Rescuing the Secretion and Lipidation Deficits of APOE4 Using HDL Mimetic Peptides in Primary Glial Cells

A THESIS

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SIDDHI S. JOSHI

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DR. LING LI

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Abstract

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases with age and genetics significantly contributing to its development. Genome-wide association studies (GWAS) have identified apolipoprotein E4 (APOE4) as the greatest genetic risk factor for developing sporadic AD; however, the exact underlying mechanism remains undeciphered.

Several clinical studies have established a strong correlation between low levels of high-density lipoproteins (HDL) and an increased risk and severity of AD. The human APOE, encoded by three alleles—ε2, ε3, ε4, is a key constituent of HDL-like particles in the brain. APOE and HDL in the brain regulate cognition through multiple processes including cholesterol homeostasis, synaptic growth, anti-inflammation, and protein clearance. Lipidation of APOE is isoform dependent (ε2>ε3>ε4) and is crucial for HDL formation and function. APOE4 exhibits altered physiological functions because of its decreased propensity to form HDL in the brain, which may be a potential mechanism driving APOE4-linked AD pathology.

We hypothesize that reversing APOE4's lipidation deficit corrects its functions and ameliorates APOE4 associated AD pathology. Previously, Chernick *et al* demonstrated that an HDL mimetic peptide 4F mitigates amyloid-β (Aβ)-induced inhibition of APOE secretion and lipidation in astrocytes, a major source of APOE and HDL in the brain. Expanding upon this work, we investigated the isoform dependent effects of 4F and a modified 4F (X-4F) in the presence or absence of aggregated Aβ. To this end, we cultured primary murine astrocytes derived from homozygous human APOE3 and APOE4 knock in (KI) mice. We also investigated the effects of 4F and X-4F on primary APOE4 KI astrocytes overexpressing mutant forms of human amyloid-β precursor protein (APP) and presenilin-1 (PS1) to model endogenous Aβ production *in vitro*.

In this study, we confirmed the well-established lipidation deficit of APOE4. We further demonstrated that HDL-mimetic peptides 4F and X-4F increase the secretion and lipidation of both APOE4 and APOE3. Importantly, 4F and X-4F improve lipidation of APOE4 to a greater extent than APOE3, reversing the lipidation deficit of APOE4. In addition, 4F and X-4F mediated enhancement of APOE secretion and lipidation persist in APOE4 KI and APOE3 KI astrocytes treated with aggregated Aβ42 as well as in APOE4 KI astrocytes overexpressing APP/PS1.

In conclusion, the present study provides additional *in vitro* evidence of HDL-mimetic peptides as potential APOE4 modulating agents. Future studies are warranted to assess the efficacy of HDL-mimetic peptides to restore the beneficial functions of HDL and APOE in the brain and to mitigate the pathogenic process of AD.

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List of Abbreviations

ABCA1, ATP-binding cassette type A1

ABCG1, ATP-binding cassette type G1

AD, Alzheimer's disease

ANOVA, Analysis of variance

apo, Apolipoprotein

APOA-I, Apolipoprotein A-I

APOE, Apolipoprotein E

APOE2, Human apolipoprotein E2

APOE3, Human apolipoprotein E3

APOE4, Human apolipoprotein E4

APOJ, Apolipoprotein J

APP, Amyloid precursor protein

Aβ, Amyloid-β peptide

BACE1, Beta-secretase 1

BBB, Blood-brain barrier

C3, Complement component 3

CAA, Cerebral amyloid angiopathy

CETP, Cholesteryl ester transfer protein

CNS, Central nervous system

CSF, Cerebrospinal fluid

DAPI, 4’,6- diamidino-2-phenylindole

DMEM, Dulbecco’s Modified Eagle Medium

EGF, Epidermal growth factor

ER, Endoplasmic reticulum

ERK, Extracellular signal-regulated kinase

GFAP, Glial fibrillary acidic protein

GWAS, Genome-wide association studies

HDL-C, High-density lipoprotein cholesterol

HDL, High-density lipoprotein

HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A

HRP, Horseradish peroxidase

HSPG, Heparin sulfate proteoglycan

IACUC, Institutional animal care and use committee

Iba1, Ionized calcium-binding adapter molecule 1

IDE, Insulin degrading enzyme

iPSC, Induced pluripotent stem cell

LCAT, Lecithin cholesterol acyltransferase

LDL, Low density lipoprotein

LDLR, Low density lipoprotein receptor

LPLAT, Lysophospholipid acyltransferases

LPS, Lipopolysaccharide

LRP1, Low density lipoprotein receptor-related protein 1

LTP, Long-term potentiation

LXR, Liver X receptor

miRNAs, MicroRNAs

mRNA, Messenger RNA

NDGGE, Non-denaturing gradient gel electrophoresis

PBS, phosphate buffered saline

PKC, Protein kinase C

PDL, Poly-D-Lysine

PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase

PL, Phospholipids

PLA, Phospholipase A

PLTP, Phospholipid transfer protein

PS1, Presenilin-1

rHDL, Recombinant high-density lipoprotein

RXR, Retinoid X receptors

SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SE, Standard error

SR-B1, Scavenge receptor B1

TGs, Triglycerides

TR, Targeted replacement

TREM2, Triggering receptor expressed on myeloid cells 2

Veh, Vehicle

VLDL, Very low-density lipoprotein

Chapter I: Introduction

1. Role of Lipids and Lipoproteins in Brain

The human brain is the second largest lipid reserve after adipose tissue, with lipids contributing to ~50% of the brain's dry weight. (Bruce et al., 2017) Major classes of lipids found in the brain include glycerophospholipids, sphingolipids, long chain unsaturated fatty acids and cholesterol. (Agranoff et al., 1999) Neutral lipids such as triglycerides and cholesteryl esters, primarily involved in energy storage are scarcely present in the brain. Instead, most lipids in the brain are polar in nature and provide structural support to neural membranes. (Skowronska-Krawczyk & Budin, 2020)

1.1 An Overview of Lipids in the Brain

Phosphatidyl-choline, phosphatidyl-serine, phosphatidyl-inositol and plasmalogens are major species of glycerophospholipids found in neural membranes. They are formed as a result of enzymatic actions of phospholipase As (PLAs), acyl-CoA synthases, trans-acylases, and lysophospholipid acyltransferases (LPLATs). (Hishikawa et al., 2014) Glycerophospholipids and their derivatives mainly function as secondary messengers in several complex signaling cascades that regulate synaptic plasticity and neurogenesis. (Farooqui et al., 2000) For instance, phosphatidyl-serine mediates the release of neurotransmitters by regulating the fusion of secretory vesicles and presynaptic membranes. Subsequently, it also activates Akt, protein kinase C (PKC) and Raf-1 signaling on the postsynaptic membrane stimulating neuronal growth. Similarly, phosphatidyl-inositol and its derivative PI (4,5) P2 activate phospholipase C (PLC) and control IP3 receptor mediated calcium efflux from endoplasmic reticulum. Elevated intracellular levels of calcium trigger the recruitment of cellular machinery to the active zone and subsequent release of neurotransmitters. (Glade & Smith, 2015; Magaquian et al., 2021; Raghu et al., 2019; Roy et al., 2022) Moieties such as ethanolamine plasmalogens also preserve the integrity of the synaptic membranes by providing protection against free radical oxidation. (Su et al., 2019; West et al., 2020)

Species of sphingolipids found in the brain are ceramides, sphingomyelin and gangliosides. Ceramide is a focal point in the metabolism of sphingolipids, which involves several enzymes such as ceramidase, sphingosine kinase, glucocerebrosidases, galactosylceramidase, and glucosylceramide synthase. (Alaamery et al., 2021) Sphingolipids have varying functions in

the brain. For instance, stress signals such as tumor necrosis factor-alpha and amyloid beta trigger ceramides to activate c-Jun N-terminal kinase (JNKs), ultimately causing apoptosis, neuroinflammation and oxidative stress. On the contrary, sphingosine-1-phosphate (S1P) has been reported to promote cellular proliferation. (Alessenko & Albi, 2020) Several species of gangliosides have been discovered in neural stem cells. They are highly expressed during the generation of neurons and astrocytes, suggesting a role in neurodevelopment. (Palmano et al., 2015)

The brain is also enriched in polyunsaturated fatty acids (PUFA), mainly omega-3 fatty acids such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), as well as omega-6 fatty acids such as linoleic acid (LA) and arachidonic acid (ARA). These are seldom present in their "free form"; instead, they are esterified to phospholipid membranes. (Joffre & Joffre, 2019) Phospholipase As delink PUFAs from membranes, subjecting them to more enzymatic action of cycloxygenesases, lipoxygenases and cytochrome P450. PUFA derivatives are key players in neuroinflammation affecting metabolic phenotypes of microglia and astrocytes; ARA derivatives being pro-inflammatory in nature while EPA and DHA derivatives being anti-inflammatory and/or pro-resolving mediators. (Bazinet & Layé, 2014)

1.2 Brain Cholesterol and its Metabolism

The brain is the most cholesterol-enriched organ of the human body, containing almost 25% of the total cholesterol. Unesterified or "free" cholesterol is the major sterol in the adult brain, while small amounts of desmosterol and cholesteryl esters are also present. (Simons & Ehehalt, 2002) The blood-brain barrier (BBB) separates the cholesterol pool in the brain and the periphery. Therefore, glial cells solely account for in-situ biosynthesis of cholesterol in the brain. (Vitali et al., 2014) Neurons, despite having a lower rate of cholesterol turnover than astrocytes, lack compensatory mechanisms to produce enough cholesterol. Thus, neurons depend on cholesterol supplied by astrocytes to build and repair membranes of axons, dendritic spines, and synaptic vesicles (Zhang & Liu, 2015).

Cholesterol biosynthesis is transcriptionally regulated by the membrane-bound sterol regulatory element-binding protein 2 (SREBP-2). Low levels of cholesterol stimulate SREBP-2 to undergo further processing in the ER-Golgi and translocate to the nucleus. (Madison, 2016) SREBP-2 activation results in an increased transcription of genes involved in cholesterol

synthesis and uptake such as HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS), mevalonate kinase (MVK) and LDL receptor (LDLR). (C. Wang et al., 2019) Cholesterol biosynthesis occurs via the mevalonate pathway that involves the conversion of acetyl-CoA to HMG CoA. HMGCR then converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate. This step is considered the rate-limiting enzyme in cholesterol synthesis. (Zhang & Liu, 2015) ABCA1, a cholesterol efflux transporter expressed on astrocytic membranes, facilitates the binding of newly synthesized cholesterol and other lipid moieties to apolipoproteins in the brain such as APOE and APOJ, resulting in the formation of HDL-like particles in the brain. (Ito et al., 2014)HDL-like particles transport cholesterol to neurons. Neuronal uptake of cholesterol occurs via interactions of HDL with LRP1 and LDLR receptors, after which it is converted to free cholesterol in the endolysosome. (Nieweg et al., 2009; Turri et al., 2022)

Surplus cholesterol is metabolized in different ways by neurons; it is re-esterified and stored in lipid droplets, or converted to 24S-hydroxycholesterol by cytochrome P450 oxidase Cyp46a1 or released as a component of HDLs. (Matsuda et al., 2013) Oxysterols, being endogenous ligands of nuclear LXR receptors, counter-regulate cholesterol synthesis transcriptionally. Activated LXRs bind to RXRs to form heterodimers that induce the transcription of genes such as ABCA1, ABCG1, SREBP1c, improving cholesterol efflux and transcriptional control of cholesterol synthesis. (Martín et al., 2014) Furthermore, APOA-I-HDLs and oxysterols are also transported across the BBB by endothelial cells via the activity of receptors from LDLR family, SR-B1 and ABCG4. Once they enter the blood plasma, they bind to plasma lipoproteins and are transported to the liver for metabolism. (Pifferi et al., 2021)

1.3 Apolipoproteins and HDL-like Particles in Brain

Lipoproteins are an assembly of apolipoproteins and lipids including phospholipids, cholesterol, cholesteryl ester, and triglycerides. Lipoproteins are classified into chylomicrons, VLDL, LDL and HDLs, based on their size, density and composition.(Feingold & Grunfeld, 2021) In the peripheral system, lipoproteins have been studied extensively in the cardiovascular field. HDLs in the periphery are involved in the removal of excess free cholesterol from tissues and blood vessels, transporting it back to the liver where it is metabolized. They also exhibit anti-inflammatory and anti-oxidative properties. (Bhargava et al., 2022; Rader, 2006). In the central nervous system (CNS), HDLs are present in the cerebrospinal fluid (CSF) and the brain

parenchyma, where they mediate various functions, including cholesterol trafficking across cells, protein clearance, anti-inflammation and cognition. (Zhang & Liu, 2015) However, the mechanisms by which HDLs exert these effects in the brain are poorly understood. Glia derived brain HDLs and CSF HDLs differ in terms of their size and composition. Glial HDLs are larger, discoidal particles that contain APOE and APOJ, phosphatidylcholine, sphingomyelin and, unesterified cholesterol. In contrast, CSF HDLs are smaller, spherical particles containing APOE, APOA-I, phosphatidylethanolamine and esterified cholesterol as their key constituents.(Koch et al., 2001; Mahley, 2016)

