Apolipoprotein E and Mitochondria-associated Endoplasmic Reticulum Dysfunction

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ABSTRACT

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Despite the tremendous advances of the last century, the cause of Alzheimer disease (AD) remains unclear. Genetic analysis of families with Alzheimer disease has revealed a disease-associated variant of the *APOE* gene, which encodes apolipoprotein E, a transporter of lipids in the blood and central nervous system. The effect of the AD-associated isotype, termed ApoE-E4, on disease risk has been validated, though it is unclear by what mechanism apoE-E4 confers AD risk. Mitochondria have long been implicated in AD pathogenesis, as the canonical histopathological findings of amyloid plaques and tau tangles occur in the setting of mitochondrial dysfunction. The disrupted processes include calcium homeostasis, cholesterol metabolism, phospholipid synthesis, and mitochondrial dynamics, and are all regulated by a subcompartment of the ER that is in physical contact with mitochondria. This compartment, called the mitochondria-associated ER membrane, or MAM, has been found to be overactive in AD patient cell lines and cell models of AD. Given that MAM is dysfunctional in AD and that ApoE-ε4 is the most important risk factor for AD, this dissertation examines if ApoE4 contributes to the MAM dysfunction seen in AD.

The MAM dysfunction seen in AD patients and in cell models of AD has been best characterized in the context of familial AD, and it is the purpose of this study to extend those findings to the more common, sporadic, form of the disease. Familial AD is the result of

autosomal dominant mutations in one of three genes, amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*). APP is the protein from which amyloid-beta, the component of amyloid plaques, is cleaved. The presenilins constitute the enzymatic core of the γ-secretase complex, which cleaves amyloid-beta from a precursor APP molecule. Both PSEN1 (PS1) and PSEN2 (PS2) localize at the MAM, and their action is speculated to influence MAM activity. Fibroblasts from familial AD patients, which contained mutations in *APP*, *PSEN1* or *PSEN2*, showed a marked increase in MAM activity when compared to that of age-matched controls. In mouse embryonic fibroblasts, one can recapitulate this increased MAM activity by knocking out presenilins 1 and 2. In these Psen1/2 double knockout (DKO) cells, the typical measures of MAM function, i.e. increased cholesteryl ester and phosphatidylethanolamine synthesis, calcium transport from ER to mitochondria, and co-localization of ER and mitochondria by confocal and electron microscopy, mimicked the same phenotype found in fibroblasts obtained from familial AD patients, which suggests that the presenilins are negative regulators of ER-mitochondrial apposition. The precise mechanism by which they regulate the ER-mitochondria interface, whether directly as part of a tethering complex, or indirectly though the metabolism of APP-derived substrates, is unclear.

While the effect of familial AD mutations on MAM has been characterized, the mechanism of mitochondrial dysfunction seen in the more common sporadic form of the disease remains obscure. Sporadic AD patients harbor no mutations in *APP*, *PSEN1*, or *PSEN2*, but rather inherit mutations in other genes which do not guarantee the development of the disease, but are instead considered risk factors. The most important of these risk factors, in terms of both amount of AD risk conferred and prevalence in the population, is ApoE. Embedded in the phospholipid monolayer of lipoproteins, ApoE is involved in the transport of phospholipids,

cholesterol, and cholesteryl esters in plasma and the central nervous system (CNS). In the CNS, it is the most abundant apolipoprotein, and is secreted primarily by astrocytes and taken up by neurons. Once endocytosed, ApoE can follow three different pathways: degradation by the lysosome, intracellular retention in early endosomes, or re-lipidation and re-secretion out of the cell. Our approach takes advantage of the physiological role of ApoE as part of a high density-like lipoprotein particle (HDL). Using astrocytes from ApoE targeted gene replacement mice in which murine APOE has been replaced by either human APOE-E3 or human APOE-E4, cultured media containing ApoE3 and ApoE4-lipoproteins can be produced and applied to target cells that do not express ApoE, such as neurons or fibroblasts. These target cells can then be analyzed for MAM activity.

To examine the contribution of ApoE towards MAM dysfunction, target cells, either neurons or fibroblasts, were grown in the presence of astrocyte conditioned media (ACM) from ApoE targeted gene replacement mice. Several measures of phospholipid and cholesteryl ester synthesis were performed to analyzed MAM function. To confirm that the alterations in phospholipid synthesis were the result of altered MAM activity, the same assay was performed in cells in which a protein tethers that bind mitochondria and ER were genetically ablated. Finally, to confirm that the effects seen were the result of the HDL particles and not the result of other components of the ACM, lipoproteins were extracted from ACM by density ultracentrifugation and applied to fibroblasts. In all of the assays performed, ApoE4 conditioned media or ApoE4 isolated lipoproteins were able to induce a significant increase in MAM activity, whereas ApoE4 from recombinant sources did not. These data suggest a contribution of ApoE4 towards MAM dysfunction seen in AD.

The mechanism of these ApoE4 induced MAM alterations remains to be deduced. One may speculate that given the role of ApoE in cholesterol transport outside of the cell, its intracellular retention may impact the distribution of cholesterol within the cell. MAM is a cholesterol rich subdomain with lipid raft-like properties, and any change in the cholesterol content or lipid nature of this membrane may alter its activity. To test this hypothesis, MAM was biochemically extracted from ApoE3 and ApoE4 treated cells and analyzed for cholesterol and lipidomic content. The results described in this thesis demonstrate an AD-like effect in wild-type cells when treated with ApoE-E4, and that the mechanism for these alterations may be due to disturbances in cholesterol distribution in the MAM.

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DEDICATION

I dedicate all of my work to the memory of my late wife, Hannah Burke Tambini. I cannot express how much you have meant to me and how close your memory is to me still.

BACKGROUND

Alzheimer disease

Alzheimer disease (AD) is the most common form of dementia in the elderly. Clinically, it is characterized by memory loss and impaired cognition that worsens irreversibly with age (McKhann et al. 1984). Patients display behavioral changes, become dependent on caregivers, and typically die from secondary effects of the disease, i.e. ulcers, myocardial infarctions, or pneumonia. As health has improved and the average lifespan has extended since the first description of the disease in 1906, so too has the incidence of AD, owing to the increase in aged populations. Current estimates place worldwide incidence at 35 million, with 5.5 million AD cases in the United States (Querfurth and LaFerla 2010). The odds of developing the disease after age 85 are one in three.

AD was first described in 1906 by the German neuropathologist Alois Alzheimer after one of his patients, Auguste Deter, died after a clinical course of worsening memory loss and dementia (Maurer et al. 1997). While a finding of dementia in an elderly patient would have been unremarkable at the time, Deter was only in her late forties when her symptoms began. Upon autopsy and histopathological analysis of her brain tissue, Alzheimer noted gross atrophy, presence of extracellular "numerous small miliary foci" (senile plaques) and intracellular fibrils notable for their "characteristic thickness and peculiar impregnability" (neurofibrillary tangles) (Maurer et al. 1997). Interestingly, Alzheimer also noted lipid changes in affected brains, though these changes received far less attention than the plaques and tangles (Di Paolo and Kim 2011). Four years after this initial description, Alzheimer's colleague Emil Kraepelin termed these pathological changes "Alzheimer's disease," and for many decades, the disease was limited to

these findings in the clinical setting of dementia in young to middle-aged patients. Subsequent analysis of the brains of elderly senile patients revealed similar Alzheimer-type pathology and this led pathologists to reclassify the disease to include any patient with plaques and tangles (Katzman 1976).

Given their striking appearance and disease-association, plaques and tangles became a subject of intense investigation. The first clue about the identity of the main component of cortical senile plaques came from the observation that similar deposits were present in blood vessels of AD brains. Like senile plaques, these deposits displayed birefringent green fluorescence when stained with Congo Red under polarized light, suggesting a β sheet-rich or "amyloid" composition. The identity of the main proteinaceous component, hindered by the insolublity of the plaques, was solved in 1984 by Glenner and Wong. The protein, called "amyloid beta" or Aβ, was found to be a fragment of a larger protein, or "amyloid precursor protein," which is expressed ubiquitously in all cells (Glenner and Wong 1984). From this initial landmark finding flowed a series of investigations aimed at determining the enzymes that metabolize APP to produce Aβ. Subsequently, it was determined that the intracellular tangles were composed of paired helical filaments of hyperphosphorylated tau, a microtubule stabilizing protein (Goedert et al. 1988). Interestingly, the presence of tau tangles, though directly proportional to disease intensity, is not pathognomonic for AD. Tau inclusions are also seen in other neurodegenerative diseases, i.e. tauopathies, such as dementia pugilistica, frontotemporal dementia, progressive supranuclear palsy, and corticobasilar degeneration (Lee et al. 2001).

The amyloid precursor protein (APP) is a type 1 (N-terminus luminal) membrane protein present on the cell surface and in intracellular membranes along the secretory and endocytic pathways (Kang et al. 1987). Although the cellular function of APP is unclear, its domain

composition is well known and its crystal structure has been partially solved (Rossjohn et al. 1999, Xue et al. 2011). APP consists of two extracellular/luminal domains, a transmembrane region, and a cytosolic domain (Zheng and Koo 2006) **(Fig 1)**. The first extracellular domain E1 begins with a signal peptide sequence which is cleaved off upon translocation of APP into the endoplasmic reticulum (ER). E1 binds copper and heparin and is joined to the second extracellular domain E2 by an acidic linker. Some alternatively spliced isoforms of APP, APP751 and APP770, contain a Kunitz-type protease inhibitor (KPI) and OX-2 domain at this linker region; however, the neuronal isoform, APP695, does not. The E2 domain has been hypothesized to have ferroxidase activity, although this idea has been recently challenged (Honarmand Ebrahimi et al. 2013).

The amyloidogenic cleavage of APP is a complex process that is governed by different enzymes that are spatially and temporally regulated. It is important to note that though the quantity of Aβ and its tendency to aggregate is altered in AD, its production is a constitutive event that occurs throughout life and in many different tissues (Haass et al. 1992). The first of two cleavages that result in the production of Aβ occurs in the juxtamembrane region at the β-site by β-secretase (BACE1) (Vassar et al. 1999) **(Fig 2)**. This processing occurs intracellularly after APP has been endocytosed from the plasma membrane into low-pH compartments. Cleavage liberates the soluble ectodomain (sAPPβ) (595 aa), and the smaller 99-aa membranous fragment (C99) undergoes a transmembrane cleavage by γ-secretase, a rhomboid protease composed of presenilin 1 and 2 (PS1/2), nicastrin (NCSTN), presenilin-enhancer (PEN2), and defective anterior-pharynx 1 (APH1) (Wolfe 2008). γ-Secretase cleavage releases Aβ (~40 aa) and the cytosolic ~50-aa APP intracellular domain (AICD) is simultaneously released and traffics to the nucleus to affect transcription (Cao and Sudhof 2001, Leissring et al. 2002). The

majority of Aβ produced is 40 aa long, though carboxypeptidase activity of γ-secretase is not precise, and will sometimes result in a more aggregation-prone 42aa species (Fernandez et al. 2014).

A majority of APP is not metabolized to produce Aβ, and instead is cleaved via the non-amyloidogenic pathway (Haass et al. 1993). Instead of processing by BACE1, APP is cleaved at the plasma membrane by α-secretase to release sAPPα (579 aa) and an 83-aa transmembrane fragment (C83). α-Secretase cleavage occurs within Aβ, thus precluding Aβ generation. Upon γ-secretase of C83, p3, a fragment of unknown importance, is released as well as AICD, as in the amyloidogenic pathway. What determines whether APP is cleaved along the amyloidogenic or non-amyloidogenic pathway is not completely understood.

GENETICS OF AD

AD can be classified into two types that share the clinical presentation and neuropathological hallmarks, but differ by age of onset. Early onset, or familial AD (FAD), occurs before the age of 65. FAD is inherited in an autosomal dominant manner and is the result of mutations in APP, PS1, or PS2. Additionally, as APP resides on chromosome 21, many Down syndrome patients develop early onset AD (Wisniewski et al. 1985). In APP, AD mutations cluster in three regions at the C-terminus of the protein. Mutations near the β-secretase site increase β-cleavage of APP (Citron et al. 1992). Recently, a rare APP mutation that decreases β-cleavage and reduces AD risk has been found in Scandinavian populations (Jonsson et al. 2012). Mutations within Aβ affect its tendency to aggregate (Baumketner et al. 2008), while mutations at the γ-secretase site increase the relative abundance of Aβ42 to Aβ40 (De Jonghe et al. 2001). Mutations within PS1/2 result in a partial loss of function, in that amyloid is

still produced, though there is less overall, and the Aβ42/40 ratio is increased (Bentahir et al. 2006). The confluence of amyloid pathology and mutations in amyloid-producing genes provides the intellectual basis for the amyloid cascade hypothesis, which posits that, over time, aberrant metabolism of APP generates neurotoxic oligomeric assemblies and plaques (Hardy and Selkoe 2002).

Late onset, or sporadic, AD (SAD) occurs after age 65 and constitutes more than 95% of AD cases (Tanzi 2012). Though SAD displays the same clinical course and neuropathological changes as FAD, the mode of inheritance is much more complex. SAD is not inherited in an autosomal dominant fashion. Instead, the genetic inheritance of SAD is determined by "risk factors," i.e. polymorphisms that do not by themselves guarantee AD, but can increase the chances of developing the disease. Unlike FAD, the genes that have been reported in genome-wide studies of SAD involve a number of different cellular processes and not just amyloid production (Hollingworth et al. 2012). The most important gene in terms of prevalence in population and conferral of disease-risk is *APOLIPOPROTEIN E (APOE)*, which encodes ApoE, a member of a class of proteins that regulates transport of lipids and cholesterol in the plasma and CNS (Corder et al. 1993). *APOE* exists in three major isoforms that are defined by two coding-region mutations: ApoE2 contains cysteine at position 112 and 158, ApoE3 contains cysteine at 112 and arginine at 158, and ApoE4 contains arginine at both positions. Each isoform confers a varying degree of risk (Farrer et al. 1997). ApoE2 is protective for AD, though interestingly, this isoform increases risk for Type III hyperlipoproteinemia, in which excess lipoproteins that have failed to be cleared from plasma become deposited in skin and blood vessels (Weisgraber et al. 1982). Individuals with ApoE3 genotypes are said to be at no increased risk for the disease, and though many will eventually develop the disease. Those who

inherit ApoE4 are at a 4-12x greater risk for developing AD, depending if both alleles are present (Holtzman et al. 2012).

Other polymorphisms found to increase AD risk include those in bridging integrator 1 (*BIN1*), clusterin (*CLU*), ATP-binding cassette sub-family A member 7 (*ABCA7*), complement receptor 1 (*CR1*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), and others (Harold et al. 2009, Hollingworth et al. 2011). In addition to any effect on amyloid production, these genes have diverse functions, including regulation of inflammation, lipid metabolism, and endocytic trafficking. SAD is clearly a multifactorial disease.

APOE

ApoE is a 34-kDa protein synthesized primarily by macrophages, liver, and astrocytes. In the plasma, ApoE is studded on the membrane of lipoproteins, which are assemblies of phospholipids, cholesterol, cholesteryl esters, and triglycerides that circulate throughout the body. The density and composition of plasma lipoproteins vary considerably, while in the CNS, ApoE is the primary apolipoprotein and part of high density lipoprotein (HDL)-like particles (Mahley 1988). After synthesis in the ER and cleavage of its signal peptide, ApoE traffics along the secretory pathway and, in a process regulated by the lipid transporters ATP-binding cassette subfamily A1(ABCA1) and subfamily G1 (ABCG1), becomes associated with cholesterol, triglycerides, and phospholipids at the plasma membrane (Wahrle et al. 2004, Lammers et al. 2009). The nascent lipoprotein is then secreted, with the lipid-binding C-terminal of ApoE covalently attached to lipids in the lipoprotein membrane, while the N-terminal receptor binding domain of ApoE is exposed to the extracellular milieu. In the periphery, ApoE-containing lipoprotein can then travel long distances and may be endocytosed by any cell expressing a

lipoprotein receptor, class of proteins which includes low density lipoprotein receptor (LDLR) family, very low density lipoprotein receptor (VLDLR) family, LDLR-related protein 1 (LRP1), and ApoE receptor 2 (APOER2), among others (Holtzman et al. 2012). In the brain, astrocytes and, to a limited extent, microglia, are the main producers of ApoE, and neurons are the target cells. Neurons typically do not produce ApoE, although there are some reports of neuronal ApoE production during starvation or after treatment with the excitotoxin kainate (Xu et al. 2006). The ApoE-containing lipoprotein is endocytosed via a receptor and trafficked to the endolysosome, where it is hydrolyzed and separated from the lipid component. ApoE may either be degraded in the lysosome or recycled via endosomes back to the cell surface, where it may be re-lipidated via ABCA1 and secreted (Fazio et al. 2000).

