

Chapter 2

Apolipoprotein E

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Introduction

For the past two decades the epsilon 4 ($\epsilon 4$) allele of the Apolipoprotein E (*APOE*) gene has remained the only well established and greatest genetic risk factor for late-onset Alzheimer's disease (LOAD). ApoE is a 299-amino acid, 34.2 kDa, glycoprotein that has been implicated in multiple biological functions which will be described in this chapter. However, it is the evidence of its effect on β -amyloid metabolism that most strongly links this protein to the pathogenesis of LOAD.

APOE's Genetic Link to AD

The *APOE* gene is located on chromosome 19q13.32. It has four exons, three of which are transcribed into the 1,180 nucleotides long *APOE* mRNA. Two single nucleotide polymorphisms (SNPs) in exon 4 (rs429358—a thymine to cytosine; rs7412—a cytosine to thymine) give rise to three common *APOE* haplotypes known as the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles, which in turn encode three different protein isoforms (ApoE2, ApoE3, and ApoE4) composed by cysteine to arginine and arginine to cysteine amino acid changes at positions 112 and 158 (ApoE2: Cys-112, Cys-158; ApoE3: Cys-112, Arg-158; ApoE4: Arg-112, Arg-158), as shown in Fig. 2.1.

In 1991, Pericak-Vance et al. reported genetic linkage in LOAD families to a locus on chromosome 19 [1]. Two years later, several groups published genetic and molecular evidence which demonstrated that the *APOE* gene was responsible for this linkage signal [2–4]. Over the next 20 years, numerous reports of replicable association between the *APOE* $\epsilon 4$ allele and the risk of AD have been published.

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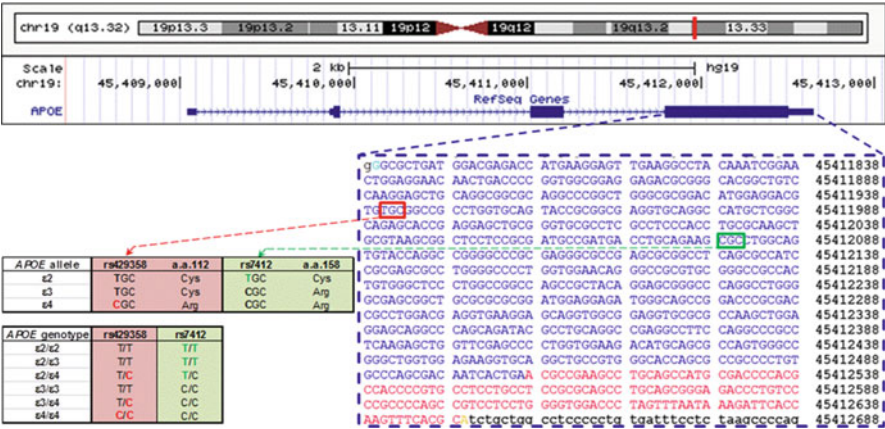


Fig. 2.1 Chromosomal position, gene structure, and composition of the *APOE* ε2, ε3, and ε4 alleles. The chromosomal location (19q13.32), genomic position (chr19:45409039–45412650), and gene structure of the *APOE* gene are shown above the exon 4 sequence [UCSC genome browser (<http://genome.ucsc.edu>) human genome build 19, Feb. 2009 assembly]. The coding sequence of exon 4 is shown in *blue font* while the 3′ untranslated region is shown in *red*. SNP rs429358 is shown within the *red box* and rs7412 is shown within the *green box*. These two SNPs have relatively low minor allele frequencies (MAF), with rs429358-C having a MAF of approximately 15 % and rs7412-T having a MAF of approximately 9 %, as reported in dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>, for “American Caucasians”. The ε2, ε3, and ε4 alleles give rise to six commonly observed genotypes: ε2/ε2, ε2/ε3, ε3/ε3, ε3/ε4, ε4/ε4