Apolipoproteins are the key constituents of lipoproteins that bind to and stabilize HDL lipid assemblies. Apolipoproteins found in the CNS include APOE, APOA-I, APOJ and APOD. (Hottman et al., 2014) However, APOE is the major apolipoprotein present in the brain whereas APOA-I is largely found in CSF via SR-BI receptor-mediated transcytosis across the BBB from the systemic circulation. (Slot et al., 2017) Astrocytes are the primary producers of APOE in the brain. Although, microglia, oligodendrocytes, pericytes, endothelial cells and neurons under stress can also secrete APOE to a lesser extent. APOE is synthesized in the endoplasmic reticulum, following which it undergoes post-translational modifications, glycosylation and sialylation, in the Golgi apparatus. It is then transported to the plasma membranes and released from vesicles.(Liao et al., 2017)

ATP-binding cassette transporters (ABCAs) expressed on glial cell membranes participate in the efflux of phospholipids and free cholesterol to form nascent HDLs. (Jin et al., 2019) APOE's association with ABCA1 at the membrane stabilizes ABCA1, promoting its phosphorylation and preventing subsequent calpain mediated degradation. (Arakawa et al., 2004; Okoro et al., 2016) Nascent HDLs undergo maturation by the catalytic action of several enzymes, including CETP, PTP and LCAT, which converts free cholesterol to cholesteryl esters and transport cholesterol or phospholipids. These mature HDLs primarily function as cholesterol transporters, delivering it to neurons for axonal growth and synaptogenesis. (Hottman et al., 2014) APOE containing HDLs also activate protein uptake pathways through APOE's interaction with lipoprotein receptors such as the LDLR, the very low-density lipoprotein receptor (VLDLR), the apoE receptor 2 (apoER2), the low-density lipoprotein receptor-related protein 1 (LRP1) and TREM2 widely expressed in the brain. (Vitali et al., 2014; H. Wang & Eckel, 2014; Zhang & Liu, 2015)

1.4 Lipid Rafts and Lipid Droplets in Brain

Other lipid assemblies in the brain include lipid rafts and lipid droplets. Lipids are assembled with other cytoplasmic proteins in the outer leaflet of neural membranes in distinct clusters. Such microdomains, known as "lipid rafts" ensure a tightly organized structure for lipid-protein interactions in the otherwise fluid membranes. (Calder & Yaqoob, 2007) Lipid rafts contain lipid molecules such as phospholipids, glycosphingolipids, cholesterol, palmitoyl-ated or myristoyl-ated cytoplasmic proteins and glycosylphosphatidylinositol (GPI)-anchored proteins. (McGuinn & Mahoney, 2014) Lipids in lipid rafts contain saturated fatty acid side chains, conferring an orderly structure to the microdomain. Cytoplasmic proteins in lipid rafts participate in various signaling cascades such as Src family kinases, G proteins, endothelial nitric oxide synthase (eNOS) and β-secretase (BACE) involved several trafficking, cellular proliferation, and neurotransmission. Lipid rafts can be either planar or caveolae; depending on scaffolding protein present in the microdomain. Flotillin is associated with a planar raft and caveolin is associated with caveolae. (Sezgin et al., 2017; Simons & Ehehalt, 2002)

Lipid droplets (LDs) are intracellular organelles that store neutral lipids such as triglycerides and cholesterol esters. Lipid droplets contain a phospholipid monolayer embedded with enzymes involved in lipid metabolism. (Olzmann & Carvalho, 2019) For instance, perilipins that regulate lipolysis, protect LDs from degradation. Other proteins in the LD proteome include lipases such as adipose triglyceride lipase (ATGL), lipid biosynthetic enzymes such as acyl-CoA synthetase long chain family member 3 (ACSL3), glycerol-3-phosphate acyltransferase 4 (GPAT4) and diacylglycerol O-acyltransferase 2 (DGAT2) and proteins involved in ubiquitin-dependent proteolysis such as ancient ubiquitous protein 1 (AUP1). (Roberts & Olzmann, 2020). In a healthy brain, lipid droplets facilitate temporary storage of triglycerides and cholesterol esters until they undergo lipolysis or lipophagy. Lipolysis results in the release of energy for metabolic processes and, lipid derivatives that are incorporated into new membranes. LDs are also implicated in ER protein homeostasis and protect cells against lipotoxicity by sequestering free fatty acids that can disrupt membrane integrity. (Onal et al., 2017; Ralhan et al., 2021; Yang et al., 2022)

In summary, lipids and lipoproteins play important role in the regulation of brain function. The overall pathways of HDL metabolism in the brain are summarized schematically in **Figure 1.1**

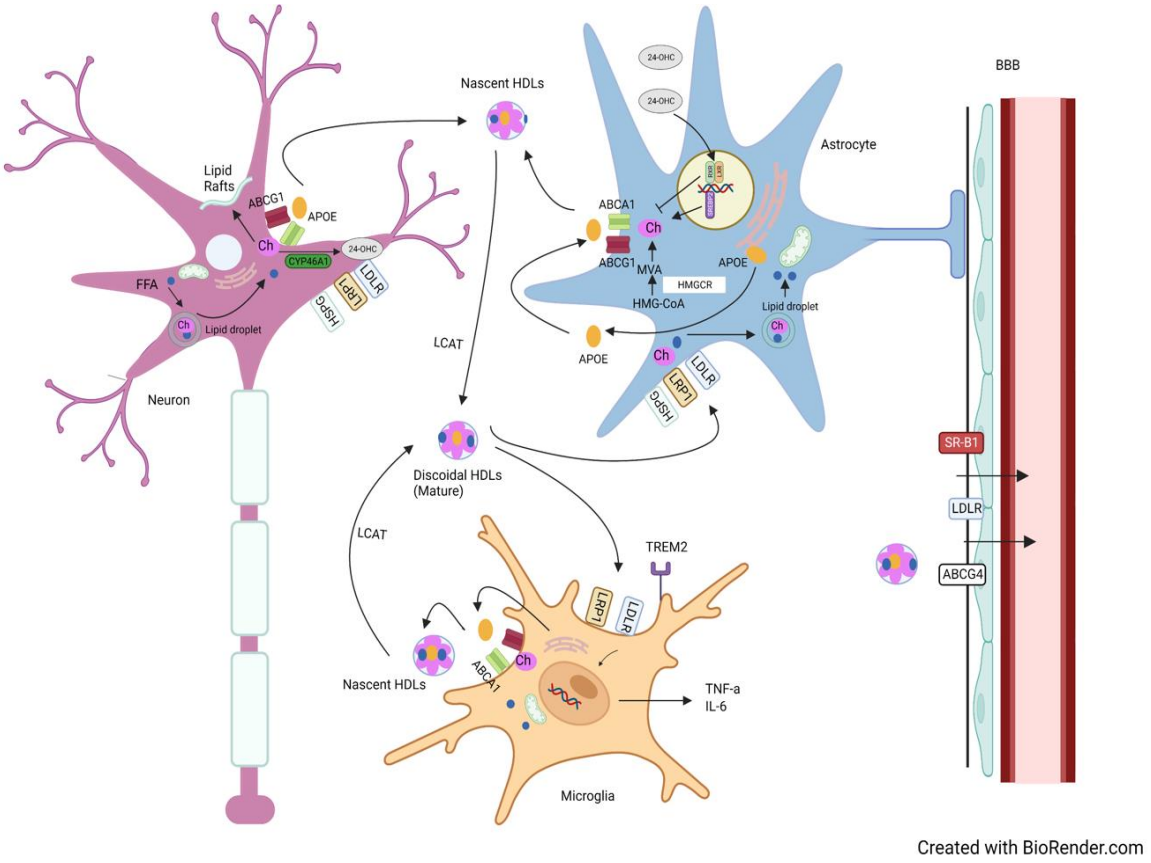


Figure 1.1 An Overview of APOE, Cholesterol and HDL Metabolism in the Brain. Cholesterol metabolism is transcriptionally regulated by LXR/RXR and SREBP2. Synthesized cholesterol is loaded onto APOE secreted by glia by ABC transporters to form nascent HDL-like particles in the brain. Nascent HDL-like particles form mature HDL-like particles under the action of LCAT that supply cholesterol and other lipids to other cells. Free fatty acids in neurons, released during energy production are temporarily stored in lipid droplets until eliminated via HDL-like particles and transported for cellular uptake. Ch: Cholesterol, FFA: Free Fatty Acid

2. Impact of APOE4 on Neuropathology of Alzheimer's Disease

2.1 Overview of Neuropathology of Alzheimer's Disease

Alzheimer's Disease (AD) is one of the most prevalent neurodegenerative diseases with age and genetics significantly contributing to its development. The incidence of AD doubles every 5 years after the age of 65 years.(Deture & Dickson, 2019) The main clinical symptoms of AD are cognitive decline and memory loss.

Extracellular senile plaques of amyloid beta (Aβ) and neurofibrillary tangles (NFTs) composed of phosphorylated tau are hallmarks of AD neuropathology. Apart from plaques and neuronal

tangles, AD brains have been characterized by an overall cortical atrophy, astrogliosis, activated microglia that surround dense Aβ plaques and cerebral amyloid angiopathy (CAA). (Sengoku, 2020; Serrano-Pozo et al., 2011)

Familial, early-onset AD is caused by mutations in the amyloid-β precursor protein (APP) and presenilin genes PSEN1 and PSEN2. However, the etiology of sporadic, late onset AD (LOAD) is still unclear with several genetic, environmental and lifestyle related factors playing a role in its incidence. GWAS have identified variants of the *APOE* gene as one of the largest genetic risk factors for LOAD. The human APOE gene exists as three polymorphic alleles—ε2, ε3 and ε4 with global frequencies of 8.4%, 77.9% and 13.7%, respectively. Inheriting one copy of the ε4 allele increases the risk of AD by 3-folds whereas inheriting both copies dramatically increase the risk by nearly 15-fold in Caucasian individuals. (Gharbi-Meliani et al., 2021; Liu et al., 2013) On the contrary, inheriting the APOE2 allele decreases the risk of AD relative to APOE3. (Hannon et al., 2020; Z. Li et al., 2020)

The 3 human APOE isoforms differ in amino acids present in the 158^{th} and 112^{th} position. APOE2 has Cys residues at both positions; APOE3 has a Cys residue at 112 and an Arg residue at 158 whereas APOE4 has Arg residues at both positions. (Fernández-Calle et al., 2022)These structural differences impact the lipidation state of APOE, with the degree of lipidation being APOE4 < APOE3 < APOE2. Lipidation of APOE is extremely crucial for its interactions with receptors, including LDLR, LRP1, VLDL, TREM2 and APOER2, and influences the role of APOE in various processes, including autophagy, cholesterol trafficking and neuronal plasticity. (Raulin et al., 2022) Hence, isoform specific differences have been observed in several features of AD, including Aβ and tau pathology, neuroinflammatory response, synaptic and cerebrovascular function, and lipid metabolism. (Liao et al., 2017) The impact of APOE4 on AD neuropathology is illustrated in **Figure 1.2 (**see next sections)

2.2 APOE4 and amyloid beta (Aβ) pathology

Aβ plaque deposits in AD brains have been classified into dense and diffuse plaques. Dense plaques have a compact nucleus and fibrillar structure, and are surrounded by dystrophic neurites and activated microglia. On the contrary, diffuse plaques are loose, amorphous deposits lacking a fibrillar structure and do not elicit microglial activation. (Serrano-Pozo et al., 2011)

APOE has been known to co-localize in plaques and initiate Aβ seeding. Several studies have reported lower levels of $A\beta_{42}$ in the CSF, an overall increased and early onset of amyloid deposition in APOE4 carriers than in non-carriers as well as in mouse models overexpressing APOE4. (Gharbi-Meliani et al., 2021b; Honda et al., 2023; Mahan et al., 2022)Evidence also suggests that APOE4 promotes Aβ aggregation, although its exact mechanism remains unclear.(Hashimoto et al., 2012; Honda et al., 2023) For instance, Baek et al reported a deposition of dense, fibrillar amyloid plaques, instead of diffuse plaques in brain tissues of homozygous APOE4 carriers. APOE4 genotype also increased levels of oligomeric Aβ as compared to APOE3 and APOE2 in post mortem human brain tissue. (Baek et al., 2020) Liu et al specifically highlighted the pathogenic role of astrocyte derived APOE4 in driving amyloid deposition during the seeding phase. An increased expression of astrocytic APOE4 during the seeding stage enhanced amyloid deposition and neuritic dystrophy in APP/PS1 mice, an effect that was absent in APP/PS1 mice overexpressing astrocytic APOE3 and astrocytic APOE4 in the plaque growing phase. (Liu et al., 2017) Overexpression of ABCA1, responsible for lipidating APOE, reduced Aβ deposition in PDAPP mice in contrast to an increased plaque burden observed in ABCA1-deficient APP mice. (Wahrle et al., 2005, 2008) This may suggest that correcting APOE4's lipidation deficit may mitigate APOE4-induced high plaque burden.