The main function of ApoE is to redistribute cholesterol and lipids. Though viable, ApoE knockout mice display hyperlipidemia, hypercholesterolemia, and atherosclerosis (Zhang et al. 1992). In these animals, lipoproteins are still produced but are inadequately cleared from plasma ApoE and are deposited systemically. Though cognitive defects are mild, these mice display synaptic dysfunction and reduced long term potentiation (Veinbergs et al. 1998). Recently, a case report has described an ApoE-null patient who presented with severe hyperlipidemia and systemic fatty deposits, with no evidence of cognitive dysfunction (Mak et al. 2014). This latter observation, while important to note, is not particularly meaningful, since the patient was 40 years old, and therefore too young to develop SAD. While the knockout model may be most useful for models of hyperlipidemia and atherosclerosis, a targeted replacement model was generated to examine the effect of each human ApoE polymorphism on cognition and lipid homeostasis **(Fig 3)**. ApoE targeted gene replacement (ApoE-TR) mice have exons 2-4 of each isoform under the murine ApoE promoter (Sullivan et al. 1997, Knouff et al. 1999). Relative to

ApoE3-TR mice, ApoE4-TR mice do not display striking cognitive phenotypes or Alzheimer-type neuropathology, though some have reported altered spatial cognition, spine density, and hyperlipidemia on a high-fat diet (Rodriguez et al. 2013). It is important to note, however, that wild type mice do not develop Alzheimer-type neuropathology, and that much genetic manipulation is required to elicit these phenotypes.

The impact that each isoform has on Aβ metabolism in the context of APP overproduction has been studied extensively. In crosses of PDAPP mice, which overexpress the familial APP V717F mutation, and ApoE-TR mice, an isoform-dependent effect on number of plaques and cognitive impairment (E2<E3<E4) was observed (Castellano et al. 2011). Further experiments demonstrated that the increase in plaque formation was not the result of Aβ overproduction, but of impaired Aβ clearance. This clearance is unlikely the result of a direct interaction of ApoE and Aβ, but may be the result of competition between ApoE and Aβ for receptor-mediated binding of lipoprotein receptors (Verghese et al. 2013). The failure of ApoE4 mice to clear amyloid suggests that a stimulation of ApoE production would ameliorate plaque pathology. To test this hypothesis, production of endogenous ApoE was stimulated pharmacologically by the retinoid X receptor agonist bexarotene. Strikingly, in APP transgenic mice, bexarotene treatment resulted in clearance of plaques and a concomitant improvement in cognition (Cramer et al. 2012), though subsequent replication studies failed to find a consistent effect (Fitz et al. 2013, Price et al. 2013, Tesseur et al. 2013, Veeraraghavalu et al. 2013). Complicating the issue is that bexarotene, in addition to increasing the production of ApoE, affects its lipidation state as well (Boehm-Cagan and Michaelson 2014). Given these contradictory results, the therapeutic value of increasing ApoE expression is unclear.

Amyloid-independent explanations of how ApoE contributes to AD risk have also been proposed. The differences in the relative stability of each isoform may underlie ApoE4 toxicity. In neurons, ApoE4 has been found to undergo proteolysis and the resulting N-terminal fragment translocates to mitochondria and impairs oxidative phosphorylation (Huang et al. 2001). These fragments have also been found to associate with tangles, cause amyloid to aggregate, and increase the amount of reactive oxygen species. It is unclear if these toxic fragments are the result of ApoE imported from astrocytes or if there is a small amount of ApoE constitutively present in neurons or expressed during injury. Aside from the toxicity of the ApoE fragments, the polymorphisms which define each isoform of ApoE may affect the normal physiological function of ApoE. ApoE2, for example, has been found to weakly bind very low density lipoprotein (VLDL) receptors, and it is this loss of function that may explain the incidence of hyperlipidemia in ApoE2 homozygotes (Rodriguez et al. 2013). The relative binding affinities of ApoE3 and ApoE4 are comparable, and this is not thought to underlie AD risk (Yamamoto et al. 2008). A major difference has been observed in the trafficking and function of each isoform after receptor mediated endocytosis. Upon internalization by target cells, ApoE-containing HDL-like particles traffic to the endolysosomal compartments, where ApoE is separated from its lipid cargo and is further trafficked for degradation or recycling. This recycling of ApoE back to the cell surface and its subsequent lipidation and re-secretion are associated with cholesterol efflux from the cell into the extracellular space (Langer et al. 2000, Heeren et al. 2003). ApoE4 tends to get trapped in recycling endosomes and cholesterol efflux is impaired in cells treated with ApoE4-containing lipoproteins (Heeren et al. 2004). Thus, the differential distribution and handling of cholesterol by the ApoE isoforms may underlie AD risk.

Neuronal cholesterol metabolism plays a critical role in neurodegeneration (Benarroch 2008). The brain contains 25% of the total body cholesterol, with most used for myelin synthesis. This cholesterol is synthesized *de novo*, as plasma lipoproteins are unable to cross the blood brain barrier. Free cholesterol is a component of cellular membranes, and dysregulation of its metabolism affects a diverse array of processes (e.g. synaptic function, neurite outgrowth, apoptosis, and spine density). Within membranes, cholesterol is not distributed homogeneously but instead clusters in domains, i.e. "lipid rafts" which are enriched in cholesterol and sphingolipids and are resistant to solubilization by detergents (Simons and Toomre 2000). In addition to their unique lipid profile, these subdomains contain specialized proteins and are a privileged site for certain processes/reactions. The amount and distribution of cholesterol within neurons is kept under tight homeostatic control. Excess cholesterol can be converted to cholesteryl esters (CE) by Acyl-CoA:cholesterol acyltransferase 1 (ACAT1; gene *SOAT1*) and stored intracellularly in the form of lipid droplets (Rusinol et al. 1994). Lipid droplets are cytoplasmic, phospholipid membrane bound spherical bodies that may contain either cholesteryl esters or triglycerides. Cholesteryl ester hydrolases liberate free cholesterol from lipid droplets. In addition to storage as lipid droplets, neurons may eliminate excess cholesterol by oxidizing it. Cholesterol oxidases (e.g., CYP46A1) produce 24-hydroxycholesterol, which unlike its lipid-bound substrate, is soluble and diffuses from neurons to the bloodstream (Lund et al. 1999). In this way, excess cholesterol is removed from the brain.

Perturbations of cholesterol homeostasis have been implicated in neurodegenerative diseases. Mutations of the cholesterol transporter Neimann-Pick disease, type C (NPC1) protein result in an accumulation of cholesterol within the endolysosomal system (Nixon 2004). The sequestration of cholesterol causes a deficiency in the rest of the cell, as well as altered

endosomal trafficking, but it is unclear which of these phenomena causes the neurodegeneration seen in Niemann-Pick disease.

Cholesterol metabolism plays a very important role in AD (Di Paolo and Kim 2011). APP was found to bind cholesterol and both β- and γ-secretase activities occur in lipid rafts (Riddell et al. 2001, Urano et al. 2005, Barrett et al. 2012). Dietary cholesterol overload was sufficient to produce amyloid plaques in rabbits (Sparks et al. 1994). Moreover, cholesterol:cholesteryl ester ratio regulates Aβ production (Huttunen et al. 2007). Genetic ablation or pharmacological inhibition of ACAT1 greatly reduces Aβ synthesis (Puglielli et al. 2001, Puglielli et al. 2003).

MAM

The cause of AD is unknown. The confluence of plaque pathology and familial mutations in amyloid-generating genes pointed the field towards Aβ as the ultimate cause of neurodegeneration in AD (Hardy and Allsop 1991). This view, while currently informing many AD investigations, has engendered many revisions as therapies that test this hypothesis have failed in clinical trials. Several attempts to decrease Aβ have proven ineffective at stalling cognitive decline. These include vaccines against Aβ, passive immunization against Aβ, and pharmacological inhibition of γ-secretase. Interestingly, one γ-secretase inhibitor actually worsened cognitive function compared to placebo, though there has been some concerns raised about its effectiveness at lowering Aβ in the clinical trials (Doody et al. 2013, De Strooper 2014). In retrospect, there are reasons to have doubted the therapeutic effectiveness of targeting amyloid (Herrup 2015). The pathological changes that occur in AD precede cognitive decline by several years, so clearly the mere presence of plaques is insufficient to cause the disease. The

distribution and quantity of plaques does not correlate to disease severity. Indeed, some elderly individuals have a very high plaque load but never develop the disease (Jack et al. 2009). These observations have caused some to reevaluate the amyloid cascade hypothesis, and shift the focus to the oligomeric precursors of the plaques (Hardy and Selkoe 2002). However, it is unclear if these oligomeric assemblies of Aβ exist physiologically in high enough concentrations to cause cytotoxicity, and if so, how a dynamic assembly of amyloid is able to evoke the stereotyped pattern of neurodegeneration seen in AD. Amyloid is clearly important - the pathology and genetic data indicate as much - however the underlying biology of AD is complex and much attention must be paid to the conditions in which AD-associated pathological changes occur.

One of the early changes that occurs concurrently with amyloid deposition and tightly correlates with cognitive decline is altered glucose metabolism (Hoyer 1992). Positron emission tomography (PET) scans reveal cerebral hypometabolism in patients who go on to develop AD (Mosconi et al. 2008). These observations, as well as the close association of AD with aging, suggest that changes to mitochondrial function are an important and early event in AD. Mitochondria, in addition to their well-known role in oxidative phosphorylation, associate with other organelles to perform specialized functions. Mitochondria form dynamic, reversible associations with the ER at specialized contact sites called the mitochondria-associated ER membrane (MAM) (Vance 1990). MAM is physically contiguous with the ER, but differs in its protein composition and has the properties of a lipid raft (Hayashi et al. 2010, Area-Gomez et al. 2012). Several important functions are regulated by MAM, including calcium homeostasis, phospholipid trafficking, cholesteryl ester synthesis, and mitochondrial dynamics (Schon and Area-Gomez 2013). In cells from AD patients as well as in AD models, there is a significant increase in the apposition of ER and mitochondria, and several of these MAM functions are

highly upregulated (Area-Gomez et al. 2012). This forms the basis of the MAM hypothesis, which places altered mitochondria-ER communication at the heart of AD pathogenesis (Schon and Area-Gomez 2010, Schon and Area-Gomez 2013).

Amyloid production. MAM was first implicated in the pathogenesis of AD as the result of localization studies of PS1/2. The presenilins had been reported to localize in the plasma membrane, endosomes, Golgi, nuclear envelope, ER, and mitochondria (Area-Gomez et al. 2009). The localization of PS1/2 to mitochondria would be notable, since these proteins do not possess a canonical mitochondrial targeting sequence. Confounding any analysis of mitochondrial localization of proteins is the tendency for MAM to co-purify with mitochondria during biochemical fractionation. It is possible to generate fractions enriched in mitochondria that are free of ER, as well as fractions that contain MAM and are free of any contaminating mitochondria and bulk ER. Analysis of purified mitochondria and MAM shows an enrichment of PS1/2 at the MAM compared to isolated mitochondria (Area-Gomez et al. 2009). All the components of the γ-secretase complex, i.e. PS1/2, PEN2, APH1, and NCSTN, localize and are active at MAM, as measured by Aβ production is isolated fractions (Area-Gomez et al. 2012).

Calcium Homeostasis. The maintenance of intracellular levels of calcium ions (Ca^{2+}) is critical to neuronal function (Berridge et al. 2003). Ca^{2+} enters the cell through calcium channels in the plasma membrane and is then removed from the cytoplasm by Sarco/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA). SERCA pumps Ca^{2+} into the ER, which acts as an internal calcium store. This Ca^{2+} can then be released from the ER by the inositol triphosphate receptor type 3 (IP3R3) upon binding IP_3 or cytosolic Ca^{2+}. The increase of cytosolic Ca^{2+} affects a number of cellular functions, including neurotransmitter release, intracellular transport, and apoptosis. The regulation of intracellular Ca^{2+} is therefore critical, and mitochondria maintain

this homeostasis by absorbing excess Ca^{2+}. Given the role of ER in Ca^{2+} release and mitochondria in Ca^{2+} buffering, the spatial proximity of these two organelles is essential for Ca^{2+} homeostasis (Hayashi et al. 2009). Many proteins that regulate this homeostasis, such as SERCA, IP3R3, and others are found at MAM, and the direct transfer of Ca^{2+} from ER to mitochondria has been observed at MAM (Hayashi and Su 2007, Garcia-Perez et al. 2008, Szabadkai and Duchen 2008). In AD, Ca^{2+} homeostasis is greatly perturbed, both as a result of Aβ generation and more directly by the presenilins, which may act as Ca^{2+} leak channels (Bezprozvanny and Mattson 2008). FAD mutations in PS1/2 eliminate this "leak channel" function, and the increase in ER [Ca^{2+}] results in an amplified Ca^{2+} release upon ligand binding to IP3R (Zampese et al. 2011). Mitochondria become overloaded and are unable to adequately buffer the excess Ca^{2+} which leads to cytotoxicity.

Phospholipid Synthesis. In addition to buffering cytosolic Ca^{2+}, the interface between ER and mitochondria is a platform for phospholipid synthesis. In the MAM, phosphatidylserine (PtdSer) synthase (PSS1) exchanges the polar head group of phosphatidylcholine (PtdCho) with serine to produce PtdSer (Vance 1990). This PtdSer is then transferred across the ER to the mitochondria by an unknown mechanism, where it is decarboxylated to produce phosphatidylethanolamine (PtdEtn). The mitochondrial PtdEtn is then translocated back to the ER where it can be distributed to the rest of the cellular membranes. This MAM-based PtdEtn is a major source of PtdEtn in eukaryotic cells, and genetic ablation of mitofusin 2 (Mfn2), the molecular tether that forms MAM, greatly reduces PtdEtn production (de Brito and Scorrano 2008, Area-Gomez et al. 2012). In SAD and FAD patient fibroblasts and in PS1/2 DKO mouse embryonic fibroblasts (MEFs), PtdSer and PtdEtn synthesis is greatly increased, which reflects an increase in MAM function (Area-Gomez et al. 2012).

Cholesterol esterification. MAM, a cholesterol-rich compartment, contains several regulators of cholesterol metabolism, including those controlling cholesterol esterification. Excess cholesterol can be converted into cholesteryl esters by ACAT1 at the MAM and stored in cytoplasmic lipid droplets which bud from the ER (Walther and Farese 2012). Perturbations of cholesterol metabolism are a feature of AD (Puglielli et al. 2003). Patient skin fibroblasts and presenilin DKO MEFs contain many more lipid droplets than nondemented and wild-type controls, respectively (Pani et al. 2009, Area-Gomez et al. 2012). These lipid droplets are composed mostly of CEs, and CE synthesis in these cells is markedly increased. Genetic ablation of ACAT1 or pharmacological reduction of CE synthesis by ACAT1 inhibition greatly reduces Aβ (Puglielli et al. 2001).

Mitochondrial dynamics. Mitochondria are not static organelles. In response to cellular conditions, they undergo fusion or fission (Chen and Chan 2009). Damaged mitochondria or mitochondria with mutated DNA will typically undergo fission which isolates the defective organelles from functional ones. Autophagic elimination of these mitochondria constitutes a form of "quality control" (Youle and Narendra 2011). Mitochondria fusion, on the other hand, distributes the contents of mitochondria and normalizes mitochondrial function. These processes are highly regulated and require the concerted action of several proteins located on the inner and outer mitochondrial membranes. To undergo fusion, two mitochondria associate via homodimerization of mitofusin 1 (MFN1) or 2 (MFN2) or heterodimerization of MFN1 and MFN2 on the outer membrane of each mitochondrion. After fusion of the outer membranes, the inner membranes fuse via the optic atrophy 1 (OPA1) protein. Aside from its location on the outer mitochondria membrane, Mfn2 is also present on the ER, with its N- and C-terminal domains facing the cytosol. This ER-Mfn2 may homodimerize with mitochondrial Mfn2 or

heterodimerize with mitochondrial Mfn1 to form ER-mitochondria connections (de Brito and Scorrano 2008). Genetic ablation of Mfn2 drastically reduces but does not completely eliminate the association of ER and mitochondria, indicating the existence of other determinants of MAM aside from Mfn2.

Mitochondrial fission is mediated by the recruitment of dynamin related protein 1 (DRP1; gene *DNML1*) from the cytosol to the outer mitochondrial membrane (Chen and Chan 2009). Drp1 oligomerizes around the mitochondrion to form a contractile ring, whose action squeezes the mitochondrion until two daughter mitochondria form. Recently it has been found that the perpendicular point of contact between ER and mitochondria determines the site of Drp1 recruitment and subsequent mitochondrial division (Friedman et al. 2011). MAM therefore plays a role in mitochondrial fission/dynamics. In AD, mitochondria are in closer association with ER and are found in higher proportions in the perinuclear region of cell, though it is unclear if there is an overall trend towards increased fusion or fission. Conflicting studies have found both increased fragmentation of mitochondria and fused mitochondria in AD patients and models (Wang et al. 2008, Wang et al. 2009). Despite this uncertainty, both mitochondrial fusion and fission are regulated by MAM, and MAM dysfunction may underlie the morphological changes seen in mitochondria in AD (Kornmann et al. 2011).

Rationale and organization of this thesis

Previous work in the lab demonstrated that in SAD and FAD patient cell lines and in presenilin DKO cell lines, mitochondria-ER communication is greatly increased (Area-Gomez et al. 2012). These observations form the basis of the MAM hypothesis, namely, that AD pathogenesis is due to increased mitochondria-ER communication (Schon and Area-Gomez 2010, Schon and Area-Gomez 2013). If this hypothesis is indeed correct, other AD genes should

result in the same MAM phenotype. The obvious candidate to test is APOE, both because it is the most prevalent and validated SAD risk factor and because it confers the greatest degree of risk. Chapter 1 describes how ApoE4 increases MAM function relative to ApoE3. Using an astrocyte-condition media (ACM) model, I demonstrate that ApoE4 ACM-treatment of wild-type cells upregulates MAM function as measured by cholesteryl ester and phospholipid synthesis. Chapter 2 is a study of the compositional changes in MAM that occur in ApoE3 and ApoE4 ACM-treated cells. These data are the preliminary results of an ongoing project that aims to understand mechanistically how ApoE4 evokes this MAM phenotype. Chapter 3 describes future experiments that will elucidate the role of ApoE in MAM dysfunction. The appendix contains the manuscript that initially describes the increased ER-mitochondria communication in AD (Area-Gomez et al. 2012), on which I am a coauthor.

