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Apolipoprotein E Sets the Stage: Response to Injury Triggers Neuropathology, Including Alzheimer's Disease

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Abstract

Apolipoprotein (apo) E4 is the major genetic risk factor for Alzheimer's disease and is associated with poor clinical outcome following traumatic brain injury and other neuropathological disorders. Protein instability and an isoform-specific apoE property called domain interaction are responsible for these neuropathological effects. ApoE4 is the most neurotoxic isoform and can induce neuropathology through various cellular pathways. Neuronal damage or stress induces apoE synthesis as part of the repair response; however, when apoE4 is expressed in neurons, its unique conformation makes it susceptible to proteolysis, resulting in the generation of neurotoxic fragments. These fragments cause pathological mitochondrial dysfunction and cytoskeletal alterations. Here, we review data supporting the hypothesis that apoE4 (> apoE3 > apoE2) has direct neurotoxic effects and highlight studies showing that blocking domain interaction reverses these detrimental effects.

Introduction

Neuronal Injury Induces ApoE Synthesis: A “Hair-Trigger” Mechanism Allows for Rapid Production

Apolipoprotein (apo) E was originally described in the early 1970s as a protein constituent of cholesterol- and triglyceride-rich plasma lipoproteins synthesized by the liver. Its expression is induced by cholesterol-rich diets in a large variety of animals and is enriched in lipoproteins in humans with the genetic disorder type III hyperlipoproteinemia (Mahley, 1988; Mahley and Rall, 2000; Mahley et al., 2009). ApoE circulates in the blood as a protein component of very low density lipoproteins, chylomicron remnants, and a subclass of high

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density lipoproteins, as well as in the cerebrospinal fluid and central nervous system interstitial fluid on small particles and disks resembling high density lipoproteins. ApoE is responsible for the transport of cholesterol and other lipids, as well as for mediating the clearance of plasma lipoproteins by serving as a critical ligand for lipoprotein uptake by the low density lipoprotein (LDL) receptor and LDL receptor–related protein family members. Furthermore, apoE participates in the redistribution of lipids to cells that require cholesterol and phospholipids for reparative processes throughout the body, including the central nervous system.

Human apoE is a polymorphic protein arising from three alleles at a single gene locus on chromosome 19 (Mahley, 1988; Mahley and Rall, 2000; Mahley et al., 2009). The three major isoforms—apoE2, apoE3, and apoE4—differ from one another by single amino acid interchanges at just two residues; however, these minor changes have profound effects on the structure and function of apoE at both the molecular and cellular levels and, as a consequence, on their association with specific diseases, including Alzheimer's disease (AD).

The pioneering work of Roses and associates during the early 1990s established, through a genetic linkage study, the very strong association between apoE4 and AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). Expression of the apoE4 allele significantly increases the risk of developing AD during one's lifetime (by 4- to 12-fold compared with apoE3/3 individuals) and decreases the age of onset (by approximately 8 years to 15 years in apoE4 heterozygotes and homozygotes, respectively). It is now established that apoE4 is a major AD gene with semidominant inheritance in apoE4 homozygotes, equivalent to the BRAC1 gene for breast cancer (Genin et al., 2011), making it the strongest genetic risk factor for AD by far (Farrer et al., 1997). Although the data are not as strong as with AD, apoE4 has also been associated with progression or poor clinical outcomes in traumatic brain injury (TBI) (Chamelian et al., 2004; Crawford et al., 2002; Friedman et al., 1999; Gandy and DeKosky, 2012; Mayeux et al., 1995; Nicoll et al., 1996; Teasdale et al., 1997), multiple sclerosis (Chapman et al., 2001; Fazekas et al., 2001), stroke (Alberts et al., 1995; McCarron et al., 1999; Slioter et al., 1997), frontotemporal dementia (Agosta et al., 2009), and Parkinson's disease (Harhangi et al., 2000; Li et al., 2004; Martinez et al., 2005; Parsian et al., 2002). Furthermore, apoE4 is not rare—approximately 25% of all individuals are carriers of this allele—making the potential detrimental effects of apoE4 expression all too common. Indeed, the apoE4 allele is heavily enriched in AD patients, with 65–80% of all AD patients carrying at least one copy (Farrer et al., 1997).

The neuropathological effects of apoE4—the least stable of the three isoforms and the most tightly associated with AD—are myriad and include (for review, see refs. Huang, 2010; Kim et al., 2009; Mahley et al., 2006): 1) impaired neurite outgrowth; 2) cytoskeletal disruption and hyperphosphorylation of tau; 3) mitochondrial dysfunction in neurons, including altered membrane potential, reduced mitochondrial motility, and decreased mitochondrial respiratory enzyme levels and activity; 4) impaired synaptogenesis; 5) increased amyloid β ($A\beta$) production; 6) increased lysosomal leakage and apoptosis in neurons; 7) brain neuropathology and impaired learning and memory in mice; and 8) altered $A\beta$ peptide clearance and/or deposition.

The premise of this review is that the structural differences among the apoE isoforms determine their roles in the onset and progression of AD and other neurodegenerative diseases, and that modulation of the abnormal structure of apoE4—by converting it to a more apoE3-like (or apoE2-like) structure—will reverse the apoE4-associated detrimental effects in the central nervous system (Mahley and Huang, 2012). First, however, we discuss how apoE may indirectly impact neuropathology in AD through modulation of A β metabolism, before moving on to present the apoE hypothesis more fully and the most recent evidence describing the direct effects of apoE (apoE4 > apoE3 > apoE2) in the pathogenesis of neurodegenerative disorders.

ApoE Modulation of the A β Pathway

The amyloid hypothesis focuses on the effects of the A β peptide and its different assemblies in causing neuropathology, disrupting synaptic connections and forming plaques (Hardy, 2006; Palop et al., 2006; Palop and Mucke, 2010; Selkoe, 2011). Importantly, it is established that there are apoE isoform-specific effects on the A β pathway (Huang and Mucke, 2012; Kim et al., 2009; Selkoe, 2011) and that apoE4 expression is associated with a significant increase in amyloid plaques at earlier ages compared with apoE3 or apoE2. Furthermore, apoE4 is known to impair A β clearance (Bien-Ly et al., 2011; Castellano et al., 2011; Deane et al., 2008; Kim et al., 2011) and accelerate amyloid synthesis (Ye et al., 2005), as well as amyloid fibril formation and deposition (Bales et al., 1999; Bien-Ly et al., 2011; Sanan et al., 1994; Wisniewski et al., 1995).

ApoE protein levels in the cerebrospinal fluid and brain have been correlated with A β levels and related to apoE isoform-specific effects (Beffert et al., 1999). For example, comparison of human apoE3 and apoE4 knock-in mice demonstrated that apoE4 levels were 30–40% lower than apoE3 levels in the cortex, hippocampus, and cerebellum (Ramaswamy et al., 2005). One explanation for this reduction in apoE4 expression was revealed by Zhong et al. (2009), who demonstrated that apoE4 domain interaction activates the endoplasmic reticulum (ER) stress response in astrocytes, which results in the degradation of apoE4. Could this suggest that increasing apoE levels is protective?

Consistent with the postulate that increasing apoE levels could be beneficial, Cramer et al. (2012) demonstrated that induction of mouse apoE expression in an AD mouse model using the RXR agonist bexarotene led to a short-term reduction in soluble A β and plaque loads (i.e., within 72 h of initiating treatment). However, after 3 months of oral treatment they observed no change in amyloid burden. While these are potentially important observations, a number of questions remain. First, can one equate the effect of increasing mouse apoE to that of the human apoE isoforms? Mouse apoE is neither structurally nor functionally equivalent to human apoE3 or apoE4 (Zhong and Weisgraber, 2009) and behaves differently from the human isoforms with respect to A β clearance (Bien-Ly et al., 2011). Importantly, it was recently reported that genetically increasing either human apoE3 or apoE4 levels increased A β accumulation (Bien-Ly et al., 2012; Kim et al., 2011). Second, would it be beneficial to increase apoE4 levels in the brains of patients? As discussed later, numerous studies demonstrate that apoE4 has detrimental effects in the central nervous system (CNS).

Finally, bexarotene is known to regulate numerous genes related to lipid metabolism, thus further complicating the interpretation of the data.

Others have emphasized the protective role for apoE3 in the context of amyloid metabolism, postulating that apoE4 lacks the beneficial effects of apoE3. Clearly apoE3 does possess beneficial effects (Kim et al., 2009; Mahley et al., 2006). For example, apoE3 is more effective than apoE4 in mediating A β clearance from mouse brains (Kim et al., 2009). It also has been demonstrated that apoE3 suppresses inflammation better than apoE4 (Lynch et al., 2003). In contrast, apoE4 has been shown to stimulate pro-inflammatory cytokines and exacerbate inflammation to a greater extent than apoE3 (Guo et al., 2004). Interestingly, apoE mimetics, which are small peptides corresponding to the apoE receptor-binding region, appear to mimic the anti-inflammatory activity of apoE3 and have been shown to improve cognitive performance and neuronal survival in TBI mouse models (Vitek et al., 2012). However, the mechanism by which these peptides work remains to be defined.