Aβ acts as a ligand for several APOE receptors such as LDLR, LRP1 and HSPGs expressed on neurons and glia. (Fu et al., 2016; Liu et al., 2016) Thus, APOE may also compete with Aβ for receptor binding, inhibiting Aβ uptake and clearance via these receptors. Structural alterations in APOE4 increase its preference for LDL receptors in comparison to APOE3 and APOE2. (Fernández-Calle et al., 2022; Liu et al., 2013) APOE4 also inhibits the activity of enzymes such as the insulin-degrading enzyme, matrix metalloprotease 9 and neprilysin that degrade Aβ and protect neuronal cells from Aβ mediated cytotoxicity. (Cook et al., 2003; Farris et al., 2003; Graykowski et al., 2020)A recent study by Tachibana et al highlighted the involvement of neuronal LRP1 in APOE4 mediated Aβ seeding. By knocking out neuronal LRP1 from APP/PS1 mice expressing APOE4, the authors demonstrated a reversal of the increased amyloid burden. A possible explanation for this effect could be attributed to the increased accumulation of poorly lipidated APOE4-Aβ aggregates in the endosomes, impairing LRP-1 mediated Aβ degradation. Knocking out LRP1 may have resulted in an increased association of APOE4 at the surface, promoting endosomal Aβ degradation. (Kim et al., 2009; Strickland & Holtzman, 2019; Tachibana et al., 2019)

In conclusion, APOE influences both amyloid deposition and clearance in an isoform dependent manner. Improving APOE4's lipidation may inhibit its propensity to aggregate and alter its interaction with APOE receptors, facilitating the clearance of Aβ.

2.3 APOE4 and tau pathology

Neurofibrillary tangles in AD can be classified into intraneuronal NFTs and extracellular NFTs. Tau, a microtubule-associated protein, undergoes hyperphosphorylation to form insoluble aggregates in the cell body of neurons. These are then released into the extracellular space after the neurons die, where they are engulfed by microglia. (Lindsley & Hooker, 2018)

APOE4 carriers have been shown to have increased concentrations of total tau and phosphorylated tau in the CSF compared to non-carriers in a gene dose-dependent manner. (Benson et al., 2022). APOE4 status also increased phosphorylated tau levels in iPSC-derived cerebral organoids from AD patients. (J. Zhao et al., 2020) Shi et al observed an increased brain atrophy in an APOE4 P301S tau mouse model in comparison to APOE2 and APOE3 tau mice and that tau accumulation in the presence of APOE4 may make neurons more susceptible to death. Gratuze *et al* suggested a role for microgliosis in exacerbating tau mediated neurodegeneration in the presence of APOE4. (Gratuze et al., 2023; Shi et al., 2017; Sun et al., 2023)

Recent evidence has highlighted the role of cell type specific APOE4 in the development of tau pathology. Genetic ablation of astrocyte-derived APOE4 and neuronal APOE4 in models of tauopathy significantly decreased the accumulation of phosphorylated tau, tau mediated neurodegeneration and microglial phagocytosis. (Koutsodendris et al., 2023a; C. Wang et al., 2021) The deleterious effects of neuronal APOE4 may be attributed to its proteolysis that occurs during its secretion. The subsequently generated APOE4 fragments exhibit cytotoxicity, increase phosphorylation of tau, and exacerbate AD-related neurodegeneration. (Benson et al., 2022; Brecht et al., 2004)

The differential effects of APOE isoforms on tau pathology have not been studied as extensively as the amyloid pathology in AD. Further investigations are needed to elucidate the effect of APOE's lipidation status on tau pathology.

2.4 APOE4 and neuroinflammation

Reactive gliosis is a feature of AD brains. Microglia and astrocytes cluster around dense Aβ plaques to phagocytose them. Plaque associated glia have a pro-inflammatory phenotype described as "disease associated" (DA) glia. DA microglia exhibit a downregulation of homeostatic genes such as *Tmem119, P2ry12, CX3CR1* and upregulation of genes linked to microglial activation, phagocytosis, and lipid metabolism such as *Clec7a, CST7, APOE, LPL*. Reactive astrocytes release pro-inflammatory molecules such as ATP, CX3CL1, and C3 while reactive microglia secrete IL-1, TNF-α, and C1q, resulting in neuronal death. (Chun et al., 2018; Rodríguez-Giraldo et al., 2022)

APOE4 exacerbates neuroinflammation in AD and is associated with driving glia towards a disease associated transcriptional profile. (Lin et al., 2018)Cortical levels of IL-1β were significantly greater in E4FAD mice relative to E3FAD mice. APOE4 microglia in AD have been reported to exhibit features such as altered morphology, reduced Aβ phagocytosis and an increased secretion of TNFα. (Ferrari-Souza et al., 2023; Kloske & Wilcock, 2020). Similarly, APOE4 astrocytes in AD have been reported to display features such as impaired Aβ uptake, increased cholesterol accumulation resulting in the formation of lipid droplets and poor neuronal support in comparison to APOE3 astrocytes. (Fernandez et al., 2019; J. Zhao et al., 2017) *APOE4* has also been linked to an increased activity of NF-κB and decreased activity of Transgelin 3 (TAGLN3) in human astrocytes, resulting in a pro-inflammatory phenotype. This might be a possible mechanism through which APOE4 drives inflammation. (Arnaud et al., 2022; Rodriguez et al., 2014)

The relationship between APOE and microglial response has been extensively studied in the context of tauopathy. Recent evidence suggests that APOE4 driven microglial activation may precede tau pathology and accelerate tau mediated neurodegeneration. (Parhizkar & Holtzman, 2022; Shi et al., 2019; Tcw & Arranz, 2023) Shi *et al* showed that genetic depletion of microglia in APOE4 P301S tau mice blocked the progression of phosphorylated tau and subsequent neurodegeneration. Importantly, the authors also reported no difference on tau histopathology in the absence of microglia in APOE3 and APOE4 tau mice. A drastic reduction in the viability of P301S expressing neurons cocultured with APOE4 mixed glia as compared to APOE2 and APOE3 co-cultures was observed. APOE4 mixed glia also secreted higher levels of TNF-α than APOE2 and APOE3 mixed glia. (Shi et al., 2017, 2019)

Several studies have also highlighted the role of cell type specific APOE4 in AD associated inflammation. Transcriptomic analysis has highlighted a role for neuronal APOE4 in promoting a disease associated phenotype in glia. Removal of neuronal APOE4 decreased the amounts of DAA and DAM while increasing the amounts of disease protective glia in a mouse model of tauopathy. The authors also reported an attenuation of tau mediated neurodegeneration and microgliosis. (Koutsodendris et al., 2023) Wang *et al* reported that genetic deletion of astrocytic *APOE4* decreased DAM and attenuated microglia-dependent synaptic loss in tauopathy. (C. Wang et al., 2021)

Besides APOE4, GWAS studies have identified variations in TREM2 as risk factors for LOAD. TREM2 is a receptor expressed on microglia involved in its immune response. APOE is a ligand of TREM2. The connection between APOE4 and TREM2 mediated microglial response in AD pathology is under investigation. (Chun et al., 2018; Jain et al., 2023; Wolfe et al., 2019)

2.5 APOE4 and cholesterol dysregulation in AD

Poor lipidation of APOE4 can be attributed to its structural modifications or a faulty cellular machinery responsible for lipid efflux. (Staurenghi et al., 2021)Transporters from the ABC family are a key component of this machinery. Expression of ABCA1 at glial surfaces is controlled by ADP-ribosylation factor 6 (ARF6). ARF6 promotes the degradation of ABCA1 in the endolysosomes that are involved in recycling membrane associated ABCA1. (Chen et al., 2022) Rawat *et al* found that APOE4 promoted greater expression of ARF6 in comparison to APOE3. Thus, a reduced surface expression of ABCA1 resulted in a lower ABCA1-mediated cholesterol efflux associated with APOE4. (Chen et al., 2022; Mukhamedova et al., 2016; Rawat et al., 2019) Several studies have also reported a reduced secretion of APOE4 from astrocytes in AD. Insufficient levels of APOE4 available for lipidation would result in reduced HDL-like particles being formed in the brain. (Martinez et al., 2023; Sullivan et al., 2011)

Astrocytic APOE can regulate neuronal cholesterol homeostasis through multiple routes. (Asante et al., 2022) Lipidated APOE deliver microRNAs involved in cholesterol biosynthesis and histone acetylation to neurons. Compared to HDLs containing APOE3, HDLs containing APOE4 have lesser amounts of these microRNAs, resulting in lower cholesterol biosynthesis in neurons and a decreased transcription of genes involved in memory consolidation. Moreover, APOE4-carrying human astrocytes oversupply cholesterol to promote expansion of

neuronal lipid rafts. This evidence reiterates the role of APOE on APP processing as these lipid rafts serve as a site for APP cleavage and Aβ generation, (S.-I. Lee et al., 2021; X. Li et al., 2021; H. Wang et al., 2021)

Furthermore, accumulation of lipid droplets has also been observed in brain samples of AD patients. Astrocytes under stress accumulate lipid droplets as a source of energy. Farmer *et al* demonstrated that astrocytes accumulate LDs in an isoform dependent manner, APOE4>APOE3. (Farmer et al., 2020) APOE4 reduces the sequestration of fatty acids in neuronal lipid droplets. Under healthy conditions, excessive fatty acids from neurons are transported to astrocytes for uptake. However, APOE4 expressing astrocytes have a decreased capacity to shuttle fatty acids from neuronal lipid droplets. Thus, APOE4 may induce lipotoxicity through lipid accumulation in neurons and glia. (Guoyuan Qi et al., 2021; Ioannou et al., 2019; Smolič et al., 2021)

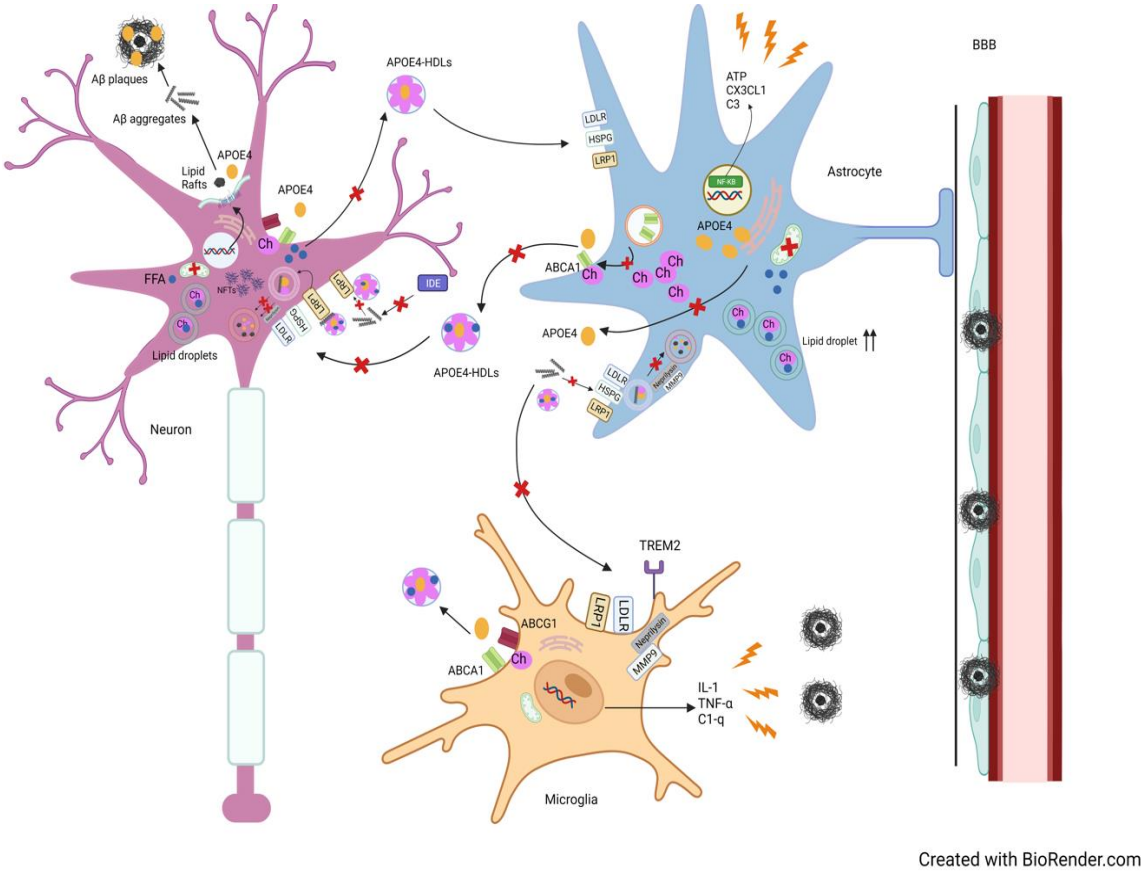


Figure 1.2 Impact of APOE4 on AD neuropathology. APOE4 promotes tau phosphorylation, APP transcription, Aβ aggregation and is co-deposited in Aβ plaques. APOE4 preferentially binds to LRP1, LDLR and HSPGs, competing with Aβ for receptor mediated uptake. Following uptake, APOE4-Aβ deposits are trapped in endolysosomes, resulting in decreased lysosomal

degradation. Aβ degradation by other enzymes such as IDE, neprilysin and MMP9 is also impaired. APOE4 reduces cholesterol efflux and the formation of HDL-like particles, affecting lipid trafficking. APOE4 reduces fatty acid sequestration in neuronal lipid droplets and decreases the shuttling of excess fatty acids to astrocytes via APOE4-HDL-like particles. APOE4 is associated with mitochondrial dysfunction and an inflammatory phenotype of neuroglia. Ch: Cholesterol, FFA: Free fatty acid