Figure 1

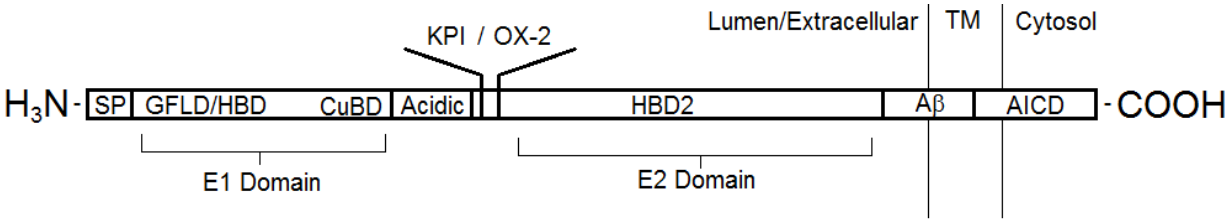

Figure 1. Domain organization of the Amyloid Precursor Protein

The Amyloid Precursor Protein (APP) contains two extracellular/luminal domains, a transmembrane region, and a cytosolic domain. A signal peptide (SP) directs the nascent APP peptide to the endoplasmic reticulum. The first extracellular domain (E1) is composed of a growth factor like (GFLD)/ heparin-binding domain (HBD) and a cysteine-rich, copper binding domain (CuBD). The E1 domain is adjacent to an acidic region, followed by a Kunitz-type Protease Inhibitor (KPI) and OX-2 domain, present in some isoforms by alternative splicing. The second extracellular domain (E2) also contains a heparin-binding domain. The N-terminal region of amyloid beta (Aβ) is located in the extracellular space/lumen and the C-terminus is located within the transmembrane domain of APP. The cytosolic portion of APP includes the APP intracellular domain (AICD).

Figure 2

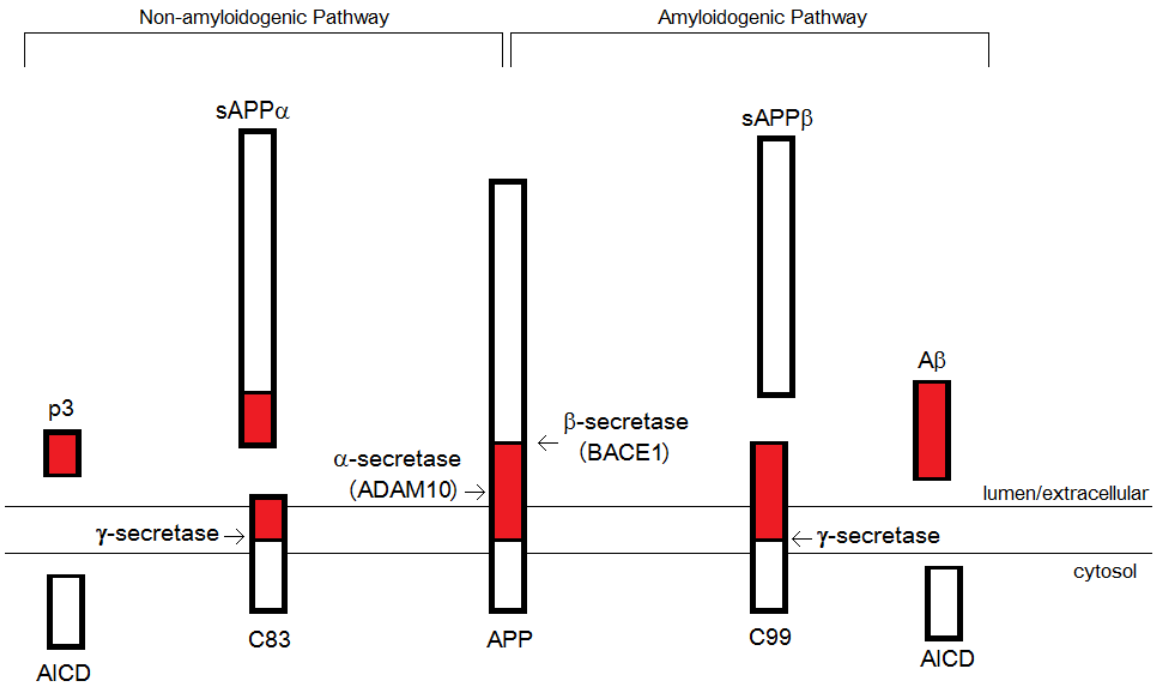

Figure 2. Amyloid Precursor Protein metabolism

APP can be metabolized via the amyloidogenic or non-amyloidogenic pathway. In the amyloidogenic pathway, APP is cleaved sequentially by β-secretase to produce C99, which is further processed by γ-secretase to yield Aβ and AICD. In the non-amyloidogenic pathway, APP is first cut by α-secretase to produce C83, which is then also cleaved by γ-secretase to yield p3 and AICD. Cleavage of APP by α-secretase precludes the formation of Aβ.

Figure 3

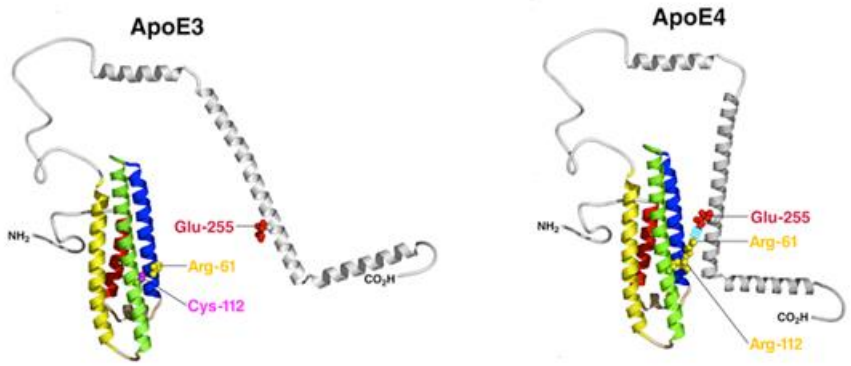

Figure 3. ApoE domain organization

ApoE contains two domains, an N-terminal receptor-binding domain (colored) and a C-terminal lipid-binding domain (gray). ApoE3 contains cysteine at position 112, while ApoE4 contains arginine. An ApoE4-specific domain interaction between Arg-61 of the receptor-binding domain and Glu-255 of the lipid-binding domain has been found reported. Adapted from Huang (2010).

CHAPTER 1. THE EFFECT OF APOE ON MAM FUNCTION

(Manuscript I)

INTRODUCTION

The finding that the presenilins and other components of the γ-secretase complex are enriched at the MAM, and that AD mutants and presenilin knock-outs (KOs) have drastically increased MAM function prompted us to investigate the effect of other AD genes on MAM function. ApoE poses a particular challenge in that it is not normally expressed in the target cell population, i.e. neurons, affected in AD. Very interestingly, apolipoproteins traffic though the MAM in astrocytes ((Vance 1990), our unpublished data), but the cell biology of ApoE synthesis and ApoE receptor-mediated uptake are likely to be different. The physiologically relevant question to ask is "how does ApoE affect MAM in target cells?" since it is in these cells that pathological changes are seen in AD. It should be noted that some studies have found that target cells express ApoE under starvation and apoptosis (Quinn et al. 2004), or upon treatment with the excitotoxin kainate (Xu et al. 2006). Therefore, the interpretation of any observations made in neurons or other target cells expressing ectopic ApoE, (i.e. by transfection or infection) would be of limited value. Other studies have examined the effect of lipid-free ApoE applied to target cells, but this does not reflect the fact that most of the ApoE found in the CNS is lipidated (Yamamoto et al. 2008). Because ApoE is mainly present in the CNS as part of HDL-like particles, we chose to use a system whose source of ApoE is derived from astrocytes and is

incorporated into a lipoprotein particle. Immortalized astrocytes from targeted gene replacement mice constitutively secrete human ApoE3 or ApoE4 as part of a lipoprotein into the tissue culture media. This astrocyte conditioned medium (ACM) can then be applied to other cells which express ApoE receptors. Fibroblasts were selected as target cells because they have been previously shown to demonstrate AD-related changes in MAM function (Area-Gomez et al. 2012).

Because MAM formation is a dynamic and reversible process, it is a difficult compartment to analyze. Biochemical fractionation of MAM is possible, though the presence of markers enriched in MAM does not specifically indicate the extent of communication between ER and mitochondria. The most meaningful way to study the apposition of ER and mitochondria is to measure the activity of functions that require MAM. Phospholipid and cholesteryl ester synthesis are both such functions and are well-established markers of ER-mitochondria communication (Schon and Area-Gomez 2013). Each can be experimentally analyzed by several different methods. To confirm that any MAM phenotype found is actually the result of increased ER-mitochondria communication, the effect of ApoE on phospholipid and cholesteryl ester synthesis was also examined in Mfn2-KOs, in which ER-mitochondrial apposition is reduced, as a control (de Brito and Scorrano 2008). If any ApoE-mediated increase in these functions acts through MAM, the abolition or mitigation of the phenotype would be expected in MAM-deficient cells.

The following manuscript describes an increase in MAM function in cells treated with ApoE4 ACM.

Manuscript I

ApoE4 upregulates the activity of mitochondria-associated ER membranes

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Keywords: ApoE, cholesterol, cholesteryl esters, endoplasmic reticulum, lipoproteins, MAM, mitochondria, phospholipid

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Abstract

In addition to the appearance of senile plaques and neurofibrillary tangles, Alzheimer disease (AD) is characterized by aberrant lipid metabolism and early mitochondrial dysfunction. We recently showed that there was increased functionality of mitochondria-associated endoplasmic reticulum (ER) membranes (MAM), a subdomain of the ER involved in lipid and cholesterol homeostasis, in presenilin-deficient cells and in fibroblasts from familial and sporadic AD patients. Individuals carrying the E4 allele of apolipoprotein E (ApoE4) are at increased risk for developing AD compared to those carrying ApoE3. While the reason for this increased risk is unknown, we hypothesized that it might be associated with elevated MAM function. Using an astrocyte-conditioned media (ACM) model, we now show that ER-mitochondrial communication and MAM function - as measured by the synthesis of phospholipids and of cholesteryl esters, respectively - are increased significantly in cells treated with ApoE4-containing ACM as compared to those treated with ApoE3-containing ACM. Notably, this effect was seen with lipoprotein-enriched preparations, but not with lipid-free ApoE protein. These data are consistent with a role of upregulated MAM function in the pathogenesis of AD, and may help explain, in part, the contribution of ApoE4 as a risk factor in the disease.

Introduction

Alzheimer disease (AD), the most common form of dementia in the elderly, is characterized by progressive neuronal loss accompanied by the formation of extracellular plaques containing β-amyloid (Aβ) and of intracellular tangles composed of hyperphosphorylated tau (Goedert and Spillantini 2006). Aβ results from the processing of the C-terminus of the amyloid precursor protein (APP), first by β-secretase (BACE1) and then by γ-secretase. The active components of

γ-secretase are presenilin-1 (PS1) and/or presenilin-2 (PS2), which are aspartyl proteases (Goedert and Spillantini 2006).

In addition to plaques and tangles, AD is associated with an array of biochemical alterations, including perturbations in cholesterol homeostasis (Pani et al. 2009, Stefani and Liguri 2009), phospholipid metabolism (Pettegrew et al. 2001), calcium trafficking (Bezprozvanny and Mattson 2008), and mitochondrial function (Wang et al. 2009). Notably, these functions share a common feature in that they are associated with a subcompartment of the endoplasmic reticulum (ER) known as mitochondria-associated ER membranes (MAM). MAM is a region of the ER that communicates with mitochondria, both physically and biochemically, but it has different biophysical properties than bulk ER (e.g. it is a lipid raft-like domain (Hayashi and Fujimoto 2010, Williamson et al. 2011, Area-Gomez et al. 2012)). We recently showed that PS1 and PS2, and γ-secretase activity itself, are localized predominantly in MAM (Area-Gomez et al. 2009), and that pathogenic mutations in, or genetic ablation of, PS1 and PS2 increase MAM activity significantly (Area-Gomez et al. 2012). The convergence of these upregulated MAM functions in AD, and the finding that MAM contains active γ-secretase complexes, has led us to propose that MAM dysfunction is a key event in the pathogenesis AD (Schon and Area-Gomez 2010, Area-Gomez et al. 2012, Schon and Area-Gomez 2013).

Mutations in APP, PS1, or PS2 result in the early-onset or familial form of AD (FAD), although these mutations account for only 1% of total AD incidence; the vast majority of AD cases are late-onset or sporadic (SAD), and are not inherited in an autosomal dominant manner, as is the case with FAD (Tanzi 2012). Nevertheless, SAD is characterized by the inheritance of

genetically-determined risk factors, polymorphisms that do not positively determine AD but rather confer an increased likelihood of the disease.

Different isoforms of *APOE* are the most common and validated of these risk factors (Holtzman et al. 2012). *APOE* encodes apolipoprotein E (ApoE), a component of the lipoproteins that transport cholesterol and lipids throughout the body. In the brain, cholesterol is synthesized mainly by astrocytes but not neurons, with astrocyte-derived cholesterol delivered to neurons via high-density lipoproteins (HDL) (Pitas et al. 1987, Lund-Katz and Phillips 2010). The □4 variant of *APOE*, denoted here as ApoE4 (arginines at positions 112 and 158; allele frequency in Europeans of ~10-30% (Gerdes 2003)), confers an increased gene dose-dependent risk for developing AD of ~4-fold with one copy and ~12-fold with 2 copies compared to individuals carrying ApoE3, the most common isotype (Cys112/Arg158; allele frequency of ~70-90%), while the rarest variant, ApoE2 (Cys112/Cys158; allele frequency of ~3-12%), is protective against AD (Farrer et al. 1997). While it is unclear exactly how the two coding mutations that determine the *APOE* genotype modulate AD risk, a proposed mechanism implicates differential ApoE-mediated aggregation and clearance of Aβ (Holtzman et al. 2012), but it is still unknown whether the normal physiological function of ApoE in cholesterol and lipid homeostasis relates to increased AD risk (Holtzman et al. 2012).

Given that ApoE4 is the main genetic risk factor for AD, and that MAM alterations in cholesterol and lipid homeostasis are features of AD, we investigated the possibility that relative to ApoE3, ApoE4 exerts effects that lead to MAM dysfunction. Specifically, we compared MAM activity of cells treated with astrocyte-conditioned media (ACM) generated from gene replacement mouse astrocytes expressing either ApoE3 or ApoE4. We found that, compared to

ApoE3 ACM, ApoE4 ACM increased the MAM activity of target cells significantly. These findings imply that ApoE can modulate ER-mitochondria communication, which may help explain, in part, the contribution of ApoE4 to the risk of developing AD.

Results

We developed a protocol to treat fibroblasts with ACM derived from knock-in mice expressing either human ApoE3 or ApoE4 under the control of the endogenous mouse ApoE promoter (DeMattos et al. 2001). We cultured the astrocytes for three days (Hayashi et al. 2007), and then incubated human fibroblasts, which, like neurons, normally do not express ApoE (Xu et al. 2006) (**Fig S1**), in ACM for one day (Harris et al. 2004) (see scheme in **Fig 4A**). Prior to all experiments, we measured the amount of ApoE present in the ACM, which typically contained more ApoE3 than ApoE4, as expected (Ramaswamy et al. 2005, Ulrich et al. 2013) **(Fig S1)**, and then applied the ACM's on an "equal-ApoE" basis. Following treatment with the ACM's, we measured various aspects of MAM function.

ApoE4 ACM treatment increases MAM-mediated phospholipid synthesis relative to ApoE3 ACM

Phospholipid synthesis is a major function of MAM (Voelker 2005). In particular, phosphatidylserine (PtdSer) is synthesized in MAM (Hayashi et al. 2009). PtdSer is then transported from the ER to mitochondria, where it is decarboxylated to produce phosphatidylethanolamine (PtdEtn); PtdEtn can then be transported back to the ER, where it is either methylated to produce phosphatidylcholine (PtdCho) or distributed to other membranes of the cell (Vance 2008). Although some PtdEtn is produced on the cytosolic face of the ER by CDP-ethanolamine exchange via the Kennedy pathway (Gibellini and Smith 2010), the majority

of PtdEtn is produced by the conversion of PtdSer to PtdEtn in mitochondria (Voelker 2005). The conversion of PtdSer to PtdEtn is an established marker for ER-mitochondrial communication (Voelker 2005). We had previously shown that presenilin-mutant cells, including AD fibroblasts, synthesized significantly more PtdSer and PtdEtn via the MAM pathway than did wild-type controls (Area-Gomez et al. 2012). We therefore incubated normal human fibroblasts in ACM medium containing ^{3}H-Ser and measured the incorporation of the label into newly-synthesized ^{3}H-PtdSer and ^{3}H-PtdEtn (Schumacher et al. 2002).