It is now recognized that there are multiple factors acting through various pathways to cause cognitive decline and neurodegeneration in AD (Huang, 2010; Huang and Mucke, 2012; Kim et al., 2009; Mahley et al., 2006). ApoE clearly interacts with the A β pathway; however, there is abundant evidence showing that apoE (apoE4 > apoE3 > apoE2) also can directly impact the pathogenesis of AD and other neurodegenerative disorders independent of A β . As clinical trials targeting the lowering of amyloid and the A β peptide fail to impact AD cognitive decline and neurodegeneration (Selkoe, 2011), alternative mechanisms must be considered, and the critical importance of apoE in pathogenesis further acknowledged.

Direct Effects of ApoE in Causing Neuropathology

Under normal physiological conditions, apoE is synthesized primarily by astrocytes in the brain to support lipid transport and membrane repair processes (Mahley, 1988). In contrast, apoE synthesis in neurons usually occurs in response to neuronal insult or injury. Each of these processes is designed to protect neuronal integrity and promote neuronal repair, respectively, but in fact can be directly injurious when apoE4 is expressed in neurons. Indeed, apoE4 expression is associated with poor clinical outcome or accelerated/more severe progression in numerous neurological disorders. This finding strongly suggests that while apoE participates in general cellular pathway(s) designed to respond adaptively to various environmental, metabolic, or genetic stimuli, its expression can also increase the likelihood of neurodegeneration. Several pathways for this have been suggested.

ApoE is known to exhibit isoform-specific effects on blood-brain barrier (BBB) integrity in mouse models (Bell et al., 2012). In either target replacement mice or glial fibrillary acidic protein promoter transgenic mice, apoE4 expression increases the BBB's susceptibility to injury by activating the proinflammatory cytokine cyclophilin A in pericytes and triggering the NF- κ B/matrix metalloproteinase 9 pathway. Interestingly, BBB breakdown is independent of A β . The subsequent neuronal damage that occurs appears to result from the leakage of blood-derived proteins—including immunoglobulin G, thrombin, and fibrin—into the brain. The extravasation of blood proteins has been implicated in the activation of numerous neurotoxic pathways. For example, in a mouse model of multiple sclerosis, fibrinogen leakage and fibrin deposition in the brain activates microglia and leads to

neuropathology (Adams et al., 2007; Akassoglou et al., 2004; Davalos and Akassoglou, 2012). As will be discussed, BBB leakage could represent an additional insult linked to neuronal injury and the induction of neuronal apoE synthesis and neurotoxicity.

ApoE is also involved in maintaining and regulating synaptic activity and strength in knock-in mice and hippocampal slices. Specifically, apoE4 reduces neuronal cell-surface expression of the apoE receptor 2, as well as the *N*-methyl-D-aspartate and AMPA receptors, by sequestering them in an intracellular compartment (Chen et al., 2010). It is postulated that the apoE isoform-specific effect on lipoprotein and glutamate receptor trafficking contributes to AD neuropathology by impairing synaptic activity. In addition, other LDL receptor family members have been implicated in AD, owing to their roles in modulating the intracellular trafficking and processing of the amyloid precursor protein (Cam and Bu, 2006).

The effects of astrocyte-derived apoE in the brain are a point of ongoing study, and it remains to be described whether astrocyte-derived apoE impacts neuronal health and pathology differently from the apoE that is synthesized within neurons. The remainder of this review will describe how induction of neuronal apoE (apoE4 > apoE3 > apoE2) in response to injury sets the stage for neuropathology and subsequent neurodegeneration.

The ApoE Hypothesis: Relationship between ApoE Structure, Function, and Expression in Health and Disease

The apoE hypothesis posits that apoE genotype sets the stage for neuropathology in an isoform-dependent manner (apoE4 > apoE3 > apoE2), and “second hits” that directly induce neuronal injury or stress initiate a pathological response to injury when apoE4 is synthesized in neurons (Huang, 2010; Huang and Mucke, 2012; Mahley and Huang, 2012; Mahley et al., 2006). With respect to AD, these second hits could include aging, ischemia, trauma, inflammation, oxidative stress, or toxins like the A β peptide and its different assemblies. TBI causes direct damage to neurons, whereas following stroke the second hit may be ischemia. Given the nature of many neurological diseases, where multiple pathologies occur over a protracted period, the possibility for second hits is very high. Other genetic disorders and metabolic disturbances, such as diabetes, can also be injurious factors that contribute to apoE4's neurotoxic effects.

In response to injury, neurons induce the synthesis of apoE, presumably to participate in lipid transport and redistribution for membrane repair and remodeling. However, because of varying degrees of structural instability and tendency to assume domain interaction across the apoE isoforms (which we discuss in greater detail below), apoE can be recognized as structurally abnormal by neurons and undergo proteolytic cleavage (apoE4 > apoE3 > apoE2). The neurotoxic fragments that are generated cause mitochondrial dysfunction and cytoskeletal alterations. In the sections to follow, we describe in more detail the data supporting the apoE hypothesis (Figure 1).

As mentioned previously, in the brain apoE is primarily synthesized by astrocytes under normal physiological conditions (Mahley, 1988) and the neuropathological effects of astrocyte-derived apoE are a point of ongoing study. However, apoE can also be produced by

neurons under pathological conditions resulting from neuronal cell injury or stress (Huang, 2010; Huang and Mucke, 2012; Mahley et al., 2006). Xu et al. (2006) established an enhanced green fluorescent protein (EGFP)_{apoE}-reporter mouse model in which EGFP was inserted into one allele of the apoE gene to serve as a reporter of apoE expression. The hippocampus of a normal, uninjured mouse brain showed abundant EGFP expression in astrocytes, but little expression of EGFP in neurons, indicating no or very low neuronal apoE production (Figure 2). However, following kainic acid-induced excitotoxic injury, apoE production was markedly increased in damaged hippocampal neurons, as measured using the EGFP reporter as the readout (Figure 2). In addition, both apoE mRNA and protein were expressed in hippocampal neurons following kainic acid treatment, as shown by in situ hybridization and immunochemistry, respectively (Xu et al., 2006).

Further studies revealed that the mechanism controlling the synthesis of apoE in neurons is unique and poised for rapid protein production (Xu et al., 2008). These studies found that while little apoE protein is seen in uninjured neurons, the apoE gene is still transcribed; however, in these uninjured neurons intron 3 is typically left intact in the transcribed mRNA sequence, leading to its retention and degradation in the nucleus. However, following kainic acid-induced injury, intron 3 was spliced out, resulting in mature apoE mRNA being transferred out of the nucleus for apoE protein production in neurons. In situ hybridization studies in uninjured mouse brains revealed that hippocampal neurons almost exclusively expressed intron 3-containing apoE mRNA, while hippocampal astrocytes expressed the intron 3-lacking apoE transcript in abundance (Xu et al., 2008). Laser-capture microdissection studies in the hippocampus of uninjured mice also revealed the presence of intron 3-containing apoE mRNA; however, after injury there was a dramatic switch in expression to intron 3-lacking, mature apoE mRNA (Figure 3). This phenomenon is unique to neurons, as apoE intron retention has not been observed in other apoE-synthesizing cell types. In addition, astrocyte-conditioned medium can trigger the synthesis of apoE in neurons, revealing an important “cross-talk” between neurons and glia that is likely to operate during an injury response (Harris et al., 2004b). Thus, neurons possess a unique mechanism whereby they are primed for the rapid production of apoE. The splicing and nuclear export pathways that regulate mRNA and protein production operate ubiquitously in eukaryotic cells and are modulated, in part, through stress; however, these pathways remain to be fully understood (Cullen, 2000; Fox and Lamond, 2010; Galy et al., 2004; Prasanth et al., 2005).

Why might injured neurons turn on the synthesis of apoE and appear to be primed for apoE secretion? Lipid metabolism is unique in the brain for two reasons. First, apoE is the only apolipoprotein present in the brain that binds to the LDL receptor or members of the LDL receptor family, which are responsible for delivering cholesterol and other complex lipids to central nervous system cells through receptor-mediated endocytosis (Bu, 2009; Herz and Bock, 2002; Mahley and Rall, 2000; Mahley et al., 2009). Second, complex lipids are almost totally synthesized de novo by the various brain cells and very little of these lipids, including cholesterol, are delivered from the periphery by exchange or uptake of plasma lipoproteins across the BBB (Dietschy and Turley, 2001).