A meta-analysis of published association studies indicates that the ε4 allele increases the risk of AD nearly fourfold [5] relative to ε3. On the other hand, the ε2 allele has been demonstrated to protect against AD [6]. Unequivocal proof of the as-of-yet unparalleled role of *APOE* in AD came from the replicability of its association in all of the LOAD genome-wide association studies (GWAS) that evaluated SNPs at this locus [7]. However, these GWAS were also reporting highly significant LOAD associations with other genes in the vicinity of *APOE*, such as *PVRL2*, *TOMM40* and *APOC1* [8–10]. Most GWAS reports assumed that the multiple signals at the *APOE* locus were strictly due to the tight linkage disequilibrium in this genomic region (Fig. 2.2). However, in 2010 Roses et al. challenged this thought by providing evidence of association of longer repeats in a variable-length polymorphism (poly T) in *TOMM40* with an earlier age of- onset for LOAD. Yet, other groups have been unable to replicate this finding [11–13]. This controversy was recently reviewed by Guerreiro and Hardy [14] who explained the possible reasons for the association observed with multiple genes at this locus, which are: (1) that in addition to *APOE*, another gene with an effect on AD risk exists at this locus, (2) that variants outside of the *APOE* coding region have an effect on *APOE* expression and thus influence the risk of AD, (3) that association with variants in a nearby gene are merely a reflection of the effect of *APOE* and its tight linkage disequilibrium with other variants in this region. More recently, Caselli et al. [15] reported association of the *TOMM40* short poly T allele with attenuated age-related cognitive decline, but this

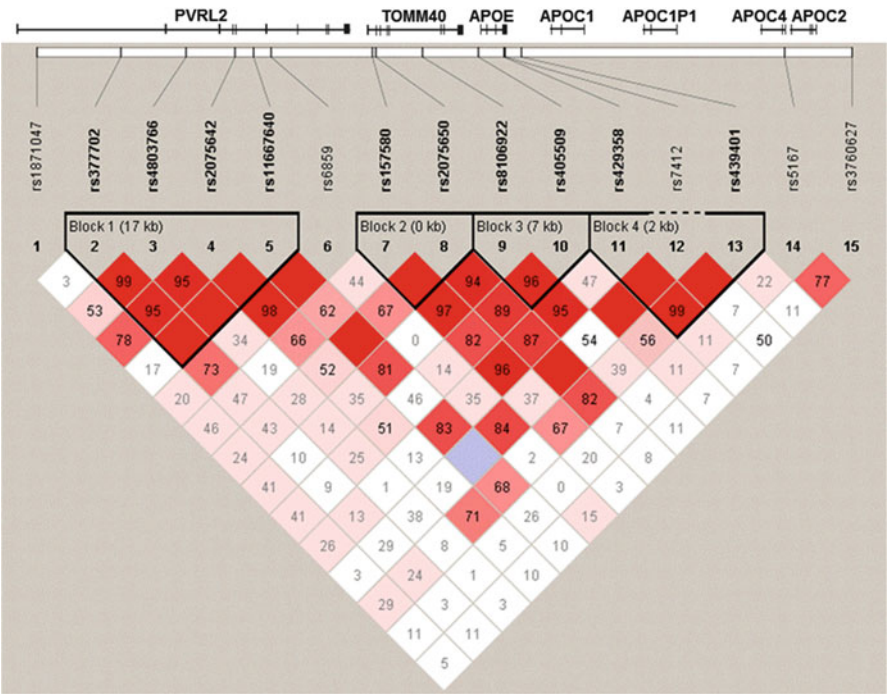


Fig. 2.2 Linkage Disequilibrium (LD) at the *APOE* locus. The LD plot was generated by Haploview 4.2 (D' LD values, haplotype blocks defined by confidence intervals) [16] using data from 844 AD cases from stage I of the Mayo Clinic AD GWAS [17]. *APOE* variants in exon 4 (rs429358 and rs7412) were not included in the SNP genotyping platform utilized in the GWAS, but data was available and included here for determination of the LD with these two SNPs. GWAS SNPs 6–10 and 13 achieved genome-wide significant ($p < 1.6 \times 10^{-7}$) association with AD status in the stage I GWAS, and all 6 SNPs replicated in stage II. Given that all SNPs that show significant association with AD at this locus are in tight LD ($D' \geq 80\%$) with one or both of the *APOE* variants in exon 4, it is necessary to adjust for the presence of the *APOE* $\epsilon 2$ and $\epsilon 4$ alleles to obtain a more accurate estimate of the actual risk conferred by the other variants. The location of genes at this locus (chr19:45,339,393–45,462,822) are shown above the LD plot and follow the UCSC genome browser (<http://genome.ucsc.edu>) annotation for human genome build 19, Feb. 2009 assembly

is the opposite effect reported by Cruchaga and colleagues in an AD clinical series [13]. Thus, resolution of the *TOMM40* controversy will require the identification of rare *TOMM40* variants that have an effect on LOAD and that are not in linkage disequilibrium with *APOE* risk-associated variants.