3. Impact of APOA-I on Neuropathology of Alzheimer's Diseases

Several cross-sectional studies have reported a lower risk and severity of LOAD and dementia in the elderly population in association with higher levels of APOA-I, the major apolipoprotein present in plasma HDLs.(Merched et al., 2000; Reitz et al., 2010; Saczynski et al., 2007) Conversely, lower levels of plasma HDLs and APOA-I have been reported in AD patients. Higher levels of plasma HDLs also correlated with a decreased amyloid burden and improved memory in cognitively healthy populations. (Endres, 2021; Kuriyama et al., 1994; Luo et al., 2023; Zuin et al., 2021; Zuliani et al., 2010)

In addition to clinical data, *in vivo* animal studies have also provided strong evidence supporting the beneficial role of APOA-I and HDLs in AD. Genetic ablation as well as deficiency of murine APOA-I decreased plasma HDL levels, worsened memory deficits and increased CAA in APP/PS1 mice. (Button et al., 2019; Lefterov et al., 2010)Conversely, APP/PS1 mice with transgenic APOA-I overexpression in the periphery exhibited increased plasma HDL levels, preserved cognitive function during aging and reduced CAA as compared to controls (Lewis et al., 2010). These mice also had a significantly decreased astrocyte activation and a trend towards decreased microglial activation, consistent with the known anti-inflammatory role of APOA-I and HDLs, documented in atherosclerosis. The effect of APOA-I on parenchymal Aβ plaque load is inconsistent; however, the protective effect of APOA-I on reducing vascular Aβ deposition has been consistently observed.(Lewis et al., 2010)

Interestingly, Fitz *et al* demonstrated that knocking-out both APOE and APOA1 in APP/PS1 mice ameliorated the amyloid pathology by increasing amyloid clearance. However, despite the protective outcome on amyloid pathology, these mice exhibited substantial behavior deficits and impaired dendrite morphology (Fitz et al., 2015)The role of APOA-I in tau pathology has been reported.

Taken together, similar to APOE, APOA-I plays an important role in cholesterol/lipid homeostasis, cerebral amyloid deposition, and neuroinflammation, and thus modulates the pathogenic process of AD.

Chapter II: HDL Mimetic Peptides to Rescue APOE4 Associated Deficits Pertinent to Alzheimer's Disease

1. Background

APOE and HDL-like particles play diverse roles in physiological processes of the brain as described in Chapter I. Many of these functions are dysregulated in an *APOE4* genetic background, either through a loss of protective function or a gain of toxic function of APOE4. Therefore, correcting the function of APOE4 and restoring the levels of HDL-like particles in the brain may prove to be an alternative therapeutic approach to ameliorate features of AD. (Massari et al., 2023; Namjoshi et al., 2011; White et al., 2014)

Supporting this hypothesis, several studies on recombinant HDL therapies have shown promising results. For instance, intravenous administration of reconstituted HDL reduced soluble brain Aβ levels in symptomatic APP/PS1. (Robert et al., 2016) Intravenous administration of recombinant APOA-I Milano in APP23 mice reduced microgliosis, Aβ deposition, and CAA. (Fernández-de Retana et al., 2017) Infusion of human APOE3 or APOE4 in APOE deficient mice improved synapto-dendritic pathology and cognitive deficits observed in controls. (Masliah et al., 1997) Another study on chronic infusion of APOE3 and APOE4 into rat brains increased the expression of α and β-APP-CTF, suggesting that APOE inhibited cleavage of APP-CTFs by γ-secretase. Importantly, APOE3 exerted this effect to a greater extent than APOE4. (Hoe et al., 2006; Irizarry et al., 2004)

However, direct supplementation of APOs in its native form is clinically unfeasible owing to its high manufacturing cost, as well as its large size and poor penetration across the BBB. Therefore, several efforts have been devoted to the synthesis of HDL mimetic peptides that are smaller in size, enabling them to cross the BBB and exhibit a greater bioavailability. Clinically tested HDL-mimetic peptides derived from the structure of APOs such as CN-105, 4F and 5A have been extensively studied in the cardiovascular field for their anti-atherogenic and anti-inflammatory properties. A common feature of HDL-mimetic peptides is their ability to form Class A amphipathic helices, responsible for lipid association in exchangeable APOs such as APOE and APOA-I (**Figure 2.1**). Amphipathic peptides can bind to lipid domains formed by ABCA1 and form a stable HDL-like scaffold during cholesterol efflux.(Anantharamaiah et al., 2007; Frank & Marcel, 2000; Islam et al., 2018; Meloni et al., 2020) The outcomes of different

HDL mimetic peptides under investigation for various CNS diseases have been summarized in **Table 1** (see end of main text)

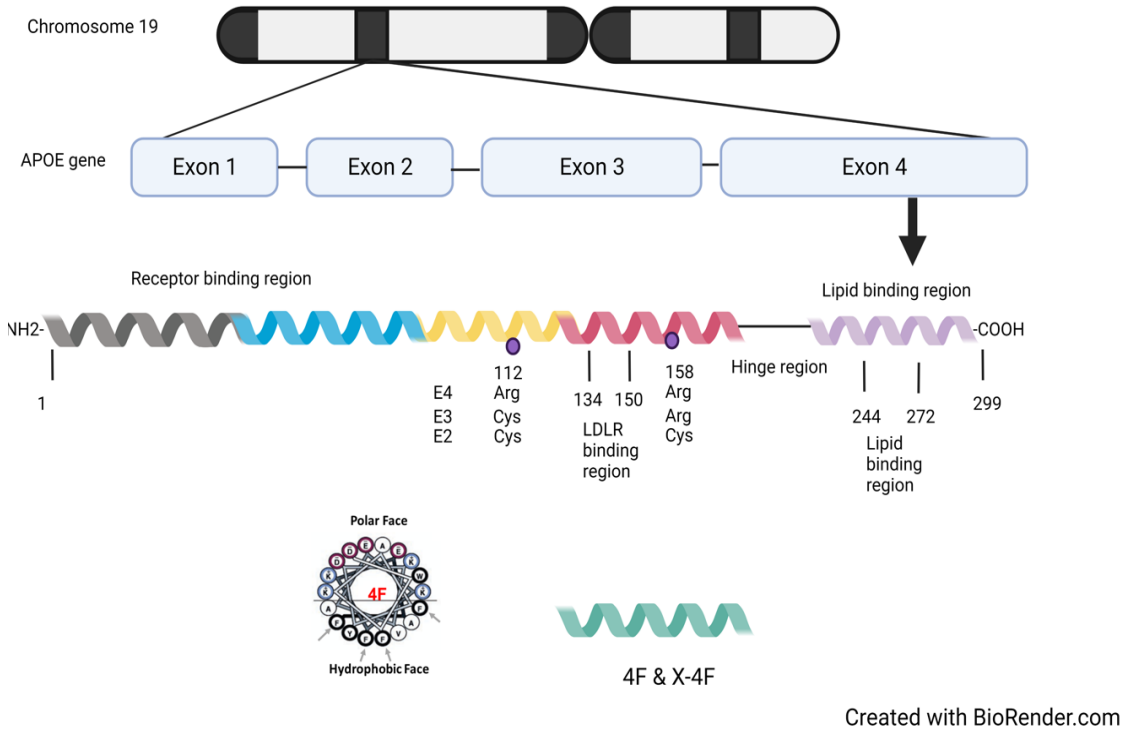


Figure 2.1. Structure of APOE and 4F. Exchangeable APOs such as APOA-I, APOE and APOC contain tandem 22-mer repeating domains. The 22-mer repeats are usually separated by Pro residues and have been associated with the formation of Class A amphipathic α-helix. They have opposing polar and nonpolar faces with positively charged amino acids appearing at the polar-nonpolar interface and negatively charged amino acids at the center of the polar face. These allow the interaction of the protein with phospholipids through its hydrophobic face, while the hydrophilic face of the helices interacts with the aqueous phase. (Frank & Marcel, 2000; Segrest et al., 1974)

4F is an 18 amino acid long peptide that contains four phenylalanine residues and blocking groups at the N and C terminals to improve lipid association and stability. (Wolska et al., 2021) The BBB penetration rate of 4F was estimated to be about 1000 folds than that of APOA-I after intravenous injection. Importantly, 4F treatment increased efflux and decreased influx of Aβ in the brain, highlighting its potential for mitigating amyloid accumulation in AD. (Swaminathan et al., 2020) L-4F is subjected to digestion by gut proteases. Therefore, only D-4F is efficacious when administered orally.(Navab et al., 2009) Previous studies involving co-administration of D-4F and pravastatin inhibited the formation of atherosclerotic lesion and

decreased hyperlipidemia induced inflammation of brain arterioles. (Navab et al., 2002) D-4F exhibited similar anti-inflammatory effects on brain arterioles and improved cognition in LDL receptor-null mice on a Western diet. (Buga et al., 2006) Expanding upon this work, the effects of D-4F were studied in AD mouse models. Oral administration of D-4F with pravastatin for 3 months improved memory deficits, inhibited hippocampal Aβ deposition, gliosis and decreased the levels of pro-inflammatory cytokines TNF-a and IL-1b.(Handattu et al., 2009) Chernick et al provided further insights into the mechanism by which 4F potentially exerts its protective effects in the brain. The authors showed an increased secretion and lipidation of murine APOE from primary glial cells upon 4F treatment and that 4F relied on ABCA1 and ER-Golgi secretory pathway to exert these effects. Importantly, 4F rescued oligomeric Aβ induced reduction in APOE secretion and lipidation. (Chernick et al., 2018)

Apart from AD models, D-4F has also exhibited positive outcomes in other CNS models such as stroke and spinal cord injury. D-4F decreased neuroinflammation and increased insulin-like growth factor-1; a neurotropic factor inducing neurite/axonal outgrowth, alleviating white-matter damage after middle artery occlusion induced stroke. D-4F also promoted the clearance of myelin debris and the reduction of foamy macrophages after spinal cord injury. (Cui et al., 2016; J. Li et al., 2022)

These findings provide a strong rationale to further investigate the potential of HDL-mimetic peptides in correcting APOE4's altered functions. We hypothesize that reversing APOE4's lipidation deficit with HDL-mimetic peptides corrects its functions and rescues APOE4 associated AD pathology. Restoring the beneficial functions of HDLs and APOE in the brain using HDL-mimetic peptides can be a promising approach to ameliorate AD features.

2. Materials and Methods

2.1 Immortalized astrocyte culture

Immortalized mouse astrocytes derived from human APOE3 and APOE4 targeted-replacement mice (Morikawa et al., 2005), generously provided by Dr. Guojun Bu (Mayo Clinic, Jacksonville, FL) and Dr. William Rebeck (Georgetown University, Washington, D.C.), respectively, were cultured in DMEM supplemented with 10% FBS, 2mM GlutaMAX, 1X antibiotic/antimycotic and 10ng/ml epidermal growth factor (EGF). The cells were plated at $2x10^5$ cells per well on poly-D-lysine (PDL) coated 12-well tissue culture plates, allowed to

attach and grow for 24 hours, and then exposed to experimental treatments. During treatment, the cells were washed twice with sterile phosphate buffered saline (PBS) before adding experimental agents in serum-free OPTI-MEM supplemented with 1X antibiotic/antimycotic for various durations.

2.2 Primary astrocyte culture

Homozygous human APOE3 and APOE4 knock in (KI) mice were purchased from The Jackson Laboratory (Stock # 029018 and 027894). Furthermore, these human homozygous APOE4 KI mice (E4/4) were bred with APP/PS1 transgenic mice to produce E4/4 and E4/4/APP/PS1 littermates, through a 3-step breeding strategy. Primary glial cultures were established from neonatal (1 to 3-day-old) pups for *in vitro* studies as described by Fagan et al., 1999 (Chernick et al., 2018). Briefly, neonatal pups were sacrificed within the first 3 days post-natal. Both sexes of the pups were used and pups were randomly allocated in the study. Brains were dissected out, then cortex and hippocampal tissue were triturated into a single-cell suspension and cultured for 14 days in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 16mM HEPES buffer, 0.1mM nonessential amino acids, 2mM GlutaMAX, and 1X antibiotic/antimycotic cocktail at which point microglia were removed by shaking. Medium was replaced and microglia were allowed to grow for an additional week, at which point they were shaken loose and removed again. The purity of astrocyte and microglial cultures was determined by immunofluorescence analysis of markers specific for astrocytes and microglia.

Cells were plated for treatment at $2x10^5$ cells per well on poly-D-lysine (PDL) coated 12-well tissue culture plates and allowed to attach and grow overnight prior to experimental treatment. In general, cells from pups of the same genotype were pooled for experiments, except that for E4/4 and E4/4/APP/PS1 littermates, cells from individual pups were cultured while their APP/PS1 genotype was determined by PCR.

All animal procedures were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC protocol # 2207-40221A) of the University of Minnesota.

