domain of apoE4 is neurotoxic [193-195], though it is not known if proteolytic cleavage occurs prior to or after interaction with Aβ. While lipidated apoE is protected from proteolysis to a greater extent than lipid-free apoE, the quantity and physiological functions of lipid-free apoE in brain is unknown [62,196]. Offering a new perspective, Hatters *et al.* reported that apoE4 forms aggregates (independent of Aβ aggregation) at substantially higher rates than apoE3 or apoE2 and is more neurotoxic to cultured neuronal cells [197]. These aggregates bear an irregular protofilament-like morphology with a high α-helical content, unlike the 'classic' amyloid fibrils that are rich in β-sheet structures. Whether this is the cause or a consequence of amyloid neuropathology associated with AD is not known. Finally, other studies point to a role of apoE4 in potentiating Aβ-induced lysosomal leakage [152,198] and/or activating the endoplasmic reticulum stress response, leading to increased apoptosis [199,200].

Genetically altered mice harboring a Thr61Arg mutation in murine apoE (creating a human apoE4-like mouse apoE) results in decreased apoE levels in the brain along with synaptic and cognitive defects [201,202]. Transgenic mice with a genetic predisposition for higher Aβ production in which murine apoE is substituted for each of the three human isoforms, develop the predicted isoform-specific differences in amyloid deposition with apoE4 > apoE3 > apoE2 [203,204]. Interestingly, however, human isoform substituted mice manifest a delay in onset of plaque formation compared to murine apoE mice [205,206]. Better understanding of the manner in which apoE interacts with Aβ, receptors and other binding partners in response to various conformational and lipidation states may provide further insight into its role in AD pathology.

A recent study employing mice bearing Thr61Arg apoE, to mimic human apoE4, demonstrated that domain interaction *per se* is associated with deficits usually noted in AD, supporting the hypothesis that apoE4 can act independent of Aβ to induce pathophysiology [207]. Furthermore, induction of domain interaction via the Thr61Arg mutation leads to endoplasmic reticulum stress and an up-regulated unfolded protein response, which in turn destines apoE for degradation. The authors proposed that endoplasmic reticulum stress results in dysfunctional astrocytes that provide sub-optimal support to neurons, which in turn respond with self-generated apoE leading to increased levels of neurotoxic fragments [193-195]. Dysfunctional astrocytes and toxic fragments likely represent early events in apoE4-associated pathophysiology independent of Aβ-related pathways (Figure 4).

Deposition of extracellular amyloid plaque, formed by soluble and insoluble assemblies of the Aβ peptide, is a hallmark of AD and is considered one of the primary events in disease pathology. Aβ is derived from the amyloid precursor protein (APP) by sequential β- and γ-secretase-dependent intramembranous proteolysis [161]. Aβ is produced and secreted under normal metabolic conditions and can be found at high levels in normal CSF and plasma [208,209]. Thus, disease pathology is thought to be driven by a net imbalance between Aβ clearance and production [210,211]. Gradual increases in Aβ production lead to its oligomerization in brain interstitial fluid and within neurons [212,213] and subsequent

fibrillization to produce amyloid plaques [161]. The dominantly-inherited, familial form of AD is associated with either increased production of Aβ (most commonly caused by mutations in APP itself, the presenilin 1 and 2 gene products that form two of the necessary components of the γ-secretase complex) [214] or increased production of the longer Aβ(1-42) peptide which is more toxic than the Aβ(1-40) peptide [215]. Non-familial forms of AD have been attributed to an imbalance in the relative clearance and aggregation of Aβ [216]. The only consistently associated genetic risk factor for non-familial AD is the ε4 allele of the *APOE* gene. Despite this, the pathology and phenotypic display manifested by familial and non-familial forms of AD are nearly indistinguishable.