As is the case with most common disease susceptibility genes, *APOE* is neither necessary nor sufficient to cause AD. In fact, it has been estimated that $\sim 1/2$ of AD patients are not carriers of the *APOE* $\epsilon 4$ allele, and that only $\sim 10\%$ of *APOE* $\epsilon 4$ carriers develop AD [18]. The same study showed that the effect of the $\epsilon 4$ allele is dose dependent with the odds ratio increasing from ~ 4 in $\epsilon 4$ heterozygotes to up to 30 in $\epsilon 4$ homozygotes, when compared to $\epsilon 3$ homozygotes. Also, typical of genes involved in common disorders, is the effect difference observed between

populations due to differences in the frequency of genetic and environmental risk factors. Although, the risk conferred by the $\epsilon 4$ allele in Asian populations is very similar to that in Caucasians (AlzGene meta-analysis odds ratios=3.99 and 3.81, respectively; lowest single study odds ratio=2.06) [5] the effect observed in Hispanic and African-American populations is much lower and sometimes even undetectable, with odds ratios ranging from 1.0 to 2.6 [10, 19–23]. However, it is possible that this difference in effect size is an artifact caused simply by the small sample size typically available from Hispanic and African American populations which decreases the power to detect association, especially in the presence of confounders that are likely to be present (i.e., study design, and differences in genetic and environmental background) [24].

ApoE Expression

In the periphery hepatic parenchymal cells are the major ApoE producing cell type however *APOE* mRNA can also be found at multiple other sites including the spleen, kidneys, lungs, peripheral nerves, smooth muscle cells, ovaries, testes, and adrenal glands. In the central nervous system (CNS), ApoE is mainly produced by astrocytes but also pericytes, microglia, and under certain pathological conditions also by neurons [25–29].

Levels of ApoE can readily be determined in various body fluids like plasma (about 40 mg/L) and cerebrospinal fluid (CSF) (about 10 mg/L) [30–32]. Expression of *APOE* is regulated by the nuclear receptors liver x receptors alpha and beta ($LXR\alpha$ and $LXR\beta$), peroxisome proliferator-activated receptor- γ (PPAR γ), and retinoid X receptors (RXRs) which ligate into pairs of PPAR γ :RXR and LXR :RXR for induction of *APOE* expression [33, 34]. The LXRs were shown to regulate peripheral ApoE production by macrophages, adipocytes, and hepatocytes [35, 36]. These receptors are also expressed in the brain and their activation leads to increased expression of ApoE in microglia and astrocytes [37]. In support, treatment of primary microglia and astrocyte cultures with bexarotene, a blood brain barrier (BBB) permeable RXR agonist induced expression of *APOE* and the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, both vital to the lipidation and regulation of lipoprotein [38]. Whether other peripheral stimuli than bexarotene can regulate centrally produced ApoE and vice versa is rather unexplored however a recent study by Liu and colleagues showed that peripherally administered insulin can increase CSF but not plasma ApoE levels [39]. Importantly, ApoE levels found in plasma and CSF are derived from separate sources as indicated by the finding that the plasma but not CSF ApoE phenotype changes into the phenotype of the graft donor upon liver transplantation [40]. This assumption was recently confirmed by results from an in vivo study where mice were subjected to adenovirus encoding human ApoE3 for peripheral production of human ApoE. The authors of that study showed that high levels of human ApoE3 were found in plasma of these mice whereas human ApoE3 levels in the CSF of the same animals were essentially undetectable [41].

Further, ApoE concentrations in both plasma and CSF have been shown to vary depending on *APOE* genotype ($\epsilon 2/\epsilon 3 > \epsilon 3/\epsilon 3 > \epsilon 2/\epsilon 4 > \epsilon 3/\epsilon 4 > \epsilon 4/\epsilon 4$) [31]. However, ApoE production was found to be similar in cultures of primary human astrocytes derived from *APOE* $\epsilon 4$ -positive versus $\epsilon 4$ -negative individuals suggesting additional mechanisms to be partly responsible for the variability in ApoE levels observed in individuals of various *APOE* genotypes [26]. For instance lower ApoE levels may not be due to altered ApoE synthesis but rather result from different ApoE turnover kinetics with ApoE4 being the most rapidly catabolized isoform relative to ApoE2 and ApoE3 (ApoE4 > ApoE3 > ApoE2). No isoform-specific turnover differences have been found between ApoE3 and ApoE4 in human CSF. Thus, the turnover rates between peripheral and central ApoE levels appear to be different suggesting not only separate synthesis but also distinct catabolism pathways for ApoE in the periphery versus the CNS [32, 42, 43].