2.3 Preparation of aggregated $A\beta_{42}$

Aggregated $A\beta_{42}$ was prepared as previously described (Chernick et al., 2018). Briefly, hexafluoroisopropanol (HFIP) treated $A\beta_{42}$ film was resuspended in fresh dry DMSO, diluted

in phenol-free F12 cell culture media, and incubated at 37°C for 24 hours for aggregation. Aliquots of the Aβ preparation were examined by LDS NuPAGE (4-12% bis-tris gels; ThermoFisher; Waltham, MA; Cat# NP0322BOX) followed by immunoblot analysis with the anti-Aβ antibody, 6E10 (BioLegend; SanDiego, CA; Cat# SIG-39340; RRID:AB_662806). This aggregated Aβ preparation was used to treat cells.

2.4 Pharmacological treatment

The peptides 4F (Ac-DWFKAFYDKVAEKFKEAF-NH2) (4F: peptide purity=95%, peptide content=), and a modified version of 4F, herein called X-4F (X-4F: peptide purity > 95%) were custom synthesized and purchased from American Peptide Company Inc (Sunnyvale, CA) and ThermoFisher Scientific (Rockford, IL), respectively. 4F and X-4F were prepared in sterile PBS and used at a range of concentrations from 0.1 μM to 5 μM. Prior to treatment, cells were washed twice with sterile phosphate buffered saline (PBS) and then treatments were performed in serum-free OPTIMEM supplemented with 1X antibiotic/antimycotic cocktail for various durations, as defined for each experiment. Aggregated Aβ preparation was used to treat cells at a concentration of 5 μM.

2.5 Gel electrophoresis and Western blot analysis

After treatments were performed, conditioned media was collected and cells were lysed in ice-cold RIPA buffer. Protease inhibitors were added to both media and cell lysate samples. Media and cell lysates were then subjected to 12% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-human APOE (Millipore, Burlington, MA; Cat# 178479; RRID:AB_564230) and tubulin (Sigma-Aldrich, St. Louis, MO; Cat# T5168; RRID:AB_477579) antibodies, followed by HRP-conjugated secondary antibody and chemiluminescence detection using Western Lighting Plus-ECL reagents (PerkinElmer, Waltham, MA; Cat# NEL103001EA).

Non-denaturing gradient gel electrophoresis (NDGGE) was used to assess APOE lipidation. Fresh media was run on 4-20% polyacrylamide tris-glycine gels (ThermoFisher; Waltham, MA; Cat# EC6021BOX) in the absence of SDS, reducing agents or sample boiling at 120V for 5 hours. Proteins were transferred to PVDF membranes at 110V for 90 minutes and probed for APOE, followed by HRP-conjugated secondary antibody and chemiluminescence detection using ECL reagents. Poorly lipidated APOE was defined as complexes smaller than 8.2 nm as

measured by NDGGE based on the high molecular weight marker (GE Healthcare; Buckinghamshire, UK; Cat# 17044501).

2.6 Mitochondrial Flux Assay

Metabolic flux analysis was performed using the Seahorse Mito Stress test as described previously (Jiang et al., 2013; Yin et al., 2012). Primary APOE3 and APOE4 KI astrocytes were cultured on poly-D-lysine coated Seahorse 96-well plates at a density of 15,000 cells per well, respectively. Astrocytes were grown in DMEM medium containing 10% FBS, 16mM HEPES buffer, 0.1mM nonessential amino acids, 2mM GlutaMAX, and 1X antibiotic/antimycotic cocktail. On the day of experiment, culture medium was replaced with pre-warmed Seahorse media (phenol free DMEM supplemented with 13.8 mM glucose, 1 mM sodium pyruvate, 4 mM glutamine; pH preadjusted to 7.4) and incubated at 37°C in a CO2-free incubator for 1 h. Three baseline measurements of OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were sampled prior to sequential injection of mitochondrial inhibitors: oligomycin A (ATP synthase complex V inhibitor) at 2 μM, FCCP (an uncoupling agent that disrupts the mitochondrial membrane potential, allowing free flow of electron through the ETC and maximal oxygen consumption by complex IV) at 1 μM, and rotenone/antimycin A (complex I inhibitor and III inhibitors, that shut down mitochondrial respiration) at 1 μM / 1 μM. OCR and ECAR were recorded by the Seahorse XFe96 instrument and normalized to the number of cells per well. The basal mitochondrial respiration was calculated as the differences between OCR before oligomycin injection and OCR after rotenone/antimycin A injection (non-mitochondrial respiration). Maximal respiration was the differences between maximal OCR value after FCCP injection and OCR after rotenone/antimycin A injection. Spare capacity ratio (SCR) is defined as the ratio of maximal respiration to basal respiration, as an indicator for the utilization of the cells' maximum bioenergetic capacity.

2.7 Statistical Analysis

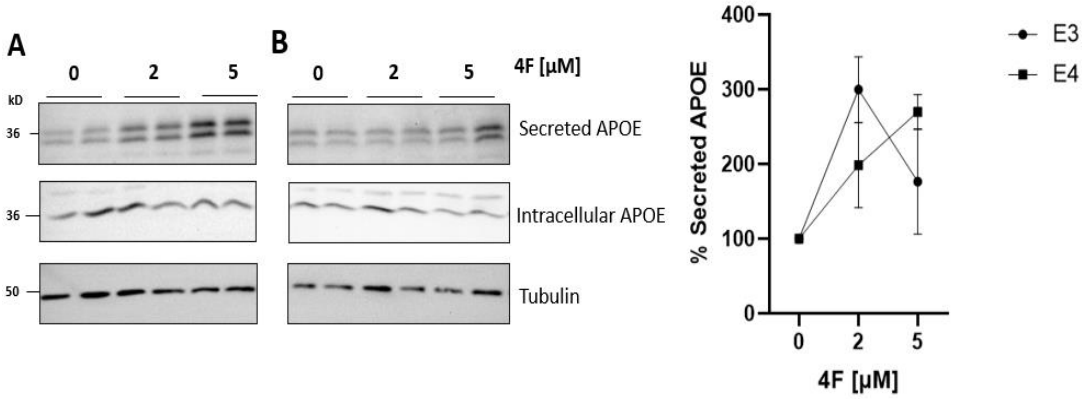
Western blot results were quantified using Image J software. Secreted APOE was analyzed as the ratio of APOE in medium to APOE in cell lysate and is expressed as a relative percent in media with the amount in the vehicle treatment set as 100%. The amount of lipidated APOE was analyzed as the ratio of lipidated APOE to poorly lipidated APOE in medium, and is expressed as relative percent in lipidated form with the amount in the vehicle treatment set as

100%. To compare the isoform specific effects on APOE secretion and lipidation, the vehicle treatment of APOE3 was set as 100%. Data were expressed as mean ± standard error (SE) from at least 2 independent experiments with each treatment in duplicate or triplicate. No sample size calculation was performed. Normality of the data was tested using Shapiro-Wilk test. Comparison of different treatments was performed by one sample t-test, Welch's t-test, ANOVA (for normally distributed data), and the Wilcoxon Rank Sum test, Mann-Whitney rank sum test (for non-normally distributed data). GraphPad Prism 9.5.1 was used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1 4F increases APOE secretion in APOE3 and APOE4 immortalized astrocytes

Chernick *et al* previously confirmed the relevance of 4F-mediated effects to human physiology using human primary astrocytes (Chernick et al., 2018). However, the differential effects of 4F on APOE isoforms has not yet been investigated. Therefore, we used immortalized astrocytes generated from human APOE3, and APOE4 targeted replacement mice (Morikawa et al., 2005) to study the isoform specific effects of 4F. Our studies show that 4F tends to increase APOE secretion in both APOE3 and APOE4 immortalized astrocytes in a concentration- and time-dependent manner (**Figure 2.2A-D**). The effect of 4F in APOE3 astrocytes was more significant than in APOE4 astrocytes.



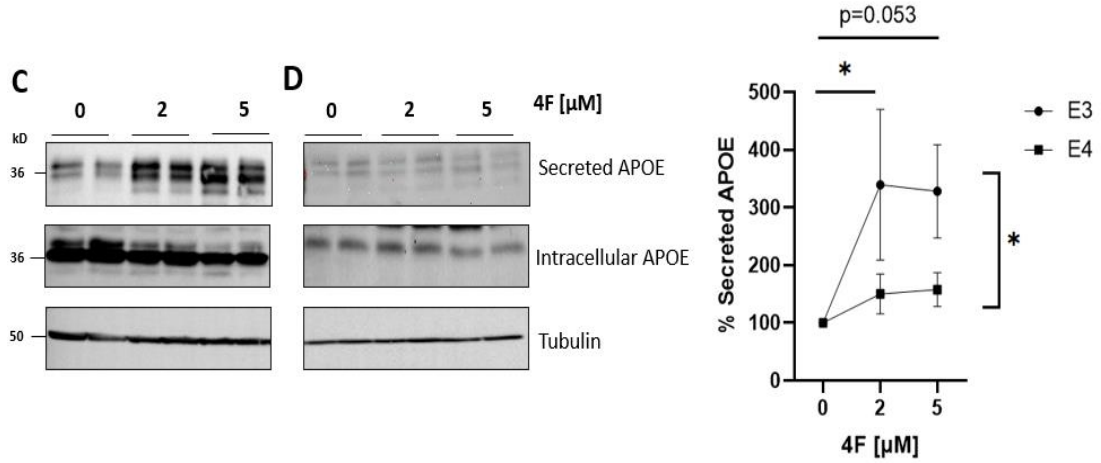


Figure 2.2 4F increases APOE secretion in APOE3 and APOE4 immortalized astrocytes. Immortalized astrocytes expressing human (A) APOE3, (B) APOE4 were treated for 6 hours with 4F at 2 and 5 μM in serum-free OPTIMEM. Immortalized astrocytes expressing human (C) APOE3, (D) APOE4 were treated for 20 hours with 4F at 2 and 5 μM in serum-free OPTIMEM. SDS-PAGE was performed on media and cell lysates. Data represents 2-3 separate experiments performed in duplicates. * = $p < .05$

3.2 X-4F increases APOE secretion in APOE3 and APOE4 immortalized astrocytes

X-4F is a modified form of the peptide 4F to potentially increase its efficacy. Like 4F, X-4F also increases APOE secretion in both cell lines, more so in APOE3 astrocytes, in a dose dependent manner (**Figure 2.3A & 2.3B**).

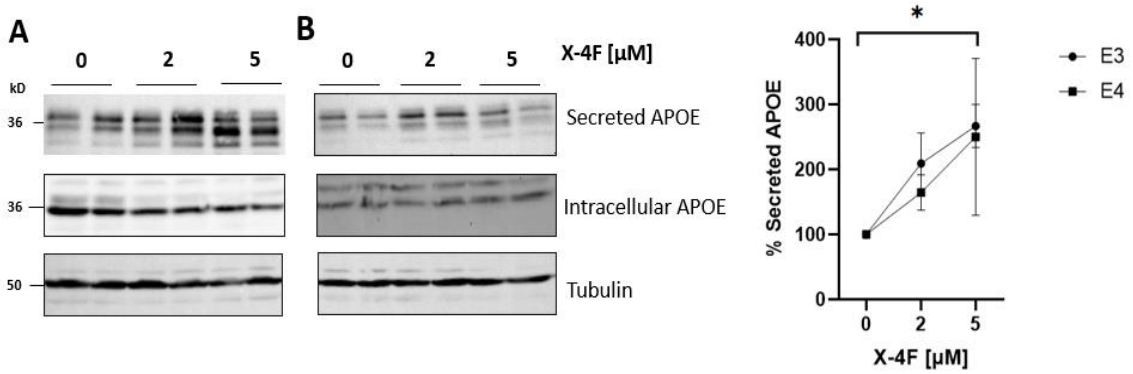


Figure 2.3 X-4F increases APOE secretion in APOE3 and APOE4 immortalized astrocytes. Immortalized astrocytes expressing human (A) APOE3, (B) APOE4 were treated for 20 hours with X-4F at 2 and 5 μM in serum-free OPTIMEM. SDS-PAGE was performed on media and cell lysates. Data represents 3 independent experiments performed in duplicates. * = $p < .05$

3.3 Characterization of APOE3 KI, APOE4 KI and APOE4 APP KI primary astrocytes

To enhance the physiological relevance of our studies, we investigated 4F and X-4F mediated effects in primary astrocyte cultures. Primary glial cultures from homozygous APOE3 KI and APOE4 KI mice were established from neonatal (1 to 3-day-old) pups. The purity of astrocyte cultures was confirmed by immunofluorescence wherein cells were probed for GFAP, a marker for astrocytes (**Figure 2.4**)