Relative to the values obtained with ApoE3 ACM, we found a significant increase in the synthesis and transport of both ^{3}H-PtdSer (fold increase of ~2.0±0.3) and ^{3}H-PtdEtn (~2.2±0.3) in human fibroblasts treated with ApoE4 ACM **(Fig 4B)**. We also performed the same experiment on explanted hippocampal neurons derived from 1- to 3-day-old mice, and found a significant increase in ApoE4-mediated PtdEtn synthesis (~1.8±0.3), while the increase in PtdSer trended to significance (~1.7±0.4) (**Fig 4B**).

To establish that the increase in phospholipid production was indeed mediated by MAM, we performed the same assay using mouse embryonic fibroblasts (MEFs) in which the mitofusin 2 gene (*Mfn2*) had been knocked out (Chen et al. 2003). Mfn2, in addition to its role in mitochondrial fusion, tethers ER to mitochondria (de Brito and Scorrano 2008); importantly, genetic ablation of *Mfn2* reduces this connectivity (de Brito and Scorrano 2008). Consistent with this loss in interorganellar communication, we had previously established that PtdSer and PtdEtn production is reduced significantly in Mfn2-KO MEFs (Area-Gomez et al. 2012). We therefore asked if the ApoE4-mediated increase in phospholipid synthesis could be abrogated in Mfn2-KO cells. Similar to what we observed in the human fibroblasts, treatment of wild-type MEFs with

ApoE4 ACM resulted in an increase in PtdSer (~1.5±0.3) and PtdEtn (~1.6±0.2) production compared to ApoE3 ACM **(Fig 4C)**. However, we found no such increase either PtdSer (~1.0±0.2) or PtdEtn (~0.9±0.1) production in the Mfn2-KO MEFs **(Fig 4C)**, strongly suggesting that the ApoE4-mediated effect on phospholipid metabolism was indeed the result of increased MAM functionality.

Phospholipids produced at the MAM, including PtdEtn, are subsequently transported to the other membranous compartments of the cell, including the plasma membrane. Cell surface PtdEtn can be detected using cinnamycin and duramycin, two highly-related lantibiotics that bind specifically to PtdEtn on the cell surface and induce cell death in a PtdEtn concentration-dependent manner (Marki et al. 1991, Makino et al. 2003). We had previously shown that the elevated levels of PtdEtn produced by presenilin-mutant cells, including AD fibroblasts, were significantly more sensitive to both cinnamycin and duramycin than were wild-type controls (Area-Gomez et al. 2012). To test if there was also increased lantibiotic sensitivity in our experimental paradigm, we applied duramycin to ACM-treated fibroblasts and measured cell death. We found that ApoE4 ACM-treated fibroblasts were significantly more sensitive to duramycin treatment (~50±20% cell death) than were ApoE3 ACM-treated cells (~13±7%) **(Fig 4D)**. The ability of duramycin to induce cell death was abrogated by the addition of exogenous PtdEtn to the medium **(Fig 4D),** supporting the specificity of the assay. Thus, these data are consistent with the ^{3}H-serine incorporation data, and support the conclusion that ApoE4 plays a role in MAM-mediated phospholipid synthesis and transport.

The stability and capacity of mitochondrial oxidative phosphorylation (OxPhos) complexes (which is reduced in AD cells (Wang et al. 2009)) can be affected by the phospholipid

composition of the mitochondrial inner membrane, especially by PtdEtn and the related molecule cardiolipin (Bottinger et al. 2012). Nevertheless, we found that respiration in ApoE4 ACM-treated cells was similar to that in ApoE3 ACM-treated cells **(Fig S2)**.

Lipoprotein fractions enriched from ApoE4 ACM increase MAM-mediated phospholipid synthesis

ApoE is present in the brain as a component of HDL-like particles that are secreted by astrocytes and are taken up by neurons by various lipoprotein receptors (Pitas et al. 1987, Lund-Katz and Phillips 2010). Following receptor-mediated endocytosis, the ApoE-containing HDL-like particles are hydrolyzed in the lysosome, and the remaining ApoE is either degraded intracellularly or re-lipidated in the secretory pathway or at the cell surface (Heeren et al. 2004). The activity of ApoE largely depends upon its lipidation state, as the lipid-free form of ApoE ("free ApoE") affects cellular activities differently than does ApoE as a component of assembled lipoproteins (Tokuda et al. 2000, Heeren et al. 2003, Heeren et al. 2004).

We therefore examined if the increase in MAM-mediated phospholipid synthesis seen in ApoE4 ACM-treated cells was the result of the effect of ApoE4 as a component of HDLs as opposed to an effect due to ApoE as the free protein. Specifically, we prepared lipoprotein-rich and lipoprotein-free fractions from ApoE3- and ApoE4 ACM by density ultracentrifugation **(**purification scheme in **Fig 5A)** (Hayashi et al. 2007), isolated successive fractions from the gradient, and then added selected fractions either containing (e.g. "fraction 5" **[Fig 5B]**) or not containing (e.g. "fraction 3" **[Fig 5B]**) ApoE **[Fig. S1]** to apolipoprotein-free medium, in order to generate a "surrogate" ACM that was then applied to the cells, followed by assays for phospholipid synthesis as before. Compared to a lipoprotein-rich fraction derived from ApoE3

ACM (i.e. "fraction 5"), lipoproteins enriched from ApoE4 ACM increased PtdEtn synthesis significantly (~2.4±0.5) **(Fig 5C)**. Importantly, this effect was not seen with a lipoprotein-negative fraction from the same preparations (i.e. "fraction 3"), nor when equimolar amounts of free (i.e. unlipidated) recombinant ApoE3 and ApoE4 were applied to the cells **(Fig 5C)**. Thus, we ascribe ApoE4's role in increasing MAM activity (as measured by phospholipid synthesis) to its function as a component of lipoproteins.

ApoE4 ACM treatment increases MAM-mediated cholesteryl ester synthesis relative to ApoE3 ACM

MAM has the properties of an intracellular lipid raft that is rich in cholesterol and sphingolipids (Hayashi and Fujimoto 2010, Williamson et al. 2011, Area-Gomez et al. 2012). Membrane cholesterol is under tight control, and excess cholesterol may either be oxidized by cholesterol hydroxylases (e.g. CYP46A1) or converted into cholesteryl esters (CE) by acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1; gene *SOAT1*), a resident MAM protein (Rusinol et al. 1994). ACAT1 controls the equilibrium between membrane-bound free cholesterol and CE stored in cytoplasmic lipid droplets (Puglielli et al. 2001). Remarkably, that equilibrium plays a key role in regulating the production of Aβ, via a currently-unknown mechanism (Puglielli et al. 2004). We also note that skin fibroblasts (Pani et al. 2009, Area-Gomez et al. 2012) and neurons (Gómez-Ramos and Asunción Morán 2007) from AD patients contain more lipid droplets than controls.

We had previously shown that presenilin-mutant cells, including AD fibroblasts, synthesized significantly more CE and lipid droplets than did wild-type controls (Area-Gomez et al. 2012). To test the contribution of ApoE to CE synthesis, we applied ACM to wild-type fibroblasts as

above, and determined CE production (via incorporation of labeled oleate into CE) and lipid droplet formation (via staining with LipidTox™). We added ^{3}H-oleate to ACM-treated fibroblasts for 24 h, extracted lipids from these samples, and separated cholesteryl esters from triglycerides by thin layer chromatography (TLC). Compared to ApoE3 ACM-treated fibroblasts, there was a significant increase in the levels of ^{3}H-cholesteryl oleate in the ApoE4 ACM-treated cells (~4.1±1.2) **(Fig 6A)**. In addition, the ApoE4 ACM-treated cells contained significantly more lipid droplets per cell (~39±4) than did the ApoE3 ACM-treated samples (~25±3) **(Fig 6B)**. LipidTox also stains triglycerides, another component of lipid droplets. Importantly, we detected essentially no difference in the incorporation of ^{3}H-oleate into ^{3}H-triglycerides **(Fig 6A)**, indicating that the ApoE4-specific increase in lipid droplets was primarily the result of elevated CE production **(Fig 6B)**.

Discussion

The role of ApoE4 as a major risk factor in AD has been enigmatic since the initial discovery of its linkage to the disease in 1993 (Strittmatter et al. 1993). Broadly speaking, studies of ApoE's role in AD pathogenesis have focused on amyloid-dependent or amyloid-independent pathways. Amyloid-dependent theories make use of the fact that ApoE, either by direct binding to Aβ (Strittmatter et al. 1993) or by competition with Aβ receptors, such as the lipoprotein receptor-related protein-1 (Sagare et al. 2013), is able to modify Aβ production (Vincent and Smith 2001) or clearance (Verghese et al. 2013). Amyloid-independent theories implicate the cytotoxicity of ApoE4 fragments (Harris et al. 2003), its role in cholesterol homeostasis (Heeren et al. 2003), neuroprotection/apoptosis (Hayashi et al. 2007), neurite outgrowth (DeMattos et al. 2001), inflammation (Guo et al. 2004), and vascular integrity (Bell et al. 2012). AD is clearly a

multifaceted disease, with changes in amyloid metabolism occurring alongside these amyloid-unrelated phenomena.

We reported previously that PS1, PS2, and γ-secretase activity are highly enriched in the MAM (Area-Gomez et al. 2009), and that ER-mitochondrial communication and MAM function, including cholesteryl ester and phospholipid synthesis, are increased dramatically in presenilin-mutant and -deficient cells and in cells from AD patients (Area-Gomez et al. 2012). Both lines of evidence support the view that PS1 and PS2, in addition to generating Aβ, are negative regulators of MAM function (Area-Gomez et al. 2012), and that these alterations play a critical role in the pathogenesis of the disease (the "MAM hypothesis") (Schon and Area-Gomez 2010, Schon and Area-Gomez 2013). If the hypothesis has any validity, it should accommodate ApoE4's role as a risk factor in the disease, i.e., ApoE4 should play a role in mediating MAM behavior, either directly or indirectly. We therefore treated cells with ApoE4 vs. ApoE3 ACM and measured two key aspects of MAM behavior, namely ER-mitochondrial communication and MAM function, as measured by the synthesis of phospholipids and of cholesteryl esters, respectively. We found that in both cases ApoE4 ACM, like the presenilin mutants, was indeed able to increase MAM functionality. We note that in general, the degree of increase, while significant, was not as pronounced as that reported previously in presenilin-mutant cells (Area-Gomez et al. 2012), consistent with ApoE4's role as a risk factor, not a determinative factor, in AD. These findings lend support to the idea that ApoE4's role as a risk factor in AD may be via its effects, at least in part, on ER-mitochondrial communication.

The mechanism of this ApoE4-mediated increase in MAM activity is unclear. However, given that ApoE4 exerted its effects on MAM when incorporated into lipoprotein particles, but

not when present as the "free" protein, we suspect that the lipoprotein metabolism pathway plays a role in ApoE4-mediated pathogenesis. Support for this idea comes from the numerous points of intersection between lipoprotein metabolism and alterations in APP metabolism by γ-secretase, which is a known regulator of MAM function (Area-Gomez et al. 2012) and which is central to AD pathology (Strittmatter et al. 1993).

Isoform-specific differences in lipoprotein metabolism, and, by association, cholesterol trafficking, may provide some insight as to how ApoE4 leads to MAM dysfunction. One of these differences is the relative affinity of each isoform for the lipoprotein receptors at the cell surface, thereby affecting the total amount of cholesterol imported (Holtzman et al. 2012). Upon receptor-mediated endocytosis, the ApoE-containing HDL-like particles are transported to lysosomes, where they are hydrolyzed. Some ApoE is degraded and some is recycled back to the cell surface (Heeren et al. 2003, Heeren et al. 2004), where it is re-lipidated by ABCA1 or ABCG1 and secreted. Notably, ApoE4 is recycled much less efficiently than is ApoE3 (Heeren et al. 2003, Heeren et al. 2004), resulting in an accumulation of cholesterol in endosomes and lysosomes, reminiscent of Neimann-Pick disease Type C, a neurodegenerative disease characterized by the accumulation of cholesterol in lysosomes. Thus, it may be that the increase in cholesteryl esters seen in ApoE4 ACM-treated cells reflects a concomitant increase in available substrate (i.e. cholesterol) at the MAM.

Cellular cholesterol handling affects APP processing at numerous points in cell metabolism (Di Paolo and Kim 2011) and vice versa (Pierrot et al. 2013). First, the internalization of cholesterol-containing lipoproteins from the extracellular space may affect the rate of APP internalization, which is a requirement for its amyloidogenic processing (Schneider et al. 2008).

Indeed, the bulk uptake of cholesterol is important in AD, as diet-induced hypercholesterolemia in rabbits resulted in amyloid pathology (Sparks et al. 1994). Second, the cholesterol content of intracellular membranes likely affects APP processing, since the C-terminal fragment of APP binds cholesterol (Beel et al. 2008, Barrett et al. 2012). Third, each of the three proteases that process APP is also influenced by cholesterol. A fraction of β-secretase (BACE1) localizes to, and is active in, lipid rafts, and an increase in cholesterol enhances the β-cleavage of APP (Marquer et al. 2011). γ-Secretase is also localized to rafts (Urano et al. 2005) and is affected by cholesterol levels, particularly by the ratio of free cholesterol:cholesteryl esters, as inhibition of ACAT1, either pharmacologically or by genetic ablation, reduces Aβ production (Puglielli et al. 2004). Conversely, the non-amyloidogenic arm of APP processing by α-secretase is inhibited by the addition of exogenous cholesterol (Bodovitz and Klein 1996). These observations point to a complex biology in which the subcellular amount and distribution of cholesterol are linked to APP metabolism and MAM function.

In summary, we have demonstrated that ApoE4-containing ACM increases MAM function relative to ApoE3. This report extends our previous findings demonstrating upregulation of MAM function in AD and may help explain why ApoE4 is a risk factor in the disease.

Materials and Methods

Cells and reagents

Telomerase (hTERT)-transformed human fibroblasts were a kind gift of Drs. Orhan Akman and Sonja Brosel (Columbia University). Immortalized *APOE3*- and *APOE4*-targeted gene replacement astrocytes (Xu et al. 1996) were prepared as described (Fagan et al. 1999). Cells were cultured in minimal essential media (MEM [lacking serine]) (Gibco 11095) or Dulbecco's

MEM (DMEM [containing serine]) (Gibco 11995-065). Immortalized cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Sigma F2442) and 1% antibiotic-antimycotic (Gibco 15240).

Mouse hippocampal neuronal culture techniques were adapted from our previous approaches (Staal et al. 2007). The hippocampus was dissected from 1-3-day-old wild-type C57BL6/J pups, the tissue digested in a papain solution (Worthington Biochemical, Lakewood, NJ) at 20 units/ml), the cells triturated, spun down, and resuspended in NbActiv4 media (Brain Bits Nb4-500) supplemented with 1% heat-inactivated FBS (Life Technologies 16140-063). Cells were plated at a density of ~320,000 cells per 35-mm diameter petri dishes precoated with laminin (Millipore, 10 µg/ml) for 1 hour. The medium was changed 3 days after plating the cells. Cultures were maintained at 37°C and 5% CO_2. For all experiments with ACM, the cultured neurons were incubated for 10 d prior to assay.

We used antibodies against ApoE (monoclonal aa140-160; Novus WUE-4 NB110-60531), clusterin (polyclonal C-terminus; Novus NBP1-19637), β-actin (monoclonal; Sigma AC-15 A5541), which were used according to the manufacturers' instructions. Thin layer chromatography (TLC) silica plates were from EMD Biosciences (5748-7). Phosphatidylserine (PtdSer) (Sigma P7769), phosphatidylethanolamine (PtdEtn) (Sigma 60660), cholesteryl palmitate (Sigma C6072), and soybean phospholipid mixtures (Sigma P3817) were used as standards for TLC in experiments with radiolabelled ^{3}H-serine (Perkin Elmer NET248005MC) and ^{3}H-oleate (Perkin Elmer, NET289001MC). Bovine serum albumin (Sigma A3803) was used to solubilize ^{3}H-oleate (Saifer and Goldman 1961).

Astrocyte conditioned media generation and lipoprotein enrichment

APOE3 and *APOE4* astrocytes were grown to confluency, washed once with MEM, and incubated in MEM for 3d. Astrocyte conditioned medium (ACM) was spun at 3000 g for 3 min to remove debris and analyzed first by western blot against ApoE to confirm the presence of lipoproteins. Prior to all experiments, we measured the amount of ApoE present in the ACM by dot blot (BioRad Bio-Dot™ Apparatus) (it typically contained more ApoE3 than ApoE4, consistent with the finding that the concentration of ApoE3 in the brain homogenates (Ramaswamy et al. 2005) and in the interstitial fluid of human ApoE knock-in mice (Ulrich et al. 2013) is ~50% greater than that of ApoE4) and then added the ACM on an "equal-ApoE" basis, by adding MEM to the ApoE3 ACM as appropriate.

Density ultracentrifugation was used to prepare enriched fractions of lipoproteins (Vance and Hayashi 2010). Briefly, 100 ml ACM was concentrated to ~4 ml using Centriprep 10K filters (EMD Millipore). Four ml of ACM concentrate was layered on top of 3 ml 1.1 g/ml, 3 ml 1.2 g/ml, 2 ml 1.3 g/ml discontinuous sucrose gradient. Samples were centrifuged at 160,000 g in a Sw44-Ti rotor (Beckman) for 48 hr at 4°C. One-ml fractions were removed from the top of the gradient, and ApoE content was analyzed by western blot.