Apolipoprotein E Normal Function

Apolipoprotein E has been demonstrated to have immunoregulatory properties, to affect synapse formation, neuronal repair and survival and to act as an antioxidant possibly due to its capacity to bind metal ions [29, 44]. ApoE has also been proposed vital for maintaining the BBB and the blood–nerve barrier as *apoE* knock-out mice exhibit functional impairment of both [45]. Many of these functions may be dependent on the lipidation state of the protein as the main function attributed to ApoE is its major role as a lipid transporter essential to normal lipid homeostasis in the periphery as well as in the CNS.

Apolipoprotein E Lipid Binding and Transport

Lipids are crucial to normal cellular function and various physiological processes like formation and maintenance of cellular membranes including membrane expansion required for axonal extension, synthesis of hormones, myelin, and neurotransmitters like acetylcholine. About 25 % of the total body cholesterol resides in the brain where myelin constitutes the major cholesterol pool. The synthesis rate of cholesterol is highest during myelination, thereafter the rate decreases but does not cease completely. In the CNS most lipids are synthesized in situ mainly by glial cells, however neurons can also synthesize lipids [46]. For instance, cultured rat neurons synthesize phospholipids in their cell bodies and distal axons whereas cholesterol is synthesized in cell bodies and proximal axons from where it is transported to the distal axons [47, 48].

Transport of hydrophilic lipids between cells and tissues through the extracellular space and the circulation requires appropriate packaging of lipids into water soluble molecules described as high density lipoproteins (HDL), intermediate

density lipoproteins (IDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons. Lipoproteins contain apolipoproteins (Apo) such as ApoA-I, ApoA-II, ApoJ (clusterin), and ApoE which stabilize the lipoprotein particles through their hydrophobic and hydrophilic domains. The lipid contents of lipoproteins are constituted by phospholipids, free cholesterol, triglycerides, and cholesteryl esters [49]. Lipid transport within the CNS and the periphery appears to employ different systems as almost no cholesterol in the CNS originates from plasma lipoproteins which do not cross the BBB. The lipoprotein particles in the two different compartments also differ to some extent as plasma ApoE is mainly found in VLDLs whereas ApoE in the CSF is part of the HDL particles [46]. Two structural domains have been described for ApoE, the receptor binding region (amino acids 136–150) at the amino- (N) terminal domain and the lipid-binding region (amino acids 244–272) at the carboxyl (C)-terminal domain. These two structural domains are linked by a flexible hinge region [50]. In contrast to ApoE2 and ApoE3, ApoE4 has an extra domain interaction formed by a salt bridge between Arg-61 of the N-terminal and Glu-255 at the C-terminal [51, 52]. This domain interaction renders ApoE4 more susceptible to proteolysis [53, 54].

Binding of ApoE to lipids and to heparan sulfate proteoglycans (HSPGs) induces a two-step conformational adaptation resulting in an active ApoE conformation able to bind to receptors of the low-density lipoprotein receptor (LDLR) family. Recent results elucidating the NMR structure of ApoE3 suggest a three structural domain topology which regulates ApoE tertiary structure to allow a single possible conformation upon binding. This conformation was proposed to provide a security mechanism which prevents partially lipidated or lipid-free ApoE from premature binding to ApoE receptors [55].