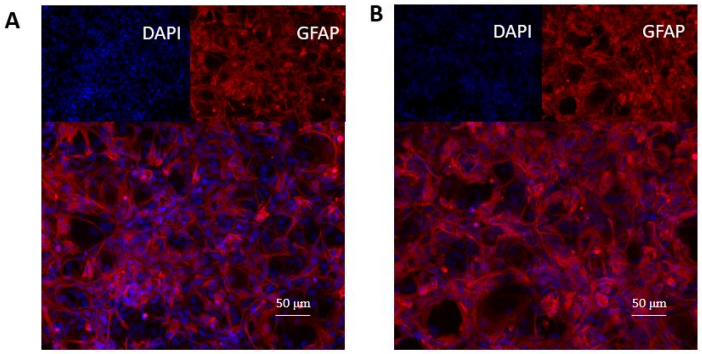
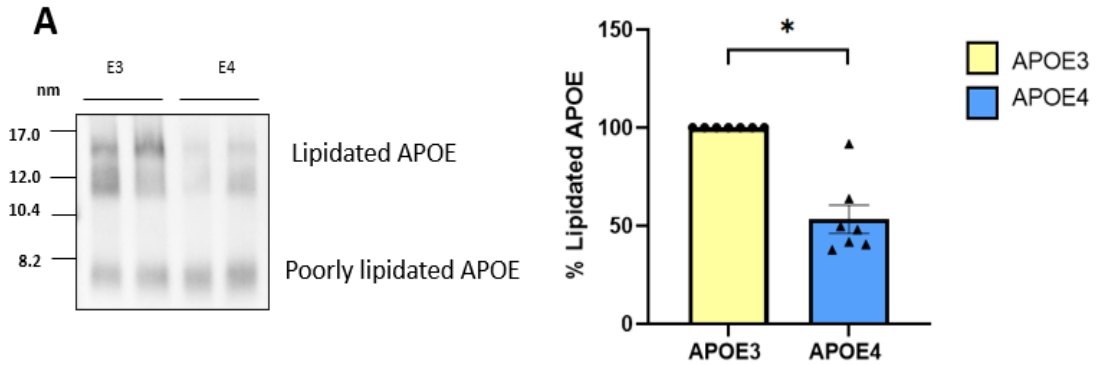


Figure 2.4 Characterization of APOE3 KI and APOE4 KI primary astrocytes. A. APOE3 KI astrocytes B. APOE4 KI astrocytes

3.4 APOE4 from primary murine astrocytes exhibits poor lipidation compared to APOE3

We confirmed the well-established lipidation deficit of APOE4 in APOE3 and APOE4 KI primary astrocytes. APOE4 exhibits only ~50% lipidation of the APOE3 control (**Figure 2.5A**). APOE4 KI primary astrocytes also seem to secrete less APOE than APOE3 KI primary astrocytes. However, this trend did not reach statistical significance, due to a small sample size and large variations (**Figure 2.5B**).



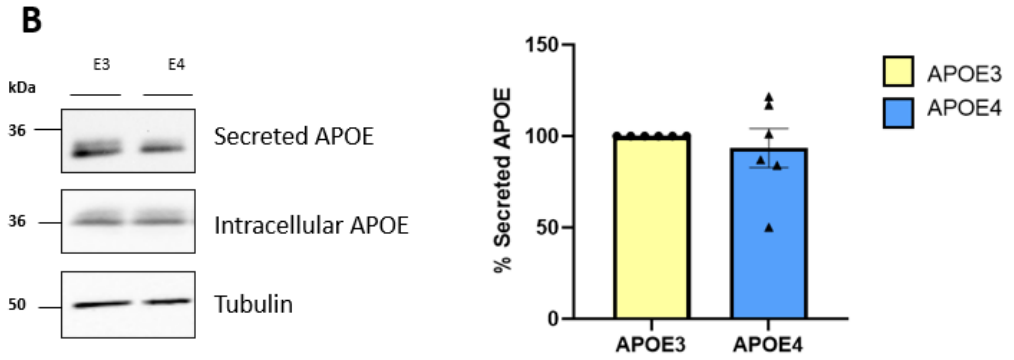


Figure 2.5 APOE4 from primary murine astrocytes exhibits poor lipidation compared to APOE3 Murine primary astrocytes expressing human APOE3 and APOE4 were treated for 20 hours in serum-free OPTIMEM. (A) NDGGE was performed on media. (B) SDS-PAGE was performed on media and cell lysates. Data represents at least 3 independent experiments performed in triplicates/duplicates. * = $p < .05$.

3.5 4F and X-4F increase APOE secretion and lipidation in APOE3 KI and APOE4 KI primary astrocytes

To confirm the impact of 4F and X-4F on APOE secretion and lipidation, primary astrocytes were subjected to treatments as in immortalized astrocytes. The results showed that the enhancing effects of 4F and X-4F on APOE secretion were reproduced in both APOE3 KI and APOE4 KI primary astrocytes (**Figure 2.6A and Fig 2.6B**). Furthermore, 4F and X-4F increased the lipidation of both APOE3 and APOE4; importantly, the treatments corrected the lipidation deficit in APOE4 (**Figure 2.6C and 2.6D**). Intriguingly, 4F-mediated effects on APOE secretion and lipidation tended to be more profound in the APOE4 genotype in comparison to APOE3. 4F increases APOE secretion and lipidation by ~4.5 fold and ~3.7 fold respectively, in the APOE4 genotype while achieving an increase of ~2.7 fold in the APOE3 control. (**Figure 2.6A and Fig 2.6C**). Interestingly X-4F improved APOE4's secretion and lipidation to a similar extent in comparison to the APOE3 control. Of note, X-4F exerted the effects at a lower concentration compared to 4F. (**Figure 2.6B and 2.6D**).

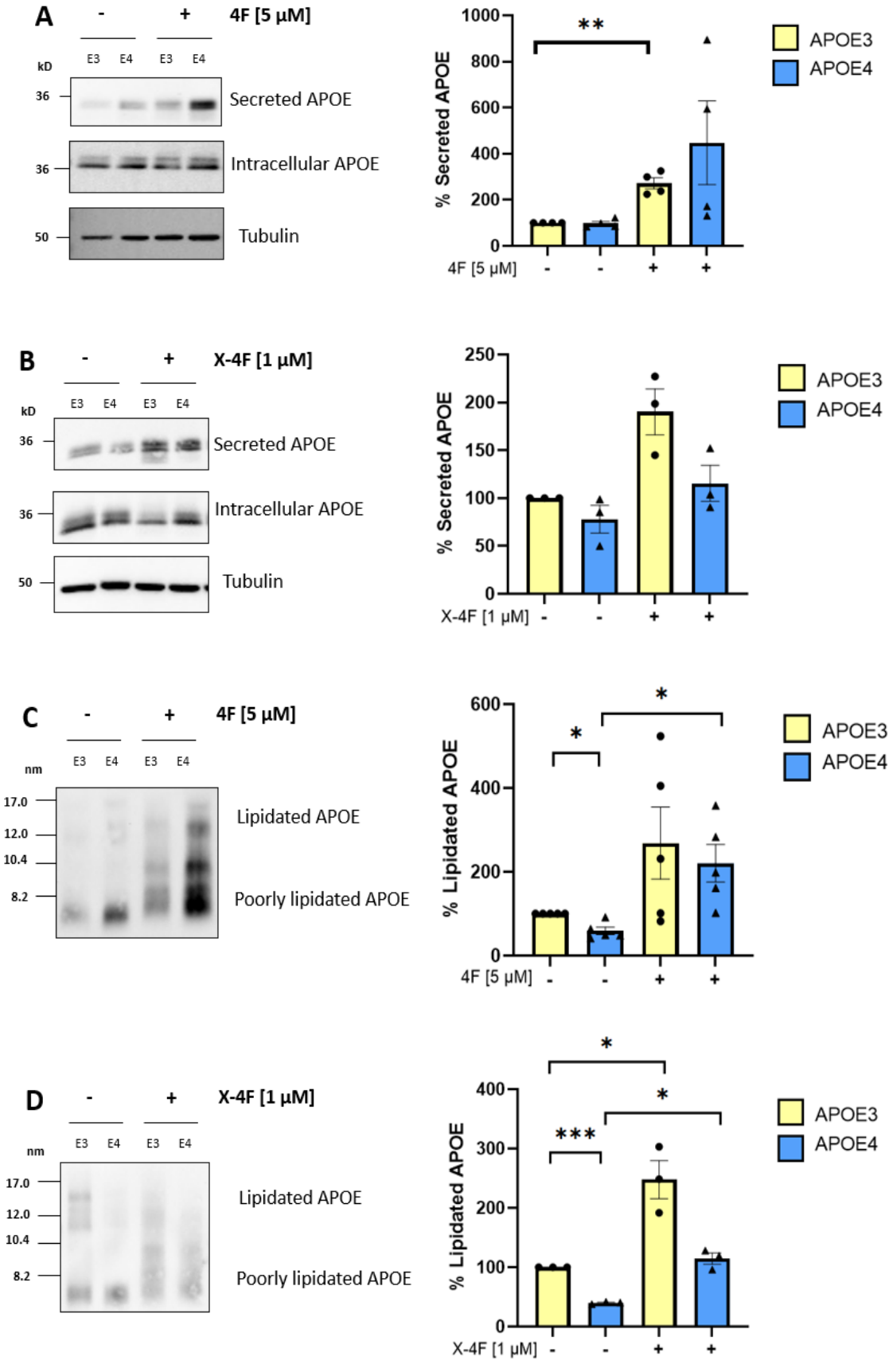

A
-
+
4F [5 µM]
E3
E4
E3
E4
kD
36
Secreted APOE
36
Intracellular APOE
50
Tubulin
% Secreted APOE
1000
800
600
400
200
0
**
4F [5 µM]
APOE3
APOE4
B
X-4F [1 µM]
% Secreted APOE
250
200
150
100
50
X-4F [1 µM]
C
4F [5 µM]
nm
17.0
12.0
10.4
8.2
Lipidated APOE
Poorly lipidated APOE
% Lipidated APOE
600
400
200
*
D
X-4F [1 µM]
***

Figure 2.6 4F and X-4F increase APOE secretion and lipidation in APOE3 KI and APOE4 KI primary astrocytes. Murine primary astrocytes expressing human APOE3 and APOE4 were treated for 20 hours with 5 uM 4F and 1 uM X-4F in serum-free OPTIMEM. (A,B) SDS-PAGE was performed on media and cell lysates. (C,D) NDGGE was performed on media. Data represents at least 3 independent experiments performed in triplicates/duplicates. * = p < .05, ** = p < .01, *** = p < .001

3.6 Characterization of aggregated Aβ42 preparations

Aggregated Aβ42 was prepared as previously described. (Chernick et al., 2018) Aliquots of the Aβ preparation were examined by LDS NuPAGE (4-12% bis-tris gels; ThermoFisher; Waltham, MA; Cat# NP0322BOX) followed by immunoblot analysis with the anti-Aβ antibody, 6E10 (**Figure 2.7**). Several studies have suggested that oligomeric forms of $A\beta_{42}$ exhibit the highest toxicity as opposed to Aβ monomers or larger, fibrillar Aβ aggregates. Oligomeric Aβ species initiate seeding and prion-like spreading, accelerating neurodegeneration in AD. (Sengupta et al., 2016) Therefore, we studied the effects of 4F and X-4F on cells treated with aggregated $A\beta_{42}$.

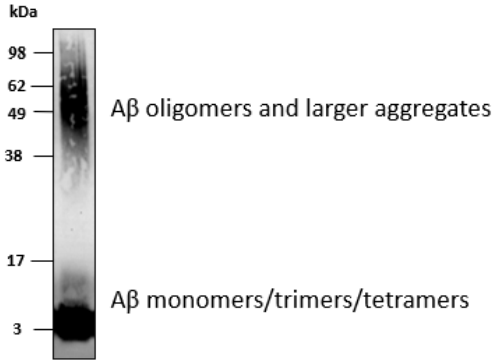
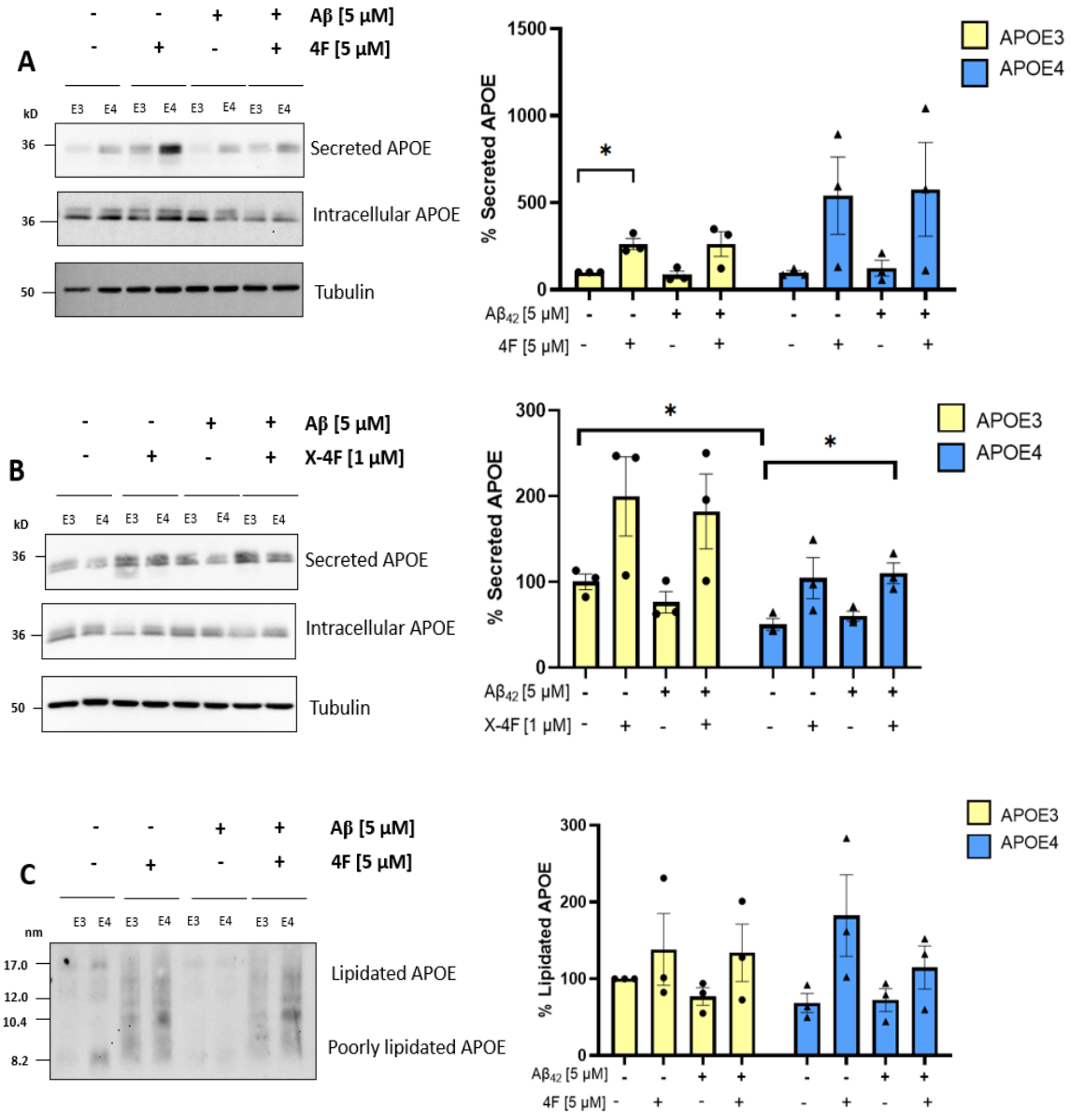


Figure 2.7 Characterization of Aβ aggregate preparations.