The observation that apoE is bound to Aβ in CSF prompted study of apoE as a candidate for acceleration of AD pathology [217-219]. Although a mechanism whereby apoE (and particularly apoE4) promotes AD pathology remains elusive, *in vitro* and *in vivo* evidence suggest interaction between apoE and Aβ is associated with disease progression. *In vitro* analysis demonstrated that Aβ and delipidated apoE4 promote fibril formation more rapidly and with higher density than those seeded with other apoE isoforms (following an aggregation rate rank order of apoE4 > apoE3 > apoE2) [163,217,220]. In contrast to delipidated protein, lipidated apoE has a different isoform dependent affinity for Aβ [221]. When the affinity of lipid-bound apoE was compared, using transfected eukaryotic cell lines, apoE3-Aβ complex levels were 20-fold higher than that for apoE4-Aβ complexes [222,223]. It has further been shown that lipidated apoE3 binds Aβ two to three times more rapidly than lipidated apoE4 [222]. Studies examining the effect of apoE on neurite extension revealed that lipidated apoE3 enhances binding to Aβ and may facilitate its clearance, thereby preventing aggregation [224]. It has been shown that Aβ binds to apoE via its C-terminal domain and Aβ binding abrogates apoE lipid binding [163,225]. These results indicate Aβ interferes with apoE function as a lipid transport protein in brain, which may contribute to AD progression by altering lipid/cholesterol homeostasis [111,226-228]. In a neuronal cell line that overexpresses APP, apoE4 increased Aβ production to a greater extent than apoE3, [153]. This isoform-specific difference was abolished when cells were treated with small interfering RNA directed against LRP1 or upon incubation with receptor-associated protein, a known inhibitor of LRP1 function. This finding suggests apoE4-specific enhancement in Aβ production or deposition is dependent upon LRP1 function. Thus, it is conceivable that apoE4 possesses defective receptor binding activity. *In vitro* studies with transfected cell lines indicate apoE and its cognate receptors play a role in APP processing and Aβ production [229,230]. A possible mechanism was suggested by studies showing that overexpression of apoE4 enhances Aβ production by promoting endocytic recycling of APP [153]. How apoE4 facilitates this recycling remains unclear. At the same time, it may be considered that clearance of Aβ is as important as its production (Figure 5). The predominant pathways for clearance of Aβ include receptor-mediated uptake by microglia and astrocytes [231-233] or LRP1 mediated transport of Aβ across the BBB [234,235].

Receptor-mediated clearance of Aβ in brain likely occurs through the action of LDL receptor family members including the LDL receptor, LRP1, apoER2, SorLA/LR11 and the VLDL receptor [138]. It has been shown that both full-length and cleaved fragments of apoE [166,191], LRP1 and other LRP1 ligands [166] colocalize, and are immunoreactive with, amyloid plaques in AD affected brain tissue. ApoE receptors have been shown to bind directly to Aβ [236] as well as through Aβ binding partner interactions, including apoE-Aβ complexes. In accordance with increased lipidated apoE3 binding to Aβ, apoE3 clears Aβ through receptor-mediated interaction more efficiently than apoE4-Aβ complexes [237]. In amyloid mouse models, expression of human apoE3 resulted in less plaque deposition than in apoE4-expressing mice [203,238,239]. In addition, post-mortem amyloid plaque load is

increased in the brains of ε4 carriers [240,241], suggesting efficient clearance of Aβ may impede amyloid formation, as suggested in Figure 5.

Though cellular and BBB export of Aβ is most certainly receptor-mediated, the responsible receptors remain controversial. Using real-time *in situ* microdialysis, Bell *et al* demonstrated that Aβ(1-42) passed more slowly across the BBB than Aβ(1-40), although both bind to LRP1 [242]. Association of Aβ(1-40) with lipid poor apoE slowed transport (and lipidated apoE complexed with Aβ blocked virtually all transport) across the BBB within the 30-minute time frame of the study. Using the same technique, an alternative study confirmed that lipidation of apoE, compared to lipid-poor apoE-Aβ or free peptide alone, dramatically slows transport of apoE-Aβ across the BBB [237]. Interestingly, this study also demonstrated that Aβ binding to apoE4 shifted receptor-mediated clearance from LRP1 to the VLDL receptor. Alternatively, apoE2 and apoE3 cleared Aβ through LRP1 and the LDL receptor at a significantly higher rate than apoE4-Aβ complexes. This apoE4-specific effect results in higher brain retention of apoE4-Aβ [237]. Taken together, these studies show that decreased amyloid deposition seen in apoE-null mice may be the result of enhanced transport of free Aβ across the BBB.

3.2 Tau protein and apoE

Tau protein is integrally bound to cellular microtubules and acts to stabilize these structures. Phosphorylation of tau, however, results in formation of paired helical filaments that lack microtubule stabilizing capability. Hyperphosphorylated tau is the primary component of pathological neurofibrillary tangles and is toxic to neurons. Studies demonstrating that a reduction in tau prevents Aβ-dependent cognitive impairments in an amyloid mouse model, suggests tau may be required for Aβ-induced neuronal dysfunction [243]. However, whether hyperphosphorylated tau is a primary cause of AD-associated dementia or simply a marker for the disease remains unclear since it is equally plausible that destabilization of microtubules by hyperphosphorylated tau interferes with cognition independent of, or downstream to, induction of AD. Transgenic overexpression of apoE4 in mice resulted in increased tau phosphorylation in neurons, but not astrocytes [244,245]. However, the pathophysiological significance of this finding is unclear since neurons only express apoE following injury [105,107], possibly implying that the contribution of apoE to tau hyperphosphorylation in neurons may be limited to conditions of excess stress or cellular damage. While it has been shown that apoE3 interacts with unphosphorylated tau more strongly than apoE4, the degree to which full-length apoE isoforms bind and associate with phosphorylated tau remains to be determined [246]. Also, it is not clear how apoE and tau, which normally partition to distinct subcellular locations, physically interact. One hypothesis is that proteolytic cleavage of apoE generates C-terminal truncation fragments that dissociate in the cytosol and interact with tau [245]. Indeed, apoE proteolytic fragments may have enhanced toxicity and intracellular activity compared to the intact protein [247].