Similar to the formation of HDLs in plasma, two pathways have been suggested for the generation of ApoE-containing HDLs by glia cells in the CNS, either by direct secretion of lipidated ApoE or by secretion of lipid-poor ApoE which becomes lipidated extracellularly. Contrary to the spherical HDLs found in plasma, the astrocyte secreted nascent ApoE-containing lipoproteins are lipid-poor and discoidal in shape. The latter lipoproteins contain primarily phospholipids and unesterified cholesterol. In the brain, members of the ATP-binding cassette (ABC) transporter family are key to formation and regulation of lipoproteins containing ApoE. Specifically ABCA1, expressed in both neurons and glial cells, is essential to the production of normally-lipidated ApoE by astrocytes *in vitro* and *in vivo*. This transporter binds to ApoE and mediates lipid efflux to the ApoE containing lipoproteins. Deficiency in ABCA1 leads to reduced size of the astrocyte secreted lipoproteins and less cholesterol efflux to these particles. The ABCA1–ApoE interaction is not ApoE isoform dependent as ApoE2, ApoE3, and ApoE4 have similar affinity to ABCA1. Also, the ABCA1-mediated efflux of cholesterol appears to be ApoE isoform independent. On the other hand accumulating evidence suggests that ApoE isoform determines the amount of lipid associated with ApoE. In cultured primary neurons and astrocytes exogenously added recombinant ApoE can induce isoform-dependent (ApoE2>ApoE3>ApoE4) efflux of phospholipids and cholesterol yielding HDL size lipoproteins. In support, two- to fourfold more cholesterol and

phospholipids can be effluxed to ApoE3 compared to ApoE4 in cultures of rat cortical astrocytes and neurons. Thus, current understanding fosters the notion that more lipids are effluxed by astrocytes to ApoE3 than ApoE4 which makes the latter isoform less potent as a lipid supplier to, for instance, neurons [46].

ApoE Lipid Delivery and Receptors

Apolipoprotein E mediates lipid transport and delivery, thus lipid metabolism, by acting as a receptor ligand for various ApoE receptors of the LDLR family [56] expressed by various cell types in the periphery and in the brain. These receptors share similar characteristics and two of them, the LDLR and the LDLR-related protein-1 (LRP1) regulate ApoE levels and have been proposed as the two major metabolic receptors for ApoE in the brain. This assumption was based on the findings that cells lacking the LDLR exhibit impaired endocytosis of astrocyte-derived ApoE-containing lipoproteins and further, CSF levels of ApoE were 50 % higher in LDLR knock-out mice compared to mice expressing the LDLR. In support the LDLR becomes up-regulated by cholesterol deficiency and down-regulated upon cholesterol enrichment [57]. In order for cells to acquire lipids from the extracellular space ApoE-containing lipoproteins can be endocytosed and intracellularly dissociated from their receptors releasing lipids and allowing recycling of the receptors. Several of the high affinity ApoE receptors, LRP1, LDLR, very low density lipoprotein receptor (VLDLR) and the ApoE receptor-2 (APOER2) are expressed by neurons. For instance the VLDLR mediates uptake of ApoE-containing lipoproteins by neuronal growth cones where it is proposed to aid lipid acquisition to cover the need for lipids upon cell membrane expansion. However, several LDLR family members are also expressed on glial cells including astrocytes, oligodendrocytes, and microglia. In fact, the LDLR is expressed at higher levels in glial cells than in neurons, whereas LRP1 is more highly expressed in neurons compared to glial cells [46]. The potency of ApoE to bind to its receptors was described to be affected by both ApoE isoform and lipidation state. LRP1 readily binds lipid-associated ApoE and has been reported to only weakly bind lipid-free ApoE4, whereas lipid-free ApoE2 and ApoE3 were not recognized by the receptor. Apolipoprotein E needs to be associated with lipids also in order to be recognized by the LDLR which readily binds to ApoE3 and ApoE4, but only weakly to ApoE2 [58], whilst the VLDLR recognizes all ApoE isoforms even if not associated with lipids [59]. In line with these results it has been shown that astrocyte-secreted ApoE particles, which are known to be lipid-poor, have higher affinity for LDLR than LRP1. In contrast however recombinant lipid-free ApoE and ApoE-containing HDL particles, isolated from the CSF, bind more readily to LRP1. The former finding introduces a controversy in regard to the low affinity of LRP1 for lipid-poor ApoE and suggests that the active ApoE conformation recognized by the receptor may be imposed by mechanisms other than lipid-association [56]. The different affinities of the ApoE receptors for