Analysis of phospholipid synthesis

Fibroblasts were grown to confluency (5 replica plates per condition) and washed once with MEM. ACM, recombinant ApoE, or enriched lipoprotein fractions containing 2.5 μCi/ml of ^{3}H-serine (30.5 Ci/mmol) were applied to the cells grown in 60-mm dishes for 24 h. The cells were washed twice with 1 ml ice-cold phosphate-buffered saline (PBS) and collected on ice in 1 ml PBS, pelleted at 3000 g for 3 m at 4ºC, and resuspended in 0.5 ml water. Twenty μl of suspension was stored at -20°C for protein quantification by Bradford analysis. Total lipids were

extracted as follows: 3 volumes of 2:1 chloroform:methanol v/v were added to the samples which were then vortexed and centrifuged at 8000 g for 5 m, and the aqueous phase was discarded. The organic phase was washed with 2 volumes of 1:1 methanol/water v/v, centrifuged at 8000 g for 5 min, and then washed again in the same manner. The organic phase was then transferred to a fresh tube and incubated at 50ºC until dry. After resuspension in 40 µl of chloroform, total lipids were spotted onto a TLC plate. Plates were run through a series of two solvents, first 84:15:1 petroleum ether/diethyl ether/acetic acid v/v/v, and then 60:50:1:4 chloroform/methanol/acetic acid/water v/v/v/v. Separated phospholipids and the corresponding standards were visualized after overnight exposure to iodine vapor. Phospholipid species were cut from the plate, suspended in scintillation liquid (Ultima Gold XR Perkin Elmer), and counted by scintillation to detect ^{3}H (Packard Tri-Carb 2900TR).

Duramycin assay

Fibroblasts were treated with ACM for 24 h, washed once with MEM, and treated with the specified concentration of duramycin (Sigma D3168) or duramycin and PtdEtn diluted in MEM (1:3 duramycin:PtdEtn mol/mol) for 20 m at 37°C. Cells were washed twice with PBS and stained with the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies L3224) according to the manufacturer's instructions. Cells were visualized by fluorescent microscopy and hand-scored. Percent death was calculated as a ratio of ethidium-stained red cells to total cells.

Analysis of cholesteryl ester synthesis

Fibroblasts were grown to confluency and washed once with MEM. ACM containing 2.5 µCi/ml of ^{3}H-oleate (54.6 Ci/mmol) solubilized in MEM-2%BSA were applied to the cells for 24 h. Sample processing and total lipid extraction were performed as described above. TLC plates

were run using 190:9:1 chloroform/methanol/acetic acid v/v/v as a solvent. After exposure to iodine vapors, spots corresponding to the cholesteryl ester standards were scraped and counted by scintillation as described above.

Lipid droplet staining

Fibroblasts were grown to ~80% confluency on glass coverslips, washed once with MEM, and treated with either ApoE3 or ApoE4 ACM for 24 h. Cells were washed once in PBS, and lipid droplets were stained with HCS LipidTox™ Deep Green neutral lipid stain (Invitrogen H34475) according to the manufacturer's instructions. Lipid droplets were visualized by fluorescent microscopy and the number of lipid droplets per cell was quantified by hand.

Mitochondrial respiration measurements

Mitochondrial respiration was determined using the Seahorse XF-24 analyzer (Seahorse Bioscience). WT fibroblasts were seeded at 8 x 10^4 cells per well in a XF24 cell culture microplate and treated with ACM for 24 h. Cells were washed with XF Assay Media (Seahorse Bioscience), and pre-incubated for 1 h at 37°C and 0% CO_2 in XF Assay Media, supplemented with 2 mM pyruvate and 25 mM glucose. For the assay, cells were treated sequentially with 1 µM oligomycin, 0.75 µM carbonyl cyanide-4-trifluoromethoxy phenylhydrazone, and 1 µM rotenone plus 1 µM antimycin A.

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Author Contributions

MDT, EA-G and EAS designed the research, MDT, MP and HY performed the experiments, MDT, MP and EAS analyzed the data, EK, DS, DH and HY provided key reagents and critical advice on their use and preparation, MDT and EAS wrote the manuscript.

Conflict of interest

The authors declare they have no conflict of interest.

Figure 4

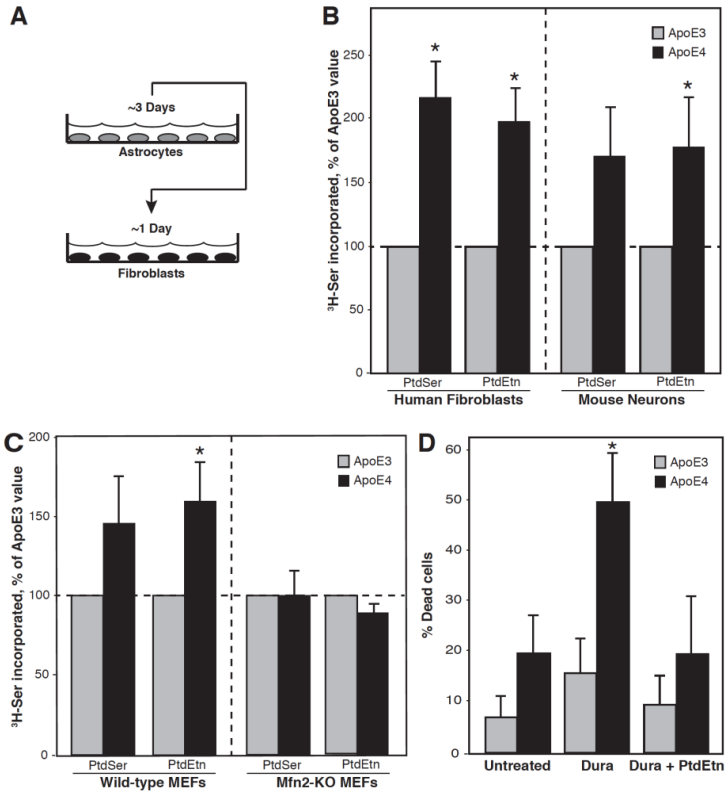


Figure 4. Phospholipid synthesis in ApoE ACM-treated cells.

A Experimental scheme. See text for details.

B Phospholipid transport/synthesis, as measured by ^{3}H-serine incorporation, in human fibroblasts (mean±SE; n=9, with 5 replicates/experiment) and in primary mouse hippocampal neurons (mean±SE; n=3, with 4 or 5 replicates/experiment). *, $p<0.05$ vs E3.

C Comparison of ^{3}H-serine incorporation in ApoE ACM-treated WT- and Mfn2-KO MEFs (mean±SE; n=3, with 3 replicates/experiment).

D Duramycin sensitivity in ApoE ACM-treated fibroblasts. Note increased sensitivity after 20 min to 5 µM duramycin in ApoE4-treated cells, which was blocked upon the addition of 15 µM exogenous PtdEtn (mean±SD; n=3, with 3 replicates/experiment).

Figure 5

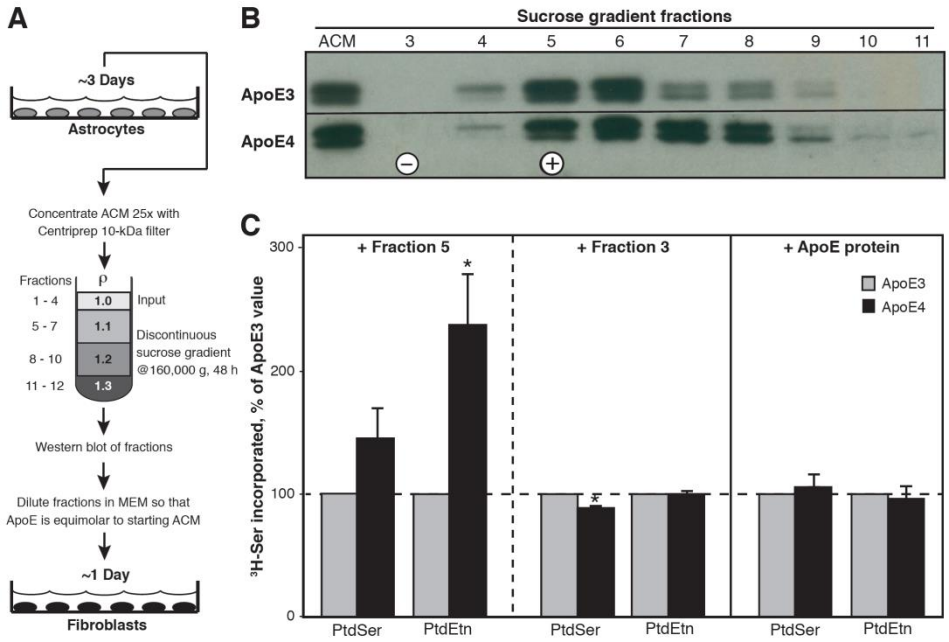


Figure 5. Effect of lipoprotein-rich and -poor fractions on phospholipid synthesis.

A Experimental scheme. See text for details.

B Fractions from the discontinuous gradient were blotted with anti-ApoE (WUE-4) antibody; the doublet is a characteristic feature on ApoE western blots due to ApoE sialylation (Pitas et al. 1987).

C An ApoE-containing fraction (fraction 5 [+]), an ApoE-negative fraction (fraction 3 [-]), and recombinant lipid-free ApoE protein were applied to human fibroblasts. Note increase in phospholipid synthesis using an ApoE4-containing lipoprotein fraction, but not with either an ApoE4-negative fraction or with lipoprotein-free recombinant ApoE3 or ApoE4 protein (mean ± SE; n=3, with 3 replicates/experiment).

Figure 6

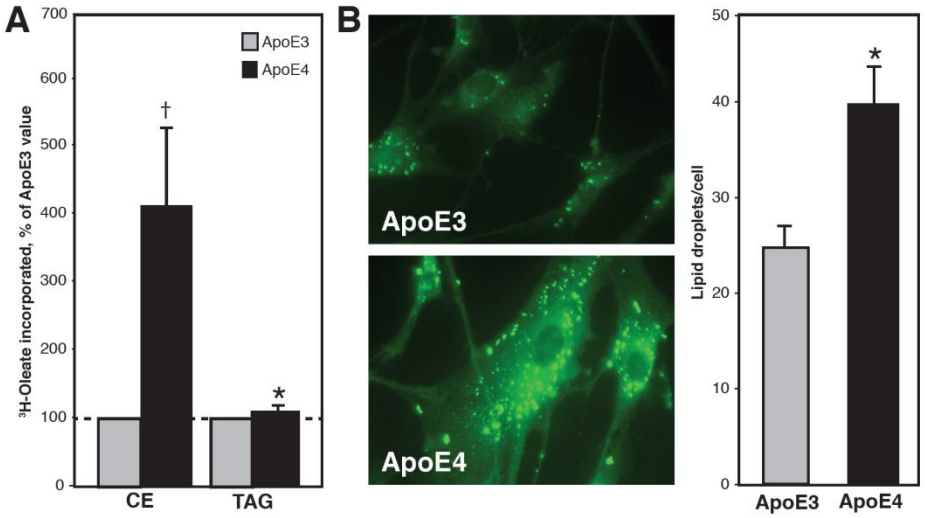
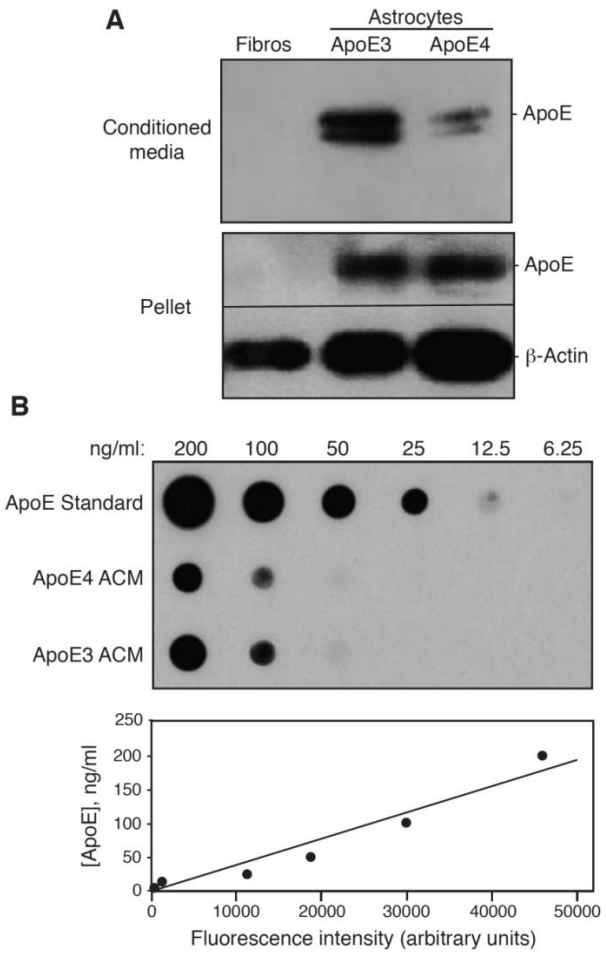


Figure 6. Cholesteryl ester production in ApoE ACM-treated cells.

A Cholesteryl ester synthesis, as measured by ^{3}H-oleate incorporation. Note increased CE in ApoE4-treated cells, whereas there was essentially no difference in triglyceride (TAG) production (mean±SE; n=3. with 3 replicates/experiment). †, p=0.06 vs E3.

B ApoE ACM-treated fibroblasts were stained with LipidTox green neutral lipid stain, and lipid droplets were visualized (example at left) and counted (right). Note significantly more lipid droplets in ApoE4-treated cells (mean ± SD, n=3).

Figure S1

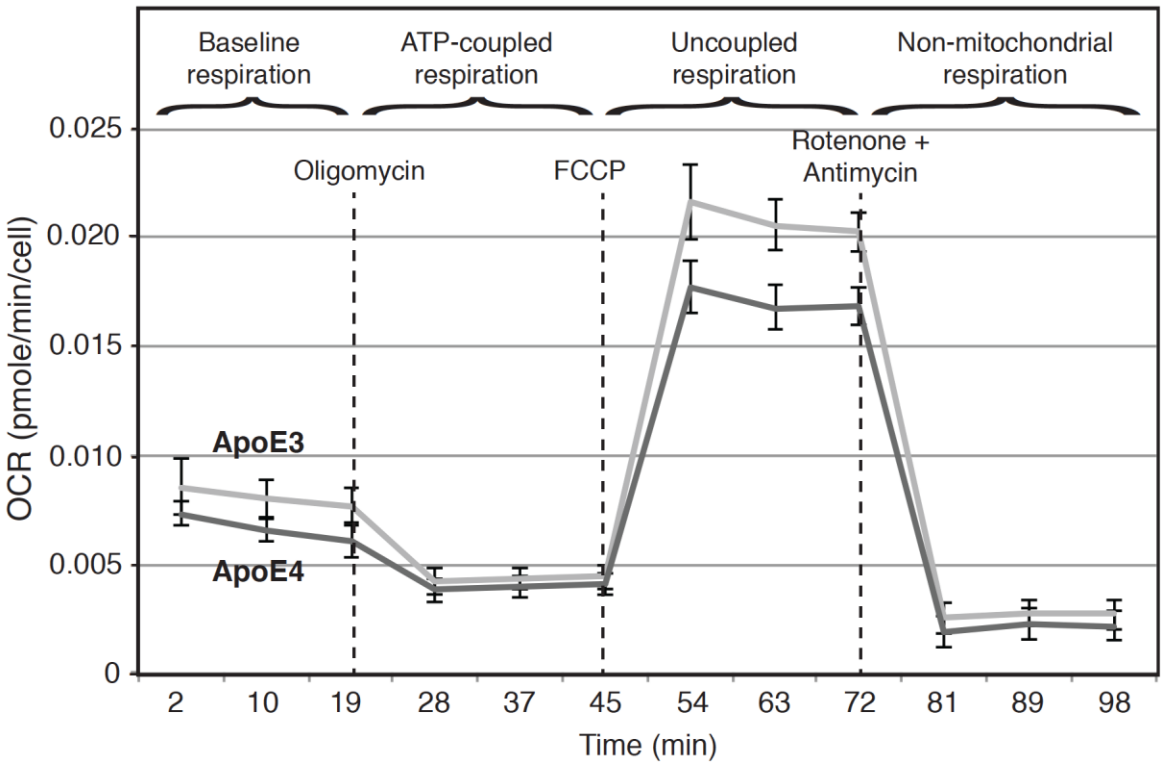


Supplementary Fig. 1. Quantification of ApoE.

A The ApoE content of whole cell lysates (30 µg total protein loaded in each lane) and conditioned media (15 µl loaded in each lane) from cultured human fibroblasts and astrocytes from ApoE3- and ApoE4-targeted gene replacement mice were analyzed by Western blotting.

B ApoE content of ACM and fibroblast-conditioned media was analyzed by dot blot (upper panel). The amount of ApoE was calculated by comparison to a serial dilution of recombinant ApoE standard (lower panel). From this standard curve the ApoE content of ApoE3 and ApoE4 ACM was determined to be ~125 and ~100 ng/ml, respectively.

Figure S2



Supplementary Figure 2. Bioenergetics of ApoE ACM-treated fibroblasts.

ApoE ACM-treated human fibroblasts were analyzed using a Seahorse XF-24 Flux Analyzer. Oxygen consumption rate (OCR) was determined (in pmole O_2/min/cell) after the addition of oligomycin (to inhibit ATP synthesis), FCCP (to uncouple ATP synthesis from respiration), and rotenone and antimycin A (to inhibit complexes I and III, respectively). The overall pattern of respiration was essentially similar between ApoE3 and ApoE4 ACM-treated cells, although maximal respiration of ApoE4 ACM-treated cells (the plateau between FCCP and rotenone/antimycin addition) was somewhat lower than that of ApoE3 ACM-treated cells (mean±SD, n=3).