The redistribution of complex lipids for membrane repair and other metabolic roles undoubtedly relies on apoE through a process termed secretion-capture (Ji et al., 1994; Mahley and Ji, 1999; Mahley et al., 2009), in which secreted apoE scavenges lipids from the local environment and targets them to cells requiring lipids for normal metabolism or membrane repair. The secretion-capture role for apoE was first demonstrated in peripheral nerve injury and regeneration (Boyles et al., 1989; Ignatius et al., 1987; Mahley, 1988) and later in the CNS following hippocampal injury (Poirier et al., 1991). When the sciatic nerve was injured, macrophages responding to the injury rapidly began secreting very large quantities of apoE (200-fold over the level seen in the uninjured nerve) and “capturing” the lipids in the local environment of the injured nerve. ApoE–lipid complexes were shown to be delivered to the growth cones of the regenerating nerves and to Schwann cells for myelin formation through lipoprotein receptor uptake. The secretion-capture process has been further established in the liver, where apoE captures lipoproteins and targets them for receptor-mediated uptake. In fact, apoE secreted by hepatocytes and macrophages has been shown to bind to cell-surface heparan sulfate proteoglycans where it is available to capture lipids and lipoproteins; the heparan sulfate proteoglycans themselves acting as a receptor or part of a receptor complex (Ji et al., 1994; Mahley and Ji, 1999). Thus, apoE secreted by injured neurons may be serving this critical role in lipid redistribution in the repair process. Alternatively, or in addition, apoE may have a role in cell signaling, as has also been suggested (Hayashi et al., 2007; Herz and Bock, 2002).

Although apoE may play an important role in repairing damaged neuronal membranes, it is also associated with neurodegeneration. This assertion is supported by a vast array of structural, molecular, cellular, and behavioral data showing that the three isoforms of apoE display key variations in their protein structure and stability that, in turn, differentially impact neuropathology.

Structural Differences among the Isoforms Set the Stage for Neuropathology

The single amino acid interchanges that distinguish the apoE isoforms result in differences in protein stability as well as the propensity to display a unique structural property called domain interaction (Dong et al., 1994; Huang, 2010; Mahley et al., 2006; Zhong and Weisgraber, 2009). ApoE2 has a cysteine residue at position 158 whereas apoE3 and apoE4 each have arginine. While this substitution in apoE2 results in defective lipoprotein-receptor binding and the development of the lipid disorder type III hyperlipoproteinemia (Mahley, 1988; Mahley et al., 1999; Mahley and Rall, 2000), it also confers greater protein stability, which likely is associated with its protective effect against Alzheimer's disease (AD).

ApoE4, which is strongly linked to AD (Roses, 1996), uniquely possesses an arginine at residue 112, whereas both apoE2 and apoE3 have cysteine at this site. This single amino acid difference in apoE4 is associated with protein instability and domain interaction (Dong et al., 1994; Zhong and Weisgraber, 2009). ApoE3, the most common isoform in humans, has a cysteine residue at position 112 and an arginine at position 158, is more stable than apoE4, and is less likely to display domain interaction than apoE4.

As illustrated in Figure 4, in apoE4 the arginine at residue 112 causes the side chain of arginine-61 to be solvent-exposed, to extend away from the helical bundle in the N-terminal

domain, and to interact with glutamic acid-255 in the C-terminal domain through ionic bonding. Although all isoforms may display domain interaction to some extent, the amino acid substitutions of apoE4 encourage domain interaction much more than the apoE3 and apoE2 isoforms (apoE4 > apoE3 > apoE2). Importantly, this apoE4 property has been established biophysically by fluorescence resonance energy transfer (FRET) and electron paramagnetic resonance spectroscopy (Dong et al., 1994; Hatters et al., 2005; Xu et al., 2004; Zhong and Weisgraber, 2009). The distance between arginine-61 and glutamic acid-255 was ~10 Å in apoE4 and greater than 22 Å in apoE3 (Hatters et al., 2005).

Recently, the nuclear magnetic resonance structure for full-length apoE3 was determined (Chen et al., 2011b). The structure reveals a unique topology, which is postulated to shield the LDL receptor-binding region by the C-terminal domain and prevent binding to the receptor in the non-lipid form. However, in order to prevent tetramer formation and aggregation, it was necessary to introduce five non-conservative mutations into the C-terminal domain (F257A/W264R/V269A/L279Q/V287E). Unfortunately, these rather severe changes undoubtedly alter the C-terminal domain and likely distort the conformation and intramolecular interactions with this domain. Thus, the nuclear magnetic resonance structure may not represent a physiological and functional structure of apoE.

In all, these data show how relatively minor changes in apoE sequence yield significant differences in apoE's ability to promote neuronal health versus neuronal damage, especially when apoE synthesis is increased after injury. The apoE hypothesis therefore suggests that the isoform-dependent neuronal response to increased apoE expression is a critical step in laying the groundwork for future pathology. In the following sections, we describe the apoE4-specific effects on neuronal function and how they affect neuronal integrity at the cellular level.

Impaired Neuronal Intracellular Trafficking of ApoE (ApoE4 > ApoE3)

Fluorescence recovery after photobleaching experiments revealed impaired intracellular trafficking of apoE4 through the ER and Golgi apparatus in cultured cells (Brodbeck et al., 2011). Neuro-2a cells expressing EGFP-apoE3 or EGFP-apoE4 were photobleached with an argon laser, and the extent and rate of recovery of fluorescence were used as a measure of transit through the secretory pathway. Although the trafficking of apoE4 through the ER and Golgi apparatus was significantly impaired compared with apoE3 (Figure 5A), blocking domain interaction by site-directed mutagenesis (i.e., mutation of arginine-61 to threonine) or by exposure to small-molecule structure correctors restored normal trafficking properties to apoE4 (Figure 5B and 5C) and led to decreased neurotoxic fragment formation. These domain interaction-blocking approaches will be discussed in more detail below. Thus, it is envisioned that 1) the impaired transit of apoE4 occurs because of its abnormal structure, because blocking domain interaction restores the transit, 2) the abnormal structure and trafficking likely target the protein for proteolysis, and 3) small-molecule structure correctors likely target apoE as it is synthesized or soon after entering the ER lumen. Such findings suggest that one way to resolve the negative effects of apoE4 expression is to convert apoE4's structure to be more apoE3-like.

ApoE (ApoE4 > ApoE3) Susceptibility to Neuron-Specific Proteolysis and the Generation of Neurotoxic Fragments

The cellular mechanisms and organelles that promote the clearance of abnormally folded proteins are ubiquitous, and abnormal forms of apoE, especially apoE4, can indeed be targeted for proteolysis. In fact, neurotoxic fragments are generated only by neurons, and not by astrocytes or other apoE-synthesizing cells (Brecht et al., 2004; Harris et al., 2003; Huang et al., 2001). Why, then, are neurons less effective than other cell types at completely degrading and clearing misfolded apoE? It is possible that, because apoE is an avid lipid-binding protein, lipid-based interactions may protect some domains from proteolytic cleavage, thus resulting in the accumulation of a spectrum of neurotoxic fragments. While full-length apoE is 34 kDa, a fragment pattern of bands ranging from 29–30 kDa to 12–14 kDa is consistently seen in extracts from cultured neurons expressing apoE4, apoE4 transgenic mice, and in the brains and cerebrospinal fluid from humans with AD (Brecht et al., 2004; Harris et al., 2003; Huang et al., 2001; Jones et al., 2011). Furthermore, more of these fragments are observed in AD patients expressing the apoE4 allele compared with normal, non-demented apoE4-carrying humans (Figure 6) (Harris et al., 2003; Jones et al., 2011).

Although the unique protease that is responsible for apoE4 fragmentation remains to be identified, it is thought to be a chymotrypsin-like serine protease (Harris et al., 2003). This protease, most likely residing in the ER or Golgi apparatus, generates the unique series of fragments ranging from 29–30 kDa to 12 kDa (Huang, 2010; Huang and Mucke, 2012; Mahley et al., 2006). The 29–30-kDa fragments result from cleavage at methionine-272 and leucine-268, respectively, and subsequent cleavage results in the generation of smaller fragments, primarily in the 12–20 kDa range. All of the neurotoxic fragments lack the C-terminal 27–30 amino acids and contain the lipid-binding region of apoE (residues 240–270) (Brecht et al., 2004; Chang et al., 2005; Harris et al., 2003; Huang et al., 2001; Jones et al., 2011). In addition to containing the lipid-binding region, the neurotoxic fragments also contain the LDL receptor-binding region of apoE (residues 136–150). The secondary cleavage events remove varying lengths of peptide from the N terminus. As mentioned above, these fragments are generated in the ER or Golgi apparatus, and yet many of their effects are seen in the cytosol. The cleavage of the C terminus allows this translocation and several of the subsequent cytosolic effects.

ApoE Fragments Escape the Secretory Pathway and Enter the Cytosol

How do the apoE4 fragments generated by neuron-specific proteolysis leave the ER or Golgi compartments and enter the cytosol? Cleaving off the C-terminal 27–30 amino acids exposes specific regions of apoE that are not accessible in the intact protein. This allows for apoE4 translocation into the cytosol, thereby facilitating mitochondrial localization and causing neurotoxicity (Chang et al., 2005). However, deletion of the lipid-binding region (residues 240–270) in a fragment encompassing residues 1–191 did not inhibit translocation into the cytosol, but this fragment also did not interact with mitochondria or cause neurotoxicity. Finally, removal of the portion of apoE that includes the LDL receptor-binding region (residues 136–150) prevented translocation (Figure 7), as did mutations of critical arginine and lysine residues in this region (Chang et al., 2005).