Amyloid-Beta-Dependent Pathways

ApoE has repeatedly been linked to A β pathology, one of the two neuropathological characteristics of AD. Both ApoE3 and ApoE4 bind synthetic A β in vitro with the latter exhibiting higher A β affinity [61]. Apolipoprotein E also acts as a strong inducer of A β polymerization with ApoE4 as the most potent catalyst of amyloid filament formation [62]. In vivo, ApoE has been identified as an amyloid-associated protein in most types of A β plaques characterizing the brains from AD patients, from diffuse irregular shaped plaques to primitive neuritic plaques [63]. Importantly, nondemented elderly *APOE* ϵ 4 carriers were shown more likely to have more A β immunoreactive deposits compared to noncarriers, and *APOE* ϵ 4 homozygous individuals also exhibited augmented cerebral amyloid angiopathy [64]. In support, Morris et al. recently demonstrated a gene dose effect for *APOE* ϵ 4 on cerebral amyloid deposition using imaging with Pittsburgh Compound-B (PIB) in cognitively normal individuals. Increasing numbers of *APOE* ϵ 4 alleles was associated with greater mean PIB cortical binding and greater reduction in CSF levels of A β 1-42, both considered markers of cerebral A β deposition [65]. Thus even before onset of clinical dementia *APOE* ϵ 4 can be linked to increased A β deposition. The phenotype of increased amyloid pathology in *APOE* ϵ 4 carriers can be recapitulated in transgenic AD mouse models expressing human ApoE, where ApoE4 expressing mice displayed higher amyloid plaque load compared to ApoE3 mice [66, 67]. The demonstrated increase in cerebral A β deposition found in *APOE* ϵ 4 individuals even in the absence of dementia symptoms, as well as in AD mouse models, may be due to either altered A β production or A β clearance, or a combination of both, as effects on both pathways have been reported.

Increased A β production can be induced by ApoE, specifically by ApoE4, as suggested by Ye and colleagues. These authors showed that lipid-poor ApoE4 increased the production of A β more profoundly than ApoE3 in cultured rat neuroblastoma cells stably transfected with human wild-type APP695 (B103-APP). The authors further showed that the ApoE4 domain interaction, which does not occur in ApoE3, was vital to the observed increased in A β production as well as a functioning LRP pathway, as interference with it abolished the difference between ApoE4 and ApoE3 in regard to increasing A β production [54]. Using the PDAPP mouse model of AD and lentiviral vectors to induce expression of human ApoE, Dodart and colleagues further showed that expression of human ApoE4 increased hippocampal A β 1-42 concentrations and amyloid burden in these mice. In contrast, markedly reduced hippocampal amyloid burden was found in the same AD mouse model when expressing human ApoE2. These results prompted the authors to conclude that gene delivery of ApoE2 may reduce A β burden and prevent neuritic plaque formation [67]. A recent study by Cramer and colleagues further demonstrated ApoE-dependent enhancement of A β clearance and reversed cognitive deficits in AD mouse models upon oral treatment with the RXR agonist bexarotene, suggesting a vital role for ApoE in A β clearance. However, the mouse models used in this

particular study expressed murine ApoE which most closely resembles human ApoE3 [38].

The clearance pathways of A β include (a) transport across the BBB for systemic degradation of A β in the liver and kidneys [68] (b) cellular uptake and degradation primarily by astrocytes and microglia [69, 70] as well as (c) extracellular proteolysis mainly by insulin-degrading enzyme and neprilysin [71]. Apolipoprotein E has been demonstrated to influence all three pathways in various ways. For instance, both ApoE2 and ApoE3 mediate transport of A β across the BBB via LRP1 whereas ApoE4 redirects A β -ApoE complexes from the LRP1 pathway to VLDLR suggesting less A β efflux across the BBB in *APOE* ϵ 4 carriers [68, 72]. Moreover, the effect of ApoE on glia cell mediated A β clearance has been extensively studied however with inconsistent results. By use of ApoE-/- mouse astrocytes Koistinaho and colleagues demonstrated an important role of ApoE in astrocytic degradation and clearance of deposited A β [73]. The authors reported that ApoE is essential to colocalization, internalization, and degradation of A β deposits in brain sections as adult ApoE-/- astrocytes did not degrade deposited A β whereas wildtype astrocytes did. Jiang et al. also showed beneficial and dose-dependent effects of ApoE on in vitro A β degradation by ApoE-/- microglia. Exogenously added human ApoE, harvested from immortalized human *APOE* knock-in mouse astrocyte cultures, increased intracellular A β degradation by neprilysin and related enzymes in an isoform-dependent manner (ApoE2>ApoE3 \geq ApoE4). The authors also showed that extracellular degradation of A β by insulin-degrading enzyme was facilitated by ApoE [37]. The beneficial effect of ApoE on microglial A β degradation was recently shown to be mediated by the cholesterol efflux function of ApoE as reduced cellular cholesterol levels promoted lysosomal A β degradation [74]. In support increased murine ApoE production, mediated by bexarotene treatment of both primary murine astrocytes and microglia, facilitated degradation of soluble A β 1-42 [38]. Results from studies on primary human cells proposed that ApoE in combination with both fibrillar and oligomeric A β can increase the expression of neprilysin in primary human astrocyte cultures derived from nondemented controls but not from AD patients [75]. Another study on primary human astrocytes suggested that ApoE reduced oligomeric but not fibrillar A β cell-association [76]. In support, Bruinsma et al. reported a significant reduction of cell surface accumulation and internalization of the Dutch A β Glu22Gln1-40 peptide in primary human astrocytes treated with ApoE3 conditioned medium from *APOE* ϵ 3/ ϵ 3 primary human brain pericytes [26]. The same study in addition reported protective effects of ApoE from A β Glu22Gln1-40 induced cell death in both human pericyte and astrocyte cultures. The contradicting results on ApoE effects on astrocytic A β clearance may be due to inherent differences between astrocytes derived from rodents and humans [77] and even more likely due to the use of different ApoE isoforms differentially lipidated in these studies.