CHAPTER 2. THE EFFECT OF APOE ON MAM LIPID COMPOSITION

INTRODUCTION

As a lipid raft, the MAM is enriched in cholesterol and sphingolipids, and its function largely depends on its cholesterol and lipidic content. MAM contains about 7x the amount of cholesterol found in bulk membranes (Fujimoto et al. 2012). Chemical perturbation of the amount of cholesterol in MAM affects its activity. Methyl-beta-cyclodextrin (MβCD) is a cyclic oligosaccharide with a hydrophobic center that reversibly binds cholesterol, sequestering it from membranes (Christian et al. 1997). Addition of MβCD to cell lysates will extract membrane cholesterol indiscriminately, though given the high cholesterol content of MAM, this compartment will be affected disproportionately. MβCD treatment has been found to have a dual effect on MAM, in that it reduces the amounts of some MAM-resident proteins (e.g. the sigma-1 receptor (σ1R)), yet increases the synthesis of PtdEtn, indicating increased ER-mitochondria connectivity (Fujimoto et al. 2012). These data would seem to indicate that cholesterol is a negative regulator of MAM activity. This idea is difficult to imagine because cholesterol itself is a substrate for MAM activity, as measured by cholesterol ester synthesis by ACAT1. Presumably the increase in cholesteryl ester synthesis and lipid droplet formation seen in AD fibroblasts, PS1/2 DKO MEFs, and recently in ApoE4 ACM-treated fibroblasts, is the result of increase in available substrate. To determine MAM cholesterol levels, we biochemically isolated MAM from ApoE3 and ApoE4 ACM-treated fibroblasts and measured membrane cholesterol by the Amplex Red cholesterol assay. A decrease in cholesterol content of ApoE4 ACM-treated would be consistent with the observations of increased MAM activity

upon cholesterol depletion, while an increase in cholesterol would indicate a more simple "substrate availability" relationship between cholesterol and MAM function.

Lipids play a very important role in AD, and this has been known from the earliest studies of the disease. Alzheimer noted, in addition to the now canonical plaques and tangles, the appearance of "lipoid granules" in AD neurons (Di Paolo and Kim 2011). Less attention has been paid to these changes in lipid metabolism, since lipids are not directly encoded genetically and fewer methodologies exist for their analysis. Recently, however, several studies have found AD-specific changes in the lipidome. These changes are useful as diagnostic biomarkers of AD. Analysis of the plasma lipidome predicts conversion of mild cognitive impairment to clinical dementia. Patients who progress to AD have lower levels of PtdCho and acylcarnitine than non-progressing controls (Mapstone et al. 2014). Other studies have found reduced amounts of long chain cholesteryl esters in plasma of AD patients (Proitsi et al. 2015). Note that the source of plasma cholesteryl esters is different from that of the intracellular cholesteryl esters measured in chapter 1 of this thesis. Plasma cholesteryl esters are formed on the surface of lipoproteins by lecithin: cholesterol acyl transferase (LCAT), and not by the intracellular MAM-resident ACAT1 (Fielding et al. 1972). Plasma studies, while useful as diagnostic indicators, may not speak to the mechanism of AD, given the co-morbidity of vascular changes in the elderly (e.g. atherosclerosis, vasculitis, inflammation) that may affect AD without causing it. Brain tissue lipidomics, however, offers a more powerful insight into AD pathogenesis. Similar changes in lipid patterns occur in the brains of AD patients and AD mice. These changes include increases in cholesteryl esters, which are consistent with our findings of increased MAM activity, as well as increases in sphingomyelin and the ganglioside GM3 (Chan et al. 2012).

As with cholesterol, the distribution of lipids is not homogenous within the ER. MAM is enriched in sphingolipids, a class of lipids with a sphingosine backbone. The relative abundance of these lipids has implications for cellular function. One example of the importance of the lipidic content of MAM is the role ceramide plays in apoptosis. Ceramide, the sphingolipid with the most basic side chain, consisting only of a hydrogen atom **(Fig 7)**, is produced *de novo* in MAM (Bionda et al. 2004). Highly insoluble, ceramide does not diffuse horizontally through cellular membranes but instead tends to stay wherever it is produced. At MAM, ceramide has been shown to translocate from ER to mitochondria, where, if present in high enough concentrations, it forms a pore and in so doing permeabilizes the outer mitochondrial membrane (Lee et al. 2011). Pro-apoptoptic factors, such as cytochrome c, can then leak out of the intermembrane space and into the cytosol and induce apoptosis (Elmore 2007). Therefore, the contact site between ER and mitochondria is critical for the induction of apoptosis.

We hypothesize that ApoE4 ACM treatment increases MAM activity by changing its lipidome. To test this hypothesis, we treated SH-SY5Y neuroblastoma cells with ApoE3 and ApoE4 ACM, and prepared MAM though biochemical fractionation of these treated cells. The lipid profiles of isolated MAM, crude mitochondria, and whole cell lysate were then analyzed by liquid chromatography-mass spectrometry. The advantage of this approach is that it is unbiased and reveals changes not only in classes of lipids, but in the length and saturation of their acyl chains as well.

MATERIALS AND METHODS

Cell culture. Telomerase (hTERT)-transformed human fibroblasts were a kind gift of Drs. Orhan Akman and Sonja Brosel (Columbia University). Immortalized APOE3- and APOE4-

targeted gene replacement astrocytes (Xu et al. 1996) were prepared as described (Fagan et al. 1999). SH-SY5Y cells were a kind gift of Shuobo Zhang (Columbia). Fibroblasts and astrocytes were cultured in Dulbecco's minimal essential media (DMEM Gibco 11995-065) containing 10% fetal bovine serum (FBS) (Sigma F2442) and 1% antibiotic-antimycotic (Gibco 15240-062). SH-SY5Y were cultured in OptiMEM (Gibco 31985) supplemented with 10% FBS, 1 mM pyruvate, 2 mM glutamine, and 1X nonessential amino acids (Gibco 11140). All cells were incubated at 37°C in 5% CO_2. We used antibodies against ApoE (monoclonal aa140-160; Novus WUE-4 NB110-60531), and β-actin (monoclonal, Sigma AC-15 A5541).

Antibodies. We purchased antibodies against ApoE (monoclonal aa140-160; Novus WUE-4 NB110-60531), β-actin (monoclonal, Sigma AC-15 A5541), and Erlin-2 (polyclonal ~aa315, Cell Signaling #2959), which were used according to the manufacturers' instructions. Western blotting was performed as described (Area-Gomez et al. 2012).

Generation of astrocyte conditioned media. APOE3 and APOE4 astrocytes were grown to confluency, washed once with MEM, and incubated in MEM for 3d. Astrocyte conditioned media (ACM) was spun to remove debris and analyzed by western blot against ApoE to confirm presence of lipoproteins. All E3 and E4 astrocyte conditioned media preparations were diluted to equimolar amounts of ApoE as determined by western blot.

Subcellular fractionation. Fibroblasts or SH-SY5Y cells were grown to confluency in five 175 cm^2 flasks. Cells were washed once with PBS, trypsinized, and centrifuged at 3000 x g for 3 min. The pellets were washed once in PBS, and resuspended in 4X volumes of homogenization buffer (250 mmol/L mannitol, 5 mmol/L HEPES, and 0.5 mmol/L EGTA at pH 7.4, Roche

complete protease inhibitor cocktail, cat # 04693159001). From this point on, all steps were performed in a 4°C cold room or on ice. Cells were homogenized with several strokes in a glass/glass dounce homogenizer, and an aliquot of whole cell lysate was stored for analysis. The remaining lysate was centrifuged twice at 600 x g to remove intact cells, cell debris, and nuclei. To pellet mitochondria, the supernatant was centrifuged for 15 min at 10,500 × g. The crude mitochondrial pellet was resuspended in 400 µl homogenization buffer, and an aliquot was stored for future analysis. The crude mitochondria homogenate was layered on top of a 30% Percoll gradient (Sigma P4937) and ultracentrifuged for 30 minutes at 95,000 × g in a SW55-Ti Beckman Coulter ultracentrifuge rotor. The upper band, which contains MAM and mitochondria associated to ER, was collected and diluted sevenfold in homogenization buffer to remove any Percoll. The samples were centrifuged at 6300 × g for 10 minutes to remove any mitochondria. The MAM-containing supernatant was ultracentrifuged for 1 hr at 100,000 x g in a SW55-Ti rotor, and MAM was collected at the bottom of the tube. MAM was resuspended in homogenization buffer and all samples were analyzed for total protein content by Bradford analysis (Biorad 500-0205).

Cholesterol quantification. Cholesterol quantification was performed using the Amplex® Red Cholesterol Assay Kit (Life Technologies A12216). One µg total protein from each subcellular fraction was analyzed per well in a 96-well plate. Cholesterol values from whole cell lysate and MAM were normalized to β-actin and ERLIN-2 western blot intensities, respectively.

Lipid analysis using High Performance Liquid Chromatography-Mass Spectrometry. Analysis of lipid profiles was performed as described. Lipids were extracted from SH-SY5Y

subcellular fractions via the Bligh/Dyer method and analyzed using an Agilent 1200 HPLC system and an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometer (3200Qtrap). Polar lipids were separated by normal phase HPLC on a silica column (Phenomenex Luna) using two phases: chloroform:methanol:ammonium hydroxide (89.5:10:0.5) and chloroform:methanol:ammonium hydroxide:water (55:39:0.5:5.5). Phases were run as described previously (Chan et al. 2012). Non polar lipids were separated on an Agilent Zorbax Eclipse XDB-C18 column using a chloroform: methanol:0.1M ammonium acetate (100:100:4) phase. Free cholesterol was also separated on XDB-C18 column using a chloroform:methanol 1:1 phase.

RESULTS

MAM isolated from ApoE4 ACM-treated fibroblasts contain less cholesterol than MAM from ApoE3 ACM-treated cells

The amount of cholesterol in lipid rafts is related to lipid raft function. ApoE has been previously shown to alter the efflux of cholesterol from the cell in an isotype-dependent manner (Heeren et al. 2003). It is possible that ApoE4 differentially alters the distribution of cellular cholesterol, and that these alterations underlie the MAM dysfunction seen in ApoE4 ACM-treated cells. To determine what effect ApoE-ACM treatment has on MAM cholesterol composition, we incubated wild-type fibroblasts in ApoE3- or ApoE4-ACM for 24 h, replicating the conditions shown to previously induce MAM dysfunction by ApoE4-ACM treatment. Total cell lysate and MAM fractions were isolated from these cells, and total membrane cholesterol was determined by the Amplex® Red Cholesterol Assay. As expected, each MAM fraction contained more (~4X) cholesterol, compared to whole cell lysates, independent of ApoE

treatment **(Fig 8)**. Compared to ApoE3 ACM-treated cells, MAM isolated from ApoE4 ACM-treated cells contained 46% less cholesterol **(Fig 8)**. This difference in cholesterol was not seen in the whole cell lysates, suggesting the cholesterol differences in MAM are the result of altered cholesterol distribution rather than a simple increase in bulk import.

MAM isolated from ApoE4 ACM-treated fibroblasts has a different lipid profile than MAM from ApoE3 ACM-treated cells

To determine the effect of ApoE ACM treatment on the lipid profile of MAM, we isolated MAM from ApoE3 and ApoE4 ACM-treated SH-SY5Y cells and characterized the lipid content by HPLC-mass spectrometry. SH-SY5Y cells were chosen for analysis because the rapid growth of this neuroblastoma line ensured enough material for biochemical fractionation of MAM. It must be noted, however, that unlike fibroblasts, SH-SY5Y express ApoE and this ApoE is detectable by western analysis of SH-SY5Y whole cell lysate (Soulie et al. 1999) (our unpublished findings). This ApoE could not be detected in media conditioned by SH-SY5Y, implying that although ApoE is expressed in neuroblastoma cell lines, it is not incorporated into lipoproteins and secreted.

The analyzed lipids were grouped according to class, and each class was further divided by acyl chain length and degree of saturation. The lipid profiles from whole cell lysates and crude mitochondria fractions ACM-treated cells were similar when comparing the effect of ApoE3 and ApoE4 ACM treatment, with no statistically significant class-wide alterations in lipid content. However, MAM fractions from ApoE4 ACM-treated cells showed a statistically significant, class-wide decrease in plasmalogen phosphatidylethanolamine (PEp) **(Fig 9)**. Within each lipid class, there were significant changes in the relative amounts of several lipid species in MAM.

Some of these changes reflect the functional alterations seen ApoE4 ACM-treated MAM, in particular, the changes seen in cholesteryl esters and triglycerides, the main components of lipid droplets **(Fig 10)**. For example, of the 21 cholesteryl ester species analyzed, 19 were increased in ApoE4 ACM-treated MAM fractions, with three (22:2, 22:3, and 24:4) reaching statistical significance. Triglyceride levels remained unchanged or slightly decreased, with 29 of the 42 species decreased, 2 of which were statistically significant (54:7/18:1 and 52:5/18:1). The largest consistent change was the decrease in PEp in MAM fractions from cells treated with ApoE4 ACM **(Fig 9)**. Of the 23 PEp species analyzed, 21 were decreased in MAM derived from ApoE4 ACM-treated cells, with 5 species reaching statistical significance. Some lipid classes showed extreme increases in certain species and decreases in others. Ceramides, for example, were unaffected overall, but contained the largest increases (Cer d18:0/20:0 was increased significantly by ~750%, Cer d18:0/18:1 was increased by ~300%) and decreases (Cer d18:0/24:0 was decreased by ~50%, Cer d18:0/24:1 was decreased by ~50%) **(Fig 11)**. Other lipid classes, including free cholesterol, showed no consistent trend in changes in lipid content.

DISCUSSION

Astrocyte conditioned media increases MAM function of target cells in an ApoE isotype-dependent manner, but the mechanism of those changes in unclear. One possibility is that ApoE alters the distribution of cholesterol and lipids within target cells, and that since MAM is a lipid raft, its activity may be modified by a change in its composition. ApoE, as part of an HDL-like particle, acts as a lipid transporter between cells, carrying phospholipids, cholesterol, and cholesteryl esters from astrocytes to neurons (Holtzman et al. 2012). Little is understood about how ApoE alters the distribution of cholesterol and lipids *within* cells, or how a lipid transporter

that acts at the cell surface might exert an effect on MAM, an internal, mostly perinuclear, cellular compartment. The isoform-specific differences in ApoE trafficking and recycling in target cells provide some rationale as to how such intracellular changes are possible (Heeren et al. 2003, Heeren et al. 2004). After receptor mediated endocytosis, the ApoE-containing lipoprotein is trafficked to early endosomes. Upon fusion with a lysosome, the lipid and protein components of the lipoprotein are separated. A fraction of ApoE is degraded, while the remainder is sent to recycling endosomes, re-lipidated by ABCA1, and re-secreted at the cell surface. Its secretion is usually, but not always, associated with cholesterol efflux from the cell (Hara et al. 2003, Rellin et al. 2008). ApoE3 is recycled efficiently, whereas ApoE4 has been found to remain in recycling endosomes for a longer time and result in a reduced cholesterol efflux (Minagawa et al. 2009). This has two consequences. The first is a lyso-endosomal accumulation of cholesterol. The accumulation of cholesterol in this compartment may be cytotoxic *per se*, as it similar to the changes seen in Niemann–Pick type C, a neurodegenerative lysosomal storage disorder that is the result of mutations in NPC1 or NPC2 (Pentchev et al. 1987). In Neimann-Pick disease, APOE genotype modulates the severity of the disease, with ApoE2 resulting in a less severe phenotype and ApoE4 resulting in a more severe phenotype, suggesting a common pathway (Fu et al. 2012). The second consequence of the inefficient recycling of ApoE4 is a reduction in the turnover of lipoprotein receptors and membrane proteins (Dumanis et al. 2013). This results in a decrease in synaptic plasticity and long term potentiation (Dumanis et al. 2009).

Given the ability of ApoE4 to alter intracellular cholesterol distribution, we wished to see what affect ApoE4 has on MAM. An increase in the cholesterol content of MAM might explain why more cholesteryl esters are present in ApoE4 ACM-treated fibroblasts. Intriguingly, the

opposite was seen, in that MAM isolated from ApoE4-treated fibroblasts contained ~50% less free cholesterol than MAM derived from ApoE3 ACM-treated cells. This implies that the lipid droplets formed in ApoE4-treated cells are not simply the result of an increase of available free cholesterol at MAM. Indeed, further analysis needs to be performed to determine if the lipid droplet formation is the cause of cholesterol depletion of MAM in ApoE4-ACM treated cells. If the addition of ACAT inhibitors is able to restore the amount of MAM cholesterol in ApoE4-ACM treated cells to ApoE3-control levels, this would suggest that the lipid droplets are being formed at the expense of MAM cholesterol, which is not replenished. This scenario is unlikely, given the ratio of cholesterol:cholesteryl esters in the cell is extremely high. In fibroblasts, the majority of cholesterol is in the unesterified form, and the addition of cholesterol esterase, which converts cholesterol esters to free cholesterol, to the reaction mixture did not change the amount of cholesterol detected. Other pathways of cholesterol metabolism need to be accounted for in our system. It is possible that the decrease in cholesterol seen in MAM from ApoE4-ACM treated cells is the result of cholesterol oxidation. Oxidized cholesterol can diffuse out of the cell and across the blood brain barrier into the peripheral circulation (Benarroch 2008). There is evidence for increased oxidation of cholesterol in AD, since plasma 24-hydroxycholesterol levels have been found to be increased in patients (Lutjohann et al. 2000, Bryleva et al. 2010) . The remaining possibility for the decreased amount of cholesterol in MAM from ApoE4 ACM-treated cells is that the cholesterol in the endolysosomal vesicles is never distributed to the ER. Whatever the mechanism of cholesterol depletion in MAM, there is precedent that such depletion may affect its activity.