These studies show that a minimal structure supporting translocation, mitochondrial localization, and neurotoxicity requires the presence of both the receptor- and lipid-binding regions of apoE (Chang et al., 2005). The charged arginine and lysine residues in the 136–150 region are critical for translocation, a region that is similar to the protein-translocation domains of other proteins, including viral proteins. The hydrophobicity of the lipid-binding region (residues 240–270) is certainly involved in mitochondrial interaction and subsequent neurotoxicity, because mutation of critical conserved residues in this region, or deletion of this region altogether, blocked mitochondrial localization. Importantly, these truncation variants generated in the laboratory are likely counterparts to the spectrum of toxic fragments observed in the brain (Figure 6) and cerebrospinal fluid of human AD patients, making the results highly relevant to our understanding of human AD pathology.

ApoE4-Associated Neuronal Mitochondrial Dysfunction

Mitochondrial dysfunction is a hallmark of several neurodegenerative diseases, including AD (Atamna and Frey II, 2007; Parihar and Brewer, 2007). Functional brain imaging with fluorodeoxy-glucose (^{18}F), a radiotracer used to study glucose utilization as a surrogate measure of mitochondrial activity, revealed that the cerebral metabolic rate was reduced in both AD patients and in cognitively normal apoE4 carriers in their 20s and 30s, several decades before the typical onset of dementia (Reiman, 2007; Reiman et al., 2004). Gene-expression profiles in postmortem human brain tissue showed a significant association between apoE genotype and the expression levels of multiple mitochondrial respiratory enzymes (Conejero-Goldberg et al., 2011), specifically, a reduction in the expression of electron transport-chain genes in apoE4 carriers (Liang et al., 2008). These results suggest that in humans, apoE genotype can influence brain metabolism and possibly mitochondrial function, and that alterations in these processes may be linked to the onset and/or progression of AD.

We have shown that primary neurons from apoE4 transgenic mice had reduced levels of numerous mitochondrial respiratory enzymes compared with apoE3-expressing cells (Chen et al., 2011a). However, there were no differences in the mitochondrial complexes in astrocytes expressing apoE4 driven by the glial fibrillary acidic protein promoter, revealing that apoE4's effects are neuron-specific. In addition, apoE4 expression in Neuro-2a cells led to reduced levels of mitochondrial complexes I, IV, and V, as well as a reduction in functional respiratory capacity (Chen et al., 2011a). The addition of an uncoupling agent led to a 98% increase in the maximal oxygen-consumption rate in apoE3-expressing cells compared with only a 50% increase in the apoE4-expressing cells (Chen et al., 2011a).

In cells transfected with the apoE4 variant lacking the C-terminal 27 amino acids, the fragment was found to be expressed in a granular distribution that localized preferentially to mitochondria (Chang et al., 2005); this led to mitochondrial dysfunction, as demonstrated by loss of membrane integrity and electropotential. Again, the active fragments that altered mitochondrial membrane electropotential contained the receptor- and lipid-binding regions. Nakamura et al. (2009) demonstrated that apoE4 lacking the C-terminal 27 amino acids bound directly to several components of the electron transport-chain enzymes and reduced respiratory activity. Likewise, apoE4, and especially the apoE4 fragments, have been shown

to impair mitochondrial dynamics and synaptogenesis (Brodbeck et al., 2011; Chen et al., 2012). For example, mitochondrial motility was reduced by 35% and 57% by apoE4 and apoE4 fragments lacking the C-terminal 27 amino acids, respectively, in transfected PC12 cells. Furthermore, dendritic spine densities were reduced in apoE4-expressing primary neurons by 26% and in apoE4 fragment-expressing neurons by 46%, compared with apoE3 (Brodbeck et al., 2008). These abnormalities, which could be related to mitochondrial dysfunction, were domain-interaction dependent, as these effects could be reversed by either blocking domain interaction by site-directed mutagenesis (apoE4-R61T) or by treatment with small-molecule structure correctors.

How and where the apoE fragments interact with mitochondria remains to be determined. As discussed, the mitochondrial binding of apoE fragments requires the absence of the C terminus and the presence of the lipid-binding region. The unique lipid composition of mitochondria could allow the fragments to interact directly with the mitochondrial surface through a hydrophobic lipid interaction. On the other hand, there are numerous mitochondrial outer membrane proteins, the function of which could be altered by an interaction with apoE fragments. For example, apoE fragment interaction with the voltage-dependent anion channel (also known as mitochondrial porin), which controls the entry and exit of mitochondrial metabolites, could disrupt multiple functions ascribed to this channel (Shoshan-Barmatz et al., 2010).

A link between apoE and a specific mitochondrial protein has been suggested. In AD patients, Roses (2010) demonstrated an age-of-onset-associated polymorphism in the translocase of the outer mitochondrial-membrane (*TOMM40*) gene, which is in the region of the apoE locus and is in strong linkage disequilibrium. Variable-length poly-T polymorphisms appear to alter the age-of-onset of AD. For example, apoE3, in the context of the longer *TOMM40* poly-T repeats, is associated with an earlier age-of-onset than apoE3 individuals with shorter repeats. Such polymorphisms could modulate the apoE isoform-specific effects on AD. *TOMM40* is a part of the mitochondrial machinery that controls protein translocation into the mitochondria (Kutik et al., 2007; Pfanner and Wiedemann, 2002; Rapaport, 2005). Specific pre- or internal sequences within a protein, or interactions with transfer chaperones—such as HSP90- and HSP70-class chaperones—participate in the recognition and translocation of proteins into the mitochondria. It has been postulated that apoE-*TOMM40* protein interactions may alter mitochondrial function, possibly causing cytochrome c release and apoptosis (Roses, 2010). This observation has been confirmed in some studies but not in others (Cruchaga et al., 2011; Maruszak et al., 2012). In fact, a recent large study of more than 11,000 AD patients and 10,000 cognitively normal controls from 15 genome-wide association studies demonstrated that the apoE alleles ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) accounted for essentially all the risk and age-of-onset of AD (Jun et al., 2012). The inherited susceptibility was not associated with neighboring genes, including *TOMM40* and apoC1. These data suggest that the genetic role of *TOMM40* should be reassessed; thus, the mechanism whereby apoE alters mitochondrial function remains to be determined.

ApoE-Associated Alterations in the Neuronal Cytoskeleton

The neuronal cytoskeleton is composed of microtubules, neurofilaments, and microfilaments. Microtubules, polymeric structures composed of α - and β -tubulin, are critical for neurite extension and organelle trafficking, including the distribution of mitochondria to the sites of newly forming synapses. They are associated with a heterogeneous set of microtubule-associated proteins, including tau, that modulate their structure and function. Until recently, the major function of tau was thought to be its ability to stabilize microtubules (Lee et al., 2001; Morris et al., 2011; Sydow et al., 2011). Now, it appears that its role may be more dynamic, possibly participating in intracellular signal transduction, among other roles. Importantly, tau is highly susceptible to hyperphosphorylation and the formation of intracellular NFTs, both of which are hallmark neuropathologies that critically promote the damage observed in AD and TBI (McKee et al., 2009; Morris et al., 2011).

ApoE4 Alters Neuronal Microtubules and Impairs Neurite Outgrowth—ApoE4 has been shown to directly alter microtubule structure and to stimulate tau hyperphosphorylation and NFT formation (Huang, 2010; Huang and Mucke, 2012). Exposure of neuronal cultures to exogenous apoE4-lipid complexes caused significant cytoskeletal disruption and impaired neurite outgrowth compared with apoE3-containing complexes (Bellosta et al., 1995; Nathan et al., 1994; Nathan et al., 1995). ApoE4-treated Neuro-2a cells also had fewer intracellular microtubules, as identified by immunocytochemical localization of β -tubulin and by electron microscopy (Nathan et al., 1995), and these effects were associated with impaired neurite outgrowth in apoE4-treated cells. These results were replicated in apoE3- or apoE4-transfected Neuro-2a cells expressing nanogram quantities of apoE (Bellosta et al., 1995).

ApoE4 Stimulates Tau Phosphorylation and NFT-like Formation—Increased tau phosphorylation has been observed in transgenic mice expressing apoE4 in neurons, but not in those expressing apoE4 in astrocytes, suggesting a cellular source-dependent effect of apoE4 on tau phosphorylation (Harris et al., 2003; Tesseur et al., 2000), occurring in parallel with the generation of apoE fragments in neurons (Andrews-Zwilling et al., 2010; Harris et al., 2003). There is evidence that apoE4 stimulates tau phosphorylation by activating the extracellular signal-regulated protein kinase pathway in the hippocampus (Harris et al., 2004a), although other signaling pathways may also be involved. Intraneuronal phospho-tau inclusions are prominent in the hippocampus and form NFT-like structures composed of apoE4, phosphotau, and neurofilaments; by electron microscopy, these inclusions are visualized as tightly packed, straight filaments that closely associate with mitochondria (Harris et al., 2003). In addition to such insights into the cell biological impacts of apoE fragments, transgenic animal experiments are uncovering how such neurotoxicity at the cellular level corresponds to neuronal function and behavior.