4. Concluding Remarks

It has become increasingly clear that, in addition to its role in the maintenance and physiology of the cardiovascular system, apoE also plays a central role in healthy and pathophysiological processes in the brain. While apoE is critical for the regulation of cholesterol homeostasis in the peripheral circulation, its role in the brain appears to involve not only cholesterol transport but also intracellular exchange of metabolites between neurons and glial cells through processes that appear to be required for maintenance of healthy brain tissue. As the structural properties of apoE, its various isoforms and lipidation states continue to be defined, new appreciation is gained for its physiological and pathophysiological functions in both the brain and periphery. A concerted effort that draws upon expertise in structural biology, cell biology, animal physiology and genetic engineering

will continue to enhance knowledge of the relationships that exist between apoE and complex disease processes that have an untold impact on the human condition.

Acknowledgments

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Abbreviations

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AD	Alzheimer's disease
apo	apolipoprotein
FRET	fluorescence resonance energy transfer
LDL	low-density lipoprotein
NMR	nuclear magnetic resonance
VLDL	very low density lipoprotein
DMPC	dimyristoylphosphatidylcholine
DPPC	dipalmitoylphosphotidylcholine
CNS	central nervous system
BBB	blood brain barrier
CSF	cerebro spinal fluid
Aβ	amyloid beta
APP	amyloid precursor protein
LXR	Liver-X-Receptor
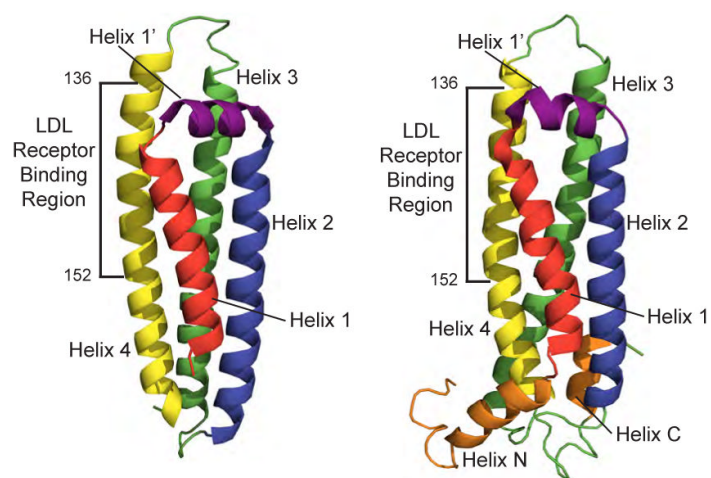
Figure 1. ApoE3 NT structures by X-ray crystallography and NMR
X-ray (left) and solution NMR (right) structures of the lipid-free N-terminal helix bundle of apoE3 displaying residues 23-164 and 1-183, respectively (PDB codes 1LPE [31] and 2KC3 [32]).
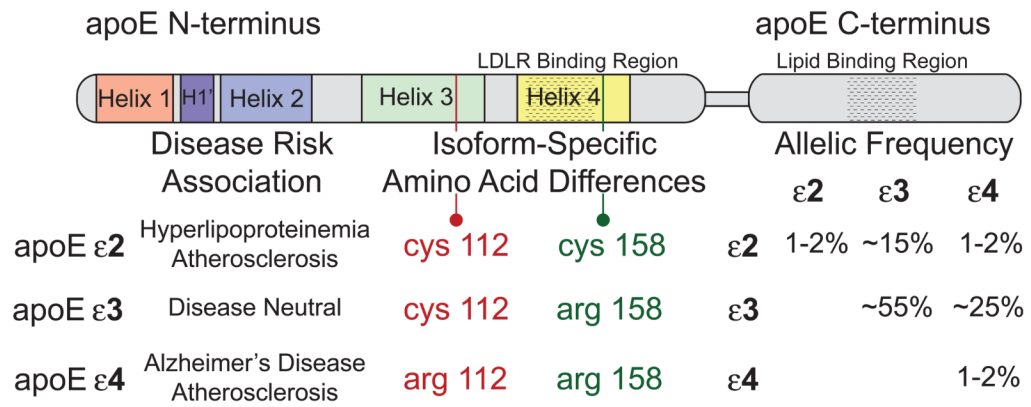
Figure 2. ApoE isoform-specific differences