Whether due to increased A β production or altered A β clearance, the resulting increased levels of A β appear neurotoxic, with the oligomeric A β species exhibiting the most neurotoxic effects [78]. The neurotoxic effects of A β have been proposed to be susceptible to ApoE influence. At physiological levels of ApoE in the CSF, all

ApoE isoforms were shown to be protective against neurotoxic effects of A β 25-35 on a rat neuronal cell line. However, superphysiological levels of ApoE4 and ApoE3 increased A β neurotoxicity with ApoE4 exhibiting the strongest effect [44]. Moreover, results from experiments using cocultures of primary neurons from wild-type mice and glia cells from human ApoE2, ApoE3, ApoE4-targeted replacement mice showed a significant increase in toxic effects of oligomeric A β 1-42 on neurons cultured in the presence of ApoE4 secreting glia compared to *apoE* knock-out, ApoE2, and ApoE3 glia cells [79]. In support, a recent study combining in vitro work on primary mouse neuronal cultures and array tomography of brain tissue from AD patients and nondemented controls further suggested that ApoE4 enhances the toxicity of A β oligomers by increasing its levels and enhancing its colocalization with synapses more than fivefold [80]. Thus an increasing body of evidence suggests concentration-dependent modulatory effects of ApoE, especially ApoE4, on A β neurotoxicity.

Amyloid-Beta Independent Pathways

Next to A β pathology the most prominent features of the AD brain include loss of synapses, tau pathology, neuroinflammation, and metabolic alterations. Synapse loss remains the strongest correlate to decreased cognitive performance in AD patients [81–83] and recently it was also demonstrated that individuals with amnesic mild cognitive impairment (aMCI) exhibit 36 % fewer synapses in the inferior temporal gyrus compared to cognitively intact individuals [84]. Interestingly, ApoE has been implicated in synaptogenesis, neuronal outgrowth, and sprouting. Whereas ApoE3, in the presence of VLDL, increased neurite outgrowth in cultures of dorsal root ganglion neurons, ApoE4 decreased neurite outgrowth in the same cultures [85]. In the absence of neuropathological events like gliosis and tau pathology Wang and colleagues demonstrated significantly reduced excitatory synaptic transmission and dendritic arborization of neurons in the lateral amygdala of human *APOE* targeted replacement mice expressing *APOE* ϵ 4. These changes were observed already in young mice and the authors proposed that the cognitive defects in *APOE* ϵ 4 carriers may be due to inherent defects in synaptic function appearing earlier than any age-dependent markers of neuropathology [86]. Using the same animal model, Dumanis et al. proposed age-dependent effects of *APOE* ϵ 4 leading to reduced neuronal spine density in the cortex of these mice. Mice expressing human ApoE4 exhibited reduced spine density already at 4 weeks and at the age of 1 year these mice had more than 50 % reduced spine density compared to mice expressing human ApoE2 and ApoE3 [87]. These results are in line with a previous preclinical study showing lower density of dendritic spines in human *APOE* ϵ 4 transgenic mice versus *APOE* ϵ 3 mice. The authors of this particular study further showed an inverse dose-dependent *APOE* ϵ 4 allele effect on spine density of neurons in the dentate gyrus of both AD patients and aged controls [88]. The mechanisms underlying the *APOE* ϵ 4 effects on neuronal outgrowth and synapse density remain elusive but it

has been shown that ApoE4-enriched beta-VLDL particles show less accumulation and retention in murine neuroblastoma Neuro-2a cells, mediated primarily by cell surface heparan sulfate proteoglycans, than ApoE2- and ApoE3-enriched VLDL particles. These results suggest differential intracellular handling of the different ApoE isoforms [89].