Neurotoxic Effects of ApoE Fragments

The expression of truncated apoE4 in transgenic mice provides insights into how and where the fragments cause neurotoxicity. Transgenic mice expressing a variant of apoE4 that lacks the C-terminal 27 amino acids [apoE4(1–272)], driven by the Thy1.2 promoter, had

significant hippocampal neurodegeneration and neuronal loss (Harris et al., 2003). However, not every fragment of apoE is toxic, because expression of apoE4(1–240)—the form lacking the lipid-binding region—was not found to trigger hippocampal neurodegeneration. The loss of hippocampal neurons in the apoE4(1–272) mice was found to correlate with impaired learning and memory behaviors as early as 6 months of age, as assessed by the Morris water maze test (Andrews-Zwilling et al., 2010; Harris et al., 2003). Likewise, when full-length apoE4 was specifically expressed in neurons (NSE-apoE4 transgenic mice), these mice displayed early-onset impaired learning and memory (Buttini et al., 1999; Raber et al., 1998; Raber et al., 2000) that correlated with multiple neuropathological effects, including loss of synaptic connections and neurodegeneration (Buttini et al., 1999). These effects are likely mediated through apoE4 fragment generation (Brecht et al., 2004). In addition to neurodegenerative changes in the brain, the apoE4(1–272) mice display other pathologies, including neurofibrillary tangle (NFT)—structures in the hippocampus rich in hyperphosphorylated tau, which were elevated by about sixfold over levels seen in nontransgenic mice (Andrews-Zwilling et al., 2010; Harris et al., 2003).

ApoE Fragments Induce Neuropathology in Specific Neuronal Subtypes

Andrews-Zwilling et al. (2010) showed that apoE4 knock-in mice display an age-dependent decrease in GABAergic interneurons selectively in the hilus of the hippocampus, and that this decrease was associated with impaired learning and memory behaviors in these mice. Transgenic expression of an apoE4 truncation mutant lacking the C-terminal 27 amino acids led to an even more marked decrease in GABAergic interneuron levels, along with pronounced hyperphosphorylation of tau in the hippocampus (Andrews-Zwilling et al., 2010). Studies by Li et al. (2009) have also revealed a link between apoE4 expression and the impaired generation of new neurons, demonstrating that apoE4 knock-in mice have reduced hilar GABAergic interneurons, leading to impaired hippocampal neurogenesis. Based on these data, it has been postulated that the parallel decreases in interneuron levels and hippocampal neurogenesis are responsible for the behavioral impairments seen in apoE4 transgenic mice (Andrews-Zwilling et al., 2010; Huang and Mucke, 2012; Li et al., 2009). In support of this hypothesis, optogenetic manipulations of hilar GABAergic interneurons confirmed that functional inhibition of this specific neuronal population results in spatial learning and memory deficits, as seen in apoE4 knock-in mice (Andrews-Zwilling et al., 2012).

These effects on hippocampal GABAergic neurons and memory behaviors are consistent across studies, but nonetheless reveal interesting differences depending on whether apoE4 is expressed selectively in neurons versus ubiquitously in the brain. For example, apoE4 knock-in mice showed impaired learning and memory behaviors, but at a later time point (16 months of age) compared with neuron-specific (i.e., Thy1.2- or NSE-promoter-driven) apoE4 mice (Andrews-Zwilling et al., 2010). Although not directly proven, it is reasonable to speculate that the delayed impairment in the apoE4 knock-in mice reflects the age-related and comparatively delayed accumulation of neurotoxic fragments in neurons, while neuron-specific apoE-expressing mice accumulate these fragments much earlier.

Having uncovered many of the isoform-specific detrimental effects of apoE, new findings examine whether such effects can be reversed by correcting apoE4's abnormal protein structure, an approach that can potentially open new avenues for therapeutics.

Domain Interaction In Vitro and In Vivo

Interestingly, all animal species except humans have threonine at the site equivalent to residue 61 (Mahley and Rall, 2000; Weisgraber, 1994; Zhong and Weisgraber, 2009). Lower species do not display isoforms, and almost all—including mice and rats—have an arginine at the site equivalent to residue 158, making it human apoE4-like in sequence. However, because these apoE orthologs expressed in lower species lack an arginine-61 equivalent, they also lack domain interaction. Thus, they are not equivalent to either apoE3 or apoE4 structurally or functionally (Mahley and Rall, 2000; Weisgraber, 1994; Zhong and Weisgraber, 2009).

Raffai et al. (2001) created a “humanized” apoE mouse line, in which the threonine located at the residue equivalent to 61 in human apoE was replaced by gene targeting with an arginine, thus allowing mouse apoE to have domain interaction. Importantly, these mice developed some characteristics resembling human apoE4 knock-in mice, including loss of synaptic marker immunolabeling in the hippocampus and a mild memory deficit in the Morris water maze test (Zhong et al., 2008).

We identified several small-molecule structure correctors that block apoE4 domain interaction using cellular FRET assays (Chen et al., 2012; Mahley and Huang, 2012). Additional assays were also used to establish the downstream functional effects of blocking apoE4 domain interaction with structure correctors (Brodbeck et al., 2011; Chen et al., 2011a; Chen et al., 2012).

The FRET assay to measure domain interaction relied on the creation of human apoE variants tagged at both the N and C termini with fluorescent constructs. When we tagged either apoE3 or apoE4 with yellow fluorescent protein (YFP) at the N terminus and cyan fluorescent protein (CFP) at the C terminus and transfected these constructs into Neuro-2a cells, we observed significantly enhanced FRET signal in the apoE4-expressing cells compared with their apoE3-expressing counterparts (Figure 8) (Xu et al., 2004), confirming that this FRET approach can detect apoE4 domain interaction, and that domain interaction is a property of apoE4 that occurs intracellularly.

In a complementary set of assays (Chen et al., 2012), apoE4 was instead tagged with GFP at the N terminus and *E. coli* dihydrofolate reductase (eDHFR) at the C terminus. The construct was expressed in Neuro-2a cells, and an eDHFR high-affinity ligand (trimethoprim) conjugated with hexachlorofluorescein served as the acceptor fluorophore. The GFP-apoE4-eDHFR gave a significantly higher FRET signal than the apoE3 construct, reflecting a closer proximity of the N- and C-terminal domains in apoE4 (Chen et al., 2012). Furthermore, a screen for small molecules capable of blocking domain interaction identified several that dose-dependently decreased the FRET signal (Figures 9A and 9B) (Chen et al., 2012), serving as proof-of-concept that apoE4 is a promising target for the development of small molecule-based therapeutics.

Detrimental Effects of ApoE4 Can Be Reversed by Blocking Domain Interaction

Blocking domain interaction in apoE4 reverses many of its detrimental effects, both in vitro and in vivo (Mahley and Huang, 2012). This can be accomplished by site-directed mutagenesis in which arginine-61 is exchanged for threonine, thereby preventing the ionic interaction, or by small-molecule structure correctors that interact with apoE in the vicinity of arginine-61 to prevent or retard domain interaction.

Importantly, blocking domain interaction by site-directed mutagenesis or small-molecule structure correctors markedly reduced proteolysis and fragment formation. Mitochondrial dysfunction was no longer observed in cells expressing an apoE4 variant that lacked the ability to undergo domain interaction (apoE4-R61T). Furthermore, a small-molecule structure corrector restored the level of complex IV mitochondrial cytochrome c oxidase in apoE4-expressing cells to levels seen in apoE3-expressing cells (Figure 9C) (Chen et al., 2012). These studies were expanded to identify potent apoE4 structure correctors that could restore the level of mitochondrial cytochrome c oxidase with the potential to be used in vivo. A class of such small-molecule compounds that displays a significant structure-activity relationship has been identified (Chen et al., 2012).

As described, blocking apoE4 domain interaction restores neurite outgrowth, mitochondrial motility, and synaptic density (Brodbeck et al., 2011; Chen et al., 2011a). Thus, apoE4 domain interaction is a critical structural element that modulates both the physiological and pathophysiological functions of apoE4 (Mahley and Huang, 2012).

Hypothesis: ApoE Sets the Stage and Response to Neuronal Injury Triggers Neuropathology

The studies reviewed here, which comprise only a subset of the work done on apoE4 in the central nervous system, overwhelmingly point to a critical direct role for apoE4 in AD-mediated neurodegeneration. Based upon these studies, we propose the following model (Figure 10) to illustrate this hypothesis.

Figure 10 (1): What is well established is that neuronal injury or stress, caused by a variety of injurious agents, induces the synthesis of apoE by neurons. The structural properties of each apoE isoform dictate its propensity to undergo domain interaction (apoE4 > apoE3 > apoE2), which leads to apoE isoform-dependent proteolysis and the generation of neurotoxic fragments. In turn, these fragments cause mitochondrial dysfunction and cytoskeletal alterations, leading to neurodegeneration (Huang, 2010; Huang and Mucke, 2012; Mahley et al., 2006).