Lipidomic analysis of ApoE3 and ApoE4 ACM-treated cells confirms an increase in ACAT activity seen previously by lipid droplet staining and radiolabeled oleate incorporation

into cholesteryl oleate. We had demonstrated that ApoE4 ACM treatment induces lipid droplet formation and that cholesteryl ester synthesis was increased, relative to triglyceride synthesis. In MAM fractions from SH-SY5Y cells, we now confirm, using HPLC-MS, a broad increase in cholesteryl esters, not limited to acyl chain length or degree of saturation. Similar to the TLC analysis, triglyceride levels were reduced in MAM from ApoE4 ACM-treated cells. However, other results from our lipidomics analysis failed to replicate previous observations. Lipidomic analysis of MAM derived from ApoE3 and ApoE4 ACM-treated SH-SY5Y cells showed no difference in free cholesterol levels. This stands in contrast to the decrease in free cholesterol seen in MAM derived from ApoE4 ACM-treated fibroblasts. This discrepancy might be explained by the differences in ApoE expression in fibroblasts and SH-SY5Y cells. Fibroblasts do not express ApoE, whereas SH-SY5Y cells, despite their neuronal lineage, do express ApoE, though they do not secrete lipoproteins (Soulie et al. 1999). SH-SY5Y ApoE is retained in the cell body and is not detectable in the media. Though this expression pattern has been reported, the physiological relevance of intracellular ApoE in neuroblastoma cells lines is unclear. This endogenous expression of ApoE may mask any effects of astrocyte-derived ApoE might have on intracellular cholesterol distribution. Replicate studies will be performed using different target cells, preferably neuronal cells that do not express ApoE and that grow at a rate that would allow the generation of MAM fractions large enough to submit to lipidomic analysis.

One advantage to examining the entire lipidome of our ApoE ACM-treated samples is that it allows for an unbiased look at ApoE-induced changes and may detect alterations not traditionally associated with MAM. One such example is the class-wide ApoE4-mediated decrease seen in ethanolamine plasmalogen (PEp) levels. Though both PtdEtn and PEp contain ethanolamine as a polar head group, each species differs structurally **(Fig).** Plasmalogens

contain a vinyl ether linkage at the sn-1 position and an esther linkage at the sn-2 position and are synthesized by a different pathways than the phosphatidic acid-derived phospholipids, i.e. PtdEtn, PtdCho, PtdSer, etc. (Braverman and Moser 2012). PEp synthesis begins in the peroxisome, where dihydroxyacetone phosphate (DHAP) is esterified at the sn-1 position and subsequently reduced to form alkyl-DHAP (Brites et al. 2004). Alkyl-DHAP traffics from the peroxisome to the ER, where a series of further reactions (including a second esterification, replacement of the phosphate group with ethanolamine, and vinyl bond formation) catalyze the synthesis of PEp (Brites et al. 2004). In this study, of the 23 PEp species analyzed, 21 were decreased in MAM from ApoE4 ACM-treated cells. There are several possibilities that may explain this result. The upregulation of mitochondria-ER communication may affect the ability of other organelles to communicate with the ER. This would limit the transfer of alkyl-DHAP from peroxisomes to ER. Alternatively, the increase in ER-mitochondria contact sites may deplete the ER of plasmalogens. Presumably, the MAM is the source of mitochondrial plasmalogens, as mitochondria contain plasmalogens yet are incapable of plasmalogen synthesis. This possibility is less likely, given that we see no increase in PEp in mitochondrial fractions concomitant with the decrease seen in MAM of ApoE4 ACM-treated cells. Whatever the cause of the decrease in plasmalogen synthesis, the observation that there is less PEp in ApoE4 ACM-treated cells is consistent with the idea that MAM is upregulated in our system. Support for this idea comes from observation that PEp and PtdEtn are in homeostatic balance, and that a decrease in plasmalogens is accompanied by a concomitant increase in PtdEtn (Dorninger et al. 2015). This effect was seen in fibroblasts derived from patients with rhizomelic chondrodysplasia punctata (RCDP), a rare disorder of plasmalogen synthesis. RCDP fibroblasts and brains from KO mice in which plasmalogen synthetic enzymes are ablated show an increase in PtdEtn

synthesis (Brodde et al. 2012). Because MAM is the major pathway for PtdEtn synthesis, it is likely that mitochondria-ER communication is upregulated in these cells. Some PtdEtn is produced on the cytosolic face of the ER by CDP-ethanolamine exchange via the Kennedy pathway (Gibellini and Smith 2010), the majority of PtdEtn is produced by the conversion of PtdSer to PtdEtn in mitochondria (Voelker 2005).

In addition to its relationship with increased MAM activity, the finding of decreased plasmalogen synthesis in ApoE4 ACM-treated cells corresponds to changes in plasmalogen levels in AD patients and models of AD. Autopsies of AD brains show a decrease in PEp in the cerebral cortex and hippocampus, which undergo neurodegenerative changes in AD, while the plasmalogen levels in the cerebellum, largely spared in AD, are unaltered (Ginsberg et al. 1995). Moreover, in affected brain regions, plasmalogen content inversely correlates with AD severity. In the periphery, PEp is also decreased in the cerebrospinal fluid and serum of AD patients as compared to cognitively normal controls (Goodenowe et al. 2007, Wood et al. 2010, Tajima et al. 2013). In transgenic mouse models of AD, in which APP and tau are mutated, a similar trend is seen. This decrease in plasmalogen content is consistent with the appearance of AD pathology, as lower membrane plasmalogen content results in an increase in the amyloidogenic processing of APP (Grimm et al. 2011, Rothhaar et al. 2012). There is no literature examining the contribution of ApoE to plasmalogen content, but given the results in our study and the close association of PEp and AD, a decrease in PEp would be suspected in *APOE4* carriers.

We had previously speculated that ApoE increases MAM function by altering the distribution of cellular cholesterol. As mentioned above, ApoE4 has been reported to remain in recycling endosomes for a longer time than ApoE3, after receptor mediated endocytosis of the ApoE-containing lipoprotein. This retention of ApoE4 is associated with reduced cholesterol

efflux and an accumulation of this cholesterol in endolysosomal vesicles, reminiscent of Neimann-Pick disease, type C. Interestingly, others have found that in RCDP fibroblasts, a model for plasmalogen synthesis disorders, cholesterol efflux is reduced, and as a result, total membrane cholesterol is increased (Mandel et al. 1998). Cholesterol efflux could be rescued upon addition of exogenous plasmalogens. Cells treated with ApoE4 ACM and cells that are deficient in plasmalogens therefore have the same cholesterol efflux impairment. Because ApoE4 ACM treatment was able to reduce plasmalogen levels, it is possible that the ApoE-mediated cholesterol efflux deficiency is mediated through plasmalogen levels.

In summary, we find that the ApoE4-mediated upregulation of MAM activity in target cells can be corroborated by lipidomic analysis. MAM from ApoE4-ACM treated cells contained more cholesteryl esters and fewer triglycerides than ApoE3-ACM treated controls, which is consistent with the earlier finding of increased lipid droplet synthesis. Our subsequent analyses have revealed some insight as to the mechanism of this ApoE4-induced upregulation of MAM. Specifically, we have found that ApoE4 ACM treatment of cells alters MAM cholesterol and PEp levels. These observations are consistent with the idea that ApoE alters MAM via cholesterol trafficking dysfunction.

Figure 7

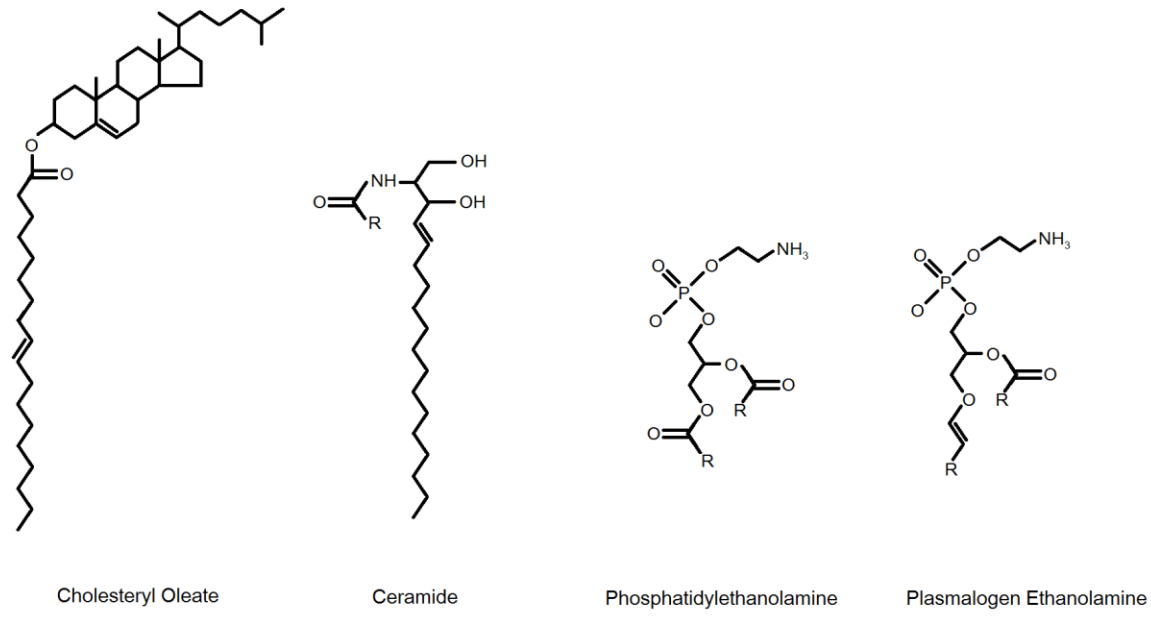


Figure 7. Lipids discussed in this thesis

Figure 8

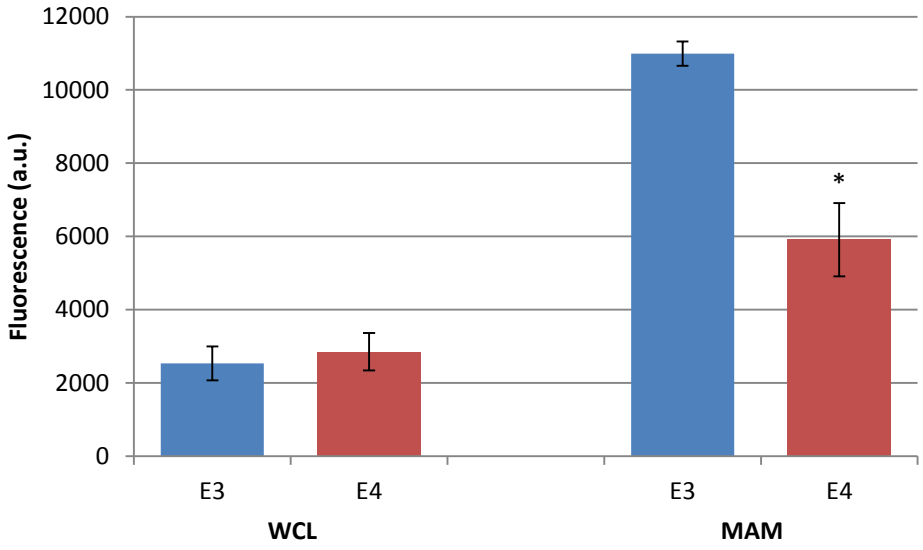


Figure 8. Cholesterol content of subcellular fractions derived from ApoE ACM-treated fibroblasts

Membrane cholesterol content, as measured by AmplexRed analysis of fractions from wild-type fibroblasts, in whole cell lysate (mean±SE; n=3, with 3 replicates/experiment), n.s. vs E3, and in MAM (mean±SE; n=3, with 3 replicates/experiment) *, $p<0.05$ vs E3. Fluorescence values were normalized to actin content for WCL and erlin-2 content for MAM as determined by western blot.

Figure 9

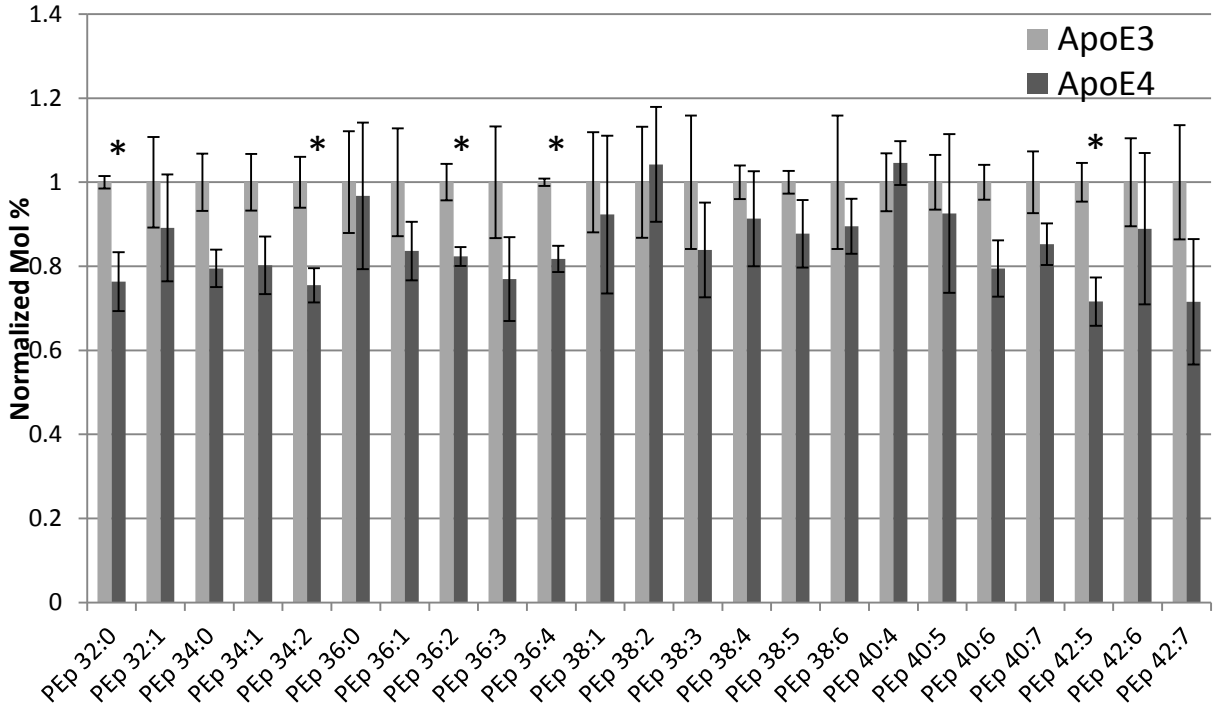


Figure 9. Ethanolamine plasmalogen content of MAM derived from ApoE ACM-treated SH-SY5Y cells

Ethanolamine plasmalogen content, as measured by HPLC-MS analysis, of MAM derived from ApoE ACM-treated SH-SY5Y cells, (mean±SE; n=3) *, $p<0.05$ vs E3.

Figure 10

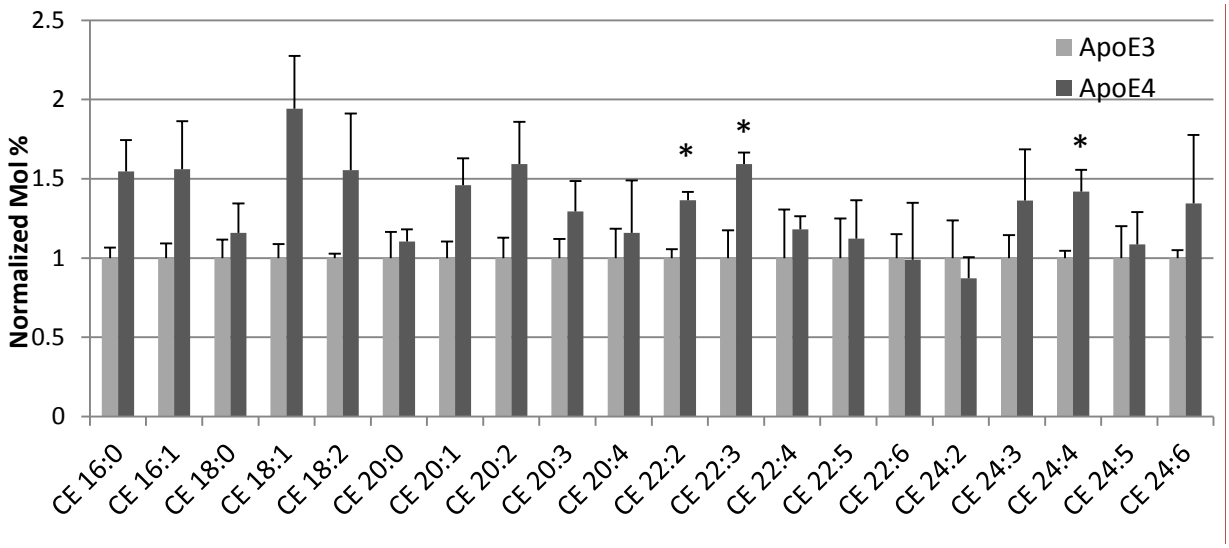


Figure 10. Cholesteryl ester content of MAM derived from ApoE ACM-treated SH-SY5Y cells

Cholesteryl ester content, as measured by HPLC-MS analysis, of MAM derived from ApoE ACM-treated SH-SY5Y cells, (mean±SE; n=3) *, $p<0.05$ vs E3.