Linear diagram of the apoE structural organization noting the N-terminal helical organization, functional interaction regions, isoform-specific differences at residues 112 and 158, genotypic frequencies of the human isoforms [248], and disease risk associations for the three isoforms.
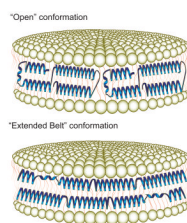
Figure 3. Models of apoE helix bundle opening upon contact with lipid surfaces
The "open" (Top panel) and "extended belt" (Bottom panel) models each permit contact of hydrophobic regions of the protein with exposed hydrophobic surface. The ultimate conformation adopted by apoE on reconstituted HDL remains unresolved.
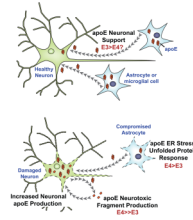
Figure 4. Aβ-independent effects of apoE4 towards neurodegeneration and pathophysiology
In healthy brain, apoE (orange) secreted from astrocytes provides support for neuronal function (Top panel). However, apoE4 increases baseline ER stress and unfolded protein response in astrocytes, slowly leading to cells that function sub-optimally (Bottom panel). As a consequence, these astrocytes are unable to provide optimal support to the neurons over a period of time. As a compensatory response, neurons generate apoE4 for self-repair, which in turn leads to increased generation of neurotoxic fragments, neurodegeneration and disease [168].
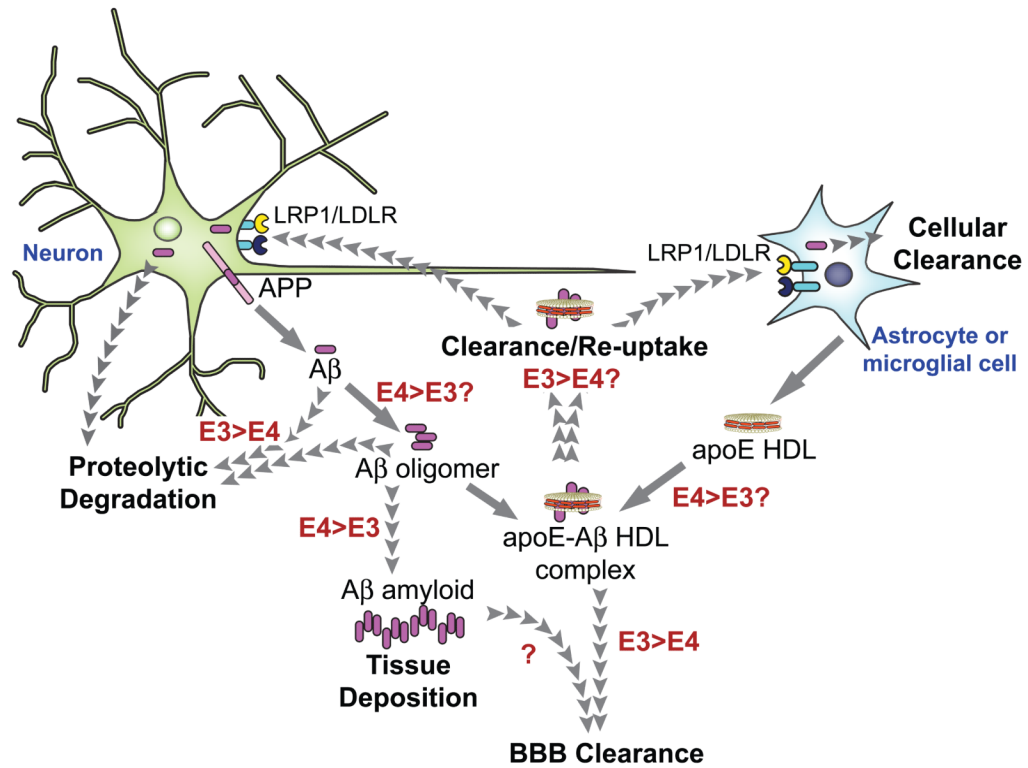
Figure 5. Schematic representation of the role of apoE3/E4-Aβ interaction in AD
ApoE isoform-specific differences (red) may influence Aβ (purple) oligomerization, deposition, transport, and/or clearance mechanisms that can influence the progression of AD. Oligomerization of Aβ released from the amyloid precursor protein (APP) in neuronal membranes has been described as a causative factor for the progression of AD and may be enhanced by apoE4 compared to apoE3. Whether apoE isoform differences affect Aβ association with apoE-HDL complexes (orange and beige) remains unclear. However clearance of apoE-Aβ HDL by lipoprotein receptors (LRP1 and LDLR) appears to be promoted to a lesser extent by apoE4 than apoE3. Astrocytes play a significant role in secreting apoE-containing HDL-sized particles.