Figure 10 (2): What is clearly more speculative is whether there is a direct effect of exogenous apoE4, i.e., astrocyte-derived apoE, on neurons and neuropathology. In some studies, exogenous apoE4 can have direct detrimental effects on neurons, e.g., by inhibiting neurite outgrowth and mitochondrial motility (Chen et al., 2012; Nathan et al., 1994). Hartman et al. (2001) demonstrated that apoE4, when produced by astrocytes, impaired working memory but did not induce AD-like neuropathology in mice. Whether internalized apoE that is sequestered in the endosomes escapes lysosomal degradation, is transferred to the ER/Golgi apparatus compartment, and undergoes proteolysis and toxic fragment

formation—or whether exogenous apoE4 acts through another mechanism independent of intracellular apoE fragment generation—is unknown. For example, in hepatocytes and macrophages, it has been established that apoE can escape lysosomal degradation and re-enter the secretory pathway (Farkas et al., 2003; Fazio et al., 2000; Zhu et al., 2005). It is possible that the detrimental effects of apoE4 observed by Chen et al. (2010) in neurons reflect the sequestration of apoE4 in endosomes and recycling to the ER/Golgi apparatus, in addition to its effects on *N*-methyl-D-aspartate receptors. Alternatively, astrocyte-derived apoE4 might directly damage the integrity of the BBB, allowing blood-derived proteins to enter the brain and injure neurons, and setting up the detrimental response to injury (Akassoglou et al., 2004; Bell et al., 2012). What is not speculative, however, is that astrocytic apoE4 can negatively impact A β clearance and/or deposition (Bien-Ly et al., 2011; Castellano et al., 2011; Kim et al., 2011) and thus induce the apoE neurotoxic pathway.

Figure 10 (3): Although much is yet to be learned about the regulation of A β and its potential roles in normal neuronal biology, it appears that A β production can be stimulated by injury to neurons through oxidative stress (Misonou et al., 2000; Paola et al., 2000; Tamagno et al., 2002) and energy deprivation (O'Connor et al., 2008; Velliquette et al., 2005). For example, exposure to low concentrations of 4-hydroxy-2,3-nonenal increased intracellular A β production by 2–6-fold in NT2-differentiated cells (Paola et al., 2000). Furthermore, glucose deprivation in cultured cells and in vivo in transgenic mice induces the phosphorylation of eIF2 α (a translation initiation factor), which increases BACE1 activity (the rate-limiting enzyme involved in A β production) and elevates A β levels (O'Connor et al., 2008). In addition, it is well established that A β injures neurons (Huang and Mucke, 2012; Palop and Mucke, 2010; Selkoe, 2011), which, based on our hypothesis, would stimulate apoE production, induce apoE neurotoxic fragment formation, and further perpetuate the toxic cycle.

Conclusion

Although much remains to be understood about how apoE function affects both health and disease states, it is clear that apoE plays a critical role in the pathogenesis of many different neurodegenerative diseases. This assertion is no surprise, given apoE's fundamental role in cellular lipid transport and metabolism. However, its widespread expression also highlights how apoE is exceptionally well poised to induce or accelerate neuronal damage in apoE4-carrying individuals. The significant risk posed by apoE4 expression, combined with its widespread presence in the population and the ever-increasing average lifespan in which apoE4 carriers may suffer from its detrimental effects—in AD, TBI, and possibly other neuropathological disorders—underscore the enormous value that can come from developing therapies to counter its neurotoxic effects.

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Abbreviations used

AD	Alzheimer's disease
Aβ	amyloid β
apo	apolipoprotein
BBB	blood–brain barrier
eDHFR	<i>E. coli</i> dihydrofolate reductase
CFP	cyan fluorescent protein
CNS	central nervous system
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
LDL	low density lipoproteins
NFT	neurofibrillary tangle
TBI	traumatic brain injury
YFP	yellow fluorescent protein

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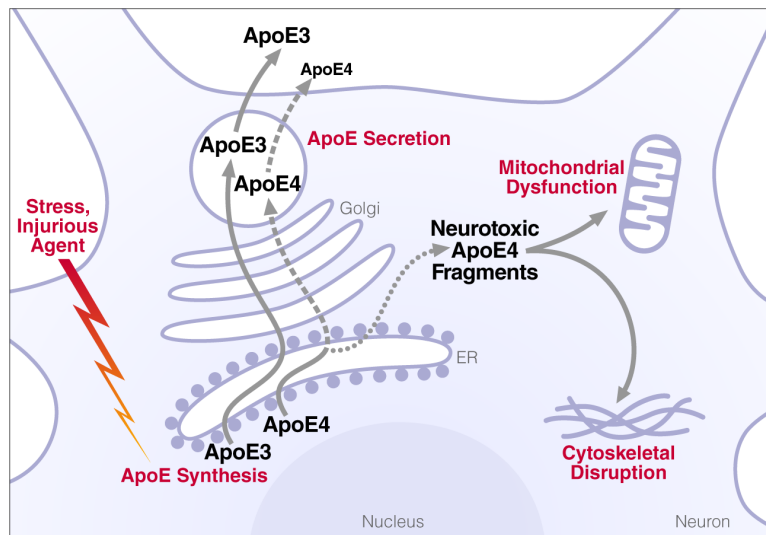


Figure 1. Injurious Agents That Stress or Damage Neurons Induce ApoE Synthesis and Initiate Neuropathology

ApoE4, because of domain interaction, displays impaired trafficking through the ER and Golgi apparatus compared with apoE3. As a result, it is targeted to a neuron-specific protease that initially cleaves off the C-terminal 27–30 amino acids, generating neurotoxic fragments. These fragments escape the secretory pathway and enter the cytosol, where they cause mitochondrial dysfunction and cytoskeletal alterations, enhance tau phosphorylation, and form NFT-like structures (Mahley et al., 2006). Figure reprinted with permission from Mahley and Huang, Small-molecule structure correctors target abnormal protein structure and function: The structure corrector rescue of apolipoprotein E4-associated neuropathology. *J. Med. Chem.* 55: 8997–9008, 2012. Copyright 2012 American Chemical Society.

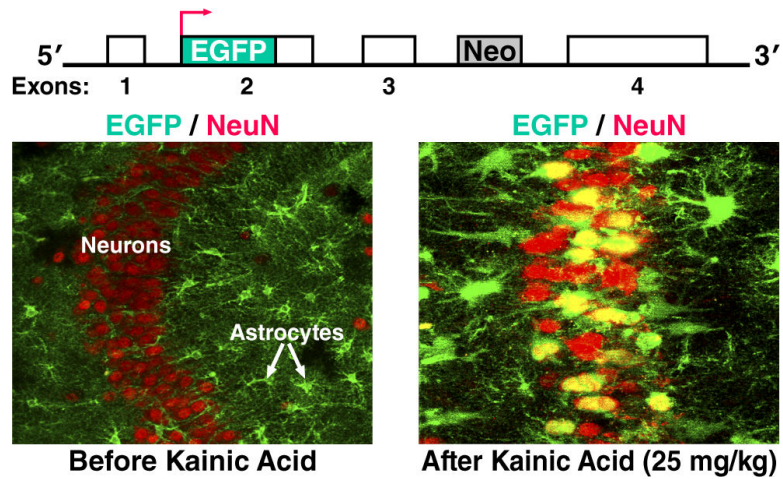


Figure 2. EGFP Inserted into One Allele of the ApoE Gene Acts as a Marker for ApoE Expression in Vivo

Top, schematic of the EGFP-apoE reporter cassette. Bottom, representative images of hippocampal sections taken from EGFP_{apoE}-reporter knockin mice. In the uninjured mouse hippocampus (before kainic acid), astrocytes display abundant apoE synthesis (bottom, left); however, after injury with kainic acid, neurons turn on the synthesis of apoE, as indicated by the colocalization of EGFP (green) and NeuN (red) (bottom, right) (Xu et al., 2006).

Modified from Figure 6, Xu, Q., Bernardo, A., Walker, D., Kanegawa, T., Mahley, R.W., and Huang, Y. Profile and regulation of apolipoprotein E (apoE) expression in the CNS in mice with targeting of green fluorescent protein gene to the apoE locus. *J. Neurosci.* 26: 4985–4994, 2006.