3.7 4F and X-4F improve APOE secretion and lipidation in APOE3 and APOE4 KI primary astrocytes, even in the presence of aggregated Aβ42

Previously, Chernick et al have demonstrated that aggregated Aβ42 inhibits the secretion and lipidation of APOE in murine primary astrocytes. These inhibitory effects of Aβ were counteracted by a 4F co-treatment. (Chernick et al. 2018) Following up on this work, we investigated the differential effects of 4F on APOE3 and APOE4 KI primary astrocytes treated with aggregated Aβ42. The data showed that aggregated Aβ42 did not significantly alter the APOE secretion and lipidation in either APOE3 or APOE4 KI primary astrocytes, unlike in primary astrocytes with mouse endogenous apoE (Chernick et al., 2018), suggesting that

human and mouse APOE may respond differently to exogenous Aβ exposure. Nevertheless, 4F mediated increase in APOE secretion and lipidation persisted in the presence of aggregated Aβ42 in APOE3 and APOE4 KI astrocytes. (**Figure 2.8A, 2.8C)** Similarly, X-4F also improved secretion and lipidation of APOE in APOE3 KI astrocytes treated with aggregated Aβ42. However, X-4F was only able to enhance secretion, but not lipidation of APOE4 in the presence of Aβ42 (**Figure 2.8B, 2.8D**).



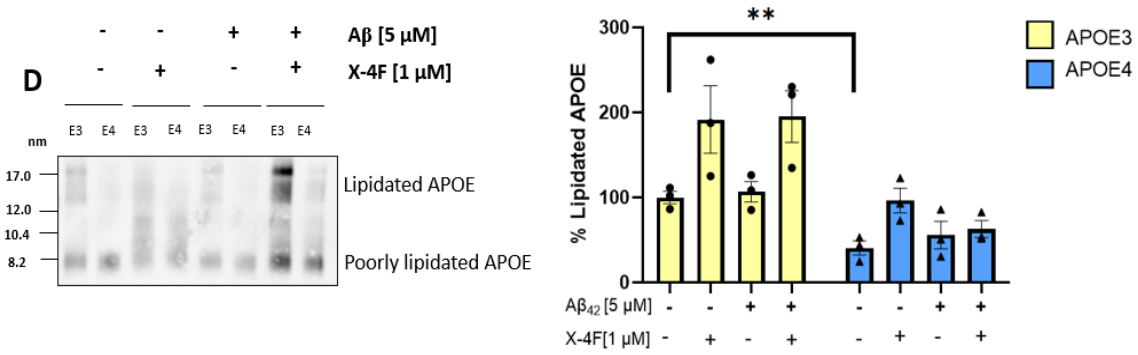


Figure 2.8 4F and X-4F increase APOE secretion and lipidation in APOE3 KI and APOE4 KI primary astrocytes, even in the presence of aggregated Aβ42. Primary mouse astrocytes expressing APOE3 and APOE4 were cultured for 20 hours with 5 µM Aβ42 aggregates in absence or presence of 5µM 4F and 1 µM X-4F in serum-free OPTI-MEM. (A, B) SDS-PAGE was performed on media and cell lysates. (C, D) NDGGE was performed on media. Data represents 3 independent experiments performed in triplicates (A, C). Data represents 1 independent experiment performed in triplicates (B, D). * = $p < .05$, ** = $p < .01$.

3.8 4F and X-4F increase APOE secretion and lipidation in APOE4 KI astrocytes overexpressing APP/PS1

Our previous experiments relied on an external supply of Aβ42 aggregates to mimic an *in vitro* AD model. Next, we studied the effects of 4F and X-4F on APOE4 KI primary murine astrocytes overexpressing mutant forms of human APP and PS1. Mutations in the APP and PS1 genes linked to FAD promote amyloidogenic APP processing and result in an increased production of Aβ, in particular Aβ42. Neurons are known to be the major producers of endogenous Aβ42 as they express high levels of the enzyme BACE1 involved in amyloidogenic APP cleavage. However, several studies have also reported the contribution of non-neuronal cells in Aβ42 formation especially astrocytes as they also express APP and BACE1. (Roßner et al., 2005; Veeraraghavalu et al., 2014; J. Zhao et al., 2011)

The results showed that APOE4 KI astrocytes overexpressing APP/PS1 tend to secrete less APOE than APOE4 KI astrocyte, but this trend did not reach statistical significance. Additionally, no differences in APOE lipidation were observed. Nevertheless, 4F and X-4F increased APOE secretion in APOE4 KI astrocytes with or without APP/PS1 overexpression, although to a lesser extent in APOE4 KI astrocytes overexpression APP/PS1 than in APOE4 KI astrocytes (**Figure 2.9A and 2.9B**). Importantly, 4F and X-4F improved APOE lipidation

in APOE4 APP/PS1 astrocytes to a comparable extent as in APOE4 KI astrocytes (**Figure 2.9C and 2.9D**).

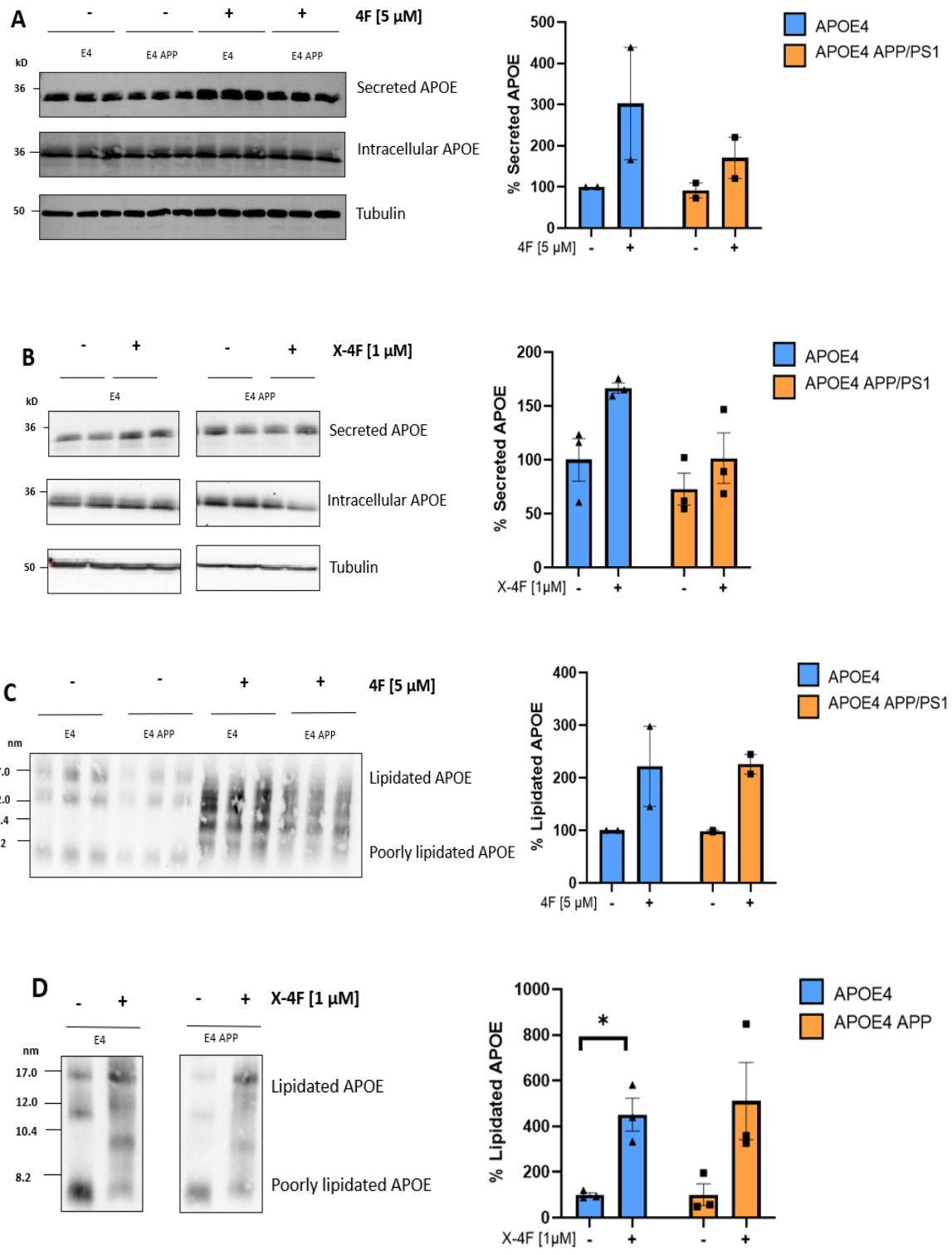


Figure 2.9 4F and X-4F increase APOE secretion and lipidation in APOE4 KI astrocytes overexpressing APP/PS1. Primary APOE4 KI astrocytes and APOE4 KI astrocytes overexpressing APP APOE4 were cultured for 20 hours with 5 μM 4F and 1 μM X-4F in serum-free OPTI-MEM. (A, B) SDS-PAGE was performed on media and cell lysates. (C, D) NDGGE was performed on media. Data represents 2 independent experiments performed in triplicates (A, C). Data represents 1 independent experiment performed in triplicates (B, D). * = $p < .05$.

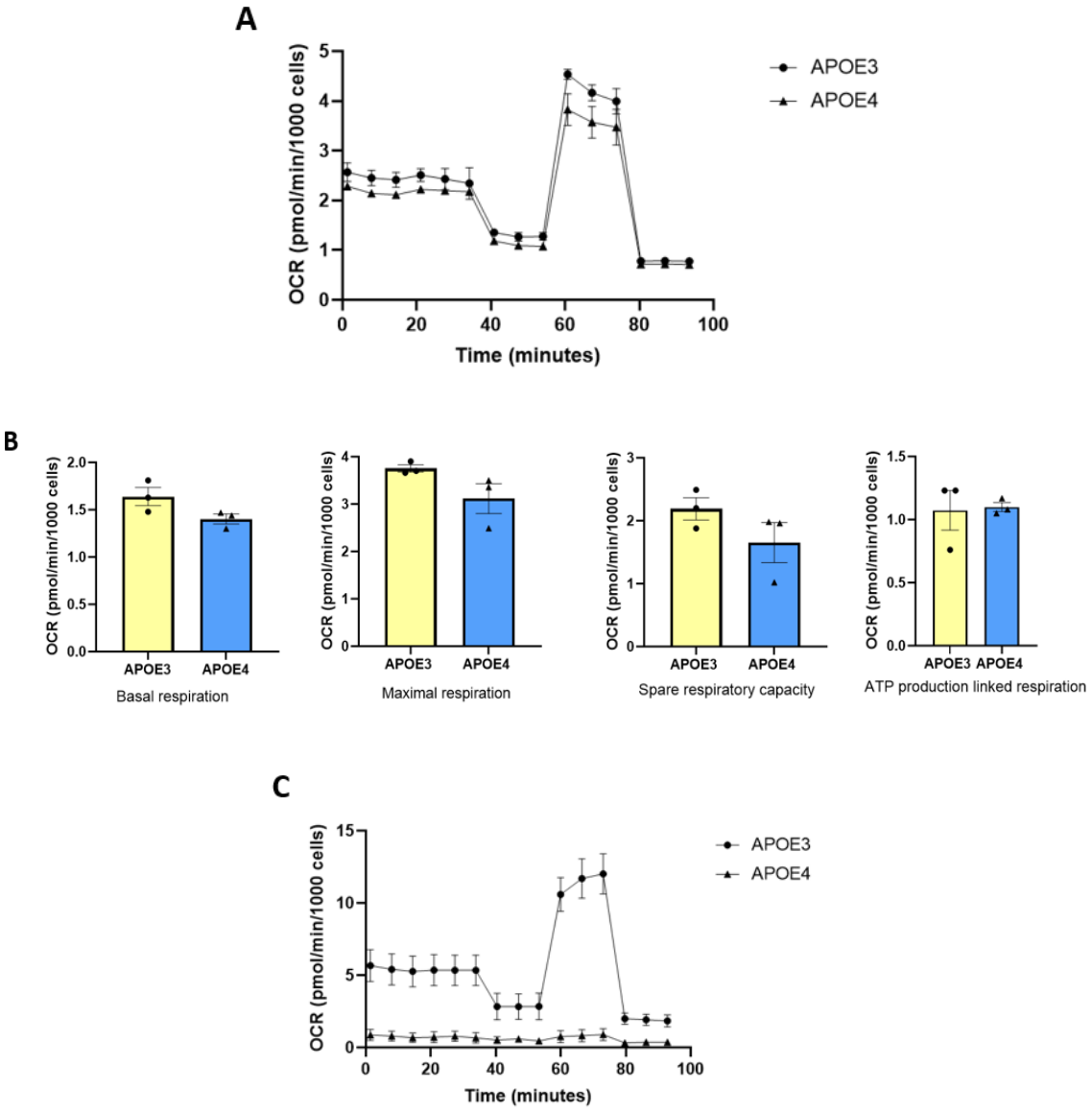
3.9 APOE4 expressing murine primary astrocytes exhibit an altered metabolic profile compared to APOE3 astrocytes