Figure 11

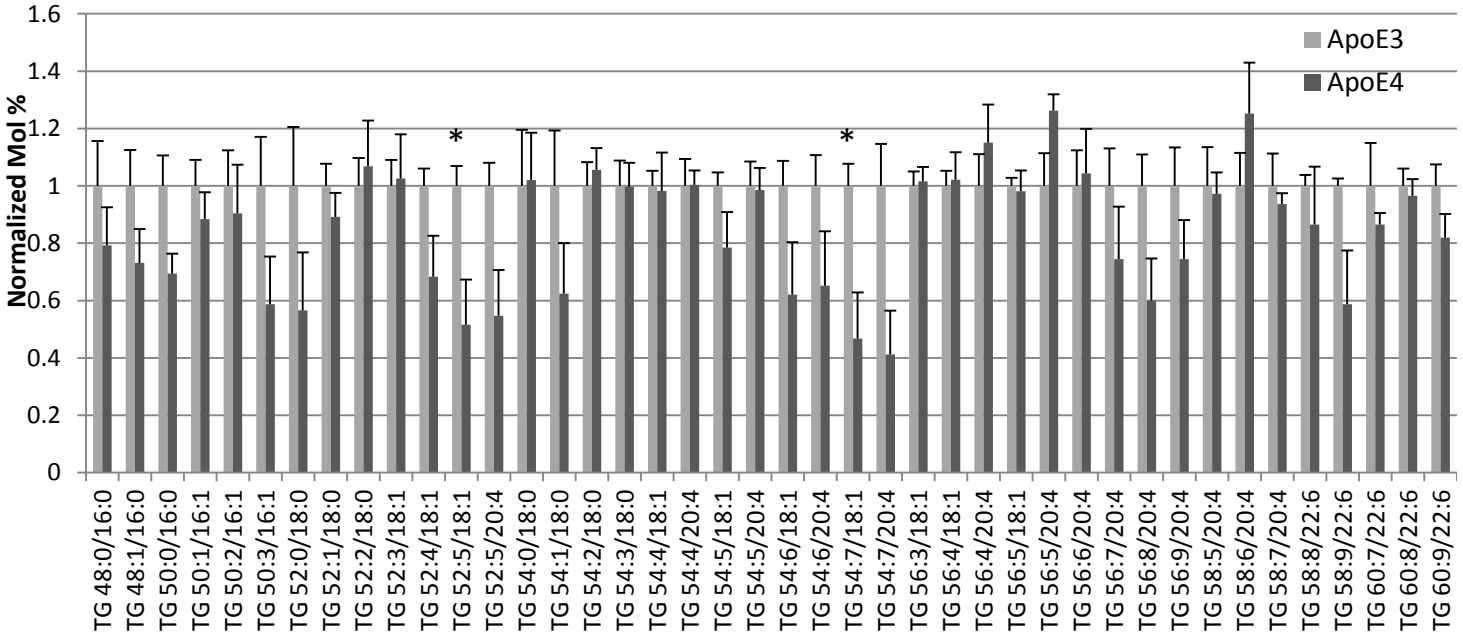


Figure 11. Triacylglyceride content of MAM derived from ApoE ACM-treated SH-SY5Y cells

Triacylglyceride content, as measured by HPLC-MS analysis, of MAM derived from ApoE ACM-treated SH-SY5Y cells, (mean±SE; n=3) *, $p<0.05$ vs E3.

Figure 12

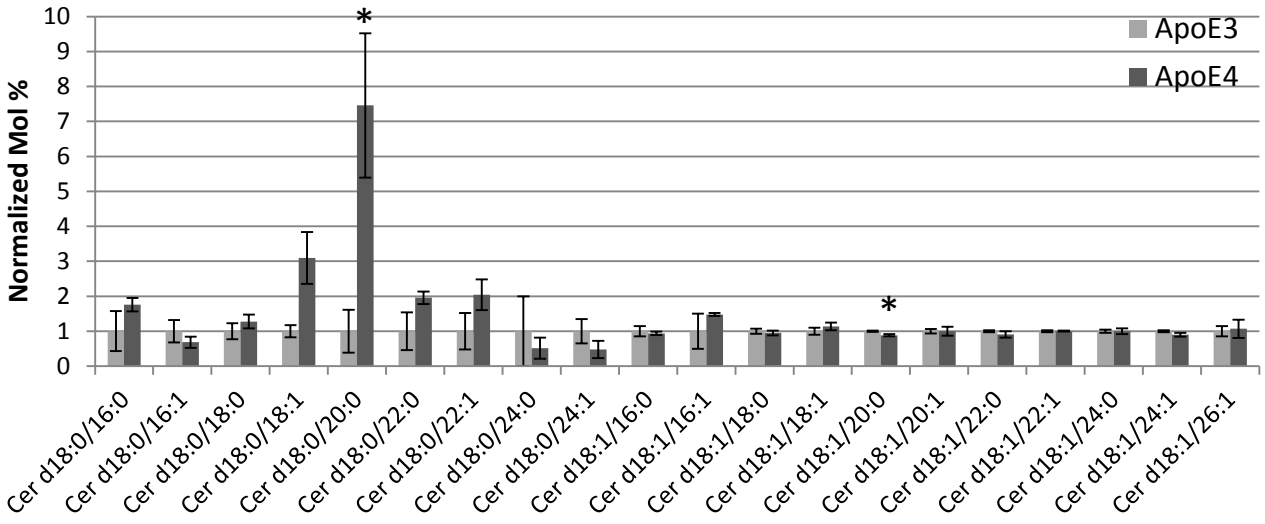


Figure 12. Ceramide content of MAM derived from ApoE ACM-treated SH-SY5Y cells

Ceramide content, as measured by HPLC-MS analysis, of MAM derived from ApoE ACM-treated SH-SY5Y cells, (mean±SE; n=3) *, $p<0.05$ vs E3.

CHAPTER 3. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Unexplained AD Pathogenesis

The pathological findings of amyloid plaques in AD brains and the genetic linkage of FAD to amyloid metabolism genes inform the "amyloid hypothesis," which states that the cytotoxicity of Aβ is the causal factor that leads to neurodegeneration in AD (Hardy and Allsop 1991). The placement of Aβ at the center of the disease would imply that each FAD mutation acted via the same mechanism: to increase amyloid production. This is not the case. Although FAD cases do indeed involve the deposition of Aβ over a patient's clinical course, each FAD mutation alters APP metabolism differently. Mutations in presenilins and in the γ-secretase cleavage site of APP generally result in a decrease in Aβ production (Fernandez et al. 2014). On the other hand, mutations near the β-cleavage of APP site result in increased production of Aβ, as does the APP gene duplication seen in trisomy 21. Mere abundance, therefore, of Aβ is an insufficient explanation of AD pathogenesis and this may explain the failure of therapeutic agents designed to reduce Aβ levels. Before the clinical trials were performed, there was some indication that the amyloid hypothesis was incomplete. Neuropathologists observed that plaque load does not correlate tightly with disease severity, that amyloid deposition begins years before clinical symptoms, and that there are many individuals who remain cognitively intact despite heavy plaque load (Herrup 2015). Accordingly, the emphasis has shifted from the cytotoxicity of insoluble plaques to the precursor oligomeric assemblies of Aβ, which correlate more closely with disease severity; the underlying premise that cell death is mediated by Aβ remains (Hardy and Selkoe 2002). Current re-evaluations of the amyloid hypothesis now interpret FAD mutations as being deleterious not by increasing amyloid production, but rather, by increasing

the ratio of Aβ42/ Aβ40. A ratio that favors the more aggregation-prone Aβ42 is believed to increase the amount of oligomeric species. Recently, however, examination of FAD mutations has found that some PSEN1/2 mutations do not alter the Aβ42/ Aβ40 ratio (Shioi et al. 2007, Robakis 2010). Inducible APP expression in mice has also demonstrated that continuous expression of APP is needed to evoke cognitive decline (Melnikova et al. 2013). Attenuating APP expression in these mice, which does not eliminate the already-formed plaques or oligomeric Aβ42, ameliorates cognitive decline. The explanatory power of Aβ is limited by these conflicts, and it may be that the simple presence of Aβ, no matter what species, is not enough to determine AD. Still unknown is the physiological function of APP and APP metabolism, and the way that they relate to AD pathogenesis.

The discovery of sporadic AD genes, while a tremendous advance in understanding AD pathogenesis, has made it difficult to reconcile the early onset and sporadic etiologies of AD. It is assumed that, because both SAD and FAD share common clinical presentations and neuropathology, they share a common neurodegenerative mechanism. In most cases of SAD, however, there are no mutations in APP, PSEN1, or PSEN2 as is the case with FAD. Moreover, many of the genes discovered are not involved in amyloid metabolism. For example, recent GWAS hits include genes involved in the immune system (CLU, CR1, ABCA7, MS4A, CD33 and EPHA1), cholesterol metabolism (CLU and ABCA7), membrane/synaptic trafficking (PICALM, BIN1, CD33, CD2AP and EPHA1) (Karch et al. 2012). Retrospective analysis of the ability of these genes to alter Aβ production or clearance is a very common approach to understanding how they impact AD, but given the uncertainty involved around what particular aspect of APP metabolism is responsible for neurodegeneration in familial AD and how mechanistically this neurodegeneration occurs, the applicability of this approach is limited. It

may be that the factors that determine sporadic AD result in cell death independently of Aβ and that the Aβ production and plaque deposition seen in AD is a downstream consequence of AD-related cell death. Proof of Aβ causality must be established in FAD before it can be used to explain the possible contributions of SAD genes.

While amyloid deposition is the most visually striking neuropathological finding, there are numerous other changes that occur in cells of AD patients. Certainly, some of these changes are present as epiphenomena, the end-result of a neurodegenerative process, but others are present early in the disease and have been reproduced in cellular models of FAD mutants. Mitochondrial dysfunction is one such cellular alteration that characterizes AD patient cell lines and AD mouse models. Refinement of biochemical and imaging techniques led to the discovery that the presenilins were present in MAM, and that rather than being purely mitochondrial in nature, the changes in AD were the result of increased communication between mitochondria and the ER (Schon and Area-Gomez 2013). The idea that there is increased MAM activity explains several different, seemingly disparate observations in AD cells, such as impaired calcium buffering, mitochondrial distribution, altered cholesterol homeostasis and phospholipid synthesis. FAD mutations supported this finding, as mutations and knockouts of the presenilins were able to mimic the MAM phenotype seen in AD patients (Area-Gomez et al. 2012). These observations provide the foundation for the MAM hypothesis, that the neurodegeneration seen in AD is the consequence of increased mitochondria and ER communication.

Proof for the MAM hypothesis is incomplete. The largest impediment of relating MAM dysfunction to neurodegeneration is lack of the appropriate animal model. The advantage of a mouse model would be to the ability to investigate the regional specificity and age-association of what effect MAM activity has on the AD. ER and mitochondria communicate in every cell and

at every age, yet neurodegeneration occurs in a subset of neurons and as a function of advanced age. The overexpression of MFN2 or transgenic expression of artificial molecular tethers between ER and mitochondria might provide insight as to how the brain responds to increased ER-mitochondria communication. A more difficult question to answer would be how MAM and presenilins interact to modify cognitive impairment and plaque deposition in AD mouse models. In PS1/2 dKO MEFs, increased ER-mitochondria connectivity can be reduced by knocking down MFN2, and inversely, the lack of ER-mitochondria connectivity in MFN2 KO MEFs can be restored by knocking down PS1 (Area-Gomez et al. 2012). Ideally, the knockout mice for each of these genes would be available for analysis, however, PS1 KO mice die at embryonic day 17 and MFN2 KO mice die at embryonic day 11.5 due to placental defects (Shen et al. 1997, Chen et al. 2003). One possibility to still take advantage of the observation that PS1 and MFN2 seem to have antagonistic effect on MAM activity is to cross an MFN2 conditional KO mouse with a PS/APP mutant mouse. In such a model, a reduction of MFN2 might modify the AD neuropathology and cognitive impairment seen in the PS/APP mice. Before such a model can be created, further work needs to be done on the function of MAM. It is not simply a quantity the result of whose increase or decrease can be easily interpreted, but rather it is a coordinated and reversible reaction to a number of conditions within the cell. Understanding the conditions that evoke its activity and how its increased activity leads to cell death is a necessary prerequisite to modeling its activity in mice.

Future Experiments to Determine the Mechanism of ApoE-mediated Increase in MAM Activity

The finding that ApoE4 ACM treatment of cells increases MAM activity extends previous reports of increased MAM in familial AD cellular models to explain the possible

involvement of MAM dysfunction in sporadic AD. However, the mechanism of this involvement in SAD is less clear than the FAD counterpart, given the different subcellular locations of ApoE and APP/γ-secretase. The presenilins, which regulate MAM function, are found in multiple locations along the secretory and endocytic pathway, but are enriched and active in MAM (Area-Gomez et al. 2009). ApoE, on the other hand, is only found in MAM as part of its synthesis in lipoprotein-producing cells, but it does not localize to MAM in target cells upon endocytosis (Rusinol et al. 1994). After endocytosis, ApoE traffics to the endolysosome system, where a fraction is degraded and the remainder is recycled back to the cell surface. This precludes the possibility of a direct interaction between ApoE and MAM, implying that its effects on MAM may be indirect. Thus, somehow, ApoE-containing lipoproteins are able to affect MAM as part of their normal trafficking pathway.

To dissect which part of the ApoE-containing lipoprotein's metabolism is responsible for the downstream increase in MAM activity, it would be useful to genetically or pharmacologically block each step of the pathway to identify what aspects are required for ApoE4 to exert its influence. It is known from work presented in Chapter 1 that lipoprotein incorporation is a requirement for ApoE4 to alter MAM activity and that lipid-free protein alone has no effect. To further extend this observation, it is possible to use competitive inhibitors of lipoprotein receptors (receptor associated proteins to compete with ApoE, or soluble receptor domains to compete with the lipoprotein receptors) or neutralizing antibodies against ApoE (Ziere et al. 1996). If blockage of receptor mediated endocytosis attenuates the ability of ApoE4 ACM to increase MAM function, this would indicate that binding at the cell surface is a required event. Sequentially, the next step after endocytosis is the hydrolysis of the lipoprotein via fusion of the lipoprotein-containing endosome with a lysosome. Several methods can be used to block the

action of lysosomes. Fusion of endosomes and lysosomes can be prevented by inhibition of phosphatidylinositol 3-kinase, which is required for Rab5 function (Christoforidis et al. 1999). Chloroquine, an antimalarial agent, prevents the acidification of lysosomes and intralysosomal proteolysis (de Duve et al. 1974). If ApoE4 affects MAM function by altering the trafficking of early or late endosomes, any lysosomal interference should not affect the differential effect each ApoE isotype has on MAM. However, if lysosomal function or the subsequent events that lysosomal dysregulation would interrupt are required, the ability of ApoE4 ACM to increase MAM function will be abolished.

After the lipid and protein components of the lipoprotein are separated in the lysosome, ApoE that has escaped degradation will traffic to recycling endosomes back to the cell surface where it is re-lipidated and re-secreted. Often this recycling of ApoE is associated with cholesterol efflux from the cell. To test whether or not the ApoE isoform-specific differences in recycling contribute to MAM dysfunction, we will selectively block endosome recycling and test if ApoE4 ACM treatment is still able to increase MAM function. Recycling endosome inhibition can be accomplished by a variety of means, including treatment with monensin, an inhibitor of endosomal trafficking, or more precisely by knocking down the recycling endosome marker Rab11a (Takahashi et al. 2012). If the recycling of ApoE by endosomes is not responsible, but it is instead the concomitant cholesterol efflux that mediates MAM dysfunction, this would be testable in target cells deficient in ABCA1 (Wahrle et al. 2004). This approach of selective pharmacological or genetic blockage of the endocytic/recycling pathway would require careful controls at each step to ensure that different proposed alterations of the endosomal pathway do not by themselves alter MAM function, as this would obscure any effect that each ApoE isoform has on MAM function. Once the precise location of where along the endocytic pathway the

different isoforms exert an effect on MAM is clarified, further experiments can be done to elucidate how each isoform might be responsible for this effect.

The preceding experimental approach, while taking advantage of the reported differences in lipoprotein metabolism and cholesterol trafficking seen in ApoE3 and ApoE4-treated cells, may not detect more global changes that might underlie the increase in MAM activity in ApoE4 ACM-treated cells. Currently, we are addressing this issue by performing RNA-seq analysis on ApoE3 and ApoE4 ACM- treated fibroblasts to detect any changes in gene expression. RNA-seq has several advantages to traditional microarray expressional analysis. Coverage of the transcriptome is increased and inclusive of alternative splicing, and hybridization artifacts are avoided. This approach, though broad, may confirm previous observations or reveal new pathways.

Conclusion

The finding of increased ER and mitochondria connectivity represents a novel understanding of how familial AD genes alter cellular function. The work presented in this thesis extends those findings of MAM dysfunction to a model of the sporadic AD risk factor ApoE.

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