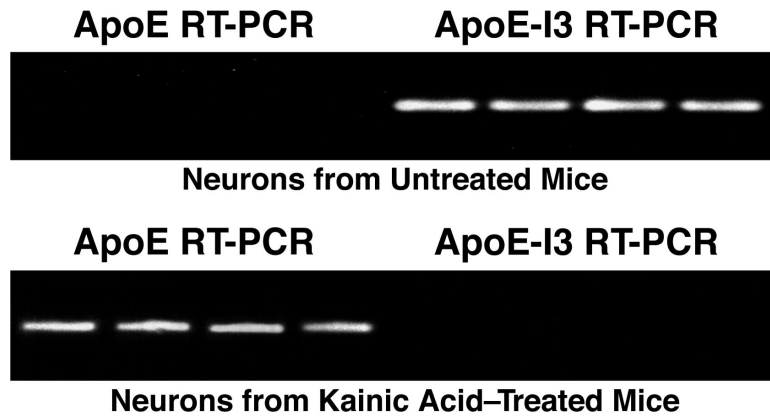


Figure 3. Kainic Acid-Induced Injury Triggers Switch from Intron 3-Containing to Mature ApoE mRNA Synthesis

EGFP_{apoE}-reporter knockin mice were treated or not with kainic acid, and CA1 hippocampal neurons were isolated by laser-capture microdissection for reverse transcription-polymerase chain reaction (RT-PCR). Neurons from a normal, uninjured mouse demonstrate the presence of apoE mRNA with intron 3 (apoE-I3), whereas after injury there is a dramatic switch to mature apoE mRNA lacking intron 3 (Xu et al., 2008). Modified from Figure 7, Xu, Q., Walker, D., Bernardo, A., Brodbeck, J., Balestra, M.E., and Huang, Y. Intron-3 retention/splicing controls neuronal expression of apolipoprotein E in the CNS. *J. Neurosci.* 28: 1452–1459, 2008.

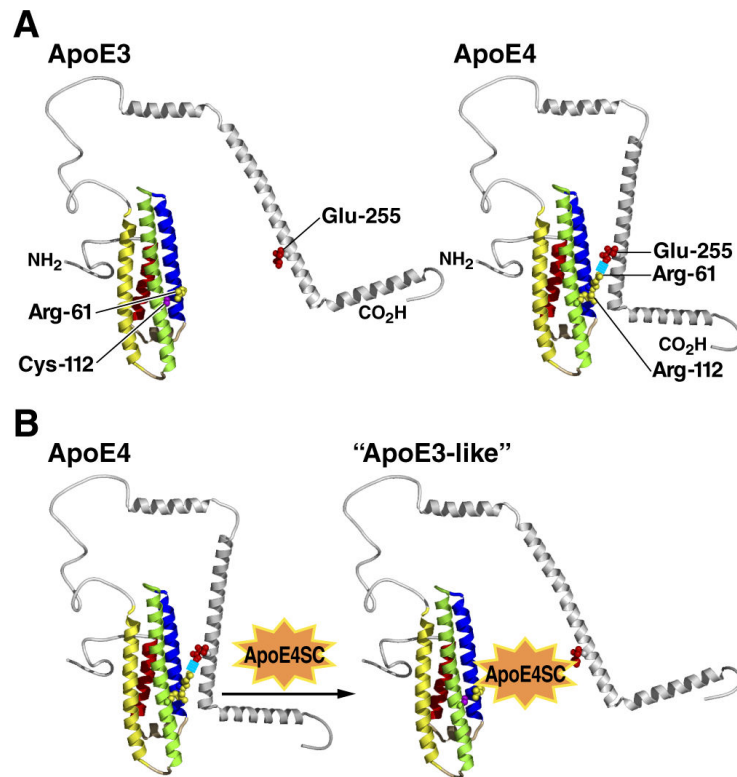


Figure 4. Models of the Structures of ApoE3 and ApoE4

(A) ApoE4 displays a unique property called domain interaction caused by the ionic interaction between arginine-61 in the N-terminal domain with glutamic acid-255 in the C-terminal domain. ApoE3 is significantly less likely to undergo domain interaction than apoE4.

(B) ApoE4 domain interaction can be blocked by a small-molecule apoE4 structure corrector that disrupts the ionic interaction between arginine-61 and glutamic acid-255. This converts apoE4 to an apoE3-like molecule both structurally and functionally (Huang, 2010; Mahley and Rall, 2000; Mahley et al., 2006, 2009). ApoE4SC, apoE4 structure corrector.

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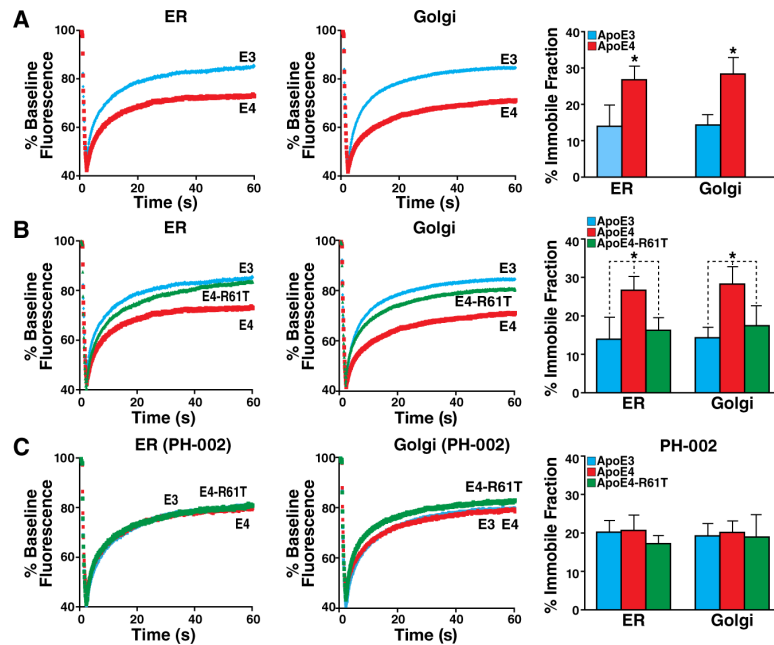


Figure 5. ApoE4 Displays Impaired Trafficking through the Secretory Pathway That Can Be Corrected by Blocking ApoE4 Domain Interaction

Fluorescence recovery after laser photobleaching experiments were performed on EGFP-apoE3- and EGFP-apoE4-expressing Neuro-2a cells. For each section, the left and center panels show representative recovery curves for the ER and Golgi apparatus, respectively, while the right-hand panel shows quantitative histograms of apoE mobility.

(A) Recovery curves show that apoE4 trafficking is decreased compared with apoE3 in both the ER (left) and Golgi apparatus (center). Right, histogram showing that the percent of the immobile fraction was greater with apoE4 than apoE3.

(B) The impaired trafficking of apoE4 was reversed by site-directed mutagenesis of arginine-61, which blocked domain interaction (apoE4-R61T). This mutation reversed the impairment in apoE4 trafficking and mobility in both the ER and Golgi apparatus.

(C) Treatment of apoE4-expressing cells with a structure corrector (PH-002) restored the trafficking of apoE4 through the secretory pathway. The structure corrector did not affect the trafficking of apoE3 or apoE4-R61T (Brodbeck et al., 2011). Modified from Figures 3, 4, 5 originally published in Brodbeck, J., McGuire, J., Liu, Z., Meyer-Franke, A., Balestra, M.E., Jeong, D.-e., Pleiss, M., McComas, C., Hess, F., Witter, D., Peterson, S., Childers, M., Goulet, M., Liverton, N., Hargreaves, R., Freedman, S., Weisgraber, K., Mahley, R.W., Huang, Y. Structure-dependent impairment of intracellular apolipoprotein E4 trafficking and its detrimental effects are rescued by small-molecule structure correctors. *J. Biol. Chem.* 2011; 286:17217–17226. © the American Society for Biochemistry and Molecular Biology.

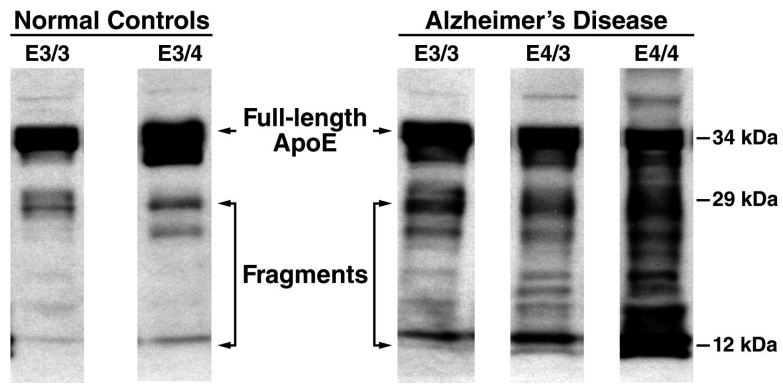


Figure 6. ApoE Fragmentation Pattern in Human Temporal Cortex in Non-Demented Controls and in Age-Matched AD Patients

Compared with full-length apoE (34 kDa), proteolytic cleavage generates an initial fragment with a molecular weight of ~29 kDa. Subsequent to this, fragments of ~12–20 kDa are generated. In AD patients, there is an apoE4 gene-dose effect on apoE fragmentation, whereby apoE4/3 subjects have more fragments than apoE3/3 subjects and the apoE4/4 subjects have the greatest amount of fragments.