APOE4 is associated with alterations in bioenergetics, mainly glucose hypometabolism.(Area-Gomez et al., 2020; Farmer et al., 2021) APOE4 linked mitochondrial dysfunction has been reported in several cell types including neurons, astrocytes and microglia. (Farmer et al., 2019; S. Lee et al., 2023; Qi et al., 2021) Several studies have demonstrated that the rates of basal respiration are isoform dependent, in the order of APOE4<APOE3<APOE2. This results in an APOE4 associated shift towards glycolysis and fatty acid oxidation as alternative sources of energy. (Orr et al., 2019; Wu et al., 2018; X. Zhang et al., 2023) To corroborate the APOE genotype effects on mitochondrial function, primary APOE4 KI astrocytes and APOE4 KI astrocytes were subjected to the Seahorse Mito Stress test/metabolic flux assay. Our preliminary data showed that APOE4 expressing primary murine astrocytes exhibit a trend of reduction in overall basal respiration as well as FCCP-stimulated respiration than control cells, suggesting a decreased ability of APOE4 expressing cells to respond to an increased energy demand or under stress. (**Figure 2.10A, 2.10B**) However, no genotype dependent difference was observed in respiration contributing to ATP production via the activity of ATP-synthase. (**Figure 2.10B**)

Next, we determined the metabolic profile of primary APOE4 and APOE3 KI astrocytes under stress with the pretreatment of OPTI-MEM, a reduced serum media. This reduced serum condition fully unmasked the metabolic deficits of APOE4 primary astrocytes. Compared with APOE3 primary astrocytes, APOE4 primary astrocytes exhibit drastic deficits in basal and maximal respiration, spare respiration capacity, and ATP production. (**Figure 2.10C, 2.10D**) These results suggest that APOE4 primary astrocytes are particularly susceptible to mitochondrial dysfunction in the stressful (e.g., reduced serum) environment.

Notably, Datta *et al* showed that 4F altered the bioenergetic profile of macrophages, improving their basal, ATP-linked and maximal OCR. (Datta et al., 2015) Further studies are required to

investigate the potential of 4F in correcting APOE4 linked mitochondrial dysfunction in astrocytes and other brain cell types.



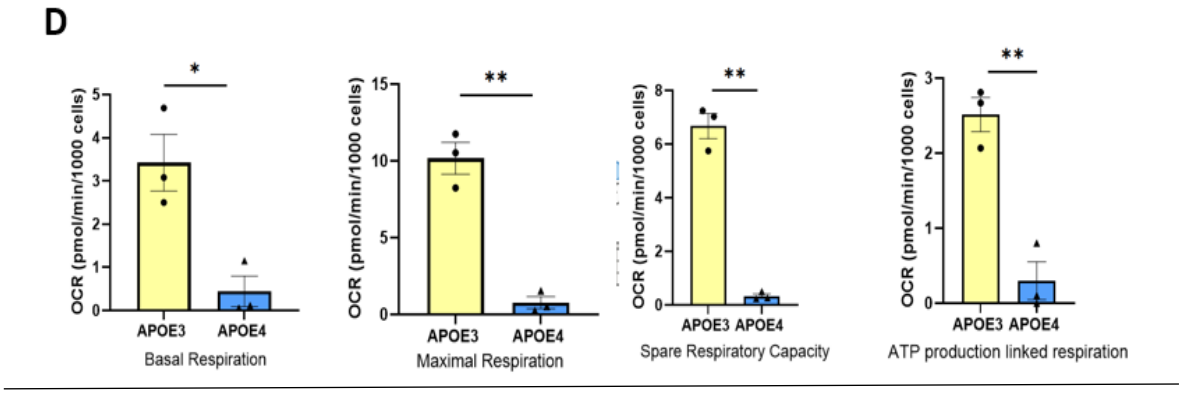


Figure 2.10 APOE4 expressing murine primary astrocytes exhibit an altered metabolic profile compared to APOE3 astrocytes. (A,B) Seahorse profiles of OCR in primary APOE3 and APOE4 KI astrocytes in serum containing media. (C,D)Seahorse profiles of OCR in primary APOE3 and APOE4 KI astrocytes in serum free OPTI-MEM media. Data represents one independent experiment performed in triplicates. * = $p < .05$, ** = $p < .01$

4. Discussion and Future Directions

APOE4 is the strongest genetic risk factor for sporadic AD. (N. Zhao et al., 2020) However, the underlying mechanisms that drive its pathogenicity in AD are poorly understood. APOE interacts with several receptors expressed in neuronal and non-neuronal cells such as LRP1, LDLR, APOER2, HSPGs and TREM2 to regulate functions such as cholesterol homeostasis, neuro-transmission, inflammation, and protein clearance in the brain. (Liu et al., 2013) Lipidation of APOE is crucial for receptor binding and functions. (Kanekiyo et al., 2014) Structural differences in APOE isoforms modify their propensity to bind to receptors as well as lipids; resulting in isoform specific alterations in the functions of APOE. (Sun et al., 2023) Several theories on loss of protective functions as well as gain of toxic functions of APOE4 have attempted to explain APOE4's role in driving AD pathology. For instance, poorer protein clearance through APOE receptors such as LRP1 and LDLR could attribute to APOE4-carrying AD patients exhibiting higher tau and amyloid accumulation, causing greater memory impairment as compared to non-APOE4 carriers. (Emrani et al., 2020) On the other hand, APOE4 may over-activate glial inflammatory responses in AD. (Parhizkar & Holtzman, 2022) In this study, we focused on lipidation deficit of APOE4 as one such loss of function exacerbating AD pathology. A direct implication of poorly lipidated APOE would be decreased levels of HDLs in the brain that are known to impact cognition through multiple mechanisms. (Hottman et al., 2014) This may explain the role of APOE4 in increasing the susceptibility of dementia and AD in aged individuals. (Gharbi-Meliani et al., 2021b)

We confirmed that APOE4 exhibits lipidation deficit compared to APOE3, a finding that has been established in the literature. (Heinsinger et al., 2016; J. Zhao et al., 2017) Furthermore, we demonstrated that HDL-mimetic peptides 4F and X-4F increase the secretion and lipidation of both APOE4 and APOE3. This suggests that the effects of HDL-mimetic peptides are not restricted to a specific APOE genotype. Importantly, 4F and X-4F improve lipidation of APOE4 to a greater extent than APOE3, reversing its lipidation deficit. 4F and X-4F mediated enhancement of APOE secretion and lipidation persist in APOE4 KI and APOE3 KI astrocytes treated with aggregated Aβ42 and in APOE4 KI astrocytes overexpressing APP/PS1.

We demonstrate that HDL-mimetic peptides 4F and X-4F modulate the lipidation of APOE4 *in vitro*. This finding opens several avenues for future investigations. For examples, could HDL-mimetic peptides 4F and X-4F correct other dysregulated functions of APOE4 such as mitochondrial dysfunction, impaired autophagy, cerebrovascular damage and pro-inflammatory responses in the context of AD? With increasing evidence on the pathogenic role of cell-type specific APOE4 in mediating AD features, it is necessary to study the effects of 4F and X-4F on other cell types, including microglia and neurons derived from mice and humans, and in co-cultures or brain organoid models as well.

Several HDL-mimetic peptides have been studied *in vivo* in the context of AD. However, these studies have only employed the amyloidogenic APP/PS1 mouse model of AD. For instance, only one study by Krishnamurthy et al demonstrated the positive outcomes of another HDL-mimetic peptide CN-105 on amyloid pathology and memory deficits in male APP/PS1/APOE4 mice (Krishnamurthy et al., 2020) Further *in vivo* investigations with 4F and X-4F in humanized APOE4-AD mouse models are necessary to solidify their clinical potential and understand the mechanism underlying their protective effects against AD.

Overall, restoring the beneficial functions of HDLs and APOE in the brain using HDL-mimetic peptides can be a promising approach to ameliorate AD features exacerbated by APOE4. The pharmacokinetic profile of 4F has been well characterized in several clinical trials for atherosclerosis. Furthermore, its smaller size and enhanced penetration across the BBB makes it a strong candidate for further investigations in the context of APOE4 associated AD.

Table 1. A summary of the treatment outcomes of HDL-mimetic peptides relevant to AD pathology

	Name	Structural Features	CNS Models	Relevant Outcomes	References
1	5A	• APOA-I mimetic • Contains 2 amphipathic helices • Each helix exhibits unequal affinity for lipids	• Myelin Damage	• Promoted ABCA1 mediated cholesterol efflux from macrophages and myelin • Enhanced phagocytic activity	(Vanherle et al., 2022)
2	APOE (141-149)	• APOE Mimetic • Lysine rich cationic peptides containing a double or triple tandem repeat of APOE's receptor-binding region	• AD	• Dose dependent effects on APP processing, in the order of trimer > dimer > monomer. • Increased α- and β-CTF-APP and decreased Aβ levels in PS1-overexpressing PS70 cells and in primary neurons • Increased sAPPα and decreased Aβ levels in wild type mice upon a single hippocampal injection of the APOE dimer	(Minami et al., 2010)
3	COG-133	• APOE-mimetic • Derived from APOE residues 133–149 located in the receptor domain	• Neuro-inflammation • Brain injury	• Suppressed the release of pro-inflammatory cytokines TNFα and IL-6 in the brain as well as periphery	(Y. Zhang et al., 2022)
4	COG-1410	• APOE-mimetic • Analog of COG133	• AD • Brain injury	• Reduced phosphorylated tau, amyloid plaques and inflammatory marker IL-6 • Selective SET antagonist	(Christensen et al., 2011; Laskowitz et al., 2007; Vitek et al., 2012; Xue et al., 2023)

	Name	Structural Features	CNS Models	Relevant Outcomes	References
5	CN-105	• APOE -mimetic • Shortest peptide comprised of 5 amino acids • Lysine rich cationic residues	• Stroke • subarachnoid hemorrhage • TBI • AD	• Attenuated amyloid pathology in male APP/PS1/APOE4 mice and reduced memory deficits only when administered early in the course of progression.	(James et al., 2022; Krishnamurthy et al., 2020; Lei et al., 2016)
6	Ac-hE18A-NH2	• APOA-I and APOE mimetic • Conjugated form of the peptide 18A and residues from binding region of APOE	• AD	• Increased APOE levels in the brain, improved cognition, decreased amyloid plaque deposition and reduced gliosis in APP/PS1 mice significantly • Blocked Aβ-oligomer uptake and the resulting inflammatory response in adult human astrocytes in co-treatment with anti-Aβ single chain variable fragment	(Handattu et al., 2013; Montoliu-Gaya et al., 2018)
7	6KApoEp	• APOE mimetic • 6 lysine residues linked to the APOE receptor binding region (133-152).	• AD	• Increased cognition and alleviated amyloid and tau pathology 5X-FAD mice • Inhibited APOE's interaction with N-terminus of APP and inhibited inflammatory responses of BV2 microglia to LPS stimulation.	(Sawmiller et al., 2019; L. Wang et al., 2019)
8	CS-6253	• APOE mimetic • Derived from the C-terminus domain involved in the formation of amphipathic helices • ABCA1 agonist	• AD	• Increase in plasma APOE levels and higher Aβ42/40 ratio with a no significant decrease in CSF Aβ42 levels upon IV administration *in vivo* • Reversal of the Aβ42 accumulation, tau hyperphosphorylation and behavioral deficits in APOE4-TR mice	(Noveir et al., 2022; Boehm-Cagan, et al., 2016; Rawat et al., 2019)

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