Recently, it has been argued that cytotoxic C-terminal truncated ApoE fragments would underlie the detrimental effects of ApoE4 on synapses and also to contribute to tau pathology. Under pathological conditions like stress and injury neurons express ApoE of which the ApoE4 isoform was reported to be more susceptible to proteolysis. Analysis of lysates from AD brain tissue showed the presence of C-terminal truncated forms of ApoE4 in association with neurofibrillary tangles (NFTs). These truncated ApoE4 species were proposed to escape the secretory pathway in neurons, to enter the cytosol and to interact with tau causing NFTs by increasing tau phosphorylation [90]. These results are in line with earlier in vivo work demonstrating altered phosphorylation of tau with significantly increased phosphorylation at two sites (Ser-235 and Ser-413) in *APOE* $\epsilon 4$ knock in mice [91]. Moreover, the cytosolic ApoE4 fragments were suggested to interact with mitochondria and induce mitochondrial dysfunction which was found greater in *APOE* $\epsilon 4$ carrying AD patients versus noncarriers. Together the effects of ApoE4 fragments were proposed to contribute to AD pathogenesis by causing mitochondrial dysfunction and synaptic deficits. Interestingly, most of these detrimental effects could be reversed when blocking the ApoE4 domain interaction either by site-directed mutagenesis (Arg-61 to Thr) or by small-molecule structure correctors, which render ApoE4 less susceptible to proteolysis [90].

Common to most neurodegenerative disorders, neuroinflammatory events mainly driven by glial cells are also prominent features of AD. Already before manifestation of dementia, inflammatory events can be traced in the CSF as suggested by a recent study by Westin et al. The authors showed that CSF levels of the pro-inflammatory chemokine monocyte chemoattractant protein-1 (CCL2) correlated with faster cognitive decline in patients with prodromal AD. Patients with higher levels of CSF CCL2 exhibited faster cognitive decline and faster progression to AD dementia suggesting inflammatory processes as an important component in the development of clinical AD [92]. However, although long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) was proposed to reduce the risk of developing AD, recently published results from the AD anti-inflammatory prevention trial (ADAPT) suggested that NSAID treatment may have adverse effects in the later stages of AD indicating that the AD-associated inflammatory processes might shift from acute to chronic with timing of treatment as an important factor to consider in prevention trials [93, 94]. Further studies also suggested that the AD risk-lowering effect of NSAID treatment may be observed in *APOE* $\epsilon 4$ carriers only [95]. Next to the described defective anti-oxidative effect of ApoE4, compared to ApoE2 and ApoE3 [44], it is well-known that ApoE isoform-dependent modulation of immune responses occurs and that inflammatory cytokines can either up- or down-regulate the production of ApoE in different tissues [29, 96]. Isoform-specific influence of ApoE microglial activation has been extensively studied and suggested a shift to a

neurotoxic microglia phenotype particularly with ApoE4 [97]. In support of a specific link between *APOE* ϵ 4 and inflammation, Zhu and colleagues recently showed increased glial activation in response to an intra-cerebroventricular pro-inflammatory challenge with lipopolysaccharide (LPS) in mice expressing *APOE* ϵ 4 versus mice expressing *APOE* ϵ 2 and *APOE* ϵ 3. Mice expressing *APOE* ϵ 4 exhibited a more prolonged increase in pro-inflammatory cytokine levels [98]. These results are in line with earlier work showing isoform-specific effects of the immuno-modulatory properties of ApoE upon intravenous administration of LPS in mice expressing *APOE* ϵ 3 and *APOE* ϵ 4. The latter mice showed greater elevation of both systemic and brain levels of pro-inflammatory cytokines, a feature which could be reversed by intravenous administration of a small ApoE-mimetic peptide (ApoE133-140) [99]. Inflammatory effects of ApoE4 fragments have also been investigated in vitro using human neuroblastoma and astrocytoma cell lines in which a specific ApoE4 fragment (ApoE4186-299) exhibited significant effects on matrix metalloproteinase 9, tissue inhibitor of metalloproteinase 1 and cytokine levels [100].

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