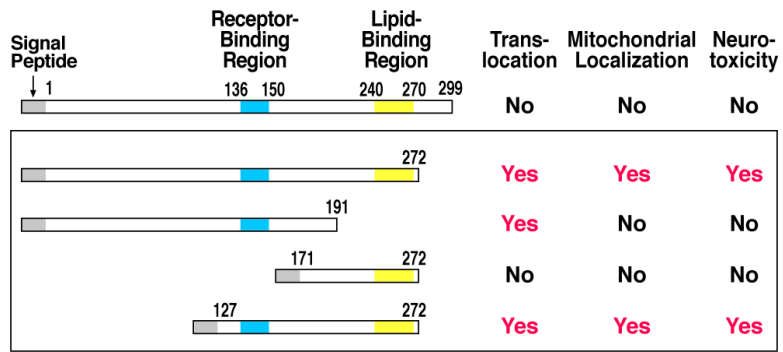


Figure 7. Regions of ApoE Responsible for Fragment Translocation into the Cytosol, Mitochondrial Targeting, and Neurotoxicity

Fragments containing the receptor-binding region (residues 136–150) and the lipid-binding region (residues 240–270) represent the minimal structure required for translocation, mitochondrial localization, and neurotoxicity (Mahley et al., 2006). Modified from Figure 4, Mahley, R.W., Weisgraber, K.H., and Huang, Y. Apolipoprotein E4: A causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 103: 5644–5651, 2006. © by the National Academy of Sciences.

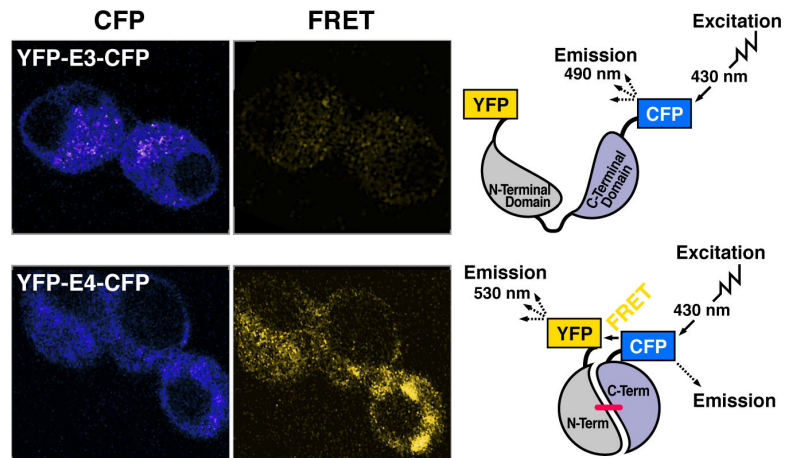


Figure 8. ApoE4-Transfected Cells Demonstrate Intracellular FRET Signal

ApoE constructs were generated to express YFP and CFP at the N and C termini, respectively, to act as reporter FRET fluorophores. When expressed in Neuro-2a cells, YFP-apoE4-CFP displays significantly more FRET signal compared to YFP-apoE3-CFP-expressing cells. This reflects the closer interaction between the N- and C-terminal domains in apoE4 and demonstrates that the biophysical property of domain interaction occurs in cells (Xu et al., 2004). Modified from Figure 1, Xu, Q., Brecht, W.J., Weisgraber, K.H., Mahley, R.W., and Huang, Y. Apolipoprotein E4 domain interaction occurs in living neuronal cells as determined by fluorescence resonance energy transfer. *J. Biol. Chem.* 279: 25511–25516, 2004. © the American Society for Biochemistry and Molecular Biology.

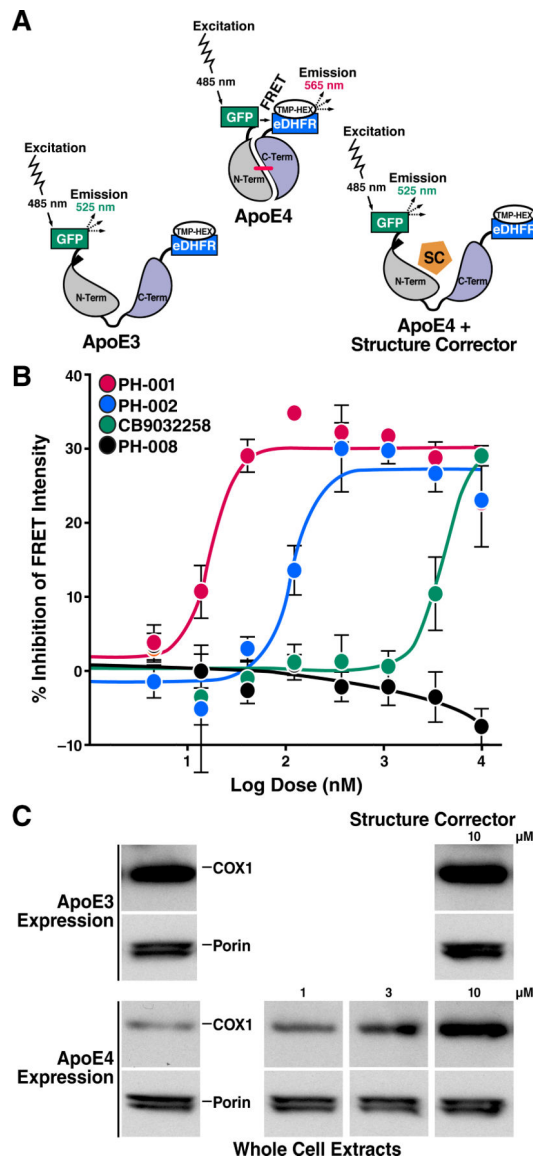


Figure 9. FRET Assay Identifies ApoE4 Structure Correctors

(A) The FRET signal is generated between the donor and acceptor fluorophores conjugated to the N and C termini of apoE. ApoE4 gives a higher FRET signal because of the closeness of the N- and C-terminal domains. The apoE4 signal is reduced by the small-molecule apoE structure corrector (SC) by disrupting domain interaction (Chen et al., 2012).

(B) Dose-response analysis reveals the relative potencies of three active and one inactive structure corrector (Chen et al., 2012).

(C) Mitochondrial cytochrome c oxidase (COX1) is reduced in Neuro-2a cells expressing apoE4 compared with apoE3. A small-molecule structure corrector (CB9032258) corrects the COX1 deficiency in the apoE4-expressing cells, but does not significantly affect apoE3-expressing cells.

(A) and (B). Modified from Figure 2, Chen, H.-K., Liu, Z., Meyer-Franke, A., Brodbeck, J., Miranda, R.D., McGuire, J.G., Pleiss, M.A., Ji, Z.-S., Balestra, M.E., Walker, D.W., Xu, Q., Jeong, D.-e., Budamagunta, M.S., Voss, J.C., Freedman, S.B., Weisgraber, K.H., Huang, Y.,

Mahley, R.W. Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 2012; 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

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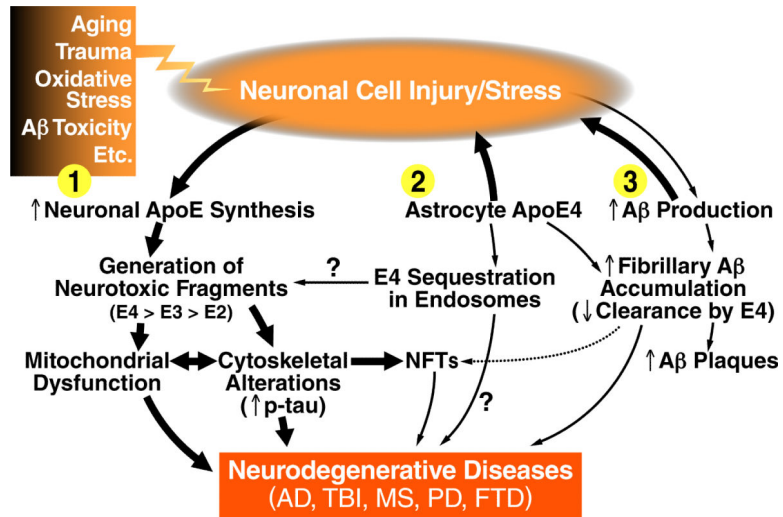


Figure 10. ApoE Sets the Stage and Response to Neuronal Injury Triggers Neuropathology
(1) Injury to neurons induces the synthesis of apoE. ApoE (apoE4 > apoE3) is susceptible to proteolytic cleavage in neurons, and the neurotoxic fragments that are generated escape the secretory pathway and cause mitochondrial dysfunction and cytoskeletal alterations. This is most likely to occur when apoE4 is expressed (apoE4 > apoE3) due to its abnormal protein conformation (instability and domain interaction). **(2)** Exogenous apoE, primarily from astrocytes, could cause neuronal injury and could generate neurotoxic fragments by being shunted to the ER/Golgi apparatus, where proteolysis could occur. In addition, exogenous apoE does impact Aβ clearance/deposition. **(3)** Aβ expression can be induced by injured/stressed neurons, and together with other injurious agents could perpetuate the toxic cycle of injury in neurons. This would include apoE synthesis followed by proteolytic cleavage, toxic fragment formation, and neuropathology.